CHAPTER - III
MATERIALS AND METHODS

1. Selection of Plant Materials:

Laying emphasis on the objective of this study, a concentrated effort has been made to review literature on the study of plants containing essential oils. The literature reviewed in this line is the work of a number of scientists like Kanjilal et al., 1932; Mukherji, 1948; Chopra et al., 1956; Jain, 1968; Guenther, 1972; Kritikar and Basu, 1975; Chaurasia and Kher, 1978; Tripathi et al., 1986; Jaiswal, 1990; Thakur, 1993; and Grace, 2002. Emphasis was laid on plants that are used regularly in our day-to-day needs. The spices have their own characteristic odors, which is due to the presence of the specific volatile oils in them. Among the different spices, ginger occupies a special niche due to factors like - natural occurrence, abundance and presence of high amount of volatile oil. Considering the above factors, it was decided to select Zingiber as the test genus. Among the Zingiber species found in Assam, Z. officinale Rosc. is used regularly as a spice. Z. zerumbet (L.) Smith. is used by some communities, but it is not commonly used for culinary purposes. It is known to possess medicinal properties. Z. capitatum Roxb. and Z. purpureum Rosc. are also known to have medicinal properties. As reported in ‘The wealth of India’, Z. casumunar Roxb. rhizomes are used as medicine. They are also
used for flavoring food preparations. The rhizome is said to possess stimulant and carminative properties and are also considered useful in diarrhea and colic. *Z. capitatum* Roxb. occurs widely all over Assam. Taking into consideration the above information the following four Zingiber species were selected for extraction of oil.

1) *Zingiber officinale* Rosc. var Sadiya - collected from Kahikuchi Research station, Kahikuchi, Guwahati.

2) *Z. zerumbet* (L) Smith - collected from Manas Reserve Forest, Assam.

3) *Z. capitatum* Roxb - collected from Manas Reserve Forest, Assam.

4) *Z. purpureum* Rosc - collected from Manas Reserve Forest, Assam.

Different parts, i.e. leaves, inflorescences and rhizomes of the four species were collected separately and tested for the presence of the essential oil.

2. **Isolation of essential oils:**

The pulverized material was subjected to hydro-distillation in order to collect the essential oils that may be present in them. The method of Guenther (1972) was followed for the purpose. The leaves and inflorescence did not yield any noticeable amounts of oil, so those parts were discarded. The rhizomes yielded noticeable amounts of oil, in all the four species of Zingiber. The rhizomes were washed properly, surface sterilized in 0.1% aq. mercuric
chloride for five minutes and then washed with sterile distilled water to remove traces of mercuric chloride. The water is soaked up with blotting papers. The fresh weights of the rhizomes were taken. They were then pulverized in a sterilized mortar and pestle and subjected to hydro-distillation in a Clevenger's Apparatus (Plate I). The oil was suspended over an aqueous layer. The oil, which is highly aromatic, was collected and then stored in the refrigerator for further experimentation.

3. Aerobiological studies and selection of test fungal studies:

Aerobiological studies were conducted at various sites of Guwahati, in order to study their aeromycofloral population, which may cause allergic disorders in human beings.

i) Sampling technique:

The aeromycofloral population at the seven different sites was studied by using the Andersen’s sampler. A three-stage sampler was used. The sampling was carried out at an interval of 10 days and each exposure was of five (5) minutes duration. The Petri dishes, plated with Sabouraud’s medium, were placed in the sampler (three plates were used for a single exposure). All the seven sites were covered in a single day. The exposed plates were stored properly and on reaching the laboratory the exposed plates were placed in a
Clevenger’s Apparatus

PLATE - I
B.O.D incubator and maintained at a temperature of 28°C ±1°C for a period of 5-7 days, depending on the growth of the fungal colonies. The medium was treated with an antibiotic, prior to plating, to avoid the growth of bacterial colonies. One plate from each site was maintained at a high temperature range of 40°C-45°C to observe the growth of thermophilic fungal species. The plates were then studied and the fungal genera identified with the help of published literature viz – Ainsworth *et al.*, 1973; Barnett and Hunter, 1972; Ellis, 1971, '76; Ellis and Ellis, 1985; Funder, 1953; Gilman, 1959 and Tilak, 1987.

**Andersen’s Sampler (Plate II):**

The Sampler first used by Andersen (1958), is used for the estimation of culturable fungi. A three-stage sampler is used, which is provided with three sieve plates, each having 400 pores on it. Each sieve plate has a different pore size and is stacked one above the other in increasing order of pore size. The sampler sucks in 28.3 litres/minute of air through the opening at the top and impinges it successively onto the Petri dishes containing 27ml of nutrient media, placed below the sieve. Finally the air passes out impacting on the last Petri dish. The particles of similar dimension get impinged on the same plate. The sampler is AC power driven. In the present study, three Petri dishes containing Sabouraud’s Agar Medium were exposed at one time.
Andersen's Sampler

PLATE - II
Conversion factor of the Andersen's Sampler:

Suction rate of air = 28.3 l/min.

i.e. In 1 min, air taken in = 28.3 l,

So, in 10 min, air taken in = 28.3 \times 10

= 283 l

= 0.283 m³

And, in 5 min, air taken in = \frac{0.283}{10} \times 5 m³

= 0.1415 m³

(i) Now, if we consider 0.283 m³ to have 1 colony,

Then, 1 m³ will have = \frac{1}{0.283} = 3.53 colonies.

(ii) Similarly if we consider 0.1415 m³ to have 1 colony,

Then, 1 m³ will have = \frac{1}{0.1415} = 7.067 colonies.

Therefore, conversion factor for 5 min = 7.067.

(ii) Selection of test fungal species:

Four test Aspergillus species were selected, basing on the following factors-
1. The species *Aspergillus nidulans* (Eidam) Winter and *Aspergillus niger*, Van Tieghem were the most dominant, found to occur at all the sites all round the year.

2. *Aspergillus flavus* Link and *Aspergillus fumigatus* Fresenius are two thermophilic species.

3. All the four species are known to be harmful to man, being responsible for a number of diseases, together known as aspergilloses. They cause a number of allergic disorders like asthma, mycoses, other respiratory problems and a serious disorder, diagnosed as ABPA (Allergic Broncho Pulmonary Aspergillosis), of the respiratory tract.

The identities of the four Aspergillus species were confirmed at the Microbial Type Culture Collection & Gene Bank (MTCC) of the Institute of Microbial Technology (IMTECH), Chandigarh.

4. **Culture Medium:**

Two media namely Sabouraud’s Agar medium and Czapeck’s Dox Agar medium were used during the course of this study.

The Sabouraud’s medium is very sensitive, so it was used to trap and culture the aeromycoflora at the different sites. The Czapeck’s medium was used in the later stages, for testing the antifungal efficacy of the oils. It has been
used to minimize contamination. Both the media are treated with an antibiotic to avoid bacterial contamination. The compositions of the two media are:

(i) Czapek’s Dox Agar Medium (pH - 7.3):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>30.0g</td>
<td>3.0%</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>2.0g</td>
<td>0.2%</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>1.0g</td>
<td>0.1%</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.5g</td>
<td>0.05%</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>0.5g</td>
<td>0.05%</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.01g</td>
<td>0.001%</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0g</td>
<td>1.5%</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

(ii) Sabouraud’s Agar Medium (pH - 5.6):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>40 g</td>
<td>4%</td>
</tr>
<tr>
<td>Peptone</td>
<td>10 g</td>
<td>1%</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
<td>2%</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5g</td>
<td>0.05%</td>
</tr>
<tr>
<td>Rose Bengal Dye</td>
<td>0.03g</td>
<td>0.003%</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
<td></td>
</tr>
</tbody>
</table>
(5) Preparation of inoculum:

The test fungi were cultured in Petri plates containing 10 ml of Czapeck's culture medium. Mycelial discs of 5 mm diameter cut from the periphery of 7-day-old cultures, along with the adhering agar, served as inoculum. Care was taken to maintain a regular supply of pure, seven day old cultures all through the period of experiments. The cultures were maintained at 28°C ± 1°C in a B.O.D. incubator (Singh et al., 1997).

(6) Assessment of antifungal efficacy:

The oils were tested for their antifungal efficacy against the test fungi by the poisoned food technique of Grover and Moore, 1962 and fungi toxicity of the oil vapors were tested using the inverted Petri plate technique of Rao and Srivastava, 1994.

In the poisoned food technique, 0.01 ml of the oil was dissolved in 0.05 ml of acetone in sterile Petri dishes and mixed thoroughly in 9.5 ml of medium to obtain 1000 ppm concentration. Control sets were set up in a similar manner using sterile distilled water in place of oil. The inoculated Petri dishes were then incubated in a B.O.D. incubator at 28°C ± 1°C for 6 days.

In the inverted Petri plate technique, inoculated plates are inverted and pre-sterilized filter paper discs (15 mm diameter, Whatman No-44), are
aseptically transferred to the center of the inverted lids. Then, the desired amount of oil, dissolved in 0.5ml of acetone, is pipetted aseptically in the center of the filter paper discs. In the control set, the oil was replaced by an equal amount of sterilized distilled water taken in 0.5ml acetone. Three replicates are used in each test.

(i) **Dose calculation of vapors of essential oil:**

The dose of vapors of the essential oils was calculated following Rao and Srivastava (1994), as ppm, i.e. parts of oil (vol) per million parts of medium free space in the Petri plates (vol), available for the oil vapors' diffusion. A 80mm diameter Petri plate, used for the experiments has a total average inner volume of (60+2) ml. Each Petri plate contains 10ml medium, so the available space in the inverted Petri plate, for the diffusion of the oil vapors is 50ml.

Therefore, ppm dose of oil = [amount of oil (ml) in petri-plate] x 20,000.

The percent inhibition of mycelial growth was calculated using Arora and Dwivedi’s (1979) formula:

\[
\text{Percent inhibition of mycelial growth} = \left[ \frac{(G_c - G_t)}{G_c} \right] \times 100
\]

Where, \(G_c\) = mycelial growth in terms of colony diameter, in control set.

\(G_t\) = mycelial growth in terms of colony diameter, in treatment set.
(ii) **Minimum inhibitory concentration (MIC):**

The minimum inhibitory concentration of the oils was ascertained by the Poisoned food technique of Grover and Moore (1962). Different concentrations of the oil were used viz. 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ppm with respect to the medium. The different concentrations were tested separately against each test fungal species. The percent inhibition of the fungal colony was noted in comparison to the control set.

**Fungitoxicity (Fungicidal or fungistatic):**

The nature of fungi toxicity, i.e. fungicidal or fungistatic, of the oils, was tested by the technique adopted by Thompson (1989). The fungal discs whose growth was completely inhibited were re-inoculated in fresh medium after washing them thoroughly in distilled water and their growth revival noted.

(iii) **Effect of temperature:**

The effect of temperature on the fungi toxicity of the oils was evaluated following Mishra *et al.*, 1993. The oils were subjected to different temperatures, viz; 25°C, 50°C, 75°C and 100°C before testing their antifungal efficacy at 500ppm dose.
(iv) Shelf-life:

The shelf life of the oils were ascertained by storing the oils for different periods and testing them against the fungi viz. testing the oil after storing for 1 month, 2 months, 3 months, 6 months, 12 months, 18 months, 24 months, 30 months and 36 months, following the technique of Mishra et al., (1993).