2. Materials and Methods
2. MATERIALS AND METHODS

Cyathial nectaries at various stages of development were collected from 13 plant species belonging to the genus *Euphorbia* from Vallabh Vidyanagar and surrounding areas. These materials were either used fresh or fixed in FAA (Formalin-acetic acid-alcohol) (Johansen, 1940).

1. *Euphorbia geniculata* L.
2. *E. heterophylla* L.
4. *E. antiquorum* L.
5. *E. neriifolia* L.
6. *E. tortilis* L.
8. *E. cyparissias* L.
10. *E. hirta* L.
11. *E. parviflora* L.
13. *E. thymifolia* L.

1. **Light microscopy**

   i) **Bright field microscopy:** The FAA fixed materials were stored in ethanol. Tertiary-Butyl-alcohol series was employed for dehydration, followed by embedding with paraffin wax (Merck 52-54°C) (Berlyn and Miksche, 1976). Longitudinal and transverse sections of 6-8 μm thick were cut with A.O. Spencer's 820 rotary microtome and stained with tannic acid and ferric chloride followed by Safranin 'O' and Fast green 'FCF' combination or Delafield's haematoxylin (Sass, 1952). The mature cyathia were cleared following the method of Rao et al. (1980).

   ii) **Phase contrast microscopy:** Unstained or stained paraffin embedded sections were viewed under phase contrast microscopy fitted with a permanent green filter.
iii) **Epi-fluorescence microscopy:** Paraffin sections were used to localize specific parts and metabolites as given below:

<table>
<thead>
<tr>
<th>Parts/Metabolites</th>
<th>Stains</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cuticle/Lipid</td>
<td>Auramine 'O'/</td>
<td>Pearse, 1968</td>
</tr>
<tr>
<td></td>
<td>Rhodamine</td>
<td></td>
</tr>
<tr>
<td>2. Xylem</td>
<td>Auramine 'O'</td>
<td></td>
</tr>
<tr>
<td>3. Phloem/Starch</td>
<td>Aniline blue</td>
<td>Currier, 1951</td>
</tr>
<tr>
<td>4. Basic protein</td>
<td>Dansyl Chloride</td>
<td>Ringertz, 1968</td>
</tr>
</tbody>
</table>

Stained sections were observed under Carl-zeiss epi-fluorescence microscope fitted with HBO-50 mercury lamp using filters of various wave lengths and types as under:

1)  
- G 365  BP 436  
- FT 365  FT 460  
- LP 420  LP 470  

2)  
- BP 546  450  -  490  
- FT 580  FT  -  510  
- LP 590  LP  -  520
Histocchemical tests

Starch, proteins, ascorbic acids and tannins were localized using Paraffin embedded material while fresh sections were employed to localize lipids and enzymes.

The following techniques were followed:

1. Periodic acid - Schiff's reaction (PAS) for starch and other insoluble polysaccharides (Jensen, 1962).
2. Feulgen reaction (Jensen, 1962).
5. Silver nitrate (AgNO₃) method for ascorbic acid (Dave et al., 1969).

6. Cytochrome oxidase activity was identified following the method of Mia and Pathak (1965).

7. Acid phosphatase activity was detected by Gomori method (1950).

8. DAB technique for peroxidase (Graham and Karnovsky, 1966).


3. **Scanning Electron Microscopy (SEM)**

Nectaries were dehydrated in graded ethanol series and fixed on aluminium stubs using double sided adhesive tape. Pollengrains were directly dusted on a double sided adhesive tape fixed on aluminium stubs. Materials were coated with a mixture of gold and palladium and scanned with a Cambridge Stereoscan S4 10 Scanning Electron Microscope at Ahmedabad Textile Industries Research Association (ATIRA), Ahmedabad.
Transmission Electron Microscopy (TEM):

Nectaries at different developmental stages were prefixed in ice cold freshly prepared 4% glutaraldehyde and 5% sucrose in 0.1 M phosphate buffer pH 7.2 for 4 hours. The materials were cut into smaller pieces and post-fixed in 2% Osmium tetraoxide at 20°C for 12 hours followed by several washes with cold phosphate buffer (pH7.2). Tissues were dehydrated through an acetone series and infiltrated with Spurr's low viscosity embedding medium (Spurr, 1969). The nectary tissues were then flat embedded and polymerized at 70°C for 12 hours.

Thick sections (.5-1 u) were cut with glass knives and stained in 0.05% toluidine blue 'O' in sodium-tetraborate (O'Brien and McCully, 1969). Silver sections were cut with Dupont diamand knife using a Reichert OMU3 ultramicrotome and mounted on copper grids. Standard staining procedures were performed using Uranylacetate and lead citrate (Reynolds, 1963). Sections were scanned and photographed with a Philips EM 400 electron microscope at 80-100 KV.

Measurements, Drawing and Photography:

Measurements of the nectaries (Sections) were
taken with a calibrated Carl-zeiss Visopan microscope. Measurements were taken of 20 specimens for each nectary. A Carl-zeiss microscope fitted with camera lucida was used for drawings. Carl-zeiss photomicroscope-I or Carl-zeiss epi-fluorescence microscope with or without compensatory filters were used for bright field study. For phase contrast microscope fitted with an Expo-Star auto exposure unit and planapochromatic objectives were used. Epi-fluorescence photomicrographs were taken with Carl-zeiss epi-fluorescence microscope fitted with a HBO 50 mercury lamp using various filters.

ORWO NP 22/ORWO NP 55 black and white negatives and Konica colour negative were used. Yashica FX-3 super camera equipped with a close-up converter-cum adopter was used for field photography. Freshly excised/fixed nectaries were used for Tessovar photographs (Carl-zeiss). For SEM, ORWO NP 55 negative films and TEM, Eastman Kodak fine grain release positive film 5302 were used for photography.

Analysis of Sugars and amino acids of nectar samples by thin layer chromatography (TLC):

The nectar samples were collected with the help of micro pipettes from herbaceous plants in the early morning and between 10.00 a.m. to 12.00 noon in cacti-like species and stored below 4°C until needed.
The silica gel plates were prepared at 0.2 mm thickness and the plates were activated by spraying a solution of 0.02 M sodium acetate and keeping in an oven at 90°C for 30 minutes prior to use. The nectar samples were diluted with distilled water in the ratio of 1:1 and spotted along with sugar or an amino acid standards on the silica gel plate.

A mixture of n-Butanol, glacial acetic acid and water (4:1:5 V/V) was used as solvent system for both. These plates were kept inside the saturated chambers till the solvent reached the top and plates were air dried.

For sugars, a mixture of solution containing diphenylamine (2 gms), aniline (2ml) and phosphoric acid (10ml) in 100ml of acetone was sprayed. For amino acids, ninhydrin (300 mg)dissolved in 100 ml of acetone was sprayed. Both the Rf values and colour of the spots were taken for the identification of the compounds (Harborne, 1973).