3. MATERIALS AND METHODS

The plant materials (Solanum species) for the present study were collected from different parts of South India. The collection of plants includes both wild and cultivated species. Several field trips were undertaken at different seasons of the year to different parts of South India and collected plants from Botanical gardens of Tamil Nadu & Kerala. The details of the name of the taxa, wild or cultivated and locality were tabulated (Table – 1). The collected plants were identified with the help of the Flora of the Presidency of Madras (Gamble, 1935), Flora of Tamilnadu carnatic (Mathew, 1982) and Flowering Plants of Kerala, a check list (Sasidharan, 2006).

Table 1: List of plants selected for the study

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Name of the taxa</th>
<th>Wild/ cultivated</th>
<th>Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>S. aculeatissimum, Jacq.</td>
<td>Cultivated</td>
<td>Thovalai</td>
</tr>
<tr>
<td>2.</td>
<td>S. anguivi, Lam.</td>
<td>Wild</td>
<td>Pechiparai</td>
</tr>
<tr>
<td>3.</td>
<td>S. diphyllum, Linn.</td>
<td>Cultivated</td>
<td>Pallode</td>
</tr>
<tr>
<td>4.</td>
<td>S. macranthum, Dunal.</td>
<td>Cultivated</td>
<td>Cheruvarakonam</td>
</tr>
<tr>
<td>5.</td>
<td>S. marginatum, Linn.</td>
<td>Wild</td>
<td>Cheruvarakonam</td>
</tr>
<tr>
<td>6.</td>
<td>S. melongena, Linn.</td>
<td>Cultivated</td>
<td>Eathamozhy</td>
</tr>
<tr>
<td>7.</td>
<td>S. melongena. var.esculentum, Linn.</td>
<td>Cultivated</td>
<td>Karungal</td>
</tr>
<tr>
<td>8.</td>
<td>S. nigrum, Linn.</td>
<td>Wild</td>
<td>Nagercoil</td>
</tr>
<tr>
<td>9.</td>
<td>S. peruvianam, Linn.</td>
<td>Wild</td>
<td>Thovalai</td>
</tr>
<tr>
<td>10.</td>
<td>S. seafortianam, Andr.</td>
<td>Cultivated</td>
<td>Pallodu</td>
</tr>
<tr>
<td>11.</td>
<td>S. torvum, Sw.</td>
<td>Cultivated</td>
<td>Eathamozhy</td>
</tr>
<tr>
<td>12.</td>
<td>S. trilobatum, Linn.</td>
<td>Cultivated</td>
<td>Nagercoil</td>
</tr>
<tr>
<td>13.</td>
<td>S. tuberosum, Linn.</td>
<td>Cultivated</td>
<td>Kodaikanal</td>
</tr>
<tr>
<td>14.</td>
<td>S. viarum, Dunal.</td>
<td>Cultivated</td>
<td>Pechiparai</td>
</tr>
<tr>
<td>15.</td>
<td>S. xanthocarpum, Schrad.</td>
<td>Wild</td>
<td>Kanyakumari</td>
</tr>
</tbody>
</table>
The above species of *Solanum* have been collected for the study. The voucher specimens have been deposited as herbarium in the Botany department. The collected *Solanum* species were subjected to systematic study based on the following features.

1. **Morphological studies**

2. **Anatomical studies**

3. **Palynological studies**

4. **Phytochemical studies**

5. **Bio-molecular studies and**

6. **Bio-systematical consideration**

1. **Morphological studies**

   The detailed morphological studies were carried out in 15 South Indian species of *Solanum* (Table 2). This study included habit, nature of stem, leaf, flower, calyx, corolla, androecium, gynoecium, seed, fruit and flowering season. The length and breadth of foliar and floral parts were measured by using centimeter scale, ocular micrometer and weight (seed) were measured by using electronic balance. The morphological identification was based on the Flora of the Presidency of Madras (Gamble, 1935), Flora of Tamilnadu Carnatic (Mathew, 1982) and Flowering Plants of Kerala-a check list (Sasidharan, 2006). All the selected morphological characters were observed. The significance of these morphological characters in establishing the phylogentic relation between taxa and their taxonomic considerations were analyzed.
2. Anatomical studies

In the present study anatomical studies deals with the epidermal studies of 15 species of *Solanum*. This epidermal characters such as type and number of stomata, number of epidermal cells, stomatal frequency, stomatal index, length and breadth of stomata in upper and lower side, nature, type, length and breadth of leaf, trichome and stomata of calyx and corolla, leaf venation major and minor, shape, length and breadth of areole were observed.

2.1. Stomatal Analysis

The stomatal analyses were carried out based on Ahmad (1964). Epidermal peels of leaf, calyx and corolla were taken mechanically from upper and lower epidermises using a razor blade. They were first washed in distilled water and stained in 1% aqueous safranin and again washed in distilled water, mounted in 50% glycerine and sealed with paraffin wax.

For estimating the size of stomata micrometry was employed. In this ocular micrometer was used. The ocular micrometer was placed on the metal diaphragm in the eye piece of the microscope. Throughout the study, objective lens of 40x and 50x have been used. Type, frequency and index of stomata were calculated for the upper and lower leaf epidermises using laboratory microscope having 10x × 40x magnifications. The same method was followed in the stomata of calyx and corolla. Photomicrographs were taken for all the observations. Jetner – Biolux research microscope was used for micro photographing. All measurement was taken from an average of twenty readings.
Stomatal frequency and index were calculated by using the following formula

\[
\text{Stomatal Frequency} \quad (S.F) = \frac{S}{E} \times 100
\]

\[
\text{Stomatal Index} \quad (S.I) = \frac{S}{E+S} \times 100
\]

Where ‘S’ is the number of stomata per unit area and ‘E’ is the number of epidermal cells other than the stomata in the same unit area.

2.2. Trichome Analysis

Epidermal peels were taken mechanically from upper and lower epidermises using a razor blade. They were first washed in distilled water and stained in 1% aqueous safranin. Again washed in distilled water and mounted in 50% glycerine and sealed with paraffin wax (Ahmad, 1964)

The nature of epidermal trichomes present on the upper and lower surfaces of the leaf, calyx and corolla were studied. The length and breadth of trichomes were also investigated using laboratory microscope. All the measurement was taken from an average of twenty readings. Photomicrographs were taken for all plant materials by using Jetner-Biolux research microscope.

2.3. Venation and Areole

Foliar venation studies were carried out in all the selected species of *Solanum*. The venation pattern of leaves was traced out from fresh leaves that were covered by a carbon sheet facing upwards. A white paper was placed over the carbon sheet and was
rubbed with the base of a pencil. By this process an accurate impression of the vein pattern was obtained on the side of the white paper facing the carbon sheet. The pattern was then transferred to drawing sheets. The minor venation pattern was observed by clearing the leaves based on the method of Foster (1959) with modification by Hickey (1973). First the leaf materials were washed thoroughly in tap water. Then the veins of the leaves were stained by immersing the base of the petiole in safranin stain for overnight. Then washed the leaves in water and boiled them in 50% ethyl alcohol in a water bath. The materials were transferred to 5% sodium hydroxide and kept in the oven at 40 to 50°C for 6 to 10 days for clearing. After clearing, the specimens were washed thoroughly in water to remove all the traces of sodium hydroxide. The leaves were examined for their major and minor venations.

The major and minor venation pattern was studied with the help of microphotographs. The terminology and description of leaf architecture were done based on the leaf architectural studies by Hickey (1973). The data regarding the nature of major venation pattern and the nature of minor venation pattern including the nature of areole and veinlets were recorded.

3. Palynological Studies

Palynological studies were carried out in the selected 15 species of *Solanum*. palynological characters such as pollen type, aperture morphotype, exine ornamentation, spine type, grain size, shape and pollen fertility were observed.
3.1. Pollen Analysis

Mature pollen grains from mature anthers were dusted on a clean slide and stained with one percent acetocarmine. The acetolysed pollen grains were mounted in glycerine jelly and the slides were sealed with paraffin wax. Acetocarmine was found to be the most suitable stain for pollen grain studies in Solanaceae. The size of the pollen grains was measured by using ocular micrometer. Twenty readings were taken in each case. The pollen grains were micro photographed to study the shape of pollen grains and the wall ornamentation. Photomicrographs were taken for all plant materials. Jetner–Biolux research microscope was used for micro photographing.

Pollen size was calculated by taking measurements of polar axis and the maximum breadth in the equatorial view of the grain and applying the formula \( P/E \times 100 \). The shape of the pollen grain depends upon three factors (Knox, 1904; Sukhla, et al., 1998) (i) position of the microspore within the tetrad. (ii) distribution and form of apertures (iii) the mode of cellular expansion after the release of microspores from progenitor wall.

Pollen sterility was examined by smearing mature anther in 1:1 mixture of glycerine and acetocarmine. The slides were kept 30 minutes for better staining and then examined under microscope. Fully stained pollen grains were counted as fertile and partially stained or unstained was counted as sterile. Pollen sterility was calculated.
4. Phytochemical Studies

The selected species of *Solanum* leaf and root samples were shade dried for 15 days and then pulverized into fine powder using pestle and mortar. The extraction was done by solvent extraction techniques. Solvents were used successively with gradient polarity (methanol). The extracts were evaporated to complete dryness by vacuum distillation and stored in refrigeration for further use (Akingemi *et al.*, 2000; Mohanta *et al.*, 2001, Patra *et al.*, 2008).

4.1. Qualitative Analysis

The phytochemical analysis of the plant was carried out by the standard methods provided by Waterman (1993) and Odebiyin and Ramstard (1978).

4.1.1. Test for Phenolic compounds

2 ml of extract was diluted to 5 ml with distilled water. To this a few drops of neutral 5% ferric chloride solution was added. A dark green color indicates the presence of phenolic compounds.

4.1.2. Test for Flavonoids

1.5 ml of 50% methanol was added to 5 ml of extracts. Warmed the solution and metal magnesium was added. Then added 5 – 6 drops of concentrated hydrochloric acid to the solution and observed for red coloration.
4.1.3. Test for Alkaloids

To 4 ml of extract filtrates, a drop of Mayer’s reagent was added by the sides of test tubes. Creamy yellow or white precipitate indicates the test is positive for alkaloids.

4.1.4. Test for Glycosides

To 4 ml of extract solution add glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid and observe for a reddish brown coloration at the junction of 2 layers and bluish green color in upper layer.

4.1.5. Test for Steroids

5 drops of concentrated sulphuric acid (H₂SO₄) was added to 2 ml of each extract and observed for red coloration.

4.1.6. Test for Saponins

2 ml of distilled water was added to 2 ml of test solution, shake well and observed for frothing.

4.1.7. Test for Tannins

To 0.5 ml of extract solution, 1 ml of distilled water and 1 to 2 drops of ferric chloride solution was added and observed for blue or green black coloration.

4.1.8. Test for Anthraquinones

1 gm of powdered plant material was taken and extracted with 10 ml of hot water for 5 minutes and filtered. Filtrate was extracted with 10 ml of CCl₄, then CCl₄ layer was
taken off. Five ml water and 5 ml dilute ammonia solution was added. No free anthraquinones were revealed as absence of appearance of pink to cherry red color. 1 gm of second sample of the same plant materials was extracted with 10 ml of ferric chloride solution and 5 ml of hydrochloric acid, then it was heated on water bath for 10 minutes and filtered. Filtrate was cooled and treated as mentioned above.

4.2. Quantitative Analysis

4.2.1. Phenols

Plant samples was boiled for 15 min with 50 ml of Diethylether (CH₃CH₂)₂O. 5 ml of the sample was pipette into 50 ml flask, and 10 ml of distilled water was added. Then 2 ml of NH₄OH solution and 5 ml of concentrated amyl alcohol was added to the mixture. The sample was made up to the mark and left to react for 30 min for color development and measured for 505 nm wave length using a spectrophotometer (Tyler, 1994; Harborne, et al., 1973).

4.2.2. Flavonoids

Extracted 10g of the plant sample with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered and the filtrate was then transferred into a water bath. The solution was evaporated to dryness and weighed to a constant weight (Mattila and Hellstrom, 2007).
4.2.3. Alkaloids

5g of the plant sample was prepared in a beaker and 200 ml of 10% acetic acid in ethanol was added to the plant sample. The mixture is covered and allowed to stand for 4 hrs. The mixture was then filtered and the extract is allowed to become concentrated in a water bath until it reaches ¼ of the original volume. Concentrated ammonium hydroxide was added until the precipitation is complete. The whole solution is allowed to settle sodium carbonate until the effervescence leaser make up to the volume to 100 ml and centrifuge. Collect the supernatant and take 0.5 and 1 ml aliquots for analysis. Prepare the standards by taking 0, 0.2 – 1 ml of the working standard ‘0’ serves as blank. Make up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water. Then add 4 ml of anthrone reagent and heat for 8 minutes in a boiling water bath. Cool rapidly and read the green to dark green color at 630 nm. Draw a standard graph by plotting concentration of the standard on the X-axis verses absorbance on the Y-axis. From the graph calculate the amount of carbohydrates present in the sample tubes.

4.2.4. Glycosides

Glycoside content in the sample was evaluated using Buljet’s reagent as described by El – olemy et al., 1 g of the fine powder of extracted material was soaked in 10 ml 70% alcohol for 2 hrs and then filtered. The extract obtained was then purified using lead acetate and NaZHPO₄ solution before the addition of freshly prepared Buljet’s reagent. The difference between the intensity of color of the experimental and blank (distilled
water and Bulijet’s reagent) samples gives the absorbance and is proportional to the concentration of the glycosides.

**4.2.5. Steroids**

1 ml of methanolic extract of steroid solution was transferred into 10 ml volumetric flasks. Sulphuric acid (4N, 2 ml) and iron (III) Chloride (0.5% W/V, 2 ml), were added, followed by potassium hexacyanoferrate (III) solution (0.5% W/V, 0.5 ml). The mixture was heated in a water bath maintained at 70 ± 20°C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank.

**4.2.6. Saponins**

20 g of each ground plant samples were put into a conical flask and 100 ml of 20% ethanol was added to the plant sample. The above said sample is heated over a water bath for 4h at about 55°C with continuous stirring. The extracted mixture is then filtered and the residue is then re-extracted again with 200 ml of 20% ethanol. The collective residues are reduced to 40 ml over a hot water bath. The concentrated solution was transferred to a separating funnel and 20 ml of diethyl ether is added to the plant extract and shaken vigorously. The aqueous layer was recovered while the organic layer was discarded and the process of purification was repeated. 60 ml of n-Butanol was added and combined n – Butanol extract were washed twice with 10 ml of 5% sodium chloride. The remaining solution was then heated on water bath. After the evaporation, the samples were dried in oven to a constant weight.
4.2.7. Tannins

500 mg of plant sample was weighted and transferred to 50 ml flask. Then added 50 ml of distilled water and stirred for 1 h. sample was filtered into a 50 ml volumetric flask and the volume was made up to the mark. 5 ml of the filtered sample was pipette into test tube and then mixed with 2 ml of 0.1 M ferric chloride. The absorbance was measured using spectrophotometer at 395 m wavelength within 10 min (Tyler., 1994; Harborne et al., 1973).

4.2.8. Anthraquinones

50 mg of the fine powder sample was soaked in 50 ml of distilled water for 16 hours. This suspension was heated in water bath at 70°C for one hour. After the suspension was cooled, 50 ml of 50% methanol was added to it and then filtered.

The clear solution was measured by spectrophotometer at a wavelength of 450 nm compared with a standard solution containing 1 mg/ 100 ml alizarin and 1 mg/ 100 ml purpurin with the absorption maximum 450 nm.

5. Bio-molecular Studies

The bio-molecular studies based on quantitative analysis of DNA and RNA was observed to leaf & root samples of 15 South Indian species of Solanum were selected.

5.1. Isolation of genomic DNA

DNA, the genetic material can be isolated from leaf and root samples of Solanum species by phenol chloroform extraction method. The phenol extraction method gives
fairly intact and pure DNA. The principle involves breakage of cell to release DNA and subsequent treatment with detergents and enzymes to degrade most of the contaminating proteins. The digest is de-proteinised by successive phenol: Chloroform: isoamylacohol extractions and the DNA is recovered by ethanol precipitation.

5.2. RNA isolation

Collect the leaf samples as quickly as possible and always keep on ice. If dealing with sample, cut into 0.5 cm x 1 cm x 1 cm pieces. Place the piece samples into RNAse –free tubes. Add 5 x or 5-10 x volume of RNA l. Sample can be stored in RNA as follows.

5.2.1. Storage at – 80°C (recommended for long – term storage)

Incubate samples at 4°C overnight, the remove RNA later before storage at 80°C to prevent the formation of salt crystals. For sample cells, do not remove the RNA later. Sample can be thawed at room temperature and refrozen without affecting the amount or the integrity of the RNA.

5.2.2. Storage at – 20°C (recommended for long – term storage)

Incubate sample at 4°C overnight, then transfer to – 20°C. Samples will not freeze at -20°C. Samples can be brought to room temperature and refrozen without affecting the amount or the integrity of the RNA.

5.2.3. Storage at 4°C

Samples can be stored at 4°C for up to 1 month without any experimental evidence of RNA degradation.
5.2.4. Storage at 25°C (room temperature)

Samples can be stored at room temperature of up to 1 week. Leaf sample/ cells - RNA (including Homogenization)

❖ Thaw sample (if frozen)

❖ Use RNase AwayR (and 100% ethanol) to clear all tools, pipettes and bench before starting.

Use a pair of forceps (treated with RNase) to pick the sample out of the tube. Tap the sample (Several times) on the side of the tube to rid tissue of excess RNA later. Add 1 ml of TRI zol reagent per 50-100 mg of tissue. Homogenize the tissue using a plastic/ glass mortar (treated with RNase).

6. Bio-systematical Consideration

Biosystematics is referred as the use of biological criteria in refining the system of classification. Biosystematics uses experimental data in order to understand patterns of organizational variation in fundamental biological level. In this study bio-systematical consideration were based on morphological, anatomical, palynological, phytochemical and bio-molecular studies. Based on the above characters, constructed the identification key such as morphological identification key, anatomical identification key, palynological identification key, phytochemical identification key and bio-molecular identification key.