CHAPTER – 3

METHODOLOGY
MATERIALS AND METHODS

To identify antifungal properties of selected plants from Durg, Rajnandgaon and Kawardha districts of Chhattisgarh State and to identify responsible active biomolecules in the plants to inhibit human pathogenic fungi, the following methodological steps were followed:

1. Selection of plants.
2. Preparation of extracts.
3. Collection of fungal organisms.
4. Antifungal study.
5. Phytochemical evaluation.
6. Statistical analysis of the data (“t” test)

3.1 PLANTS SELECTED FOR STUDY

On the basis of available traditional knowledge in the society, ten medicinal plants were selected for study from three districts. Durg, Rajnandgaon and Kawardha of Chhattisgarh state of India with the following geographical informations.

The Latitude and Longitude of Durg district ranges between- 20°23’ and 22°02’ N and 80°46’ and 81°58’ E. The Latitude and Longitude of Rajnandgaon district is between20°07’ and 22°29’ N and 80°23’ and 81°24’ E. The Latitude and Longitude of Kawardha district is between- 21°32’ and 22°28’ N and 80°48’ and 81°48’ E.
Following four plants were collected from different geographical area of Durg district (College campus, Patan, Nagpura, Bori and Anjora).

1. *Moringa oleifera* (*Moringaceae*)

2. *Sphagneticola trilobata* (*Asteraceae*)

3. *Chrysanthemum morifolium* (*Asteraceae*)

4. *Parthenium hysterophorus* (*Asteraceae*)

Following three plants were collected from different geographical area of Rajnandgaon district (Chuikhadan, Thakurtola, Manpur and Banjari).

5. *Achyranthes aspera* (*Amaranthaceae*)

6. *Lantana indica* (*Verbenaceae*)

7. *Jatropha curcas* (*Euphorbiaceae*)

Following three plants were collected from different geographical area of Kawardha district (Bodla, Chilpi, Bhorumdev and Pondi).

8. *Curculigo orchioides* (*Amaryllidaceae*)

9. *Asparagus racemosus* (*Asparagaceae*)

10. *Cymbopogon citratus* (*Poaceae*)

All plants were taxonomically identified by Department and further verified by Botanical Survey of India, Allahabad and Botanical Survey of India, Kolkata.
Vaucher samples were deposited in the Department of Biotechnology Govt V.Y.T PG. Autonomous College, Durg, (C.G) for further needful investigation.

3.2 PREPARATION OF EXTRACTS

After collection of plants from their respective area in respective season it was sterilized and shade dried for buffer stock. Flowers, leaf, stem, root, (bark and seed were also considered for *Moringa oleifera* and *Jatropha curcas*) was separated out and stored separately for each plants. During the extraction process, the plant parts were powdered properly and then processed with Soxhlet Extraction Unit (MSW, India) All extracts were prepared in two solvents, methanol and aqueous for each plant parts in the ratio of 1:10. Freshly prepared extracts were categorized in to different concentrations (100,200,300 and 400µl) for further investigations. The extracts thus obtained were used for the invitro studies (Parihar and Bohra, 2002).

3.3 COLLECTION OF FUNGAL ORGANISMS

Following pathogenic fungal strains were collected from Microbial Type Culture Collection and Gene Bank of Institute of Microbial Technology (IMTECH) Chandigarh, India. Culture was properly maintained in Microbiology lab of Department of Biotechnology of our Res Centre. The sample for experiment procured from IMTECH, Chandigarh was following-

a) *Microsporum canis* (MTCC - 2820)

b) *Epidermophyton floccosum* (MTCC - 613)
c) *Trichophyton rubrum* (MTCC - 296)

d) *Aspergillus candidus* (MTCC - 1989)

3.4 ANTIFUNGAL STUDY

Media used:

Potato Dextrose Agar (Himedia, India) was used for *Microsporum canis* - Sabouraud Dextrose Agar (Himedia, India) for *Trichophyton rubrum* and *Epidermophyton floccosum*. Czapack Yeast extract Agar (Himedia, India) for fungus *Aspergillus candidus*.

Media preparation:

i. Potato Dextrose Agar (Himedia, India) was prepared for *Microsporum canis* culture. Their compositions are as follows:

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Gms / Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato (Infusion)</td>
<td>200.mg</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.0g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 litre</td>
</tr>
</tbody>
</table>

PH after sterilization (at 25°C) 5.6±0.2

39.0 gms of dehydrated media powder was dissolved in 1000ml of distilled water, and was heated till the medium was completely dissolved and then sterilized by autoclaving at 15lbs pressure at 121°C for 15 minutes.
ii. Sabouraud Dextrose Agar (Himedia, India) was prepared for *Trichophyton rubrum* and *Epidermophyton floccosum* culture. Their compositions are as follows-

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Gms / Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>40.000</td>
</tr>
<tr>
<td>Mycological peptone</td>
<td>10.000</td>
</tr>
<tr>
<td>Agar</td>
<td>15.000</td>
</tr>
</tbody>
</table>

PH after sterilization (at 25°C) 5.6±0.2

65 grams of dehydrated medium powder was dissolved in 1000 ml distilled water, homogenized and sterilized by autoclaving at 15 lbs pressure (121° C) for 15 minutes and further used for culture.

iii. Czapack Yeast extracts Agar (Himedia, India) was prepared for fungus *Aspergillus candidus*. Their compositions are as follows-

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Gms / Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>30.000</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.000</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>1.000</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>0.300</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.050</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.050</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.001</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>0.001</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>0.0005</td>
</tr>
</tbody>
</table>
Agar 15.000

PH after sterilization (at 25°C) 7.3 ± 0.2

51.40 grams of ingredients was suspended in 1000 ml distilled water. This medium was completely dissolved when heated to boiling. The same was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Finally, it was mixed well and poured into sterile Petri plates.

Inoculum preparation:
Tests organisms were aseptically inoculated on Petri dishes containing suitable agar medium. *Microsporum canis*, *Epidermophyton floccossum*, and *Aspergillus candidus* were incubated at 25°C for 72 hrs. whereas *Trichophyton rubrum* was incubated at 30°C for 72 hrs.

Antifungal assay:
To evaluate in-vitro antifungal property of plants, the disc diffusion method was adopted. It is a modification of the description by Bauer *et al.*, (1966) For each extract (both solvent), the parts of all plants were tested against each fungal strains considered for experiments in replicate of five. A parallel control set was also maintained. The fungal growth was determined by measuring the diameter of zone of inhibition in millimeter.

3.5 PHYTOCHEMICAL EVALUATION
Plant’s parts with positive result were subjected for phytochemical evaluation. The presence of Saponin, Tannins, Alkaloids, Terpenoids, Steroids, Flavonoids
and Cardiac glycosides were detected by using appropriate phytochemical screening methods of Harborne, (1973, 1998); Trease and Evans, (1989); Sofowora, 1993 and Brindha et al., (1981). Plants extracts were taken in separate test tubes and lyophilized in a Lyophilizer to get dried extract.

Test for saponins:

The dried plant extract was dissolved in equal amount of distilled water and shaken vigorously. Formation of froth at the upper surface of the solution indicated the presence of saponin. Amount of froth formed, determines the amount of saponin present in the extract (Sofowora, 1993).

Tests for Tannins:

The dried plant extract was dissolved in equal amount of distilled water and mixed by vigorous shaking, followed by heating for few minutes and filtered. Added 2 or 3 drops of saturated aqueous ferric chloride solution to the filtrate and observed for appearance of brown red precipitate; determines the amount of tannin present in the extract (Harborne, 1973).

Tests for Terpenoids:

The dried plant extract was mixed with 2 ml of chloroform, shaken well and added 3 ml of concentrated sulphuric acid (very carefully) A reddish brown coloured ring at the interface indicated the presence of terpenoids in the extract (Harborne, 1973).

Tests for Steroids:
The dried plant extract was mixed with 2 ml of chloroform, then it was filtered and 3 ml of concentrated sulphuric acid (very carefully) was added to filtrate. A reddish brown colour at the interface indicated the presence of steroids in the extract (Harborne, 1973).

Tests for Flavonoids:
The dried plant extract was dissolved in equal amount of distilled water and mixed by vigorously shaking and filtered, then 2-3 ml dilute aqueous ammonia solution was added followed by addition of 2 ml concentrated sulphuric acid (very carefully). A yellow coloration observed indicated the presence of flavonoids in the extract. The amount of flavonoids present in the extract was indicated by the extent of coloration (Sofowara, 1993).

Tests for Cardiac Glycosides:
Added 2 ml of glacial acetic acid, 1 or 2 drops of saturated aqueous ferric chloride solution and 1 ml of concentrated sulphuric acid (very carefully) in the dried extract of the plant and observed for appearance of reddish brown coloured ring at the interface indicating the presence of cardiac glycosides in the plant extract. (Trease and Evans, 1989).

Tests for Alkaloids:
Added 2 ml of glacial acetic acid, 2 ml of methanol and few drops of diluted ammonia solution in the dried extract of the plant and observed for appearance of
white precipitate, indicating the presence of alkaloids in the plant extract (Brindha et al., 1981).

As per need some secondary metabolites were analyzed using C\textsubscript{18} column for HPLC. HPLC separation was performed in a Dionex prep HPLC system coupled with Gynkotek GINA 50 auto sampler and Dionex UVD 3403 Photo Diode array Detector and ELSD-4-Luna (Evaporative Light Scattering Detector) C\textsubscript{18} preparative HPLC column (10μ, 250mm × 21.2mm) was used. Sep Pack DSC-18 Supleco 10g cartridge was used for prep HPLC fractionation. HMBC spectra were optimized for a 10 ng range JH-C of 9 Hz and NOSEY experiment was carried out with a mixing time of 0.8 sec.

3.6 STATISTICAL VALIDATION

For statistical validation of data to test the significance level of zone of inhibition, student “t” test was applied.