4 EXPERIMENTAL

4.1 PHARMACOGNOSTICAL STUDIES

The proper identification of the plant and its corresponding drug is the most important aspect of any crude drug research programme. The problem of identification is more pronounced, since much controversy exists due to the application of more than one or several botanical species for the same drug due to confusion regarding their vernacular name. The authentication of crude drug is more difficult than the plant material with flowers or fruits where the taxonomic method of identification is easily adaptable. In crude drugs the various tissues systems and their contents are to be studied to arrive at a broad consensus of the group of plant material. Every part of the plant has some common characters and specific characters, recognition of which help in the correct identification in powder condition.

Since, many of the drugs used in Ayurveda are sold in the market as crude drugs, it is essential to study and understand the tissues system like dermal, vascular and ground tissues, which still hold a key in arriving at the correct identification. These characters in combination with the taxonomic characters of the plant help in the proper identification of the plant materials.

The pharmacognostical and phytochemical studies of S. dulcis whole plant were performed which includes identification, morphological evaluation, microscopical study, determination of physical parameters, preliminary phytochemical analysis and column chromatography.

4.1.1 COLLECTION AND AUTHENTICATION

The plants were collected from local area of Barpali, Dist-Bargarh, Odisha during November 2009 to January 2010. The plant was authenticated as Scoparia dulcis Linn. by Botanical Survey of India, Central National Herbarium Howrah, Kolkata, India (Ref.No:CNH/I-I/38/2009/Tech II/137). An authentic voucher specimen was deposited in the Herbarium Museum, Department of Pharmacognosy, The Pharmaceutical College, Barpali. The authentication letter of BSI is given in Ph.D. Thesis, Sambalpur University, Odisha Page 59
Appendix-I. The gathered plants were cleaned which involved screening, washing and also hand picking. The unnecessary parts were removed prior to drying. The plant so collected were dried under shade at about 25-30°C and coarsely powdered by the help of mechanical process using sieve no. 40 and stored in a airtight container for further studies.

4.1.2 MACROSCOPICAL STUDIES[1, 2]

Morphological characteristics are referred to the evaluation of herbs by colour, odour, taste, size, shape and special features like touch, texture, etc. It is a technique of qualitative evaluation based on the study of morphological and sensory profiles of herbs.

A. Morphology of Leaf:

<table>
<thead>
<tr>
<th>Type of leaf</th>
<th>Simple &amp; pinnatifed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Green</td>
</tr>
<tr>
<td>Odour</td>
<td>Characteristics</td>
</tr>
<tr>
<td>Taste</td>
<td>Sweet</td>
</tr>
</tbody>
</table>

B. Morphology of Stem:

<table>
<thead>
<tr>
<th>Type of stem</th>
<th>Woody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer surface</td>
<td>Rough</td>
</tr>
<tr>
<td>Fracture</td>
<td>Irregular &amp; fibrous</td>
</tr>
<tr>
<td>Odour</td>
<td>Characteristic</td>
</tr>
<tr>
<td>Taste</td>
<td>Sweet</td>
</tr>
<tr>
<td>Colour</td>
<td>Green</td>
</tr>
</tbody>
</table>

C. Morphology of Fruit:

<table>
<thead>
<tr>
<th>Outer surface</th>
<th>Smooth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Green</td>
</tr>
<tr>
<td>Odour</td>
<td>Characteristic</td>
</tr>
<tr>
<td>Taste</td>
<td>Astringent</td>
</tr>
</tbody>
</table>
Figure 4.2. *Scoparia dulcis* Plant Morphology

A: *Scoparia dulcis* twig

B: Leaf

C: Dorsal leaf

D: Ventral leaf

E: Flower

F: Fruit

*Ph.D. Thesis, Sambalpur University, Odisha*
Figure 4.2. *Scoparia dulcis* Plant Morphology
Morphological characters of *Scoparia dulcis* Linn. [Hand-drawing]

**Figure 4.3.** Morphology hand drawing of *Scoparia dulcis*

Ph.D. Thesis, Sambalpur University, Odisha
4.1.3 **MICROSCOPICAL STUDIES**[3, 4]

This study allows more detailed examination of a crude drug to identify their histological characters. It is mostly used for qualitative evaluation of organized crude drug in entire part and in powdered form.

Microscopy of different plant parts

**Materials and Methods:**

The microscopy of *Scoparia dulcis* Linn. plant was carried out using digital microscope attached with computer system (Besto- 1688). The different organs of the plant materials were sliced fine by using microtome for its transverse section (TS) as well as longitudinal section (LS) and the finest sections were selected for study. The TS & LS were stained with phloroglucinol and HCl for staining the lignified tissues and mounted with glycerin and observed under microscope. The sections were examined under microscope using 10x eye piece and objective pieces 10, 40 and 100x. For leaf constants 5x eye piece and 10x objective piece were used.
Figure 4.4. *Scoparia dulcis* Stem microscopy

Maginification 400x
Figure 4.5. *Scoparia dulcis* Stem microscopy

Magnification 1000x
In the stem microscopy Fig 4.4 endodermis, cortex, chlorenchyma and the presence of needle shaped calcium oxalate crystals may be considered as an identifying character of *S. dulcis*. Fig 4.5 showed the surface view of xylem element with vessel, xylem elements, annular thickening vessels, bordered pitted xylem, uniseriate multicellular glandular trichomes and pith.

![Images of leaf and stem microscopies](image1.jpg)

**A: Sessile glandular trichome**  
**B: Epidermis along the midrib**

**Figure 4.6. Scoparia dulcis Leaf microscopy**  
**Magnification 1000x**

In the leaf microscopy, Fig 4.6 the thick walled loosely attached collenchymatous tissues of epidermis and the hypodermis of *S. dulcis* leaf, the upper epidermis stomata (subsidiary cells, guard cells and opening aperture of stoma) and epidermal cells, lower epidermis with numerous stomata were observed. Anisocytic stomata were fairly abundant on both surfaces of epidermis. Lower epidermis consists of large, dark and more irregular cells in comparison to upper epidermis.
Figure 4.7. TS of *Scoparia dulcis* root

Magnification 400x
Figure 4.8. *Scoparia dulcis* root tissue structure.

Magnification 1000x
Figure 4.9. *Scoparia dulcis* root tissue structure.

Figure 4.10. Microscopy of *Scoparia dulcis* flower, T.S. of ovary Magnification 400x
In the root microscopy, Fig.4.7 cortex, phloem, xylem element, trachid, phloem components and phloem cell were shown. Fig. 4.8 showed LS of cortex, cork cell and epiblema were observed. Fig.4.9 showed LS of pith cell and Fig. 4.10 showed the ovary TS in the microscopy of flower.

4.1.3.1 Leaf constants [4]

The specification of leaf constants mentioned in quantitative microscopy includes Vein-islet number, veinlet-termination, stomatal number, stomatal index and Palisade ratio.

The main importance of these values lies in identifying the species, whether the powder is authentic or adulterated.

**Determination of vein-islet number and veinlet-termination number:**

Vein-islet number:
The average number of vein-islet per square mm of the leaf surface midway between the central part of the lamina and margin.

Veinlet-termination:
The number of veinlet-termination per square mm of the leaf surface, midway between midrib of the leaf and its margin.

The vein islet-number and vein-termination are constant for a given species of the plant. This factor has proved useful for the distinction of the nearly related species.

**Materials & Methods:**

Compound microscope (5x X 10x), stage micrometer, camera lucida, black sheet.

Three to four cut portions of the leaf from the central region of lamina of 6 square mm size were boiled in methanol in a test tube. Then each portion was kept on a slide in chloral hydrate with lower portion facing upward so that the position of the vein may be observed easily. A drop of glycerin was added on to the material under consideration. 5x eye piece and power objective 10x were used for further study. Stage micrometer was focused and camera lucida was fixed. A black sheet was placed on the side of the microscope in such a way that the focus should come on paper. Then using stage micrometer 1 mm square was drawn. Image of the leaf was made to
super impose the square on the black sheet. Vein-islet and vein-termination were traced and counted. The vein-islet and vein-termination including those intersected by the bottom and left side of the square were included but those intersected by the top and right side were excluded. Six groups were counted.

**Determination of stomatal number and stomatal index**

Stomatal number:

It is the average number of stomata per square mm of epidermis. The actual number of stomata per square mm may vary for the leaf of the same plant grown in different climatic conditions, age of the plant, maturity of the leaves etc.

Stomatal index:

It is the percentage of stomata calculated by the formula given below. Stomatal number varies considerably with the age of leaf, climatic conditions, nature of soil and other external as well as internal factors but stomatal index are constant for a given species and helps in the identification of the plantspecies.

\[
SI = \frac{S}{E + S} \times 100
\]

Where, \( SI \) = Stomatal index

\( E \) = Total number of epidermal cells in the same unit area.

\( S \) = Number of stomata per unit area.

**Materials & Methods:**

Compound Microscope (5x X 10xand 5x X 10x), stage micrometer, camera lucida and black sheet.

Younger leaf of the plant is taken, washed properly in tap water and peeled carefully using new blade in such a way that chlorophyllus layer is removed. The peeled leaf part was taken in a test tube containing about 5 ml of chloral hydrate solution and heated on a water bath until the fragments were transparent. Then the leaf piece was taken on to a watch glass, few drops of ethanol was added and washed with water. Stained with a few drops of safranin and washed immediately with water and
mounted with the help of glycerin. Microscopy of stained piece was done by using compound microscope (5x X 10x and 5x X 10x), equipped with camera lucida. The epidermal cells and stomata were drawn on the black sheet and counted within the 1 mm square. The epidermal cells and stomata present more than half portion out side the square was not counted.

Determination of palisade ratio

The average number of palisade cells beneath each upper epidermal cell is termed as palisade ratio. It is of diagnostic value in differentiating the species. It remains constant within a range for a given plant species. This value does not alter in samples of different localities or according to the age of the plant. However, it differs from species to species and hence important for identification. This determination is not applicable to monocot leaves, as the differentiation in mesophyll cell is not possible in monocot plants.

Materials & Methods:

Compound microscope (5x X 10x), stage micrometer, camera lucida and black sheet.

A small piece of leaf along with lamina was taken and boiled in chloral hydrate till the leaf pieces became transparent. Then by using camera lucida, four adjacent cells of upper epidermis were traced. Then focused (10x) on the palisade layer and traced off the palisade cells beneath the four epidermal cells which were already traced. The palisade cells which were 50% or more inside the epidermal walls were also traced. Six determinations were done.
Table 4-1. Leaf Constants of *S. dulcis*

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Constants</th>
<th>Ranges</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vein- islet number</td>
<td>11.5 - 14.6</td>
<td>Not available</td>
</tr>
<tr>
<td>2</td>
<td>Veinlet termination number</td>
<td>16.1 - 20.4</td>
<td>Not available</td>
</tr>
<tr>
<td>3</td>
<td>Stomatal number</td>
<td>76-79 (upper surface)</td>
<td>Not available</td>
</tr>
<tr>
<td></td>
<td></td>
<td>82-86 (lower surface)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Number of epidermal cell</td>
<td>477-532 (upper surface)</td>
<td>Not available</td>
</tr>
<tr>
<td></td>
<td></td>
<td>435-491 (lower surface)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Stomatal index</td>
<td>12.5±2 (upper surface)</td>
<td>Not available</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.3±2 (lower surface)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Palisade ratio</td>
<td>6 – 9</td>
<td>Not available</td>
</tr>
</tbody>
</table>

Reading was taken from six observations.
4.1.4 **POWDER MICROSCOPY**

**Materials & Methods:**

Compound microscope (10×40x), slide, cover slip, brush, needle, chloral hydrate, iodine solution, HCl, Phloroglucinol etc.

**Preparation of Slides:**

Three slides were prepared. One only with chloral hydrate solution (a clearing agent) helps to see the cellular arrangements. In second slide a drop of N/10 iodine solution was added to observe starch grains. Whereas, the third slide was treated with dilute HCl and phloroglucinol to observe the lignified cells.

---

A: Lignified Cork cell in surface view  
B: Lignified Schlerenchymatous cell  
C: Sclereids  
D: Xylem Parenchyma & fibre
Figure 4.11. Powder microscopy of *Scoparia dulcis*
Magnification 400x
In the powder microscopy we observed the presence of cork cells in surface view, epidermal cell, sclereids, xylem parenchyma & fibre, pericyclic fibre, cork cell & fibre, starch granules & simple fibre, palisade layer of testa, sclerenchyma and Spiral vessels. All the reported figures A-J are shown in Fig.4.11.

4.1.5 PHYSICOCHEMICAL INVESTIGATION [6-8]

This includes the study of different physical parameters which are fairly constant for crude drugs. It includes determination of foreign organic matters, extractive values like alcohol soluble and water soluble, determination of ash values like total ash values, water soluble and acid insoluble ash values and moisture content.

These procedures normally adopted to get the qualitative information about the purity and standards of a crude drug. The values of these parameters are constant in a species. [11]

4.1.5.1 Determination of Ash Value

The residue left after incineration is called as ash. The residue originating from inorganic elements present in plant may be designed as physiological ash. It varies within a definite limit according to the type of soil, dust, and other mineral impurities or admixture of other drug, which may alter the ratio.

Ash values are helpful in determining the qualities and purities of a crude drug. Ash is done to remove all traces of organic matter from the vegetable drugs which otherwise interfere in analytical determination on incineration, crude drug normally leave an ash usually contain carbonates, phosphate and silicate of potassium, sodium, calcium and magnesium. In case where silica or calcium oxalate present in high concentration then a higher limit of acid insoluble ash is imposed. Some times mixing of sulphuric acid with the powdered crude drug is preferred before ash. This sulphated ash is normally less fusible than ordinary ash.
Materials & Methods:

Silica crucible, desiccator, digital balance and muffle furnace.

Total Ash:

Accurately 2g of the powdered crude drug (air-dried) was weighed in a tared silica crucible and incinerated by gradually increasing the temperature at 450°C ± 5°C (the ignition was repeated) until free from carbon, so that nearly white ash was obtained in the crucible. The ash was cooled in a desiccator and weighed. The percentage of ash was calculated with reference to the air dried crude drug and value was recorded in table 4-2.

Water Soluble Ash:

The total ash obtained from above section was boiled with 25 ml of water for 5 minutes and then filtered through an ash less filter paper (Whatmann 41). The filter paper was ignited in the dried tared silica crucible for 15 min at a temperature 450°C ± 5°C.

The weight of the insoluble matter was subtracted from the weight of the ash, the difference in weight represented the water soluble ash. Then the percentage of water soluble ash was calculated with reference to the air dried drug.

Acid Insoluble Ash:

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

The ash was prepared as per the above mentioned total ash procedure. The ash was cooled in a desiccator and weighed. The total ash obtained was boiled for five minutes with 25 ml of 2M HCl and filtered through ash less filter paper (whatmann 41). The filter paper was ignited in the dried tared silica crucible for 15 min at a temperature not exceeding 450°C ± 5°C. The crucible was cooled in a desiccator and weighed to get the percentage of acid-insoluble ash with reference of the air dried drug and value was recorded in table 4-2.
4.1.5.2 Determination of Moisture Content (Loss on Drying):

This procedure is used for the determination of amount of volatile matter (i.e. water drying off from the drug). Loss on drying is the loss in weight in % w/w. It was determined by means of the procedure given below. It determines the amount of volatile matter of any kind (including water) that can be driven off under the condition specified (desiccator or hot air oven).

Materials & Methods:

Evaporating dish, hot air oven, desiccator and digital balance.

10 g of shed dried coarse powder of crude drug was taken in a tared evaporating dish and dried at 105°C ± 1°C and weighed. The drying and weighing was continued at 1 hr interval, until difference between two successive weighing correspond not more than 0.25 % difference. Constant weight was reached when two consecutive weighing after drying for 30 minutes and cooling for 30 min in a desiccator, showed not more than 0.01 g difference. The percentage of moisture content was calculated with reference to the air-dried crude drug and the values were shown in the table 4-2.

4.1.5.3 Determination of Extractive Value

Extractive values of crude drugs are useful for their evaluation, especially when the constituents of a drug cannot be readily estimated by any other means. Further, these values indicate the nature of the constituents present in a crude drug.

Materials & Methods:

Distilled water, alcohol, chloroform, petrol ether, methanol, conical flask, beaker, funnel, water bath, desiccator and digital balance.

Pet. Ether Soluble Extractive Value:

Accurately 5 g of air dried coarsely powdered crude drug was macerated with 100 ml of pet ether of (40–60°C) in a closed conical flask for 24 hrs with occasional shaking for first 6 hrs. Then the liquid extract was filtered taking proper precaution against loss of solvent. Then 25 ml of liquid extract was taken in a dry tarred beaker, which was then completely dried at 105°C and weighed. The percentage of pet. ether
extractive value was calculated with reference to the air-dried crude drug and the value was recorded in table 4-2.

Chloroform Soluble Extractive Value:

Accurately 5 g of air dried coarsely powdered crude drug was macerated with 100ml of chloroform of specified strength in a closed conical flask for 24 hrs with occasional shaking for first 6 hrs. Then the liquid extract was filtered taking proper precaution against loss of solvent. Then 25 ml of liquid extract was taken in a dry tarred beaker, which was then completely dried at 105°C and weighed. The percentage of chloroform extractive value was calculated with reference to air-dried crude drug and value was recorded in table 4-2.

Alcohol Soluble Extractive Value:

Accurately 5 g of air dried coarsely powdered crude drug was macerated with 100ml of alcohol in a closed conical flask for 24 hrs with frequent shaking during first 6 hrs and allowed to stand for 18 hrs. The liquid extract was filtered rapidly, taking proper precaution against loss of solvent, evaporated 25ml of the filtrate to dryness in a dry tarred beaker and dried at 105°C, to constant weight and weighed. The percentage of alcohol soluble extractive value was calculated with reference to air-dried crude drug and the values were recorded in the table 4-2.

Methanol Soluble Extractive Value:

Accurately 5 g of air dried coarsely powdered crude drug was macerated with 100ml of methanol of specified strength in a closed conical flask for 24 hrs with occasional shaking for first 6 hrs. Then the liquid extract was filtered taking proper precaution against loss of solvent. Then 25 ml of liquid extract was taken in a dry tarred beaker, which was then completely dried at 105°C and weighed. The percentage of methanol extractive value was calculated with reference to air-dried crude drug and value was recorded in table 4-2.

Water Soluble Extractive Value:

Accurately 5 g of air dried coarsely powdered crude drug was macerated with 100ml of distilled water in a closed conical flask for 24 hrs with frequent shaking during first 6 hrs and allowed to stand for 18 hrs. The liquid extract was filtered rapidly, taking
proper precaution against loss of solvent, evaporated 25ml of the filtrate to dryness in a dry tared beaker and dried at 105°C, to constant weight and weighed. The percentage of water soluble extractive value was calculated with reference to air-dried crude drug and the values were recorded in the table 4-2.

4.1.5.4 **Foreign Organic Matter** [7]

200 g of powder drug sample was weighed and spread as a thin layer on stainless steel tray. Sample was inspected visually under microscope at 6x lens to separate the foreign organic matter (FOM) as completely as possible. Separated FOM was weighed and the percentage of FOM was calculated with respect to the total quantity of drug taken in analysis and value was recorded in table 4-2.

Table 4-2. Physical constants of *S. dulcis*

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Constant</th>
<th>Whole plant (%)</th>
<th>Standard value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ash value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Total ash</td>
<td>5.0</td>
<td>Not available</td>
</tr>
<tr>
<td>II</td>
<td>Water soluble ash</td>
<td>4.0</td>
<td>Not available</td>
</tr>
<tr>
<td>III</td>
<td>Acid insoluble ash</td>
<td>1.5</td>
<td>Not available</td>
</tr>
<tr>
<td>2.</td>
<td>Moisture content</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Moisture content</td>
<td>9.4</td>
<td>Not available</td>
</tr>
<tr>
<td>3.</td>
<td>Extractive value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Pet. Ether</td>
<td>1.6</td>
<td>Not available</td>
</tr>
<tr>
<td>II</td>
<td>Chloroform</td>
<td>2.4</td>
<td>Not available</td>
</tr>
<tr>
<td>III</td>
<td>Alcohol</td>
<td>12.0</td>
<td>Not available</td>
</tr>
<tr>
<td>IV</td>
<td>Methanol</td>
<td>16.0</td>
<td>Not available</td>
</tr>
<tr>
<td>V</td>
<td>Water</td>
<td>15.2</td>
<td>Not available</td>
</tr>
<tr>
<td>4.</td>
<td>Foreign Organic Matter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Foreign Organic Matter</td>
<td>1.2</td>
<td>Not available</td>
</tr>
</tbody>
</table>

Reading was taken from three observations.
4.1.6 PHYTOCHEMICAL STUDIES

4.1.6.1 Extraction

Extraction involves the separation of bioactive portion of the plant tissues from inactive components by using selective solvents with different extraction technique.

4.1.6.2 Maceration (Cold extraction)

The shade dried coarsely powdered (500 g) whole plant of *S. dulcis* was macerated with 1.5 litre of water, hydro alcoholic & methanol separately and kept at room temperature for five days. After five days solution was filtered and solvent was evaporated under reduced pressure, the extract was weighed and percent yield was calculated with reference to air-dried crude powdered materials, the residues were stored in refrigerator for further study.

4.1.6.3 Hot successive extraction (Soxhlet)

The shade dried coarsely powdered *S. dulcis* plant (500 g) was subjected to continuous hot extraction with water & methanol successively. The extracts were filtered, dried, weighed and percent yield was calculated with reference to air-dried powder.

Table 4-3. Extractive values of *S. dulcis*

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Solvent used</th>
<th>Whole plant (%)</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Maceration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Water</td>
<td>15</td>
<td>Not available</td>
</tr>
<tr>
<td>2</td>
<td>Methanol</td>
<td>16</td>
<td>Not available</td>
</tr>
<tr>
<td>II. Soxhlet extraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Water</td>
<td>15.5</td>
<td>Not available</td>
</tr>
<tr>
<td>4</td>
<td>Methanol</td>
<td>16.9</td>
<td>Not available</td>
</tr>
</tbody>
</table>
4.1.7 QUALITATIVE PHYTOCHEMICAL SCREENING

The plant may be considered as a biosynthetic laboratory, not only for the chemical compounds such as carbohydrates, proteins and lipids that are utilized as food by human being but also for multitude of compounds like glycosides, alkaloids, volatile oils, phenolic compounds, tannins etc, exert physiological activities. The compounds those are responsible for therapeutic effects are usually the secondary metabolites. A systematic study of a crude drug includes thorough consideration of both primary and secondary metabolites derived as a result of plant metabolism. The plant material may be subjected to preliminary phytochemical screening for the detection of various plant constituents.

Different qualitative chemical tests can be performed to establish profile of the extracts for there chemical composition. The following tests were performed on extracts to detect various phytoconstituents present in them.

Materials & Methods:

Reagents: Mayer’s, Wagner’s, Hager’s, Dragendorff’s, Fehling’s A & B, Barfoed’s, Millon’s, α-naphthol, Conc. Sulphuric acid, 10 % Ammonia, Pyridine, Sodium nitroprusside, Acetic anhydride, Ferric chloride, 10 % Lead acetate, 10 % Ammonium hydroxide solution and methanolic extract.

4.1.7.1 Tests for Alkaloids

200 mg of solvent free extracts were stirred with 50 ml of dilute hydrochloric acid and filtered. Then the filtrates were tested carefully with various alkaloidal reagents as follows:

Mayer’s test:

About 2 drops of Mayer’s reagent was added to 2 ml of filtrate by the side of the test tube. A white precipitate was obtained in methanolic extract which indicated the presence of alkaloids. But no precipitate was obtained in case of water extract.

Wagner’s test:

2 drops of Wagner’s reagent was added to 2 ml of filtrate by the side of the test tube. A reddish brown precipitate was obtained which indicate the presence of alkaloids.
Hager's test:
2 drops of Hager's reagent (saturated aqueous solution of picric acid) was added to 2ml of filtrate by the side of the test tube. Prominent yellow precipitates were obtained which indicated the presence of alkaloids.

Dragendorff's test:
2 drops of Dragendorff's reagent (Solution of Potassium Bismuth Iodide) were added to 2 ml of each filtrate by the side of the test tube. A prominent yellow precipitate was obtained in methanolic extract which indicates the presence of alkaloids. But no precipitate was obtained in case of water extract.

4.1.7.2 Tests for Carbohydrates
200 mg of extracts were dissolved in 50 ml of water and filtered. The filtrates were subjected to the following tests.

Molish's test:
2 drops of alcoholic solution of α-naphthol was added to 2 ml of each filtrate and the mixture was shaken well. Then 1 ml of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. Violet rings were obtained which indicate the presence of carbohydrates.

Fehling's test:
1 ml of each filtrate was boiled on water bath with 1 ml each of Fehling solutions A and B. Red precipitates were obtained which indicated the presence of sugar.

Barfoed's test:
1 ml of Barfoed's reagent was added to 1ml of each filtrate and heated on a boiling water bath for 2 min. Red precipitates were obtained which indicated the presence of sugar.

Benedict's test:
About 0.5 ml of Benedict's reagent was added to 0.5 ml of each filtrate. The mixtures were heated on a boiling water bath for 2 mins. Characteristic coloured precipitates were obtained which indicated the presence of sugar.
4.1.7.3 Tests for Glycosides
About 50 mg of extracts was hydrolysed with concentrated hydrochloric acid for 2 hrs on a water bath and filtered. Then the hydrolysates were subjected to the following tests.

Borntrager’s test:
About 10 ml of chloroform was added to 2 ml of each filtered hydrolysate and shaken properly. Then chloroform layers were separated and 10% ammonia solution was added. Pink colours were obtained which indicated the presence of glycosides.

Legal test:
50 mg of the extracts were dissolved in pyridine, sodium nitropruside solution were added and were made alkaline using 10% sodium hydroxide. Pink colour indicated the presence of glycosides.

4.1.7.4 Test for Saponin
50 mg of extracts were diluted with distilled water and made up to 20 ml. The suspensions were shaken in graduated cylinders for 15 mins. Stable layers of foam formed that indicated the presence of saponins.

4.1.7.5 Tests for Proteins and Amino Acids
2 mg of extracts was dissolved in 10 ml of distilled water and filtered through Whatmann No.1 filter paper. The filtrates were subjected to tests for proteins and amino acids.

Millon’s test:
Few drops of Millon’s reagent were added to 2 ml of each filtrate. White precipitates were obtained which indicated the presence of proteins.

Biuret test:
1 drop of 2% copper sulphate solution was treated with 2 ml of each filtrate. To this 1 ml of ethanol (95%) were added, followed by excess of potassium hydroxide pellets. Pink colour in the ethanolic layer was not obtained in water extract which indicated the absence of proteins. But the methanolic extract showed pink colour in the ethanolic layer indicating the presence of proteins.
Ninhydrin test:

2 drops of Ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) was added to 2 ml of aqueous filtrate of each extract. A characteristic purple colour was not appeared in water extract that showed absence of amino acids. But the methanolic extract showed characteristic purple colour indicating the presence of amino acids.

Phenolic compounds and tannins

Ferric chloride test:

50 mg of extracts were dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. Dark green colours were obtained which indicated the presence of phenolic compound.

Lead acetate test:

50 mg of extracts were dissolved in distilled water and to it 3 ml of 10% lead acetate solutions were added. Bulky white precipitates were obtained which indicated the presence of phenolic compound.

Gelatin test:

50 mg of extracts were dissolved in distilled water and 2 ml of 1% solution of gelatin containing 10% sodium chloride added to it. White precipitate indicated the presence of phenolic compound.

Flavonoids

Alkaline reagent test:

Aqueous solutions of the extracts were treated with 10% ammonium hydroxide solution. Yellow fluorescence were obtained, which showed the presence of flavonoids.
Table 4-4. Preliminary Phytochemical Tests for *S. dulcis*

<table>
<thead>
<tr>
<th>Phytochemical test</th>
<th>Water extract</th>
<th>Methanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Alkaloids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mayer’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wagner’s test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Hager’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dragendorff’s test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2. Carbohydrates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molish’s test</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Fehling’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Barfoid’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benidict’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3. Glycosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borntrager’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Legal’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4. Saponins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foam test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5. Proteins &amp; amino acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Millon’s test</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Biuret’s test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ninhydrin test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6. Phenolic compounds &amp; tannins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferric chloride test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lead acetate test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7. Flavonoids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline reagent test</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- Absent; + Present; ++ Abundant
4.1.8 CHROMATOGRAPHIC STUDIES [10, 11]

Chromatography is essentially a group of techniques for the separation of a mixture into individual components using a stationary phase and mobile phase. The main principle of the separation may be either partition or adsorption.

4.1.8.1 Thin Layer Chromatography (TLC)

Thin layer chromatography is based on the principle of adsorption i.e. when a mixture of compounds (adsorbate) dissolve in the mobile phase (eluent) moves through a column of stationary phase (adsorbent), they travel according to the relative affinities towards stationary phase.

The compounds which have more affinity towards stationary phase that travels slower. The compounds which have lesser affinity towards stationary phase that travels faster. Thus, the components are separated on a plate based on the affinity of the components towards the stationary phase or generally the compounds, which are readily soluble, but not strongly adsorbed moves up along with the solvent and those not so soluble but more strongly adsorbed moves up less readily leading to separation of compounds.

Materials & Methods:

Glass slide, Solvent tank, Iodine chamber and Silica gel G

Required quantity of Silica gel G was weighed and made homogenous slurry with sufficient distilled water. The slurry was poured on TLC glass plates by spreading technique and the uniform silica gel layer was adjusted to 0.25 mm thickness. The coated plates were allowed to dry in air and activated by heating in hot air oven at 100-105°C for 1 hour and then used for TLC. Then, with the help of a capillary tube, 2-5 μl of a 1% solution of extracts were spotted on TLC plate and dried, which was developed in TLC chamber previously saturated with solvent system.

The different spots developed in each solvent system were identified under day light and the Rf value were correspondingly calculated and tabulated.

\[ R_f \text{ value} = \frac{\text{Distance traveled by solute (cm)}}{\text{Distance traveled by solvent system (cm)}} \]
Plate 4-1. Showing 15 SPOTS under UV light of *S. dulcis* methanolic extract
Table 4-5. Rf values of S. dulcis methanolic extract

<table>
<thead>
<tr>
<th>Plate-4-I</th>
<th>Stationary phase</th>
<th>Solvent system</th>
<th>Numbers of spots</th>
<th>Spraying Reagent</th>
<th>Colour of spots</th>
<th>Rf Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract</td>
<td>Silica gel G</td>
<td>Toluene: methanol (80:20)</td>
<td>15</td>
<td>Anisaldehyde-sulphuric acid</td>
<td>Brown, Brown, Brown, Brown, Brown, Brown, Brown, Brown, Reddish brown, Reddish brown, Reddish brown, Reddish brown, Pink, Pink, Brown,</td>
<td>0.08, 0.23, 0.30, 0.34, 0.39, 0.42, 0.48, 0.60, 0.62, 0.65, 0.77, 0.84, 0.89, 0.94, 0.97</td>
</tr>
</tbody>
</table>
Plate 4-II. Showing 9 spots under UV light of *Scoparia dulcis* methanolic extract

Table 4-6. *Rf* values of *S. dulcis* methanolic extract

<table>
<thead>
<tr>
<th>Plate-4-II</th>
<th>Stationary phase</th>
<th>Solvent system</th>
<th>Numbers of spots</th>
<th>Spraying Reagent</th>
<th>Colour of spots</th>
<th>Rf Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract</td>
<td>Silica gel G</td>
<td>Toluene: methanol (75:25)</td>
<td>9</td>
<td>Anisaldehyde-sulphuric acid</td>
<td>Brown</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brown</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brown</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brown</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brown</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pink</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pink</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pink</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pink</td>
<td>0.88</td>
</tr>
</tbody>
</table>
Plate 4-III. Showing 6 spots under UV light of *S. dulcis* methanolic extract

Table 4-7. *R*$_f$ values of *S. dulcis* methanolic extract

<table>
<thead>
<tr>
<th>Plate-4-III</th>
<th>Stationary phase</th>
<th>Solvent system</th>
<th>Numbers of spots</th>
<th>Spraying Reagent</th>
<th>Colour of spots</th>
<th>Rf Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract</td>
<td>Silica gel G</td>
<td>Pet. ether: Chloroform: Methanol (50 : 40 :10)</td>
<td>6</td>
<td>Anisaldehyde-sulphuric acid</td>
<td>Brown</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brown</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brown</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brown</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Grey</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pink</td>
<td>0.85</td>
</tr>
</tbody>
</table>
Plate 4-IV. Showing 5 spots under UV light methanolic extract of *S. dulcis*

![Image of 5 spots under UV light methanolic extract of *S. dulcis*]

### Table 4-8. Rf values of *S. dulcis* methanolic extract

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Solvent system</th>
<th>Numbers of spots</th>
<th>Spraying agent</th>
<th>Colour of spots</th>
<th>Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract</td>
<td>Silica gel G</td>
<td>n-hexane: Ethylacetate (90: 10)</td>
<td>5</td>
<td>Anisaldehyde-sulphuric acid</td>
<td>Brown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brown</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pink</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Purple</td>
<td>0.99</td>
</tr>
</tbody>
</table>
4.1.8.2 Column chromatography \cite{12}

Basically, it is a liquid chromatography in which mobile phase in the form of liquid passes over the stationary phase packed in a column. The column is made up of glass. Methanolic extract of \textit{S. dulcis} was taken for the column chromatography.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{column_chromatography.jpg}
\caption{Column chromatography}
\end{figure}
Slurry Formation

Dried and solvent free methanolic extract of *S. dulcis* was taken and dissolved in a minimum quantity of methanol and then adsorbed on column silica gel and kept for drying at room temperature to get free flowing material.

- Partial size of column silica gel: 60-120 mesh
- Weight of columnsilica gel used (slurry): 10g
- Weight of extract: 5g

Packing of column

A neat and dried sintered column was taken. Solvent (n-hexane) was poured into the column and packed with slurry of silica gel prepared by suspending it into the solvent to prepare the column bed. The adsorbed methanolic extract in silica gel was then charged into the column.

- Weight of columnsilica gel: 75g
- Diameter of column: 2.5 cm
- Length of column: 40 cm

Elution of the column

The column was eluted successively with n-hexane to methanol, gradually increasing the polarity/percentage of different solvent. The fraction was collected as per TLC and UV absorption. The collected fractions were concentrated on reduced pressure and stored for further studies. A total of 253 fractions were collected and performed TLC by using different mobile phase.

The TLCs of different fractions were performed. After several TLC we got single and double spots in certain fractions. There is a possibility that these fractions may contain single or double compounds which need a regourous investigation. However, it may be possible that the pharmacological activities are due to these compounds.
4.1.8.3 TLC of column fractions:

Plate 4-V. Fraction no. 90 of methanolic extract of *S. dulcis*

<table>
<thead>
<tr>
<th>Plate no-4-V Fraction no.-90</th>
<th>Stationary phase</th>
<th>Solvent system</th>
<th>Numbers of spots</th>
<th>Detecting agent</th>
<th>Colour of spots</th>
<th>Rf Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica gel G</td>
<td>Ethyl acetate:N-hexane (90:10)</td>
<td>1</td>
<td>Anisaldehyde-sulphuric acid</td>
<td>Green</td>
<td>0.92</td>
<td></td>
</tr>
</tbody>
</table>
Plate 4-VI. Fraction no. 116 of methanolic extract of *S. dulcis*

Table 4-10. *R*$_f$ values of fraction no 116 of *S. dulcis* methanolic extract

<table>
<thead>
<tr>
<th>Plate no-4-VI</th>
<th>Stationary phase</th>
<th>Solvent system</th>
<th>Numbers of spots</th>
<th>Detecting agent</th>
<th>Colour of spots</th>
<th><em>R</em>$_f$ Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction no.-</td>
<td>Silica gel G</td>
<td>Toluene: methanol (75:25)</td>
<td>2</td>
<td>Anisaldehyde-sulphuric acid</td>
<td>Green Yellow</td>
<td>0.86 0.93</td>
</tr>
</tbody>
</table>

Ph.D. Thesis, Sambalpur University, Odisha
Page 97
Plate 4-VII. Fraction no. 117 of methanolic extract of *S. dulcis*

Table 4-11. *R*\(_f\) values of fraction no 117 of *S. dulcis* methanolic extract

<table>
<thead>
<tr>
<th>Plate no-4-VII Fraction no.-117</th>
<th>Stationary phase</th>
<th>Solvent system</th>
<th>Numbers of spots</th>
<th>Detecting agent</th>
<th>Colour of spots</th>
<th>R(_f) Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica gel G</td>
<td>Toluene: methanol (75:25)</td>
<td>1</td>
<td>Anisaldehyde-sulphuric acid</td>
<td>Green</td>
<td>0.46</td>
<td></td>
</tr>
</tbody>
</table>
4.1.9 **INSTRUMENTAL ANALYSIS [13, 14]**

4.1.9.1 **Total Carbohydrate estimation**

4.1.9.1.1 **By Phenol Sulphuric Acid Method**

In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This forms a green coloured product with phenol and has absorption maximum at 490 nm.

**Materials & Methods:**

**Instrument used:** SHIMADZU UV-Spectrophotometer (UV-1800), Centrifuge machine (Remi).

**Phenol 5%:** Redistilled (reagent grade) phenol (50 gm) was dissolved in water and diluted to make one liter.

**Sulphuric acid:** 96% reagent grade.

**Standard Glucose:** Stock- 100 mg was dissolved in 100ml of water.

**Working standard:** 10 ml of stock diluted to 100ml with distilled water (100μg/ml)

**Sample preparation:**

100 mg methanolic extract of *S. dulcis* was weighed and taken in a test tube. 5 ml of 2.5 N HCl was added to it and boiled in a water bath for three hours (for hydrolysis of sample) and cooled to room temperature. Then it was neutralized with solid sodium carbonate until the effervescence ceased. The volume was made up to 100 ml and centrifuged. The supernatant was collected and used for total carbohydrate estimation.

0.02, 0.04, 0.06, 0.08, and 0.1 ml of the working standard and 0.5 and 1 ml of drug solution were pipetted out in to a series of test tubes. The volumes were made up to 1ml in all the test tubes by distilled water. 1ml of distilled water was taken in a test tube for blank. Then 1 ml of phenol solution and 5 ml of 96% sulphuric acid were added to each tube. After 10 min. the contents in the tubes were shaken and placed in water bath at 25-30°C for 20 min. The absorbance was taken at 490 nm. Standard graph was drawn and the amount of total carbohydrate was calculated in the sample solution. The values were recorded in the table 4-12 & 4-13.

**Standard:** 0.02, 0.04, 0.06, 0.08, and 0.1 ml
Test: 1 ml

\[ y = 10.036x - 0.002 \]
\[ R^2 = 0.9952 \]

Figure 4.13. Standard plot of Carbohydrate by phenol sulphuric acid

Table 4-12. Standard plot of Carbohydrate

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Concentration (mg/ml)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.02</td>
<td>0.180</td>
</tr>
<tr>
<td>2</td>
<td>0.04</td>
<td>0.390</td>
</tr>
<tr>
<td>3</td>
<td>0.06</td>
<td>0.634</td>
</tr>
<tr>
<td>4</td>
<td>0.08</td>
<td>0.827</td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>0.968</td>
</tr>
</tbody>
</table>

Table 4-13. Test Drug S. dulcis

<table>
<thead>
<tr>
<th>Drug Sample (ml)</th>
<th>Absorbance (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.172</td>
</tr>
</tbody>
</table>

RESULT: *S. dulcis* contain 11.69 % of carbohydrate
**4.1.9.1.2 By Anthrone reagent**

Carbohydrates are first hydrolysed into simple sugar using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated into hydroxymethyl furfural. This compound forms a green colour product with anthrone and absorption is maximum at 630 nm.

**Materials & Methods:**

**Instrument used:** SHIMADZU UV-Spectrophotometer (UV-1800), Centrifuge machine (Remi).

2.5 N HCl

**Anthrone reagent:** Solution of 200 mg anthrone in 100 ml of ice-cold 95% sulphuric acid.

**Standard Glucose:** Stock- 100 mg was dissolved in 100 ml of water.

**Working standard:** 10 ml of stock solution diluted to 100 ml with distilled water (100μg/ml)

100 mg of the methanolic extract of *S. dulcis* was weighed into a test tube, hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cooled to room temperature. Then it was neutralized with solid sodium carbonate until the effervescence ceased. The volume was made up to 100 ml and centrifuged and the supernatant was collected for carbohydrate estimation.

0.02, 0.04, 0.06, 0.08, and 0.1 ml of the working standard and 0.5 and 1 ml sample extract were pipetted out in to a series of test tubes. The volume was made up to 1ml in all the test tubes by distilled water. 1 ml of distilled water was taken in a test tube as blank. Then 4 ml of anthrone reagent was added in each of the test tubes including blank and was heated for 8 min in boiling water bath. It was then cooled rapidly and read the intensity of green to dark green colour at 630 nm was found. Standard graph was drawn and the amount of carbohydrate was calculated in the sample solution. The values were recorded in the table 4-14 & 4-15.

**Standard** 0.02, 0.04, 0.06, 0.08, and 0.1 ml

**Test:** 0.5, 1mg/ml
**EXPERIMENTAL**

Carbohydrate (Anthrone) Standard

\[ y = 8.5257x - 0.012 \]

\[ R^2 = 0.998 \]

**Figure 4.14. Standard plot of Carbohydrate by Anthrone**

**Table 4-14. Standard plot of Carbohydrate (Anthrone)**

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Concentration (mg/ml)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.200</td>
<td>0.141</td>
</tr>
<tr>
<td>2</td>
<td>0.400</td>
<td>0.343</td>
</tr>
<tr>
<td>3</td>
<td>0.600</td>
<td>0.481</td>
</tr>
<tr>
<td>4</td>
<td>0.800</td>
<td>0.676</td>
</tr>
<tr>
<td>5</td>
<td>1.000</td>
<td>0.845</td>
</tr>
</tbody>
</table>

**Table 4-15. Test Drug S. dulcis**

<table>
<thead>
<tr>
<th>Drug Sample (ml)</th>
<th>Absorbance (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.830</td>
</tr>
</tbody>
</table>

**RESULT:** *S. dulcis* contains 9.87 % of carbohydrate

**4.1.9.2 Protein Estimation**

Proteins are complex nitrogenous organic substance of plant and animal origin. They are of great importance in the functioning of living cells. They are produced by and
associated with living matters. Protein contains carbon, hydrogen, oxygen, nitrogen and rarely sulphur.

Protein can be estimated by different methods as described by Lowry et al., 1951 \[^{13}\] and also by estimating the total nitrogen content. No method is 100% sensitive. Hydrolyzing the protein and estimating the amino acids alone will give the exact quantification. The method developed by Lowry et al. is sensitive enough to give a moderately constant value and hence largely followed. Protein content of enzyme extracts is usually determined by this method.

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the Biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry’s method.

**Materials & Methods:**

**Instrument used:** SHIMADZU UV-Spectrophotometer (UV-1800), Centrifuge machine (Remi)

**Reagent A:** Sodium Carbonate 2% in 0.1 N Sodium Hydroxide

**Reagent B:** Copper Sulphate 0.5% in 1% Potassium Sodium Tatrate

**Reagent C:** (Alkaline Copper solution) 50 ml of A and 1 ml of B were mixed prior to use.

**Reagent D:** Folin-ciocalteau Reagent

**Protein Solution (Stock Standard):** 50 mg of Bovine serum albumin was dissolved in distilled water and made up to 50 ml in a volumetric flask.

**Working Standard:** 10 ml of the stock solution was diluted with 50 ml of distilled water in a volumetric flask (200μg/ml).

500 mg of methanolic extract of *S. dulcis* was dissolved in 10 ml the buffer. The solution was centrifuged and collected the supernatant for protein estimation.

0.02, 0.04, 0.06, 0.08 and 0.1 ml of the working standard and sample extract were pipetted out in to a series of test tubes. The volumes were made up to 1ml in all the test tubes by distilled water. 1 ml of distilled water was taken in a test tube for blank.
5 ml of reagent C was added to each tube including the blank, mixed well and allowed to stand for 10 min. Then 0.5 ml of reagent D was added to each tube mixed well and incubated at room temp in the dark for 30 min. It turned into blue in colour. The absorbance was measured at 660 nm against a reagent blank. The standard graph was plotted and calculated the amount of protein in the extract. The values were recorded in the table 4-16 & 4-17.

**Standard:** 0.02, 0.04, 0.06, 0.08 and 0.1 ml

**Test:** 0.1, 0.2 mg/ml

![Protein Standard Graph](image)

**Figure 4.15. Standard plot of Protein**

**Table 4-16.: Standard plot of Protein**

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Concentration (mg/ml)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.02</td>
<td>0.032</td>
</tr>
<tr>
<td>2</td>
<td>0.04</td>
<td>0.056</td>
</tr>
<tr>
<td>3</td>
<td>0.06</td>
<td>0.085</td>
</tr>
<tr>
<td>4</td>
<td>0.08</td>
<td>0.108</td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>0.126</td>
</tr>
</tbody>
</table>
Table 4-17. Test Drug S. dulcis

<table>
<thead>
<tr>
<th>Drug Sample (ml)</th>
<th>Absorbance (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.012</td>
</tr>
</tbody>
</table>

**RESULT:** *S. dulcis* contains 6.23 % of protein.

### 4.1.9.3 Phenols estimation

Phenols, the aromatic compounds with hydroxyl groups are widespread in plant kingdom. They occur in all parts of the plant. Phenols are said to offer resistance to diseases and pests in plants. Grains containing high amount of polyphenols are resistant to bird attack. Phenols include an array of compounds like tannins, flavonols etc.

Total phenol estimation can be carried out with the Folin-Ciocalteau reagent.

**Principle:**

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue colour complex (molybdenum blue).

**Materials & Methods:**

**Instrument used:** SHIMADZU UV-Spectrophotometer (UV-1800), Centrifuge machine (Remi)

**80% Ethanol**

**Folin-Ciocalteau Reagent**

**Na₂CO₃, 20%**

**Stock Standard:** 100 mg of Catechol in 100 ml water

**Working standard:** 10 ml stock solution was made to 100 ml in volumetric flask for working standards (100μg/ml)
500 mg of methanolic extract of *S. dulcis* was dissolved in 50 ml of 80% ethanol. The solution was centrifuged and collected the supernatant and evaporated to dryness. Dissolved the residue with 5 ml of distilled water.

0.05, 0.1, 0.15 and 0.2 ml of the working standard solutions and 0.5 & 1ml test drug were pipetted out in to a series of test tubes. Then the volumes were made up to 3 ml in all the test tubes by distilled water. 3ml of water was taken in a test tube for blank. 0.5ml of Folin-Ciocalteau reagent was added to each tube including the blank and mixed well and allowed to stand for 10 min. After 3 min, 2 ml of 20 % Na$_2$CO$_3$ solution was added to each tube and mixed thoroughly. The tubes were placed in boiling water for exactly one min and cooled. The absorbance was measured at 650 nm against a reagent blank. The standard graph was plotted using different concentration of catechol and calculated the amount of phenols in the extract. The values were recorded in the table 4-18 & 4-19.

**Standard:** 0.5, 1.0, 1.5 and 2.0 mg/ml

**Test:** 0.5, 1 mg/ml

---

**Phenols**

![Graph](PhD_Thesis_Sambalpur_University_Odisha/Page106.png)

Figure 4.16. Standard plot for phenol
Table 4-18.: Standard plot of Phenol

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Concentration (mg/ml)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.061</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.128</td>
</tr>
<tr>
<td>3</td>
<td>0.15</td>
<td>0.197</td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>0.269</td>
</tr>
</tbody>
</table>

Table 4-19. Test Drug S. dulcis

<table>
<thead>
<tr>
<th>Drug taken (mg/ml)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.124</td>
</tr>
</tbody>
</table>

RESULT: S. dulcis contains 9.48 % of phenol

4.1.9.4 Estimation of Ascorbic acid by Colorimetric analysis

Ascorbic acid also known as vitamin C is an antiscorbutic. It is present in gooseberry, bittergourd etc. in high amounts. Generally it is present in all fresh vegetable and fruits. It is a water soluble and heat labile vitamin.

The dehydroascorbic acid alone reacts quantitatively and not the other reducing substance present in the sample extract. Thus this method gives an accurate analysis of ascorbic acid than the dye method.

Principle:

Ascorbic acid is first dehydrogenated by bromination. The dehydroascorbic acid is then treated with 2, 4-dinitrophenyl hydrazine to form osazone and dissolved in sulphuric acid to give an orange-red colour solution which is measured at 540 nm.
Materials & Methods:

- **Instrument used:** SHIMADZU UV-Spectrophotometer (UV-1800), Centrifuge machine (Remi)
- 4% oxalic acid solution
- 0.5 N sulphuric acid
- 2% 2,4 Dinitrophenyl Hydrazine (DNPH) reagent: Dissolve by heating 2g DNPH in 100 ml 0.5 N sulphuric acid
- 10% Thiourea solution
- 80% Sulphuric acid
- **Bromine water:** Dissolve 1-2 drop of liquor bromine in approximately 100ml cool water.
- **Ascorbic acid stock standard solution:** dissolve 100 mg ascorbic acid in 100 ml of 4% oxalic acid solution in a standard flask (1mg/ml).
- **Working standard:** Dilute 10 ml of the stock solution to 100 ml with 4% oxalic acid. The concentration of working standard was 100µg/ml.

100 mg of the methanolic extract of *S. dulcis* was dissolved in 25 ml 4% oxalic acid solution and Centrifuged. The supernatant was collected. 10 ml of the solution was transferred to a conical flask and bromine water was added drop wise with constant mixing. The enolic hydrogen atoms in ascorbic acid were removed by bromine. When the extract turns orange yellow due to excess bromine, it was expelled by blowing in air. The volume was made up of 25 ml with 4% oxalic acid solution.

0.2, 0.4, 0.6 and 0.8 ml of the working standard solutions and 0.5 and 1 ml brominated drug extract were pipetted out into a series of test tubes. Then the volumes were made up to 3 ml in all the test tubes by distilled water. Three ml of water was taken in a test tube as blank. 1ml of DNPH reagent was added and followed by 1-2 drop thiourea into each tube. The contents of tubes was mixed thoroughly and incubated at 37°C for 3 hrs. After incubation the orange red osazone crystal formed was dissolved by adding 7 ml of 80% sulphuric acid. The absorbance was measured at
540 nm. The standard graph was plotted and the amount of ascorbic acid present in the sample was calculated. The values were recorded in the table 4-20 & 4-21.

**Standard:** 0.200, 0.400, 0.600 and 0.800 mg/ml

**Test:** 1.6 mg/ml

![Graph of Ascorbic acid](image)

**Figure 4.17. Standard plot of Ascorbic acid**

**Table 4-20. Standard plot of Ascorbic acid**

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Concentration (mg/ml)</th>
<th>Absorbance (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.02</td>
<td>0.19</td>
</tr>
<tr>
<td>2</td>
<td>0.04</td>
<td>0.37</td>
</tr>
<tr>
<td>3</td>
<td>0.06</td>
<td>0.56</td>
</tr>
<tr>
<td>4</td>
<td>0.08</td>
<td>0.79</td>
</tr>
</tbody>
</table>

**Table 4-21. Test Drug S. dulcis**

<table>
<thead>
<tr>
<th>Drug taken (ml)</th>
<th>Absorbance (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>0.055</td>
</tr>
</tbody>
</table>

**RESULT:** S. dulcis contains 0.81% of ascorbic acid
4.1.9.5 Estimation of Tannins

Tannins and tannin-like substances are widespread in nature and are probably present in all plant materials. These are polyphenolic compounds divided into two main groups such as hydrolysable and condensed.

Hydrolysable tannins contain a polyhydric alcohol usually, if not always, glucose esterified with gallic acid or with hexahydroxydiphenic acid.

Condensed tannins are mostly flavonols and are probably polymers of flavons 3-ol (catechin) and these cannot be hydrolyzed to simple components.

Among the cereals, sorghum has been found to contain higher amounts of polyphenols. Even though high polyphenol seeds are immune to attack by birds and diseases, they display impaired nutritional quality, lower digestibility and reduction of food consumption.

The tannins are estimated by the following two methods:

(i) **Folin-Denis Method**: This is based on the non-stoichiometric oxidation of the molecules containing a phenolic hydroxyl group.

(ii) **Vanillin Hydrochloride Method**: Vanillin method is specific for dihydroxy phenols and is particularly sensitive for meta-substituted, di and tri hydroxybenzene containing molecules.

**Folin-Denis Method**

Principle:

Tannin-like compounds reduce phosphotungstomolybdic acid in alkaline solution to produce a highly coloured blue solution, the intensity of which is proportional to the amount of tannins. The intensity is measured in a spectrophotometer at 700 nm.

**Materials & Methods**:

**Instrument used**: SHIMADZU UV-Spectrophotometer (UV-1800), Centrifuge machine (Remi)
**Folin-Denis Reagent:** 100 g of sodium tungstate and 20 g phosphomolybdocic acid was dissolved in 750 ml distilled water in a suitable flask and added 50 ml phosphoric acid. The mixture was reflux for 2 hrs and made up to one litre with water.

**Sodium Carbonate Solution:** Accurately 350 g of sodium carbonate was dissolved in one litre of water at 70-80°C. Filtered through glass wool and allowed to stand overnight.

**Standard Tannic Acid Solution:** 100 mg of tannic acid was dissolved in 100 ml of distilled water.

**Working Standard Solution:** 5 ml of the stock solution was diluted to 100 ml with distilled water (50μg/ml)

125 mg of methanolic extract of *S. dulcis* was dissolved in 25 ml the distilled water. The solution was boiled for 30 min. and centrifuged at 2000 rpm for 20 min. Supernatant solution 2.5 ml was taken in 25 ml of volumetric flask and made up volume with distilled water. The solution was used for tannin estimation.

0.5, 1, 1.5, and 2 ml of the working standard and 1 ml extract and b lank were pipetted out in to a series of beaker. 5 ml of Folin-Denis reagent and 10 ml sodium carbonate solution was added and diluted to 100 ml with distilled water and was shaken well. The absorbance was measured at 700 nm after 30 min. The values were recorded in the table 4-22 & 4.23.

**Standard:** 0.050, 0.100, 0.15 and 0.2 mg/ml

**Test:** 0.5 ml
Figure 4.18. Standard plot of Tannin

Table 4-22. Standard plot of Tannin

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Concentration (mg/ml)</th>
<th>Absorbance (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.104</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.22</td>
</tr>
<tr>
<td>3</td>
<td>0.15</td>
<td>0.35</td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>0.473</td>
</tr>
</tbody>
</table>

Table 4-23. Test Drug S. dulcis

<table>
<thead>
<tr>
<th>Drug taken (ml)</th>
<th>Absorbance(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.003</td>
</tr>
</tbody>
</table>

RESULT: S. dulcis contains 1.0% of tannins
4.1.9.6 Estimation of Chlorophylls

The chlorophylls are the essential component for photosynthesis, and occur in chloroplasts as green pigment in all photosynthetic plant tissues. They are bound loosely to protein but are readily extracted in organic solvent such as acetone or ether. Chemically, each chlorophyll molecule contains a porphyrin (tetapyrole) nucleus with a chelated magnesium atom at the center and a long chain hydrocarbon (phytyl) side chain attached through a carboxylic acid group. There are at least five types of chlorophyll in plants. Chlorophylls \( a \) and \( b \) occur in higher plants, ferns and mosses. Chlorophylls \( c, d \) and \( e \) are found only in certain bacteria.

**Principle:**

Chlorophyll is extracted in 80% acetone and the absorption at 663 nm and 645 nm are read in a spectrophotometer. Using the absorption coefficients, the amount of chlorophyll is calculated.

**Materials & Methods:**

- **Instrument used:** SHIMADZU UV-Spectrophotometer (UV-1800), Centrifuge machine (Remi)
- Dilute analytical grade acetone to 80% acetone (prechilled)

1g of \( S. \ dulcis \) leaf was taken into a clean mortar and grinded the tissue to a fine pulp with the addition of 20 ml of 80% acetone. The sample was centrifuged (5,000 rpm for 5 min) and transferred the supernatant to a 100 ml volumetric flask. The procedure was repeated until the residue was colourless. The mortar and pestle was washed thoroughly with 80% acetone and the clear washings were collected in a clean washed volumetric flask. The volume was made up to 100 ml with 80% acetone and the absorbance of solution was read at 645, 663 and 652 nm against the solvent (80% acetone) blank. The values were recorded in the table 4-24.
Table 4-24. Chlorophyll reading

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Wavelength (nm)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>645</td>
<td>0.333</td>
</tr>
<tr>
<td>2</td>
<td>652</td>
<td>0.483</td>
</tr>
<tr>
<td>3</td>
<td>663</td>
<td>0.847</td>
</tr>
</tbody>
</table>

Calculation:

The amount of chlorophyll present in the extract mg chlorophyll per g tissue was calculated using the following equations:

\[
\text{mg chlorophyll a/g tissue} = 12.7 (A_{663}) - 2.69 (A_{645}) \times \frac{V}{1000} \times W
\]

\[
= 12.7 (0.847) - 2.69 (0.333) \times 100/1000 \times 1 \\
= 10.76 - 0.90 \times 0.10 \\
= 0.99 \text{ mg}
\]

\[
\text{mg chlorophyll b/g tissue} = 22.9 (A_{645}) - 4.68 (A_{663}) \times \frac{V}{1000} \times W
\]

\[
= 22.9 (0.333) - 4.68 (0.847) \times 100/1000 \times 1 \\
= 7.63 - 3.96 \times 0.10 \\
= 0.37 \text{ mg}
\]

\[
\text{mg total Chlorophyll /g tissue} = 20.2 (A_{645}) + 8.02 (A_{663}) \times \frac{V}{1000} \times W
\]

\[
= 20.2 (0.333) + 8.02 (0.847) \times 0.10 \\
= 6.73 + 6.79 \times 0.10 \\
= 1.35 \text{ mg}
\]

Where \( A \) = absorbance at specific wavelengths,
\[ V = \text{final volume of chlorophyll extract in 80} \% \text{ acetone} \]

\[ W = \text{fresh weight of tissue extract} \]

**RESULT**: Chlorophyll present in the extract is 1.35 mg total Chlorophyll /g tissue.
4.2 PHARMACOLOGICAL ACTIVITY

Institutional Animal Ethics Committee (Reg. No: 621/02/ac/CPCSEA)

Protocol Approval No. BIT/PH/IAEC/08/2011 (Approved)

4.2.1 ANTI-DIABETIC ACTIVITY

After the recommendations made by WHO on diabetes mellitus, the search for safer and more effective hypoglycemic pharmaceuticals has continued to be an important area of research. The search for novel hypoglycemic compounds from medicinal plants has become an important aspect. Keeping in view of the difficulties in testing in-vivo; several in-vitro tests have been developed in recent years.

The number of diabetic patients is rapidly rising in most parts of the world, especially in developing countries such as India, Thailand and Indonesia. In general, the control of blood glucose concentrations near the normal range is mainly based on the use of oral hypoglycaemic/antihyperglycaemic agents and insulin. However, all of these treatments have limited efficacy and are associated with undesirable side effects, leading to increasing interest in the use of medicinal plants for the alternative management of type 2 diabetes mellitus. The control of postprandial (PP) plasma glucose levels is critical in the early treatment of diabetes mellitus and for reducing chronic vascular complications.

PP hyperglycemia is one of the earliest abnormalities of glucose homeostasis associated with type-2 diabetes and is markedly exaggerated in diabetic patients with fasting hyperglycemia.

PP hyperglycemia has independent risk factors in the development of macro-vascular complications of diabetes mellitus.

A sudden increase in blood glucose levels, which causes hyperglycaemia in type 2 diabetes patients, occurs due to the hydrolysis of starch by pancreatic α-amylase and the uptake of glucose by intestinal α-glucosidases. An effective strategy for type 2
diabetes management is the strong inhibition of intestinal α-glucosidases and the mild inhibition of pancreatic α-amylase.\[22]\]

α-Amylase is present in both salivary and pancreatic secretion and is responsible for cleaving large malto-oligosaccharides to maltose, which is then a substrate for intestinal α-glucosidase. Tests for the ability of extracts and compounds to inhibit both α-amylase and α-glucosidase have been described by several workers.

As a number of anti-hyperglycemic agents have been found in plants, research into understanding the scientific basis for plant-based traditional medicines from various cultures has increased as scientists search for clues to discover new therapeutic drugs for type 2 DM.\[23-25]\]

Streptozotocin is an antimicrobial agent and has also been used as a chemotherapeutic alkylating agent and reported as diabetogenic. Again, this insulinopenia syndrome, called 'streptozotocin diabetes', is caused by the specific necrosis of the pancreatic beta cells and streptozotocin has been the agent of choice for the induction of diabetes mellitus in animals ever since.\[26]\]

In traditional Indian medicines plant and herbal extracts have been used as anti-diabetic agents.\[27]\] These plants are typically rich in phenolic compounds, which are known to interact with proteins and can inhibit enzymatic action.\[28-29]\] A numbers of medicinal plant and herbal extracts have been found to inhibit the enzymatic activity of α-glucosidase and α-amylase, and may have the potential as dietary anti-diabetic agents to control post-prandial hyperglycemia.\[30-33]\]

**Materials and Methods:**

α-Amylase, α-glucosidase and streptozotocin were purchased from Sigma Aldrich India Pvt. Ltd. Laboratory grades reagents were used in all experiments. The instrument, UV spectrophotometer (UV 1700 Simazdu) was used in experiments.

**Animals:**

All the experiments were carried out using Wistar albino rats and were obtained from animal house, Birla Institute of Technology, Mesra, Ranchi, India. The animals were
kept in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature 24± 2°C and relative humidity of 60-70 %. A 10:14 light: dark cycle was followed. All animals were allowed to free access to water and fed with standard commercial rat chow pallets (M/s. Hindustan Lever Ltd, Mumbai). All the experimental procedures and protocols used in this study were reviewed by the Institutional Ethics Committee (Reg. No:621/02/ac/CPCSEA) and were in accordance with the guidelines of the CPCSEA, Ministry of Forests and Environment, Government of India.

4.2.1.1 Effect of Scoparia dulcis on the activity of α-amylase

1g of the S. dulcis extract was mixed with 100 ml of the 2% potato starch solution in a 200 ml beaker. 1g of α-amylase was then added to this mixture and stirred vigorously at 37°C. After 30 min, 0.1 mol/l sodium hydroxide was added to terminate the α-amylase activity. The glucose content of the solution was determined by the above mentioned procedure.\textsuperscript{20}

4.2.1.2 Inhibitory assay of α-glucosidases enzyme

The inhibitory activity was determined by incubating a solution of starch (2% w/v maltose or sucrose) 1 ml with 0.2 M Tris buffer pH 8.0 and various concentration of plant extract for 5 min at 37°C. The reaction was initiated by adding 1 ml of α-glucosidase enzyme (1U/ml) to it followed by incubation. Then, the reaction mixture was heated in boiling water bath to stop the reaction. The amount of glucose liberated in measured by glucose oxidase peroxidase method.\textsuperscript{34}

Calculation of 50% inhibitor concentration (IC\textsubscript{50}):

The IC\textsubscript{50} values (inhibitor concentration at which 50% inhibition of the enzyme activity occurs) of the plants extract were determined by performing the assay as above with varying concentration of the plant extracts ranging 20 to 100 μg/ml. The IC\textsubscript{50} values were determined from plots of percent inhibition vs log inhibitor concentration and calculated by non-linear regression analysis from the mean inhibitory values.\textsuperscript{35}
Table 4-25. The percent α-amylase inhibition of MESD at varying concentrations

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration/ percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 (µg/ml)</td>
</tr>
<tr>
<td>MESD</td>
<td>38.7</td>
</tr>
<tr>
<td>Acarbose</td>
<td>39.2</td>
</tr>
</tbody>
</table>

Table 4-26. The percentage inhibition of α-glucosidase of MESD at varying concentrations

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Concentration (µg/ml)</th>
<th>Inhibition (%)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MESD</td>
<td>20</td>
<td>38.0</td>
<td>80.35 (µg/ml)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>42.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>46.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>50.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>54.3</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.19. IC<sub>50</sub> value appear at 50% inhibition
4.2.1.3 Streptozotocin induced Anti-diabetic Activity of Scoparia dulcis

**Determination of Acute Toxicity:**

Acute oral toxicity was performed in mice by following Organization for Economic Cooperation and Development (OECD) guidelines.\[^{36,37}\]

**Streptozotocin induced Anti-diabetic activity:**

The whole study was divided in four animal groups. Group I received vehicle only (Tween-80, 1%) and served as control. Group II and group III received methanolic extract of Scoparia dulcis (MESD) 200 mg/kg body weight (BW) low dose and 400 mg/kg BW therapeutic dose and group IV Glibenclamide (1.0 mg/kg) BW serve as standard (p.o.) respectively. 60 mg/kg BW streptozotocin was injected to every group animals following 18 h fast. After 48 h the blood glucose of surviving rats were determined by glucose oxidase method. Only those animals which showed hypoglycemia (blood glucose levels = 200-500 mg/dl), were considered diabetic and taken for further experimentation. The study was carried out after repeated administration of the extract for 21 consecutive days. Basal glycemia was determined in overnight fasted animals. The vehicle (1% Tween-80), MESD (200 and 400 mg/kg) and glibenclamide (1.0 mg/kg) were orally administered to the animals of group I-IV for 21 consecutive days. Blood samples collected on the 7th, 14th and 21st days were analysed for the determination of blood glucose.\[^{38}\]

**Statistics:**

The results were presented as mean ±SEM. "One-way Anova with Dunnett’s post test was performed using Graph Pad Prism version 3.00 for windows. Graph Pad Software, San Diego California USA, P<0.01 were considered significance.

In-vivo study of MESD was done on streptozotocin induced diabetic in rats. The effect of the treatment with extract and Glibenclamide on blood glucose concentration in diabetic rats after post-treatment days was shown in Table 4-27. The study was conducted by blood collection on the 7th, 14th and 21th days after induces of diabetes and analyzed for the determination of blood glucose. All the values were found significant in both treatment dose (200 and 400mg/kg) as compared to control and standard Glibenclamide.
Table 4-27. Effect of repeated oral administration of extracts and (1.0mg/kg) glibenclamide on blood glucose and serum insulin level in streptozotocin diabetic rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Post-treatment (day) Blood glucose profile (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control (vehicle)</td>
<td>342.20±15.01</td>
</tr>
<tr>
<td>MESD 200 mg/kg</td>
<td>331.00±21.46</td>
</tr>
<tr>
<td>MESD 400 mg/kg</td>
<td>319.00±16.46</td>
</tr>
<tr>
<td>Glibenclamide 1 mg/kg</td>
<td>311.00±9.44</td>
</tr>
</tbody>
</table>

Figure 4.20. Effect of oral administration for 21 days of extracts and glibenclamide in streptozotocin diabetic rats
4.2.2 **ANTIOXIDANT ACTIVITY**

There are clear cut evidences to show the role of free radicals in diabetes and studies indicate that tissue injury in diabetes may be due to free radicals. The significance of oxidative stress in the disease pathology is uncertain but is frequently proposed to be related to the hyperglycemia. Other possible sources include elevated plasma lipids leading to increased lipid oxidation and decreased levels of antioxidant defense systems.\textsuperscript{[42, 43]}

Aim of this study is to evaluate the antioxidant activity by using different models of MESD & WESD.

**Materials and Methods:**

*Scoparia dulcis* whole plant was collected from waste field of Barpali, Odisha, India, 1, 1-Diphenyl-2, picrylhydrazyl (DPPH) purchased from siga Aldrich India pvt Ltd. Laboratory grades reagents were used in all experiments. The instruments, UV spectrophotometer (UV 1700 Simazdu) and electrochemical analyzer (680B CH Instruments, USA) were used in experiments.

**Extraction of Total Extractable Polyphenol (TEP)**

The powdered sample (500 mg) was extracted sequentially with 40 ml methanol: water (50:50 v/v) and 40 ml acetone: water (70:30 v/v) at room temperature for 60 minutes each treatment. Supernatants from these extractions were combined. Concentrated to a small volume under reduced pressure at 50°C, and made up to 10 ml with the same solvents (v/v). TEP were assayed spectrophotometrically by the Folin-Ciocalteau method using gallic acid as a standard and expressed as gallic acid equivalents.\textsuperscript{[44]} The principle of reaction is that the phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue).
Methods of Antioxidant Activity

The antioxidant activity was done by the three methods:

1. DPPH.
2. Reducing Power.
3. Electrochemical measurements.

4.2.2.1 DPPH

The antioxidant activity of the TEP, based on the scavenging activity of the stable 2, 2-diphenyl-1, picrylhydrazyl (DPPH) free radicals, was determined by the method described by Kumaran et al. [45] MESD & WESD TEP (0.1 ml) was added to 3 ml of a 0.004% MeOH solution of DPPH. Water (0.1 ml) in place of TEP was used as control. Absorbance at 517 nm was determined after 30 min. Graph was plotted between absorbance and concentration with TEP and gallic acid taken as standard anti-oxidative compound.

4.2.2.2 Reducing Power Determination

The reducing power of TEP of MESD & WESD was determined according to the method described by Chartarjee et al. [46] Different amounts of TEP (100-300 μl) were mixed with phosphate buffer (2.5 ml, 0.02 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%). A higher absorbance indicates a higher reducing power.

4.2.2.3 Electrochemical Measurements

Electrochemical Measurements were conducted at 20 ± 0.1°C in a conventional three-electrode cell, cyclic voltammeter [PGSTAT model 30 (Ecochemie, Twente, Holland)] controlled by GPES 3.2 software from Ecochemie, running on a PIV personal computer. In voltammetric test, the working electrode was a glassy carbon electrode; counter electrode was a platinum wire, while the reference electrode was an aqueous Ag/AgCl electrode used for our experiment. All tests potential was scanned.
from 0.1 to 1.0 V at a sweep rate of 100 mVs'. Test solutions were prepared by MESD & WESD in 0.1 mol dm\(^{-3}\) KCl and made up the volume to 10 ml. \[47\]

Table 4-28. Absorbance vs concentration of methanol & water extract of S.dulcis & Gallic a.

<table>
<thead>
<tr>
<th>Concentration mg/ml</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gallic Acid</td>
</tr>
<tr>
<td>10</td>
<td>0.283±0.008</td>
</tr>
<tr>
<td>25</td>
<td>0.176±0.014</td>
</tr>
<tr>
<td>50</td>
<td>0.076±0.008</td>
</tr>
<tr>
<td>75</td>
<td>0.043±0.006</td>
</tr>
<tr>
<td>125</td>
<td>0.050±0.005</td>
</tr>
<tr>
<td>250</td>
<td>0.050±0.005</td>
</tr>
</tbody>
</table>

Figure 4.21. Plot of Absorbance verses concentration of methanolic & water extract of S.dulcis and Gallic acid.
Table 4-29. Reducing power of methanolic & water extract of *S. dulcis* and Gallic acid.

<table>
<thead>
<tr>
<th>Concentration mg/ml</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gallic Acid</td>
</tr>
<tr>
<td></td>
<td>MESD</td>
</tr>
<tr>
<td></td>
<td>WESD</td>
</tr>
<tr>
<td>50</td>
<td>0.400±0.011</td>
</tr>
<tr>
<td></td>
<td>0.260±0.011</td>
</tr>
<tr>
<td></td>
<td>0.220±0.011</td>
</tr>
<tr>
<td>100</td>
<td>0.496±0.020</td>
</tr>
<tr>
<td></td>
<td>0.376±0.014</td>
</tr>
<tr>
<td></td>
<td>0.300±0.011</td>
</tr>
<tr>
<td>200</td>
<td>0.636±0.008</td>
</tr>
<tr>
<td></td>
<td>0.470±0.005</td>
</tr>
<tr>
<td></td>
<td>0.366±0.006</td>
</tr>
<tr>
<td>300</td>
<td>0.740±0.011</td>
</tr>
<tr>
<td></td>
<td>0.650±0.011</td>
</tr>
<tr>
<td></td>
<td>0.533±0.017</td>
</tr>
</tbody>
</table>

Figure 4.22. Plot of reducing power of methanolic & water extract of *S. dulcis* and Gallic acid.
4.2.3 Diuretic Activity

Scoparia dulcis is well recognized in western Odisha as a traditional medicine having a diuretic effect, but no scientific data has been published supporting the claimed ethno medicinal use. Therefore, the aim of this study was to perform the acute and sub-chronic diuretic effects of orally administered methanolic extract of Scoparia dulcis in normal rats and thereby, to confirm its ethno medicinal use.

Diuretics are the drugs those increase the excretion of sodium and water from the body by an action on the kidney. Their primary effect is to decrease the re-absorption of sodium and chloride from the filtrate, increased water loss being secondary to the increased excretion of salt. This can be achieved by:

- A direct action on the cells of the nephron.
- Indirectly modifying the content of the filtrate.

Two widely used diuretics, thiazides and the high ceiling loop diuretic, furosemide, have been associated with a number of adverse effects, such as, electrolyte imbalance, metabolic alterations, development of new-onset diabetes, activation of the rennin-angiotensin–neuroendocrine systems, and impairment of sexual function. Hence, there is a need for new diuretics with lower potential for adverse effects, such as the plant-based substances which are considered to be relatively safe.

The diuretic activity can be evaluated using following two methods:-

1. Acute diuretic activity: Diuretic activity of a single dose of the plant extracts and furosemide.
2. Sub-chronic diuretic activity: Diuretic activity of the plant extracts and furosemide after sub-chronic administration.
4.2.3.1 Acute diuretic activity

Materials and Methods:

Chemicals: Furosemide (IDPL).

Treatment protocol:

Rats were numbered, weighed and then divided into 4 groups with 6 animals in each as follows:

1. Group I: served as the control group and received Tween 80 (control, vehicle for the extracts), orally.

2. Group II: received 250 mg/Kg BW of methanolic extract of *Scoparia dulcis* (MESD-250), orally.

3. Group III: received 500 mg/Kg BW of methanolic extract of *Scoparia dulcis* (MESD-500), orally.

4. Group IV: served as standard and received 10mg/kg BW of furosemide, orally.

(Methanolic extract in Tween 80 as suspension)

Before treatment, the animals were fasted for 18 hours with water ad libitum.

Each animal was placed in an individual metabolic cage 24 h prior to commencement of the experiment for adaptation. Before treatment, all animals received physiological saline\(^1\) (0.9% NaCl) at an oral dose of 5 ml/100 g body weight (BW), to impose a uniform water and salt load. Urine was collected and measured at 1, 2, 4, 6, and 24 h after the above treatment. The sodium and potassium concentrations were determined in the 24 h urine samples as well as in the plasma of rats using flame photometer (Systronics flame photometer 128).
4.2.3.2 Sub-chronic diuretic activity \([51-53]\)

**Materials and Methods:**

Chemicals:

Furosemide (IDPL). Creatinine assay kit (Bio in vivo diagnostics Pvt. Ltd. Gujrat), flame photometer (Systronics flame photometer 128)

Animals:

Treatment protocol:

The animals were numbered, weighed and then divided into 4 groups with 6 animals in each as follows:

1. Group I: received orally distilled water 10 ml/Kg BW for 8 days, and served as the control group.
2. Group II: received orally MESD 250 mg/Kg BW for 8 days.
3. Group III: received orally MESD 500 mg/Kg BW for 8 days.
4. Group IV: received orally 20 mg/Kg BW of furosemide for 8 days and served as standard.

Before treatment, the animals were fasted for 18 hours with water *ad libitum*.

Method \([54,55]\):

For each rat, 24 h urine was collected daily and its volume measured. The urinary sodium and potassium concentrations were measured in each urine specimen. Sodium and potassium levels were measured in plasma of rats on 8\textsuperscript{th} day. The concentration of creatinine in plasma and urine was determined for day 8 by the Creatinine assay kit using Jaffe alkaline picrate method. The sodium and potassium concentrations were determined using flame photometer. Creatinine clearance, as a measure of renal function, was calculated from plasma and urinary Creatinine levels using the formula:

\[
\text{Creatinine clearance} = \frac{\text{Urinary Creatinine x urine vol.}}{\text{Plasma Creatinine x time (min.)}}
\]
4.2.3.3 Acute diuretic activity:

a) Effect on urine volume:

The effect of single dose administration of methanolic extract of *Scoparia dulcis* Linn. (MESD) on the urine volume is shown in Table 4-30.

Treatment with a single dose of the *S. dulcis* extract increased diuresis at both the doses i.e. 250 mg/Kg and 500 mg/Kg BW. This was significantly higher than in the control rats at 4 h and 6 h after the dose of 250 mg/Kg and 500 mg/Kg BW (*P < 0.01* vs. control). The volume of urine excretion was almost similar in all tested groups at 24h.

The diuretic effect of MESD at the higher dose i.e. 500 mg/Kg BW was found to be highly significant at 1, 2, 4 and 6 h (*P < 0.01* vs. control) as compared to 250 mg/kg BW dose.

The diuretic effect of a single dose of the reference diuretic, furosemide was also rapid and higher than that of the plant extracts at all time periods for both the doses.

b) Effect on urinary electrolyte excretion:

Effect on sodium excretion:

The effect of single doses of furosemide and MESD at both the doses 250 mg/Kg BW and 500 mg/Kg BW on Na⁺ excretion in the 24 h urine is tabulated in Table 4-31.

Both the doses of plant extract and furosemide significantly enhanced the excretion of the Na⁺ versus controls (*P < 0.01*). Furthermore the natriuretic activity of higher doses of extract was more significant than the lower dose.

Effect on potassium excretion:

The effect of single doses of furosemide and MESD at both the doses 250 mg/Kg BW and 500 mg/Kg BW on K⁺ excretion in the 24 h urine is tabulated in Table 4-31.

The urinary K⁺ concentration was found to be significantly higher on administration of MESD 250 mg/Kg BW versus controls (*P < 0.01*), which was greater than that produced by MESD 500 mg/Kg BW.

Furosemide actually decreased K⁺ excretion as compared to the controls.
c) Effect on plasma electrolyte levels:

There was no effect of furosemide or plant extract on plasma levels of Na\(^+\) and K\(^+\) as shown in Table 4-31.

Table 4-30. Effect of MESD and Furosemide on urine volume after single dose administration.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose /Kg BW</th>
<th>Volume in ml. (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1hr</td>
</tr>
<tr>
<td>Tween 80</td>
<td>10 ml</td>
<td>0.90±0.21</td>
</tr>
<tr>
<td>MESD</td>
<td>250 mg</td>
<td>2.82±0.24*</td>
</tr>
<tr>
<td>MESD</td>
<td>500 mg</td>
<td>3.60±0.32**</td>
</tr>
<tr>
<td>Furosemide</td>
<td>20 mg</td>
<td>12.60±0.68**</td>
</tr>
</tbody>
</table>

Cumulative values are reported as mean±S.E.M. for six rats in each group. *P < 0.05; **P < 0.01 compared with controls using; a one-way analysis of variance (ANOVA) followed by Dunnett's test.

Figure 4.23. Effect of MESD and Furosemide on urine volume after single dose administration.
Table 4-31. Effect of MESD and Furosemide on urinary electrolyte excretion and plasma electrolyte levels after single dose administration.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose mg/Kg BW</th>
<th>Urinary electrolyte concentration</th>
<th>Plasma electrolyte concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Na⁺ (mmol/L)</td>
<td>K⁺ (mmol/L)</td>
</tr>
<tr>
<td>Tween 80</td>
<td>10 ml/Kg BW</td>
<td>85.65±1.56</td>
<td>60.50±2.80</td>
</tr>
<tr>
<td>MESD 250</td>
<td>250</td>
<td>105.63±2.78**</td>
<td>64.67±1.40</td>
</tr>
<tr>
<td>MESD 500</td>
<td>500</td>
<td>135.98±2.10**</td>
<td>69.65±4.50*</td>
</tr>
<tr>
<td>Furosemide</td>
<td>20</td>
<td>145.32±2.12**</td>
<td>57.25±3.67</td>
</tr>
</tbody>
</table>

Values are reported as mean±S.E.M. for six mice in each group. *P < 0.05 compared with controls using a one-way analysis of variance (ANOVA) followed by Dunnett's t-test.
4.2.3.4 Sub-chronic diuretic activity

Effect on urine volume:

The effect of sub-chronic administration of MESD on the urine volume is shown in Table 4-32.

Administration of daily doses of extracts produced significant diuresis starting on day 1 ($P < 0.01$ vs. control), which was increasing until day 3 in case of MESD 250 mg/kg BW and until day 5 in case of MESD 500 mg/kg BW after that urinary output leveled off. The diuretic effect of furosemide as well as both the doses of the plant extract were significant with reference to control from the day 1 to day 8 ($P < 0.01$ vs. control).

b) Effect on urinary electrolyte excretion:

Effect on sodium excretion:

The effect of sub-chronic administration of MESD on the urinary sodium excretion is shown in Table 4-33.

Sub-chronic administration of MESD for 8 days showed significant increase in urinary excretion of $\text{Na}^+$. When MESD was administered at the dose of 250 mg/Kg BW it showed a significant increase in urinary excretion of $\text{Na}^+$ on day 2 ($P < 0.05$ vs. control) which became more pronounced ($P < 0.01$ vs. control) from day 3 to day 8. When MESD was administered at the dose of 500 mg/Kg BW; the excretion of $\text{Na}^+$ increased significantly ($P<0.01$ vs. control) throughout the treatment from day 1 to day 8. In case of furosemide there was significant increase ($P<0.01$ vs. control) in excretion of $\text{Na}^+$ throughout the treatment from day 1 to day 8.

Effect on potassium excretion:

The effect of sub-chronic administration of MESD on the urinary potassium excretion is shown in Table 4-34. Sub-chronic administration of MESD 250 mg/kg BW for 8 days showed slight increase in urinary excretion of $\text{K}^+$ from day 4 onwards. When MESD was administered at the dose of 500 mg/Kg BW the excretion of $\text{K}^+$ was not increased significantly throughout the treatment from day 1 to day 8.
In case of furosemide also there was no significant increase in excretion of $K^+$ throughout the treatment.

c) Effect on plasma electrolyte levels:

The effect of sub-chronic administration of MESD on the plasma electrolyte levels is shown in Table 4-35.

Both MESD and Furosemide decreased the $Na^+$ level in the plasma to a significant level ($p<0.01$ vs. control). MESD at both the doses also reduced $K^+$ level in the plasma though the level was statistically insignificant.

There was no significant effect of furosemide on plasma levels of $K^+$.

d) Effect on Creatinine Clearance

The effect of sub-chronic administration of MESD on the creatinine clearance is shown in Table 4-36.

Creatinine clearance, measured on the last day of treatment, was not affected by the treatment of MESD at the dose of 250 mg/Kg BW. But there was a significant increase in Creatinine clearance on the treatment of methanolic extract of *Scoparia dulcis* (MESD) at the dose of 500 mg/Kg BW ($P < 0.01$ vs. control) and furosemide ($P < 0.01$ vs. control) in comparison to control.
Table 4-32. Effect on urine volume of sub-chronic administration of MESD and Furosemide.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>4.35±0.28</td>
<td>4.56±0.23</td>
<td>4.65±0.32</td>
<td>4.78±0.36</td>
</tr>
<tr>
<td>1 day</td>
<td>4.15±0.15</td>
<td>7.38±0.25</td>
<td>8.79±0.32</td>
<td>12.83±0.48</td>
</tr>
<tr>
<td>2 day</td>
<td>4.45±0.25</td>
<td>8.90±0.38</td>
<td>11.32±0.36</td>
<td>14.25±0.56</td>
</tr>
<tr>
<td>3 day</td>
<td>4.5±0.28</td>
<td>10.56±0.29</td>
<td>13.01±0.40</td>
<td>16.35±0.500</td>
</tr>
<tr>
<td>4 day</td>
<td>4.38±0.32</td>
<td>10.79±0.51</td>
<td>15.25±0.39</td>
<td>18.54±0.360</td>
</tr>
<tr>
<td>5 day</td>
<td>4.16±0.22</td>
<td>10.89±0.44</td>
<td>16.79±0.33</td>
<td>18.89±0.35</td>
</tr>
<tr>
<td>6 day</td>
<td>4.52±0.33</td>
<td>11.12±0.36</td>
<td>17.23±0.44</td>
<td>19.52±0.49</td>
</tr>
<tr>
<td>7 day</td>
<td>4.45±0.21</td>
<td>11.45±0.19</td>
<td>17.44±0.38</td>
<td>20.11±0.36</td>
</tr>
<tr>
<td>8 day</td>
<td>4.49±0.35</td>
<td>11.66±0.23</td>
<td>17.57±0.25</td>
<td>20.29±0.32</td>
</tr>
</tbody>
</table>

Values are reported as mean±S.E.M. for six rats in each group. *P< 0.05; **P< 0.01 compared with controls using; a one-way analysis of variance (ANOVA) followed by Dunnett’s test.

Figure 4.25. Effect on urine volume of sub-chronic administration of MESD and Furosemide.

Ph.D. Thesis, Sambalpur University, Odisha
Table 4-33. Effect on sodium excretion of sub-chronic administration of MESD and Furosemide.

<table>
<thead>
<tr>
<th>Treatment Duration</th>
<th>Tween 80 (mmol/L)</th>
<th>MESD 250 (mmol/L)</th>
<th>MESD-500 (mmol/L)</th>
<th>Furosemide (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>50.23±1.22</td>
<td>50.11±1.50</td>
<td>49.79±2.33</td>
<td>51.10±1.90</td>
</tr>
<tr>
<td>1 day</td>
<td>49.55±2.44</td>
<td>55.12±1.22</td>
<td>65.55±1.50**</td>
<td>65.90±1.30**</td>
</tr>
<tr>
<td>2 day</td>
<td>51.41±2.21</td>
<td>57.21±1.32*</td>
<td>70.11±0.80**</td>
<td>70.01±0.87**</td>
</tr>
<tr>
<td>3 day</td>
<td>49.42±2.8</td>
<td>60.02±0.88**</td>
<td>72.36±0.78**</td>
<td>71.02±0.56**</td>
</tr>
<tr>
<td>4 day</td>
<td>51.22±1.89</td>
<td>62.56±0.77**</td>
<td>76.48±0.56**</td>
<td>76.56±0.48**</td>
</tr>
<tr>
<td>5 day</td>
<td>52.24±1.28</td>
<td>63.98±0.69</td>
<td>78.56±1.20</td>
<td>81.22±0.89</td>
</tr>
<tr>
<td>6 day</td>
<td>50.22±1.23</td>
<td>65.21±0.98**</td>
<td>79.21±0.99**</td>
<td>82.12±0.88**</td>
</tr>
<tr>
<td>7 day</td>
<td>51.00±1.28</td>
<td>66.58±1.20**</td>
<td>81.22±0.98**</td>
<td>84.11±1.20**</td>
</tr>
<tr>
<td>8 day</td>
<td>53.22±1.20</td>
<td>67.90±1.10**</td>
<td>82.79±0.69**</td>
<td>85.56±0.59**</td>
</tr>
</tbody>
</table>

*The reported Na⁺ levels are reported as mean±S.E.M. for six rats in each group. *P< 0.05; **P< 0.01 compared with controls using; a one-way analysis of variance (ANOVA) followed by Dunnett’s t-test.

Figure 4.26. Effect on sodium excretion of sub-chronic administration of MESD and Furosemide.
Table 4-34. Effect on potassium excretion of sub-chronic administration of MESD and Furosemide.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tween-80 (mmol/L)</th>
<th>MESD-250 (mmol/L)</th>
<th>MESD-500 (mmol/L)</th>
<th>Furosemide (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>51.68±1.56</td>
<td>51.26±1.84</td>
<td>51.46±1.62</td>
<td>51.55±1.54</td>
</tr>
<tr>
<td>1 day</td>
<td>51.56±1.45</td>
<td>52.56±1.20</td>
<td>52.44±1.24</td>
<td>50.56±1.47</td>
</tr>
<tr>
<td>2 day</td>
<td>51.36±1.44</td>
<td>51.28±0.78</td>
<td>51.51±0.94</td>
<td>51.86±1.44</td>
</tr>
<tr>
<td>3 day</td>
<td>51.04±0.98</td>
<td>52.78±0.88</td>
<td>51.04±0.78</td>
<td>52.22±1.48</td>
</tr>
<tr>
<td>4 day</td>
<td>51.98±0.78</td>
<td>54.22±0.74</td>
<td>52.45±0.68</td>
<td>50.56±1.23</td>
</tr>
<tr>
<td>5 day</td>
<td>51.45±0.82</td>
<td>55.44±1.22</td>
<td>51.98±0.68</td>
<td>53.02±1.58</td>
</tr>
<tr>
<td>6 day</td>
<td>51.02±0.86</td>
<td>54.68±1.24</td>
<td>51.56±0.78</td>
<td>52.42±1.78</td>
</tr>
<tr>
<td>7 day</td>
<td>51.26±0.76</td>
<td>55.24±1.22</td>
<td>51.22±0.62</td>
<td>52.20±1.64</td>
</tr>
<tr>
<td>8 day</td>
<td>51.08±0.62</td>
<td>54.42±1.42</td>
<td>51.48±0.98</td>
<td>51.56±1.80</td>
</tr>
</tbody>
</table>

The reported K⁺ levels are reported as mean±S.E.M. for six rats in each group. *P<0.05; **P<0.01 compared with controls using; a one-way analysis of variance (ANOVA) followed by Dunnett’s t-test.

Figure 4.27. Effect on potassium excretion of sub-chronic administration of MESD and Furosemide.
Table 4-35. Effect on plasma electrolyte levels of sub-chronic administration of MESD and Furosemide.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Plasma electrolyte level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/Kg BW</td>
<td>Na⁺ (mmol/L)</td>
</tr>
<tr>
<td>Tween 80</td>
<td>10 ml/Kg BW</td>
<td>146.90±1.32</td>
</tr>
<tr>
<td>MESD</td>
<td>250</td>
<td>142.56±1.78</td>
</tr>
<tr>
<td>MESD</td>
<td>500</td>
<td>141.02±1.48</td>
</tr>
<tr>
<td>Furosemide</td>
<td>20</td>
<td>141.48±1.46</td>
</tr>
</tbody>
</table>

The reported Na⁺ and K⁺ levels are reported as mean±S.E.M. for six rats in each group. *P < 0.05; **P < 0.01 compared with controls using; a one-way analysis of variance (ANOVA) followed by Dunnett's t-test.

Figure 4.28. Effect on plasma electrolyte levels of sub-chronic administration of MESD and Furosemide.
Table 4-36. Effect on Creatinine Clearance of sub-chronic administration of MESD and Furosemide.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose mg/Kg BW</th>
<th>Plasma creatinine (mg/dl)</th>
<th>Urinary creatinine (g/l)</th>
<th>Creatinine clearance (ml/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80</td>
<td>10 ml/Kg BW</td>
<td>0.56±0.02</td>
<td>3.24±0.12</td>
<td>1.72±0.06</td>
</tr>
<tr>
<td>MESD</td>
<td>250</td>
<td>0.54±0.02</td>
<td>1.34±0.02</td>
<td>1.90±0.02</td>
</tr>
<tr>
<td>MESD</td>
<td>500</td>
<td>0.52±0.01</td>
<td>1.22±0.01</td>
<td>2.23±0.10</td>
</tr>
<tr>
<td>Furosemide</td>
<td>20</td>
<td>0.51±0.01</td>
<td>0.92±0.03</td>
<td>2.38±0.02</td>
</tr>
</tbody>
</table>

The reported values are reported as mean±S.E.M. for six rats in each group. *P< 0.05; **P< 0.01 compared with controls using; a one-way analysis of variance (ANOVA) followed by Dunnett’s test.

Figure 4.29. Effect on Creatinine Clearance of sub-chronic administration of MESD and Furosemide.

Statistical analysis:

The results were presented as mean ±SEM. “One-way Anova with Dunnett’s post test was performed using Graph Pad Prism version 3.00 for windows. Graph Pad Software, San Diego California USA, P<0.05 were considered significant.
4.2.4 *Antihypertensive Activity*

Cardiovascular disease (CVD) is a major health problem across the world, accounting for 30% of all deaths. CVD has reached epidemic proportions in India and is estimated to result in more than 3 million deaths each year. Hypertension is a multifactorial disease dependent on complex interactions between genetic and environmental factors, yet many of these causes are not completely understood. Hypertension is a major risk factor for cardiovascular diseases and a large body of evidence suggests oxidative stress, an increase in the production of reactive oxygen species (ROS), as a strong underlying factor in hypertension.

This brief review will examine a common experimental model of hypertension, namely the effects of chronic administration of deoxycorticosterone acetate (DOCA), reduced renal mass and a high salt diet (DOCA–salt model) characterized by neurohumoral activation and volume expansion. The DOCA–salt model is similar to other experimental models that depend on high salt intake and reduced renal mass leading to hypervolaemia. Excess DOCA, a mineralocorticoid, leads to an imbalance in renal sodium handling by increasing sodium and water reabsorption in the distal tubules in dogs. This is exacerbated by excess salt intake and reduced renal mass, leading to an increase in extracellular fluid and plasma volume. Deoxycorticosterone acetate–salt hypertension is thought to occur in several stages described an early phase (days) of DOCA treatment where sodium is retained, at the expense of potassium, followed by a second phase (weeks) characterized by sodium balance and chronic potassium depletion. Acute extracellular volume expansion lowers the blood pressure of early phase hypertensive rats, suggesting that dilution of elevated sodium levels contributes. Titze *et al.* (2005) reported that DOCA–salt rats had excess total body sodium with moderate water retention. There is an initial increase in fluid and salt intake that, for the most part, is matched by an increase in sodium and water excretion. Recently, investigators have proposed that DOCA–salt hypertension in rats develops in stages, with an abrupt increase in arterial pressure during the first 48 h followed by a delayed, slower rise in arterial pressure over the next few weeks, leading to sustained hypertension. Others have described a later phase, often termed malignant, where severe hypertension is observed 8–12 weeks.
later. In this review, we will discuss the evidence for and against the role of neural and humoral factors during early, developed and malignant DOCA–salt hypertension.\cite{74}

Angiotensin-converting enzyme (ACE) inhibitors are used to treat many conditions such as hypertension (high blood pressure) and some heart conditions. They block a protein in the body that leads to tightened blood vessels. As a result, blood vessels are more relaxed, lowering blood pressure. Several ACE inhibitors such as captopril, enalapril, lisinopril and temocapril are in clinical use for the treatment of hypertension. All of these drugs produced side effects, thus justifying the search for natural ACE inhibitors for safe and economical use. A number of compounds from plants have been identified to possess in vitro ACE inhibitor activity, including hydrolysable tannins, phenylpropanes, proanthocyanidins, flavonoids, xanthones, fatty acids, terpenoids, alkaloids, oligosaccharides and peptide amino acids, among others.\cite{1751}

Hypertension is potentiated by angiotensin II, a potent vasoconstrictor agent.\cite{1761} Angiotensin II is formed from angiotensin I, a histidyl-leucine dipeptide, by the action of angiotensin I-converting enzyme (ACE).\cite{1771} Anti-hypertensive drugs have been isolated from bovine beta-lactoglobulin and a number of plant extracts.\cite{76-81} Scoparia dulcis is a rich source of isoflavonoid phenolics, especially genistein and daidzein which have been shown to have numerous potential health benefits.\cite{82,83,93} A number of flavonoids and isoflavonoids, including genistein, have been shown to inhibit \( \alpha \)-glucosidase activity in vitro.\cite{1841}

Therefore, phenolic-rich extracts of Scoparia dulcis may have potential as a source of DOCA salts and anti-ACE agents for control of hypertension, a known complication of long-term diabetes and/or hyperglycemia. Here, methanolic extract phenolic-optimized extracts of Scoparia dulcis was assayed in vivo DOCA salts and in vitro and rabbit lung ACE.
4.2.4.1 ANTIHYPERTENSIVE ACTIVITY in vitro

ACE-inhibitory activity

Materials and Methods:

Enzymatic Assay of ACE (EC 3.4.15.1)

ACE-inhibitory activity of hydrosylate sample was determined in triplicate by a method adopted from LO & Li-Chan (2005)\(^{[85]}\) based on ACE activity assay of Cushman and Cheung (1973)\(^{[86]}\). In this assay, ACE activity is quantified by spectrophotometric absorbance at 228 nm produced by the liberation of hippuric acid (HA) from synthetic substrate hippuryl-L-histidyl-L-leucine (HHL). In the presence of ACE inhibitor at given concentration, ACE activity and HA production is depressed; therefore % ACE inhibition can be calculated as:

\[
\text{IC}_{50} = \frac{1}{\text{absorbance of inhibitor containing sample} - \text{absorbance of negative control}} \\
\times \frac{\text{absorbance of positive control} - \text{absorbance of negative control}}{X_{100}}
\]

Where the positive control is taken as 100% ACE activity, with inhibitor having been replaced with buffer and the negative control is taken as 0 % ACE activity, with ACE being added only after reaction termination. The IC\(_{50}\) value, the concentration of
peptide sample in the assay required to inhibit the activity by 50%, is commonly used to quantitatively express and compare potency of ACE-inhibitory activity between samples. In this study, IC\textsubscript{50} values of methanol and water extract, as shown in Figure 4.31. All controls were also performed in triplicate to monitor the reproducibility of the assay.

To carry out the assay, 0.01-0.25 mg/ml solutions of MESD and WESD were first mixed with 30 \( \mu \text{L} \) (2.5 mU) of ACE incubated at 37°C for 1 hour, after which 150 \( \mu \text{L} \) of 7.8 mM HHL was added and the solution was further incubated at 37°C for 1 hour. All assay components were dissolved in 0.025 M sodium tetraborate buffer containing 0.3 M NaCl (pH 8.3) for a total assay volume of 210 \( \mu \text{L} \). HCl (250 \( \mu \text{L} \), IN) was used to terminate the reaction. In order to extract HA, 1.0 ml of ethyl acetate was added. The solutions were then vortexed for 30 seconds (CM 101 Cyclo Mixer, Remi, India) and centrifuged at 2000 x g (Remi-C24BL India) for 5 minutes. After centrifugation, 0.7 ml of ethyl acetate layer was removed into a 10 mm diameter clear glass tube and was evaporated by heating at 120°C in a hot air oven for 30 minutes. The remaining HA residue was redissolved with 1.3 ml of distilled water and the absorbance was read at 228 nm in a quartz cuvette (UV 1700 Shimazu, Japan).

Where the positive control is taken as 100% ACE activity, with inhibitor having been replaced with buffer and the negative control is taken as 0 % ACE activity, with ACE being added only after reaction termination. The IC\textsubscript{50} value, the concentration of peptide sample in the assay required to inhibit the activity by 50%, is commonly used to quantitatively express and compare potency of ACE-inhibitory activity between samples. In this study, IC\textsubscript{50} values of methanol and water extract, as shown in Fig 4.31& table 4-37. All controls were also performed in triplicate to monitor the reproducibility of the assay.
Table 4-37. ACE activity of MESD & WESD and their IC₅₀

<table>
<thead>
<tr>
<th>Dose (mg/ml)</th>
<th>%ACE activity of MESD</th>
<th>IC₅₀</th>
<th>%ACE activity of WESD</th>
<th>IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>95.66±0.88</td>
<td></td>
<td>97.66±0.88</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>66.33±1.85</td>
<td>0.102 mg/ml</td>
<td>77.33±1.20</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>42.00±1.15</td>
<td></td>
<td>55.00±0.57</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>23.33±0.88</td>
<td></td>
<td>32.33±0.88</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>11.00±0.57</td>
<td></td>
<td>18.33±0.88</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>7.66±0.33</td>
<td></td>
<td>13.66±0.88</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.31. % ACE activity of MESD & WESD

y = -3.6476x + 94.369
y = -3.5839x + 85.729

ACE Methanol
ACE Water
Linear (ACE Methanol)
Linear (ACE Water)
4.2.4.2 ANTIHYPERTENSIVE ACTIVITY in vivo

Materials & Methods:

DOCA-salt induced hypertension:

Animals:

All the experiments were carried out using Wistar albino rats and were obtained from animal house, Birla Institute of Technology, Mesra, Ranchi, India and kept in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature 24± 2°C and relative humidity of 60-70 %. A 10:14 light: day cycle was followed. All animals were allowed to free access to water and fed with standard commercial rat chow pallets (M/s. Hindustan Lever Ltd, Mumbai). All the experimental procedures and protocols used in this study were reviewed by the Institutional Ethics Committee (Reg. No:621/02/ac/CPCSEA) and were in accordance with the guidelines of the CPCSEA, Ministry of Forests and Environment, Government of India.

Dosing of Animals:

The group I received vehicle only (Tween-80, 1%) and served as control. Group II and group III received 200 mg/kg BW low dose and 400mg/kg BW therapeutic dose (oral), respectively.

DOCA-salt induced hypertension and blood pressure measurements:

- Wistar rats were divided into four groups (n = 6). Normal control (NC) rats received a saline injection (0.5 ml/kg BW, twice weekly for 5 weeks, s.c., n = 6) and were age matched with treated rats. DOCA salt injection (20 mg/kg BW, twice weekly for 5 weeks, s.c., n = 6) was given to the rats and NaCl (1%, w/v) was added to their drinking water. [87]

- DOCA salt-induced hypertensive rats were divided into 4 groups, viz. DOCA salt hypertensive control, test 1 and 2 compound treated and standard drug treated rats. DOCA salt hypertensive control rats received saline injection (for 12 days, n = 6).
- Test compounds treated rats received methanolic extract of Scoparia dulcis (MESD) (200 mg and 400 mg/kg BW, for 12 days daily, i.p., n = 6) and standard drug treated rats received nifedipine (2 mg/kg BW, for 12 days daily, i.p., n = 6).

- Rats were initially trained for systolic blood pressure measurement on at least three separate occasions to establish a baseline blood pressure. Systolic pressure was measured by tail-cuff method using Non-invasive blood pressure instrument (NIBP-IICT Inc., Germany).
Table 4-38. Changes in mean systolic blood pressure after 12 days of Extract (200 and 400 mg/kg) treatment in DOCA-salt hypertensive rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Blood Pressure (Mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normotensive</td>
<td>115 ± 3.19</td>
</tr>
<tr>
<td>DOCA-Hypertensive</td>
<td>192 ± 4.95</td>
</tr>
<tr>
<td>MESD 200 mg/kg BW</td>
<td>156 ± 3.87**</td>
</tr>
<tr>
<td>MESD 400 mg/kg BW</td>
<td>139 ± 5.78**</td>
</tr>
<tr>
<td>Nifedipine treated 2mg/kg BW</td>
<td>121 ± 4.19**</td>
</tr>
</tbody>
</table>

Cumulative values are reported as mean±S.E.M. for six rats in each group. *P < 0.05; **P < 0.01 compared with DOCA-Hypertensive using; a one-way analysis of variance (ANOVA) followed by Dunnett’s test.

Figure 4.32. Changes in mean systolic blood pressure after 12 days of drug administration

![Graph showing changes in mean systolic blood pressure after 12 days of drug administration](image)

Figure 4.32. Changes in mean systolic blood pressure after 12 days of Extract (200 and 400 mg/kg) treatment in DOCA-salt hypertensive rats
4.3 REFERENCES


72. Brooks VL, Freeman KL, Qi Y. Time course of synergistic interaction between DOCA and salt on blood pressure: roles of vasopressin and hepatic......


87. Bankar GR, Nampurath GK, Nayak PG, Bhattacharya S. A possible correlation between the correction of endothelial dysfunction and normalization of high blood pressure levels by 1,3,4-oxadizole derivative, an L-type Ca2+ channel blocker in deoxycorticosterone acetate and NG-nitro-1-arginine hypertensive rats. Chemico-Biological Interactions 2010;183:327-331.


