Chapter - 6
Materials and Methods
Position of Uttar Pradesh in India

Fig. 1
Study Area

Survey of literature provided very little information on ethnomedicobotany of Aligarh, Bulandshahr, Budaun, Hathras and Farrukhabad districts. Hence, these districts were selected for intensive and systematic ethnobotanical exploration. All these districts are located in western Uttar Pradesh. Table-3 shows their longitudes and latitudes and names of villages surveyed in each district. Demographic profile of each district is given in Table-4, survey schedule is given in Table 5, the position of the state is provided in Fig. 1 and the study area is given in Fig. 2.

Collection of Ethnobotanical data:

The task of data collection was accomplished in two phases. In the first phase as many villages as possible were visited in each district to gather information on names and addresses of practicing traditional healers/medicine man/wise women. Practitioners of magicobotany were not taken into consideration. Villages of reputed traditional medicinemen/women were preferentially included in the study.

Second phase was actual data collection phase. Beginning in 1997, each district was explored for one calendar year. Several visits, spanning over the three seasons, were made to each village for interviewing the informants. Experience showed that this job was quite delicate and needed great deal of tactfulness, patience and perseverance. Some informants tended to share their knowledge readily and enthusiastically, while others showed a varying degree of reluctance and above all, an explicit and solemn assurance that the information provided by them would not be put to any misuse.
The informants were interviewed in any of the following two ways:

A. The informant accompanied the worker to field and told the uses of ethnomedicinally important plant species growing there.

B. The worker himself collected plants in field beforehand and visited the informant’s house. The informant then checked the collection and told uses of plant species known to them.

Both methods had their advantages and disadvantages. The first method considerably cut the time needed for field work and minimized the possibility of omission of claims.

The second method suited those informants who expressed their inability to make a visit to field. This method enabled the worker to plan his survey work or record the data according to taxonomic categories or plant habit.

Data collected were entered in a printed data sheet designed for this purpose (Fig. 3A). Every attempt was made to eliminate spurious claims. Only those claims separately confirmed by two or more informants, were retained and remaining were discarded. However, claims furnished by informants, locally renowned for their ethnobotanical acumen were retained as such.

Voucher herbarium specimens were prepared and were deposited at the Herbarium of Department of Botany, Aligarh Muslim University, Aligarh (India).
Forms of Medication Prevalent in the Study Area

**Decoction/Apozeme (karha, kwath):**

This is the aqueous extract of the fresh or dried plant material obtained by boiling the plant material in water. The ratio of plant material to water may vary from 1:4 to 1:8. However, the ratios of 1:4 and 1:6 are more commonly used. Required amount of plant material was chopped and mixed with prescribed volume of water in an earthen pot and boiled on constant flame until the final volume is reduced to one-fourth or half of original volume as per prescription.

**Plant Powder (Churna):**

Dried plant material was ground to fine powder in a mortar and pastel. Plants are mostly dried in shade but, a few medicinemen advise to dry them in sun.

**Paste (awleh):**

Fresh plant material was washed thoroughly and ground with a little water.

**Extract (Ras, Ark):**

Fresh and thoroughly washed plant material is pounded to pulp; this pulp is put in a piece of muslin cloth and squeezed to obtain fresh juice.
**Plant Ash (Bhasam):**

Shade dried whole plant/plant part is chopped and burnt in an earthen pot on cowdung fire to obtain its ash.

**Screening of Crude Extracts Plant for Antibacterial Activity**

Screening of crude plant extracts for their antibacterial activity involved two components (a) extraction of plant material with different polar and non-polar organic solvents and (b) determination of sensitivity of a pure bacterial culture to plant extract(s). A brief and succint description of these two components is given below.

**Protocol for Preparation of Plant Extracts**

All plant species, except *Catha edulis*, screened for antibacterial activity of their crude extracts were collected from the study area. The dried leaf material of *Catha edulis* was provided by Prof. S. Qureshi, College of Pharmacy, King Saud University, Riyadh (KSA). Crude extracts were prepared following (Robinson, 1963), the protocol is described below.

i. Freshly dried and healthy plant material was ground into fine powder in an electric grinder. Powder so obtained was stored in dessicator.

ii. Five hundred g plant powder was refluxed with 95% methyl alcohol (MeOH) in a round bottom flask on a water bath for 10 hours. Mother liquor (Crude MeOH extract) was filtered out and residual plant material was again refluxed with 95% methyl
alcohol for 10 hours. The process was repeated four times to obtain maximum yield of MeOH extract. The extract was evaporated to dryness at 50°C under reduced pressure.

iii. Dried methanolic extract was refluxed with light petrol (60-80°C) for five hours. After filtration, the residual methanolic extract was again refluxed with petrol for five hours and filtered. This process was repeated five times. Petrol was evaporated under reduced pressure to obtain petrol soluble extract.

iv. Petrol insoluble fraction of methanolic extract obtained in step (iii) was refluxed with benzene for five hours. Thereafter, it was filtered and refluxed again with benzene for five hours and filtered. The process was repeated five times. Benzene was evaporated under reduced pressure to obtain benzene soluble extract.

v. Benzene insoluble fraction obtained in step (iv) was refluxed with ethyl acetate for five hours. Thereafter, it was filtered and refluxed again with ethyl acetate for five hours and filtered. The process was repeated five times. Ethyl acetate was evaporated under reduced pressure to obtain ethyl acetate soluble extract.

vi. Ethyl acetate insoluble fraction obtained in step (v) was refluxed with methyl alcohol (95%) for five hours, filtered and was repeatedly refluxed for five times with methyl alcohol (Methanol). The methanolic soluble fraction was evaporated under reduced
pressure to obtain methanolic extract, while methanol insoluble residue was discarded. The steps are graphically presented as a flow chart in Fig. 3B.

**Preparation of aqueous extract**

Shade dried plant material (500 g) was ground to a fine powder. It was poured with distilled water, and left for 72 hours at room temperature.

The flask was then refluxed over hot water bath for 10 hours, the mother liquor was filtered. The solute was again added with solvent (distilled water) which was again refluxed and filtered, this process was repeated for 4 times. The filtrate, thus obtained, was evaporated to complete dryness on a water bath. The residue thus obtained was aqueous plant extract.

**Sterilization of plant extracts**

Plant extracts were sterilized by incubating at 37°C for 24 hours followed by heating on a water bath for 2 hours. This process was repeated for at least five times. Thereafter, a smear of this extract was stained to detect the presence of gram positive and gram negative bacteria. Whole process was repeated if any bacterial growth was detected. Sterilized plant extract was stored in stoppered glass bottles, sealed with wax and was stored in a refrigerator.

**Compounds, likely to occur in various fractions**

MeOH : Carbohydrates, glycosides, amino acids, tannins, saponins, flavonoids, higher phenolic oligomers.
<table>
<thead>
<tr>
<th>Material</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtoAc</td>
<td>Flavonoids, phenolic compounds, and alkaloids.</td>
</tr>
<tr>
<td>Benzene</td>
<td>Terpenoids, hydrocarbons.</td>
</tr>
<tr>
<td>Petrol</td>
<td>Hydrocarbons, fatty acids.</td>
</tr>
<tr>
<td>H₂O</td>
<td>Most of glycosides, carbohydrates, amino acids.</td>
</tr>
</tbody>
</table>
Fig 3B. Flow Chart for Plant Extraction

Plant Material (500 mg)
- Kept at room temperature for 72 hours in Distilled water
- Refluxed for 10 hours (4 times)
- MeOH extract (concentrated to dryness under reduced pressure)
- Water insoluble extract (Discarded)

AQUEOUS EXTRACT
- Refluxed with methanol (95%) for 10 hours (4 times)
- MeOH extract (concentrated to dryness under reduced pressure)
- Water soluble extract concentrated to dryness under reduced pressure

Petrol Insoluble
- Benzene
- Benzene Soluble (BENZENE EXTRACT)
- Benzene Insoluble

Petrol Soluble (PETROL EXTRACT)
- EtoAC
- EtoAC soluble (EtOAC EXTRACT)
- EtoAC Insoluble
- MeOH (95%)
- MeOH Insoluble (Discarded)
- MeOH Soluble (MeOH EXTRACT)
Microbiological Techniques


Preparation of Culture Media

Culture media are used to provide sources of carbon, energy, and nitrogen in the form of available carbohydrates and amino acids. Bacteria are generally grown in enriched culture media, obtained by addition of substances such as blood, serum etc. to a basal medium. Protocols for the preparation of basal and enriched media are described below.

Basal Media

Nutrient broths form the basis of most media used in microbiological studies. Meat infusion broth and nutrient agar were used to prepare enriched culture media and the methodology is given below.

Meat infusion broth

Lean finely minced meat was added to one liter water and extracted for 24 hours in a refrigerator. The meat was strained through muslin cloth and squeezed; the extract so obtained should be bright red. The surface layer of fat was removed by skimming with a piece of filter paper. The meat infusion was
boiled for 15 minutes and the brown and turbid liquid so obtained was filtered through whatman No. 1 filter paper. The volume was made upto 1 liter with water, peptone and salt were added and dissolved by heating. The filtered broth had an acidic reaction. The pH was adjusted to 7.5.

**Nutrient agar**

Composition

Peptone : 10g, Sodium chloride: 5g, Beef extracts 3g, Agar 20g, Distilled water: 100ml, pH: 7.6.

All ingredients except agar were dissolve in distilled water and pH was adjusted to 7.6. Agar was added and autoclaved at 121°C for 25 minutes. Nutrient agar was used to obtain pure colonies to be used for sensitivity test.

**Enriched culture media**

**Blood agar**

Composition

Nutrient agar: 1000 ml, Defibrinated blood : 50 ml, pH : 7.6

Autoclaved nutrient agar was allowed to cool to 45-50°C. Sterile and defibrinated blood was added to it aseptically and the flask was rotated to mix the contents thoroughly. The blood agar so prepared is poured into sterile petri plates immediately.

It is a differential medium for hemolytic organisms. Three patterns of hemolysis can occur on blood agar plates.
a. **Beta hemolysis** (complete hemolysis), where formation of a clear zone with a clear edge around the colony takes place

b. **Alpha hemolysis** (incomplete hemolysis) where production of methemoglobin occurs and a green cloudy zone around the colony is formed.

c. **Gamma hemolysis** (No hemolysis), where no change in colour surrounding the colony on blood agar takes place.

**Teepol Lactose Agar Plates or Lactose Teepol Agar or Blue Agar or Lactose Agar**

**Composition**

Nutrient Broth : 1000 ml, Agar : 15 g, Lactose : 10 g, Teepol : 1 ml, Bromothymol Blue : 20-25 ml, pH : 7.6

All ingredients were added to nutrient broth followed by the addition of bromothymol blue. The colour of agar changed to light blue. pH was adjusted to 7.6. The blue agar so obtained was autoclaved at 121°C for 21 minutes. The growth of gram negative bacteria is superior in comparison to gram positive bacteria in this medium.

**Mueller Hinton Agar**

Now a days it is used in conjunction with high potency antibiotic disks, for the determination of antibiotic sensitivity pattern. All antibiotic sensitivity tests for standard drug as well as for plant extract were performed on Mueller Hinton Agar.
Materials & Methods

Composition

Nutrient broth : 300 ml, Casein hydrolysate : 17.5 ml, Starch : 1.5 g,
Agar : 10 g, Distilled water : 1000 ml, pH : 7.6

Procedure

Starch was emulsified in small amount of water followed by casein
hydrolysate and then agar was added to the nutrient broth, all the components
were poured in distilled water, pH was adjusted at 7.6.

Adjustment of pH

The pH of a culture medium was adjusted to 7.6 by adding of few
drops a 4% Sodium hydroxide (NaOH) solution or concentrated hydrochloric
acid (Conc. Hcl) Sodium hydroxide solution was prepared by dissolving 4.0g
crystals of analytical grade salt in 100 ml distilled water.

Preparation of dilutions of plant extracts

Five dilution i.e. 1 mg/ml, 2 mg/ml, 5mg/ml, 10mg/ml and 15mg/ml
were used in this study. The dilutions were prepared by carefully weighing a
given amount of sterilized plant extract in sterilized 5 ml glass bottles. The
weighed extract was dissolved in 1 ml of suitable solvent. For the sake of
precission plant extracts were weighed on an electronic balance.
Obtaining pure cultures of bacteria

Streak plate method

This is one of the best methods to isolate individual bacterial cells on the agar surface. In this method, a loopful of bacterial cell were streaked across the agar solidified surface for nutrient medium. The plates were then incubated under favourable conditions at 37°C. The key to this method is that, by streaking, a dilution gradient (a decreasing concentration of bacterial cells) is established across the face of the plates, so that well isolated colony arises from a single bacterium and represents clone of a pure culture. Which was then incubated at 37°C for 24 hours to obtain pure colonies.

Spread plate method

A small volume of a suspension of microorganisms was placed on the centre of an agar plate and spread over the surface of agar by an sterile glass rod (sterilized by repeatedly dipping in ethanol and heating on a flame) and then flaming to burn the alcohol. By spreading the suspension over the plate, an even layer of cells was established, so that individual microorganism were separated from the other organisms in the suspension and deposited at a discrete location. Usually a single colony was poured in normal saline. In a test tube (The volume of normal saline was taken 15-20 ml) in order to have maximum dilution, during spreading. The plates were incubated at 37°C to isolate pure colonies.
Staining

Grams Staining (For gram positive and gram negative bacteria)

Method of Hucker and Conn

Solutions required

a. Huckers Crystal Violet

Solution X : Crystal violet [90% dye content] (2 g), Ethyl alcohol [95%] (20.0 ml). Solution Y : Ammonium Oxalate (0.8g), Distilled water (80.0ml). Dissolve crystal violet in ethyl alcohol and the ammonium oxalate in distilled water. Mix X and Y.

b. Grams Iodine (Logol’s Iodine)

Iodine (1.0g), Potassium Iodide (2.0g) and Distilled water (300.0ml). Dissolved potassium iodide in distilled water then add iodine crystals, dissolve.

c. Ethyl alcohol (95%)

Ethyl alcohol [100%] (95.0ml), Distilled water (5.0ml) mix together.

d. Safranine

Safranine [2.5% solution in 95% ethyl alcohol] (10.0ml), Distilled water (100.0ml) mixed together. The solution is filtered before use.

Procedure

1. A smear of bacterial culture or plant extract was prepared and heat fixed.
2. The smear was stained with solution (a) for 1 minute.

3. Solution (a) was replaced with solution (b) and allowed to stain for 1 minute.

4. The smear was washed with water.

5. The smear was stained with solution (C) for 10 seconds.

6. Wash the smear with water.

7. Final staining was done with solution (d) for 30 seconds. The slide was washed, air-dried and examined under a compound microscope.

Results

A gram-positive bacterium took violet stain while a gram negative bacteria took pink stain.

Determination of bacterial sensitivity to plant extracts

Pouring of petri dishes

Petri dishes were aseptically poured with Mueller-Hinton agar. Solidified plates were incubated over night at 37°C to remove the excess moisture and as a precaution against any contamination.

Preparation and impregnation of paper discs

Disks of standard size (6 mm) were punched out of Whatman filter paper No. 1, washed with distilled water and sterilized by placing in an oven at 160°C for 30 minutes.
The sterilized, discs were impregnated with the desired concentration of the plant extract, dried in an oven at 40°C and were placed directly on the surface of agar plates.

**Disc Diffusion Method**

Bacterial Sensitivity to various plant extracts was determined by disc diffusion method. The outline of this assay is as follows.

This is the most commonly used method to determine the bacterial sensitivity of antimicrobial agents which is in the form of a disc (6mm of paper) containing a certain concentration of plant extracts (i.e., 1mg/ml, 2mg/ml, 5mg/ml, 10mg/ml and 15mg/ml).

**Procedure**

Autoclaved culture medium was allowed to cool down to 50°C-45°C at room temperature. Thoroughly cleaned and sterilized 9.0 cm petri-dishes were poured aseptically with enough medium to make a 2.5-3.0 mm thick layer. Poured petri dishes were left at room temperature till the medium solidified and then were stored in a refrigerator.

A. A single bacterial colony isolated with a pre heated inoculating loop was transferred from pure culture to a tube containing 15-20 ml of normal saline. The loop was stirred in the saline and the tube contents were poured over the petri dishes. Excess suspension was decanted off. Alternatively A single colony was isolated with a swab, which was streaked at the agar surface.
B. The standard antibiotic discs or paper discs impregnated with plant extract were now placed on the surface inoculated petri dishes.

C. The plates were incubated at 37°C for 24 hours. Therefore, the plates were observed to record the sensitivity which was expressed as diameter of inhibition zone (DIZ mm) measured from the edge of the paper disc. Each treatment was replicated three times and each value of DIZ was expressed as mean of three readings.

**Sterilization**

**Sterilization of Glassware**

Thoroughly washed, cleaned and dried glassware were heated in oven at 160°C for 2 hour. Sterilized glassware were used immediately.
Table - 3 Names of districts studied, their geographical position and villages sampled and abbreviations

<table>
<thead>
<tr>
<th>S.No.</th>
<th>District</th>
<th>Coordinates</th>
<th>Villages studied</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Aligarh</td>
<td>(27° 34' and 28° 01' N Latitude and 77° 29'; and 78° 38'E Longitude)</td>
<td>Ashrafpur, Chamraul, Cheerat, Chilkauria, Dhanipur, Daulatabad, Elampur, Hardaspur, Harduaganj, Hasayan, Jawan, Kuwarsi, Narayanpur.</td>
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<tr>
<td>2.</td>
<td>Bulandshahr</td>
<td>(28° 04' and 28° 00' 0' Latitude and 77° 18'; and 78° 28'E Longitude)</td>
<td>Akbarabad, Akrabad, Bahlipura, Boorh, Chandpur, Dostpur, Gangerua, Herapur, Hazratpur, Jalkhera, Imlia, Khawajapur-Mirpur, Walipur.</td>
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<tr>
<td>4.</td>
<td>Farrukhabad</td>
<td>(26° 45' 45'' and 27° 00' 42'' N Latitude and 79° 10' 45'' and 80° 6'E Longitude)</td>
<td>Azmatpur, Barjhala, Chilkana, Dhansua, Deorampur, Dharampur, Madhopur, Midnapur, Moiesh, Jasmai, Khanpur, Tatiyani.</td>
</tr>
<tr>
<td>District</td>
<td>Total Population</td>
<td>Literacy %</td>
<td>Schedule Cast Population</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>% of total population</td>
</tr>
<tr>
<td>Aligarh</td>
<td>T 536,115</td>
<td>57.62</td>
<td>93,556</td>
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<tr>
<td></td>
<td>U 480,520</td>
<td>59.61</td>
<td>79,331</td>
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<tr>
<td></td>
<td>R 55,595</td>
<td>39.51</td>
<td>14,225</td>
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<td>Budaun</td>
<td>T 136,201</td>
<td>53.41</td>
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<td></td>
<td>U 116,695</td>
<td>56.96</td>
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<td>Farrukhabad</td>
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<td>R 38,597</td>
<td>43.96</td>
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<td>Hathras</td>
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<td>56.2</td>
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<td>U 113,285</td>
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<td></td>
<td>R 46,265</td>
<td>45.63</td>
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T = Total Population (U+R), U = Urban Population, R = Rural Population.
Table - 5: Survey Schedule

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<td>Hatharas</td>
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FORMAT OF DATA SHEETS

No. ..................   Date........

Informant:------------- Age -----------Sex----------Address--------

Length of Practice :------------------------

Knowledge of phytotherapy (family tradition or acquired):---------

Specialty, if any : ______________________________

Botanical Name : ________________________________

Local Name : _________________________________

Disease or condition : __________________________

Plant part used and form : ______________________

Recipe and Dosage : ____________________________

Duration of Treatment : _________________________

Food restrictions if any : ______________________

Fig. 3A