Materials and Methods
A. ETHNOMEDICO BOTANY

Initially a list of herbal healers residing in the study area was prepared by gathering the information from the local people and some local agencies. Herbal healers exclusively following their traditional methods were only considered under the present survey. A list of herbal healers belonging to different ethnic groups was prepared, which consisted of about 92 healers and elderly people. This formed the primary source of information for the present study.

Regular field trips were undertaken to different localities of the study area, during the study period between November 1999 and March 2003. Localities were selected in such a way that it should represent the entire district, covering rural areas and tribal pockets of the study area. Each locality was visited in different seasons to collect the information about the seasonal plants. During the fieldwork, frequent visits were made to the herbal practitioners and efforts were made to convince them to disclose their traditional knowledge about the healing herbs. The information about the plants was recorded by means of discussions and interviews with the informers along with the field visits during their collection hours. The information gathered for the plants were-

i. Local name(s) of the plant
ii. Parts used
iii. Method(s) of preparation
iv. Medicinal uses
However the other information such as ingredients added, methods of administration or application, dose and duration of medication and associated medicated food, if any, were recorded whenever available.

The collected plant specimens were authentically identified with the help of floras such as *Flora of Presidency of Bombay* (Cooke, 1967), *Flora of Presidency of Madras* (Gamble, 1984), *Flora of British India* (Hooker, 1978), *Flora of Karnataka* (Saldanha, 1984a) and *Flora of Udupi* (Bhat, 2003). Voucher herbarium specimens are deposited in the herbarium at P.G. Department of Botany, Karnataka University, Dharwad for future references.

Along with the first hand information collected, the literature based on similar survey works in local language was taken as secondary sources of information. Such major contributions are by Hegde (1981, 1983, 1995a, 1995b, 1997) and Gouda (1997). But in all these texts, the plants are described only with their vernacular names. This is a major obstacle in wider recognition, utilization and scientific evaluation of the reported healing herbs. Hence an attempt was made to find out the correct botanical identity of the medicinal plants described in these texts. The botanical names to the plants mentioned in these texts were sought in Kannada- Latin names index by Patwardhan (2000) and Narasimhachar (1979). Later, these plants were confirmed in the field by discussions with the local healers. However, the information about the plants, whose botanical identity is established beyond all doubts only are considered in this study.

The information gathered was further screened by consulting the important works on Indian ethnobotany and medicinal plants, such as Jain.

An attempt has been made, further, to give a simple artificial key for the easy identification of the medicinal plants mentioned in the text, based on their morphological characters to the maximum extent.

B. ANTIMICROBIAL SCREENING OF THE PLANTS

1. Plant material:

Four medicinal plants, namely *Holigarna arnottiana* Hook.f. (Anacardiaceae), *Ocimum gratissimum* L. (Lamiaceae), *Allophylus cobbe* (L.) Raeusch. (Sapindaceae) and *Centratherum anthelminticum* (L.) O.Kurz. (Asteraceae), were selected for the present study (Plate-1), on the basis of their utilization in local herbal medical system. The part of the plant, used in the medicine, is taken for the antimicrobial assay. The parts taken for the study are:

- *Holigarna arnottiana*  
  Bark
- *Ocimum gratissimum*  
  Leaves
- *Allophylus cobbe*  
  Leaves
- *Centratherum anthelminticum*  
  Leaves

The healthy leaves of *O. gratissimum*, *A. cobbe* and *C. anthelminticum* were collected from Karanatak University Campus, Dharwad, Karnataka, while the bark of *Holigarna arnottiana* was collected from Sirsi, Uttara Kannada, Karnataka. The plant parts were shade dried and
were made into fine powder in electric grinder. This powdered material was used for the further extraction procedures.

2. Chemicals and glassware:

Nutrient agar, Nutrient broth, Potato dextrose agar, Peptone water, Streptomycin, Nystatin and sterile disks were procured from Hi-Media laboratories, Mumbai, India. The solvents and DMF were from s.d.fine-Chem. Ltd. Mumbai, India. All chemicals were of analytical grade. Borosil, Mumbai, India, glassware were used for the purpose.

3. Extraction and preparation of test solutions:

The powdered plant materials were subjected to continuous extraction in Soxhlet apparatus. The solvents used were chloroform, acetone, ethanol and water in order. The extracts were evaporated to dryness and were re-dissolved in aliquots of Di Methyl Formamide (DMF) to obtain the test solutions of the concentrations 25, 20 and 15 mg/ml. Further, the effective concentrations of 250, 200 and 150 μg/disk were obtained by impregnating the sterile disks by 10 μL of each test solutions (Castello et. al., 2002).

4. Culture media and standards:

The ready-made dehydrated Nutrient agar (MM 012) was the bacterial culture media and Peptone water (M 028) was the media for bacterial broth. The fungal culture media used was Potato dextrose agar
(M 096) for the study. All these dehydrated ready-made media were procured from Hi-Media Laboratories, Mumbai, India.

Streptomycin (SD 031) was the standard used for bacteria, while Nystatin (SD 025) was the standard against fungal cultures. Both standards and Sterile discs (SD 067) were procured from Hi-Media Laboratories, Mumbai, India.

5. Micro organisms:

All the extracts were tested against four bacterial and two fungal strains. The bacterial strains used were:

- *Bacillus subtilis*  
  Gram (+)ve
- *Streptococcus faecalis*  
  Gram (+)ve
- *Escherichia coli*  
  Gram (-)ve
- *Psedomonas aeruginosa*  
  Gram (-)ve

The bacterial strains were obtained from the stock cultures of Microbiology Laboratory, Department of Biochemistry, Karnataka University, Dharwad.

The fungal strains used were:

- *Aspergillus niger*
- *Penicillium chrysogenum*

The fungal strains were procured from the stock culture maintained in Mycology Laboratory, Department of Botany, Karnataka University, Dharwad.
6. Preparation of bacterial and fungal broath:

Peptone water medium was used for bacterial broath. A loopful of bacterial inoculum was added to the sterile medium and was incubated at 37 °C for overnight to get slight to moderate turbidity, indicating the bacterial growth. This broath was used as seed for the further antibacterial assay.

The fungal spores from 48 h old fungal cultures were suspended in sterile distilled water and the same was used as seed for further antifungal studies.

7. Antimicrobial assay:

Antimicrobial activity was assayed by disk diffusion method (Bauer et al., 1966). This method depends on the radial diffusion of an antibiotic or the plant extract from the disk through semisolid agar layer in petridishes to such an extent that the growth of the microorganism is prevented in a circular area or 'zone' around the disk.

The sterile medium was poured while hot in to sterile petriplates to form a layer of 2-3 mm uniform thickness and was left for sometime to solidify. The petriplates, containing medium, were lawn cultured or seeded with bacterial broath or fungal spore suspension and allowed to dry. Then the impregnated disks were placed on the agar with flamed forceps and gently pressed down to ensure contact. The plates were kept 30 min. for pre-incubation diffusion. The plates were then incubated at 37 °C for 24 h for bacteria and 48 hrs for fungal strains. Then the zone diameters (including
the 6 mm disk) was recorded. Sterile conditions were maintained throughout the experiment.

Streptomycin and Nystatin were the standards used for bacteria and fungi respectively, while a disk impregnated with DMF served as blank.

C. ANTIMICROBIAL SCREENING OF THE CALLUS IN VITRO

1. Collection of plant material:

Young and healthy leaves of *Vernonia anthelminticum* and *Allophyllus cobbe* were collected from plants grown in Karnatak University campus, Dharwad.

2. Chemicals and glassware:

Mineral salts, vitamins, sucrose, agar, laboline, sodium hypochlorite and mercuric chloride were procured from Hi-Media laboratories, Mumbai, India. Growth regulators were purchased from Sigma Chemical Co., USA. All chemicals were of analytical grade. Glassware was procured from Borosil, Mumbai, India.

3. Growth regulator:

The auxin, 2,4-D (2, 4-Dichlorophenoxyacetic acid) was used for the induction of callus (Bhojwani and Razdan, 1996). In the preliminary standardization studies, 2,4-D was taken at the concentrations of 5, 10 and
15 μM and found that 10 μM and 15 μM were the optimum concentrations for the callus growth in *C. anthelminticum* and *A. cobbe* respectively. Thus the same concentrations were maintained throughout the further callus cultures.

4. Preparation of medium:

MS basal media was used for the present study and the composition of the same is presented in Table 1. The pH was adjusted to 5.8 using 0.1N NaOH or HCl and the medium was solidified with 0.8% agar. Hot medium was dispensed into culture tubes (150 ( 25 mm) or 100 ml conical flasks. Culture tubes and flasks were plugged with non-absorbent cotton wrapped in cheese cloth or autoclavable caps. The petri plates, culture tubes and culture flasks were sterilized by autoclaving at 120 °C under 1.06 kg/cm² pressure.

Culture tubes/flasks with medium, flasks containing sterile distilled water and autoclaved instruments were placed under UV light in the laminar airflow chamber (Micro-Filt, India) for 60-90 min before inoculation. Forceps and scalpels were dipped in 95% ethanol, flamed over spirit lamp and cooled before use at regular intervals.
Table 1. Composition of MS (Murashige and Skoog, 1962) basal media.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major salts</strong></td>
<td>mg l⁻¹</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
</tr>
<tr>
<td>CaCl₂ . 2H₂O</td>
<td>440</td>
</tr>
<tr>
<td>MgSO₄ . 7H₂O</td>
<td>370</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
</tr>
<tr>
<td><strong>Minor salts</strong></td>
<td>mg l⁻¹</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
</tr>
<tr>
<td>MnSO₄ . 4H₂O</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO₄ . 7H₂O</td>
<td>8.6</td>
</tr>
<tr>
<td>Na₂MoO₄ . 2H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO₄ . 5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl₂ . 6H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>Na₂ · EDTA</td>
<td>37.3</td>
</tr>
<tr>
<td>FeSO₄ . 7H₂O</td>
<td>27.8</td>
</tr>
<tr>
<td><strong>Vitamins and organics</strong></td>
<td>mg l⁻¹</td>
</tr>
<tr>
<td>Myo-Inositol</td>
<td>100</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20 g</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>5.8</td>
</tr>
</tbody>
</table>
5. Sterilization and inoculation:

Leaves were washed under running tap water for 10-15 min and immersed in an aqueous solution of liquid detergent, Laboline (1%; Qualigens, India) for 15 min and washed in 1% NaOCl (Ranbaxy, India) for 10-15 min and rinsed with 0.2% HgCl\textsubscript{2} for 8-10 min followed by 4-5 times rinse in sterile distilled water. The leaf segments measuring about 8-10 mm were excised from the sterilized leaves and were cultured on MS basal medium supplemented with 2,4-D.

All the aseptic manipulations, surface sterilization, dissection and inoculation of explants were carried out in the laminar airflow. The working surface was cleaned using 0.5% dettol solution and wiped with ethyl alcohol (95%).

6. Culture and Harvesting of the callus:

The cultures were maintained in a culture room at 24 ± 2 °C in 16 h photoperiod of 40 μmol m\textsuperscript{-2} s\textsuperscript{-1} light provided by cool white fluorescent tubes (Philips, India) with 70% relative humidity.

The callus was harvested after 60 days and was dried at 40 °C for 72 h in oven. The dried callus was powdered and 10 g of this powder was taken for extraction.

The extraction procedure, solvents used and antimicrobial assays are similar to those followed for the plant materials.