CHAPTER 4

ISOLATION, PURIFICATION AND CHARACTERIZATION OF ANTIMICROBIAL PRINCIPLE FROM ZINGIBER ZERUMBET
4.1 Introduction

The results of the preliminary screening proved that *Zingiber zerumbet* Smith, shown in Fig. 4.1(a) exhibited considerable antimicrobial activity against the tested microorganisms including bacteria and fungi. Since the material is nontoxic, biodegradable and easily available it has been selected for further study.

Of the various varieties of Zingiber, *Zingiber zerumbet* has the distinction of being a rich source of many novel humulenoid sesquiterpenoids (Chhabra et al., 1975) and is widely cultivated in India (The Wealth of India, 1985). The rhizomes are used as a remedy for cough, asthma, worms, leprosy and other skin diseases (Nadkarni, 1954). The rhizomes are applied to the head of children in convulsions (Perry, 1980). In Philippines, the pulverised rhizomes are used as antidiarrhoeal agent (Guerrero, 1921).

This plant is widespread in South East Asia, India and Okinawa and has found use as spice and ethnomedicine (Jain and Tarafder, 1970; Nagata, 1971; Weiner, 1971; Uhe, 1974; Das and Misra, 1987; Fransworth and Bunyapraphatsara, 1992; Singh et al., 1984). Since anti HIV (Dai et al., 1997) and cytotoxic (Matthes et al., 1980) components were isolated from *Zingiber zerumbet*, the plant finds a prominent place in modern medicine now a days.
4.2 Present scenario on the phytochemistry of Zingiber zerumbet

The rhizomes of Zingiber zerumbet, have been the subject of several previous investigations. These results proved the isolation and characterization of humulenol-II (Damodaran and Dev, 1968), Zerumbone oxide (Chhabra et al., 1975), 3"",4""-O-diacetylfzelin, diferuloylmethane, feruloyl-p- coumaroylmethane, di-p- coumaroylmethane (Matthes et al., 1980), zerumbone (Dai et al., 1997; Matthes et al., 1980; Duve, 1980; Gupta et al., 1979; Kitayama et al., 1999) and three new acetylated and one known kaempferol glycosides (Masuda et al., 1991).

The oil of Zingiber zerumbet has been analysed by Oliveros and Cantoria (1982), Nakatani et al. (1991), Dung et al. (1993) and Damodaran and Dev (1968). The oils of Indian (Nigam and Levi, 1963; Damodaran and Dev, 1968; Balakrishnan et al., 1956), French polynesian (Lechat et al., 1993), Vietnamese (Dung et al., 1993) and Malay origins (Ahmad et al., 1994) have been investigated for their chemical and bioactive constituents.

4.3 Materials and Methods

4.3.1 Collection of plant material

Rhizomes of Zingiber zerumbet shown in Fig. 4.1(b) were collected as described in 3.2.1.

4.3.2 Preparation of crude extracts

The shade-dried and powdered rhizomes of Zingiber Zerumbet (1 kg) were extracted successively with petroleum ether (60-80°C), acetone, chloroform, methanol and water in a soxhlet apparatus. Each extract was
concentrated in a rotary evaporator. These extracts were kept at 4°C until required for further experiments.

4.3.3 Antifungal assay

4.3.3.1 Antifungal activity of crude extracts and fractions separated from Zingiber zerumbet

The five crude extracts (petroleum ether (60-80°C), acetone, chloroform, methanol and aqueous) as well as the six fractions (Table 4.1) from column chromatography were tested for antifungal activity against Aspergillus flavus, Aspergillus niger, Phytophthora meadii, Trichoderma sp., Penicillium chrysogenum and Candida albicans as described in the previous chapter, 3.3.

4.3.3.2 Determination of the minimum inhibitory concentration of zerumbone

The minimum inhibitory concentration of zerumbone was carried out according to the method given by Rippon (1982).

Preparation of the stock solution of zerumbone

Dissolved 10 mg of pure zerumbone in 10 ml of a solution containing 9.5 mL sterile water and 0.5 mL dimethyl sulphoxide (DMSO). Therefore the concentration of the stock solution was 1mg mL⁻¹.

Experimental procedure

Set up five empty tubes labelled 1 to 5 and the stock solution was serially diluted with SDA to obtain solutions having concentration ranging from 62.5 μg/mL⁻¹ to 500 μg/mL⁻¹. Since agar was used each tube was slanted.
When cooled, the medium was inoculated with a standardized suspension of spores of the fungus/yeast cells. Control tubes containing no zerumbone were also inoculated. After 72 hrs of incubation, the tubes were examined for visible growth. The highest dilution (minimum concentration of zerumbone) inhibiting the visible growth was considered as the minimum inhibitory concentration. The test was done in duplicate.

4.3.4 Separation of the crude extract using Thin Layer Chromatography (TLC)

This involves the differential adsorption of substances as they pass through a uniform layer of inert adsorbent, which has been uniformly spread over a suitable supporting plate such as glass. TLC is an excellent qualitative and quantitative method for separating the components of a mixture. In TLC a thin layer of the stationary phase is formed on a suitable flat-surface such as glass, foil or plastic plate. Since the layer is thin, the movement of the mobile phase across the layer generally by simple capillary action, is rapid, there being little resistance to flow. As the mobile phase moves across the layer from one edge to the opposite, it transfers any analytes placed on the layer at a rate determined by their distribution coefficients between the stationary and mobile phases. The movement of the analyte is expressed by its retardation factor.

\[
R_f = \frac{\text{Distance moved by analyte from origin}}{\text{Distance moved by solvent from origin}}
\]
4.3.4.1 Adsorbents for TLC

In all chromatographic procedures, the optimum condition for separation is yielded through mutual harmonisation of the stationary phase and the mobile phase. In this study silica gel was used as the stationary phase.

4.3.4.2 Preparation of thin, uniform layers

Homogenous, thin uniform layers were prepared by spreading suspensions of silica gel on glass plates of size 20 x 20 cm. The suspension containing silica gel in water usually in the ratio 1:2, was run into a rectangular spreader with the possibility of simple adjustment of layer thickness between 0 and 2 mm. The spreader was passed in one movement over a row of glass plates which were held firmly on an aligning tray. The coated plates were left on the tray for pre-drying. After about 10 minutes, the plates were stacked in a drying rack and heated in the vertical position for 30 minutes at 110°C. Opened the door of the drying cabinet from time to time to allow the moist air to escape. Very active layers were obtained by heating silica gel for 3-4 hrs at 150°C.

4.3.4.3 Application of the crude extract for separation and development

The crude extract was applied to the “start” (starting point) as a spot (5 μL) using micropipettes with a capacity of 10 μL. Chromatographic development was carried out in a rectangular tank which had already been saturated with the developing solvent system i.e. n-hexane-ethyl acetate (96:4) v/v. Chamber saturation was established by pressing a smooth sheet of filter paper, about 15 x 40 cm, soaked in the solvent which has already been
placed in the trough against the sides of the chamber. The paper was again soaked in the solvent by tilting the chamber before the plate was introduced.

Separations were carried out at room temperature and in the presence of diffuse day light. The chamber was filled with the solvent system to a depth of about 0.5 cm. The lid of the chamber must fit tightly. The whole breadth of the TLC plate must be dipped into the solvent to the same depth, i.e. 0.5 cm but not so as to cover the spots at the start. For the preliminary tests, 5 cm and 10 cm plates were used.

4.3.4.4 Visualisation of substances on chromatogram

5% Con. H₂SO₄ in ethanol was taken as the visualising agent in this experiment. The reagent was applied as a fine spray in order to distribute it uniformly over the whole layer. Optimum colour development was attained by heating the plate after spraying 5% Con. H₂SO₄ in a small oven with temperature adjustable up to 250°C.

4.3.4.5 Preparative thin layer chromatography

Preparative TLC was carried out using thick (upto 1 mm) instead of thin layers of silica gel adsorbent. Separated components were recovered by scrapping off the adsorbent at the appropriate places on the developed plate, eluting the powder with solvent ether. It was centrifuged to remove silica gel. Each fraction was concentrated and antimicrobial assay was done using disc diffusion method. Since the quantity of the fractions obtained by preparative TLC was insufficient to carry out antimicrobial activity, column chromatography was done to separate the components.
4.3.5 Column chromatographic separation

Column chromatography is accomplished by applying the material to be tested to one end of a column in which the adsorptive material is packed as uniformly as possible and as the material moves, the various constituents adhere to the surface of the solid particles at different distances from the starting point in accordance with their chemical characteristics.

The dark brown petroleum ether crude extract (1 mL) was transferred to a column of silica gel (70g). The column was eluted successively with n-hexane and ethyl acetate (increasing polarity). Volumes of 25 mL were collected and concentrated. The collected volumes were monitored by TLC and divided into different groups as shown in Table 4.1.

4.3.6 Spectroscopic studies

Bruket DRX 500 NMR system was used to take the ¹HNMR and ¹³C NMR spectra of the active fraction.

4.4 Results and Discussion

4.4.1 Fractions separated from Zingiber zerumbet using TLC

The dark brown petroleum ether extract showed five prominent spots with Rₜ values 0.99, 0.92, 0.84, 0.54 and 0.38 (solvent system- petroleum ether: ethyl acetate 96:4) as shown in Table 4.1. In addition to these spots, the TLC plate showed dark colour at the origin.
Table 4.1  Fractions separated from petroleum ether extract of *Zingiber zerumbet*

<table>
<thead>
<tr>
<th>Eluent (petroleum ether : ethyl acetate) ratio</th>
<th>Fraction number</th>
<th>Group Number</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; value</th>
</tr>
</thead>
<tbody>
<tr>
<td>98:2</td>
<td>1-4</td>
<td>I</td>
<td>0.99</td>
</tr>
<tr>
<td>97.5: 2.5</td>
<td>5-10</td>
<td>II</td>
<td>0.92</td>
</tr>
<tr>
<td>97.3</td>
<td>11-22</td>
<td>III</td>
<td>0.84</td>
</tr>
<tr>
<td>96:4</td>
<td>23-29</td>
<td>IV</td>
<td>0.54</td>
</tr>
<tr>
<td>95:5</td>
<td>30-37</td>
<td>V</td>
<td>0.38</td>
</tr>
</tbody>
</table>

4.4.2 Antifungal activity

The crude extracts prepared from dried rhizomes of *Zingiber zerumbet* as well as six fractions separated from petroleum ether extract of *Zingiber zerumbet* were tested for antifungal activity and the results were as shown in Tables 4.2 and 4.3.

Table 4.2  Antifungal activity of crude extracts from *Zingiber zerumbet*

<table>
<thead>
<tr>
<th>Extract</th>
<th>Name of microorganism</th>
<th>A.f</th>
<th>A.n</th>
<th>P.m</th>
<th>T.</th>
<th>P.c</th>
<th>C.a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td></td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>MG</td>
</tr>
<tr>
<td>Acetone</td>
<td></td>
<td>NG</td>
<td>NG</td>
<td>MG</td>
<td>NG</td>
<td>MG</td>
<td>MG</td>
</tr>
<tr>
<td>Chloroform</td>
<td></td>
<td>MG</td>
<td>MG</td>
<td>ND</td>
<td>NG</td>
<td>MG</td>
<td>G</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>MG</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
</tbody>
</table>

G - Growth, MG - Moderate growth, NG - No growth, ND - Not done

T. - *Trichoderma* sp., P.c - *Penicillium chrysogenum*, C.a - *Candida albicans*
Table 4.3  Antifungal activity of six fractions separated from petroleum ether extract of Zingiber zerumbet

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Name of microorganism</th>
<th>A.f</th>
<th>A.n</th>
<th>P.m</th>
<th>T.</th>
<th>P.c</th>
<th>C.a</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>ND</td>
<td>G</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>MG</td>
<td>MG</td>
<td>MG</td>
<td>MG</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>MG</td>
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<tr>
<td>IV</td>
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<td>NG</td>
<td>MG</td>
<td>MG</td>
<td>MG</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>V</td>
<td></td>
<td>MG</td>
<td>G</td>
<td>G</td>
<td>MG</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>VI</td>
<td></td>
<td>G</td>
<td>G</td>
<td>ND</td>
<td>ND</td>
<td>G</td>
<td>G</td>
</tr>
</tbody>
</table>

G - Growth, MG - Moderate growth, NG - No growth, ND - Not done
A.f - Aspergillus flavus, A.n - Aspergillus niger, P.m - Phytophthora meadii, T. - Trichoderma sp., P.c - Penicillium chrysogenum, C.a - Candida albicans

Since the results showed the highest activity for the third fraction (third fraction was characterized as zerumbone) its minimum inhibitory concentration was determined. The results were as shown in Table 4.4. In this study, the concentrations of 125 µg mL⁻¹ and 500 µg mL⁻¹ were sufficient to inhibit Trichoderma sp. and Candida albicans respectively as shown in Fig. 4.2 (a) and (b). MIC for zerumbone to inhibit fungal growth of Aspergillus flavus, Phytophthora meadii and Penicillium chrysogenum was 250 µg mL⁻¹ (Fig. 4.3 (a), (b) and (c)).

Table 4.4  Minimum inhibitory concentration of zerumbone

<table>
<thead>
<tr>
<th>Name of fungi</th>
<th>Concentration of zerumbone in µg mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichoderma sp.</td>
<td>125</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>250</td>
</tr>
<tr>
<td>Penicillium chrysogenum</td>
<td>250</td>
</tr>
<tr>
<td>Phytophthora meadii</td>
<td>250</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>500</td>
</tr>
</tbody>
</table>
4.4.3 Characterization of fraction III

Antimicrobial assay was done after concentrating each fraction. The results proved the maximum efficacy of Group III. Fractions 11-22 on concentration, obtained a crystalline solid. It was recrystallized from hexane with a yield of 0.85% and designated as compound A \((R_t = 0.84)\) as shown in Table 4.1). Its melting point was found to be equal to 66.5-67°C.

The structure of the active fraction was elucidated by spectroscopic methods using \(^1\)HNMR and \(^{13}\)C NMR. Fig. 4.4(a) and (b) showed the \(^1\)HNMR and \(^{13}\)C NMR spectra of the active component. The data obtained was as follows.

\(^1\)HNMR: \(\delta 1.4\) (s, 3H, CH\(_3\) at C11), 1.6 (s, 3H, CH\(_3\) at C11), 1.9 (s, 3H, CH\(_3\) at C13), 2.1 (s, 3H, CH\(_3\) at C7), 2.2 (d, 1H, H at C1), 2.5 – 2.9 (m, 5H, H at C1, 4 and 5), 5.6 (d, 1H, H at C2), 6.1 (d, 1H, H at C10), 6.35 (d, 1H, H at C9), 6.39 (d, 1H, H at C6).

\(^{13}\)C NMR: \(\delta 12.46\) (CH\(_3\) at C7), 15.7 (CH\(_3\) at C3), 24.7 (CH\(_3\) at C11), 29.9 (CH\(_3\) at C11), 39.6 (C4), 42.6 (C1), 125.4 (C2), 127.8 (C9), 136.8 (C3), 138.1 (C7), 148.9 (C6), 160.7 (C10), 203.5 (C8).

Fig. 4.4 (a) \(^1\)HNMR spectrum of the active fraction (Zerumbone)
Hence the antimicrobial principle was characterized as *zerumbone*-a sesquiterpene, having the structure as shown in Fig. 4.5.

![Zerumbone](image)

**Fig. 4.5** Zerumbone

### 4.4.3.1 Properties and derivatives of Zerumbone

M.F. C_{15}H_{22}O, M. P. 66.5-67°C. Soluble in almost all organic solvents but insoluble in aqueous alkali.

On hydrogenation using Adam’s catalyst in acetic acid Zerumbone gave hexahydro derivative having M. P. = 60 – 61.5°C (Gupta et al., 1979). Kitayama et al. (1999) explained the conjugate addition reactions of zerumbone with potassium cyanide, KCN in presence of α - cyclo dextrin, methanol in presence of potassium tert-butoxide, methanol in presence of BF_{3} etherate and Bromine. By treating zerumbone with perbenzoic acid an epoxy
ketone identified as zerumbone oxide $C_{15}H_{22}O_2$, M. P. 110°C was formed as the major product (Chhabra et al., 1975). Catalytic reduction of zerumbone oxide afforded a mixture of dihydro and tetrahydro epoxy ketones (Chhabra et al., 1975). 8-Hydroxy-α-humulene was obtained from zerumbone by NaBH$_4$ reduction (Murakami et al., 1999).

Zerumbone exhibited a wide spectrum of biological activities such as anti-tumor promoter (Murakami et al., 1999; Vimala et al., 1999; Fujimoto et al., 1980), an HIV inhibitory effect (Dai et al., 1997), Cytotoxic (Dai et al., 1997; Matthes et al., 1980), antidiarrhoeal (Guerreno et al., 1921) and antitubercular activity (Ramaswamy and Sirsi, 1967). Kalsi et al. (1979) formulated a pesticide from zerumbone and they (1980) reported that zerumbone acts as plant growth regulator.