4.

MATERIALS AND METHODS
Collection of Materials:

The collection of plant material in proper season and time is essential for getting at least optimum constituents of medicinal importance. The proper period to collect the material is when the plant just begins to flower and vegetative phase of plant is almost over. During this phase the plant appears to be in maximum vigour and the parts of the plant is in the most rapid state of optimum metabolic activity (Datta and Mukherjee, 1956).

*Biophytum sensitivum* was collected from Samdoh at shady places in the month of August. The bark of *Buchanania lanzan* was removed from the roadside tree on Badnera Yeotmal highway in the month of January. Bark comes from the trunk in the form of pieces of various sizes. The leaves of *Butea superba* were collected from Samdoh forest area in the month of February. The fresh leaves of *Caesalpinia decapetala* was collected from Marumur, Chikalda. The root bark of *Capris zeylanica* was collected from Rajapeth, Amravati. The roots were removed from the soil, then washed in water and from them bark was removed in slices of varying sizes. The fresh leaves of *Desmodium polycarpum, Thevetia peruviana* and *Vigna trilobata* were collected from University campus in the month of September. The tuber of *Dioscorea hispida* was collected from Samdoh. The intact tuber was removed from the soil, then washed with water. The tuber was cut into small pieces. *Cuscuta chinensis* was collected from Vidasbha Dal Mill Paratwada. Collected material viz. leaf, stem, root, bark and tuber, were cut into pieces and some material fixed in 4% formalin for section cutting. About 150 gms. of plant material was oven dried at 40 to 50 °C. Dry material was crushed in mixture and made into powder. The dry powder was used for the study of powder behaviour, fluorescence, phytochemical test and TLC.
Table II  Potential psychoactive plants of Amravati region

The plant species, parts used, location and their psychoactive property

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Plant Name and family</th>
<th>Plant part used</th>
<th>Place Amravati District.</th>
<th>Property Shukla &amp; Jain Year - 1997</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Biophyton Sensítírum fam - Oxalidaceae</td>
<td>Leaf</td>
<td>Semadoh</td>
<td>Giddiness</td>
</tr>
<tr>
<td>2.</td>
<td>Buchanania lanzan fam - Anacardiaceae</td>
<td>Stem bark</td>
<td>Melghat forest</td>
<td>To increase the intoxicating property</td>
</tr>
<tr>
<td>3.</td>
<td>Butea superba fam - Papilionaceae</td>
<td>Leaf</td>
<td>Semadoh</td>
<td>Sedative</td>
</tr>
<tr>
<td>4.</td>
<td>Caesalpinia decapetala fam - Caesalpiniaceae</td>
<td>Leaf</td>
<td>Mariumpur</td>
<td>Hallucinogen</td>
</tr>
<tr>
<td>5.</td>
<td>Capparis zeylanica fam - Capparidaceae</td>
<td>Root bark</td>
<td>Amravati</td>
<td>Sedative</td>
</tr>
<tr>
<td>6.</td>
<td>Cuscuta chinensis fam - Convolvulaceae</td>
<td>Stem</td>
<td>Paratwada</td>
<td>CNS active</td>
</tr>
<tr>
<td>7.</td>
<td>Desmodium polycarpum fam - papilionaceae</td>
<td>Leaf</td>
<td>University campus</td>
<td>Cause hypnosis &amp; convulsion</td>
</tr>
<tr>
<td>8.</td>
<td>Dioscorea hispida fam - Dioscoreaceae</td>
<td>Tuber</td>
<td>Semadoh</td>
<td>Intoxicant</td>
</tr>
<tr>
<td>9.</td>
<td>Thvetia peruviana fam - Apocynaceae</td>
<td>Leaf</td>
<td>University campus</td>
<td>Intoxicant</td>
</tr>
<tr>
<td>10.</td>
<td>Vigna trilobata fam - Papilionaceae</td>
<td>Leaf</td>
<td>University campus</td>
<td>Sedative</td>
</tr>
</tbody>
</table>
Fish:

The fishes were collected from Baslapur in the month of August to January whenever in need. Baslapur is 25 km. away from Amravati; there is big fish farm run by Government of Maharashtra. The uniform fingerlings (number of 50 or 60 at one collection) were collected in plastic bags and were brought to laboratory. They were kept in aquarium (capacity is 40 lits.). Next day dead fishes were removed and tap water is replaced every 24 hrs. In one week period about 50% fishes were survived in laboratory condition and they take about two weeks for acclimatization. Fishes were supplied with commercial feed (spirulina special Japan Hitachi fish food with added vitamins and minerals) of 5% of the body weight.

Methodology:

Ethnobotanical study:

Tribal people of Melghat belonging to the caste Korku and Gond totally relied on medicinal plants for health care. Candidate visited different tribal areas of Melghat forest and rural people of Akola, Yeotmal and Washim district of Amravati region. Information on the traditional uses of plant and plant product useful in day to day life for health care was noted down. Remote villages of Semadoh; namely Bihali, Tille and Mariumpur from Chikalda of Melghat forest were surveyed during July 1998 to August 2000. Ethnobotanical information was collected by interviewing local medicine men, tribal chief old men, experience informant, Bhagat and Bhumka who prescribed their own herbal medicine and data was properly stored. Generally tribe men and women keep secrecy about the medicinal uses of plants, however by developing confidence with in them, some useful information on the ethnomedicinal uses of plants could be procured. The plant part used, local name, period of collection, practices, traditional preparations, doses and mode of administration of the crude drugs were recorded.

Pharmacognostical study:

Method of Trease and Evans (1972) is followed for the macro and microscopical study of crude drug of plants.
Macroscopical study: Detail macroscopical characters of the plant part of medicinal value was carried out and results are incorporated in observations chapter.

Microscopical characters:

Veinslet number: Boil 2 to 3 leaves in chlortal hydrate solution in a test tube placed in water bath, so that leaf becomes colourless. In case of difficulty soak leaves in water; treat them with chlorinated soda for bleaching and add 10% hydrochloric acid for the removal of calcium oxalate and finally treat with chlortal hydrate solution. Mount the preparation in glycerine water.

Stomatal Number and Stomatal Index: Leaf pieces boil with chlortal hydrate solution or alternatively with chlorinated soda. Peel out upper and lower epidermis separately by sharp forcep, prepare the mounts of lower and upper epidermis separately in glycerine water.

Ash value: Method of Peach and Traecy (1958) was followed. Weigh accurately about 3 gms of the powdered drug in tarterd silica crucible. Incinerate the powdered drug by gradually increasing the heat by raising temperature till it becomes free from carbon and then allow it to cool down and keep it desiccator. Weigh the ash and calculate the percentage of total ash with respective to air dried sample.

Powder behaviour: Behaviour of powdered drug was studied on treating powder with different chemical reagents. On the basis of colour imparts to different constituents, powder behaviour was determined.

Fluorescence: Methods of Chase and Pratt (1949) and Kokoski (1958) were followed for fluorescence study of powder drug under UV light.

Phytochemical Tests:

Successive solvent extraction:

About 50 gms of the dry plant powder successively extracted with the following solvents in a soxhlet extractor.
a) Petroleum ether
b) Benzene
c) Chloroform
d) Acetone
e) Ethanol
f) Water

Concentrate the liquid extract obtained in each solvent by distilling off the solvent and then evaporating to dryness on the water bath at 50 °C. Weigh the solidified extract obtained with each solvent and calculate its percentage in terms of the dry weight of plant material. Each time before extracting a residual part with the next solvent dry the residual powder in oven at 40 to 50 °C.

Detection of Alkaloids:
Prepare a extract of powdered drug in chloroform, alcohol and water; to decant it. Take a small quantity of solidified extract with a few drops of dilute hydrochloric acid and filter. Test the filtrate carefully with various reagents such as Mayers reagent (cream precipitate); Dragendorff reagent (orange brown precipitate); Hagers reagent (yellow precipitate) and Wagners reagent (reddish brown precipitate), for alkaloid tests.

Detection of Glycosides:
Hydrolyse small quantity of the extract with dilute hydrochloric acid for a period of about an hour, on water bath at 50 °C. Subject the hydrolysate to Libermann-Burchards test to detect the presence of different glycosides.

Detection of phytosterols:
Treat separately Petroleum ether, acetone and alcoholic extract with solution of alcoholic potassium hydroxide (1 %) till complete saponification takes place; to this add 5 ml of distilled water and 5 ml of ether. Evaporate the etheral extract upto half quantity by volume and then tested with Libermann-Burchards reagent. Brown colour ppt indicates presence of phytosterols.
Detection of fixed oils and fats:

Take small quantity of either Petroleum ether or benzene extract between two filter papers. Apply pressure on paper, oily spot indicating presence of fixed oils.

Detection of saponins:

Take 1 gm of alcoholic and aqueous extracts, add 1 ml of distilled water shake well, go on adding water and till it make a volume of 20 ml and further shake thoroughly in graduated cylinder for 15 minutes, appearance of one centimeter layer of foam indicating the presence of saponins.

Detection of tannins:

Take a small quantity of alcoholic and aqueous extracts separately in water, add dilute ferric chloried solution (5 %), black or green colour indicates presence of tannins.

Detection of proteins:

Proteins in alcoholic and aqueous extracts are detected by Biuret method.

TLC:

Method described by Wagners (1984) was followed. Prepare slurry by vigorously shaking 30 gms of the silica gel G with 60 ml of distilled water. The slurry must be prepared quickly and should be used immediately, because silica gel contains CaSO₄, which acts as a binder. Using special mounting board and applicator, coat a thin layer of silica gel on two sizes of glass plates 5 x 20 cm and 20 x 20 cm. Plate on which the sample is to be loaded, make small scratches length-wise on both sides at a distance of 1.5 cm from both the edges. Then scratch a small mark at a distance of 1.5 cm from the far ends on glass plate approximately equi-distance from the earlier marks on lateral sides.

Preparation of plant extracts for TLC:

Preparation of alkaloid drug: Mix one gm powdered drug with 1 ml of 10% ammonia solution, then extracted with 5 ml methanol at 60 °C on water bath.
Cool the filtrate; concentrate it again on water bath at 50 °C. Now lead it on TLC plate.

**Preparation of cardiac glycoside drug:**

One gm of powdered drug + 20 ml of 50 % ethanol, heat on water bath for 15 minute, add 10 ml of 10 % Lead acetate solution, cool and filter. Divide clear filtrate into 3 parts, add in each small quantity of acetic acid, shake with 15 ml of dichloromethane: ethanol (1 : 1), use this extract for chromatography.

**Preparation of Saponin drug:**

Extract two gms powdered drug with 10 ml of 70 % ethanol, on heating for 10 minutes on water bath. Evaporate the filtrate and collect maximum quantity and then subject it to chromatography.

**Fish:**

**Swimming Behaviour:** One gm of fresh plant material was crushed with 10 ml of distilled water and filtered by using whatman filter paper no. 1. The volume of filtrate was made to 10 ml by adding requisite quantity of distilled water. Different concentrations were prepared from 10 to 1000 ppm by taking 10 litres of water in aquarium and by adding requisite quantity of plant extract. The fish *Labeo rohita* of uniform sized (6 to 8 cm) was collected from Government fish farm Baslapur and brought to laboratory. They were kept in aquarium for a fort-night for acclimatization. The fishes were provided with commercial feed (5% body weight). Dechlorinated tap water in the aquarium was renewed every day in the morning, to keep fishes alive in normal condition.

Observation were made for any behavioural changes continuously up to 6 hrs initially and then once daily up to 96 hrs. Scales of experimental and control fishes were removed by using a sharp forceps. The scales were rinsed in GDW and then fixed in 10% formalin and processed further to prepare the permanent whole mount. Observations were made and changes in chromatophores were noted. A parallel control was also maintained for each set of experiment.
increasing doses in ppm or with increasing period of treatment, whenever fish dies that particular dose with time is noted.

**LC 50 value:**

Overdose of any psychotropic drug can become toxic, hence different ppm concentrations were prepared and lethality of each dose at constant duration i.e. 24 hrs. was recorded. Mortality was recorded after 24 hrs and data was used to calculate LC 50 value at 24 hrs. by probit mortality analysis. Log concentration against probit mortality using the method of Finney (1971) was applied.

**Estimation of Acetylcholinesterase** (Biggs et al, 1958):

**Procedure:**

Fishes exposed to the various concentrations of plant extract at 24 to 96 hrs. Control was maintained simultaneously. The whole brain of control as well as treated fishes was separated from head region. The brain tissue washed with distilled water and homogenized in chilled water.

**Preparation of Standard curve:**

Prepare in test tubes series of dilutions of acetic acid by measuring 0, 1, 2, 3, 4, ..., 14 ml of 0.015 N acid and making up the volume of each to 15 ml with disstilled water. Mix one ml solution from each test tube separately with 2 ml of buffer indicator, 1.4 ml of water and 0.1 ml of normal serum. These tubes will then represent 0, 10, 20, ..., 140 units of activity. Read on spectrophotometer and plot the standard graph against units of activity.

Take 2 ml of buffer indicator solution and 2.2 ml of water into test tube mixed and then add 0.1 ml of homogenate brain serum and 0.2 ml of acetylcholine bromide solution. Mix thoroughly and immediately read on the spectrophotometer against red filter (620). Incubate exactly for 30 min at 37 °C and read again. The difference of the two reading (U) will correspond to the amount of acid liberated. The quantity of AchE in treated was calculated by referring and equating O.D. with standard graph. The units of cholinesterase activity is defined as the micromols of acetic acid liberated from acetylcholine by 1 ml of serum in 30 min at 37 °C.