

## THE ANTIPROLIFERATIVE EFFECTS OF ZINGIBER ZERUMBET EXTRACTS AND FRACTIONS ON THE GROWTH OF HUMAN BREAST CARCINOMA CELL LINES

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*Zingiber zerumbet*, from Zingiberaceae family, is known for its medicinal properties. From the ethnopharmacology and laboratory studies, extracts and active component of this plant, zerumbone, were reported to have the antiinflammatory, antioxidant, antimicrobial and antitumour properties. However, no studies have been done to verify the antiproliferative activity of this plant extracts on breast cancer cells. The present study investigated the effects of extracts and fractions of *Z. zerumbet* on the growth of human breast carcinoma (MCF-7) cell lines. The number of living cells was evaluated by methylene blue staining technique. Our data indicated that *Z. zerumbet* extracts and some fractions exhibited antiproliferative effect on MCF-7. Bioassay-guided fractionation of petroleum ether extract, the most active crude extract, with EC<sub>50</sub> of 4.25 ± 0.05 µg/mL resulted in the isolation of two active subfractions. One of them was found to contain 2,6,10-cycloundecatrien-1-one, 2,6,9,9-tetramethyl with 94% similarity to zerumbone obtained from the GC-MS analysis. A comparative study between both subfractions and tamoxifen showed that the active subfractions of *Z. zerumbet* displayed a strong antiproliferative effect with EC<sub>50</sub> of 2.81 ± 0.24 and 2.49 ± 0.13 µg/mL, respectively, on MCF-7 but less cytotoxic to normal cells.

**Keywords:** *Zingiber zerumbet*, zerumbone, bioassay-guided fractionation, cell proliferation assay

### INTRODUCTION

Natural substances serve as the sources of most drugs and medicinal agents. Several of these substances are believed to have potential value as cancer chemopreventive or therapeutic agents. In addition, plant natural products play an important role in approximately 60 available cancer chemotherapeutic drugs (Kinghorn *et al.* 1999) and isolation of active components from plants has become a major challenge for pharmaceutical industry (Gebhardt 2000). One substance obtained from plants

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namely, *Zingiber zerumbet*, has been reported to be used in cancer chemoprevention and chemotherapy.

*Z. zerumbet*, a wild ginger belonging to Zingiberaceae family, is widely cultivated throughout the tropics for its medicinal properties. It grows in tropical and subtropical areas especially in South East Asia, Hawaiian Islands, Alaska and Puerto Rico. It is used in local traditional medicine to treat constipation in babies, relieve stomach ache and fever in children, and also to treat debility and vertigo in postpartum women (Perry and Metzger 1980). Isolation of several components from this plant has been reported previously (Damodaran and Dev 1968). However, among all the components found in this plant, zerumbone is the most active component reported to has bioactivity in both *in vitro* and *in vivo* studies.

Zerumbone is a sesquiterpene and has been proved to be one of the 4 major components in *Z. zerumbet* essential oil, percentage yield of 12.6% (Srivastara *et al.* 2000). Previous laboratory experiments have successively demonstrated the antitumour activity of zerumbone, cytotoxic effects on hepatoma (HTC) cell lines (Matthes *et al.* 1980) and also inhibited the proliferation of human colonic adenocarcinoma cell lines in dose-dependent manner, whilst the growth of normal human dermal and colon fibroblasts were less affected (Murakami *et al.* 2002). An appropriate dose of zerumbone induced a high intracellular redox potential which stopped the proliferation of cancer cells, but not the normal cells (Hoffman *et al.* 2002). These reports suggested that zerumbone is a food phytochemical which has potential chemopreventive and chemotherapeutic strategies against cancer.

Therefore, this present study was carried out to investigate the inhibitory effect of *Z. zerumbet* extracts and fractions on human breast cancer (MCF-7) cell lines, using bioassay-guided fractionation principle. The active component of the plant was isolated by the aforementioned method and its antiproliferative activity was compared with tamoxifen, the standard chemotherapeutic agent for breast cancer.

## METHODS

### Materials

Zerumbone used as a standard was obtained from Professor Hasnah Mat Sirat from Universiti Teknologi Malaysia, Skudai, Johor, Malaysia.

Tamoxifen was supplied by Sigma Chemicals Comp., St Louis, USA. Methylene blue, sodium bicarbonate, sodium dihydrogen phosphate, disodium hydrogen phosphate and sodium chloride were purchased from BDH Chemicals Ltd, Poole, England. Organic solvents used during extraction were of analytical grade, and silica gel 60 were purchased from Merck, Darmstadt, Germany.

### **Extraction and isolation**

The extraction procedure for dry powder of *Z. zerumbet* is shown in Figure 1. One kilogram dried and ground rhizome was extracted using soxhlet extractor with different organic solvents successively. Each of the crude extract was concentrated in vacuum. Ten gram of petroleum ether crude extract was fractionated on a silica 60 column using petroleum ether and diethyl ether. One hundred millilitre of fractions were collected and analysed by SiO<sub>2</sub> thin layer chromatography (TLC) using petroleum ether/diethyl ether, 3:1, as the mobile phase. Fractions with similar TLC profile were combined to give A, B, C, D, E and F major fractions. The most active fraction in the cell proliferation assay, fraction C, was eluted in the same column; and six major subfractions, CA, CB, CC, CD, CE and CF, were obtained. All the extracts and fractions were tested for their antiproliferative activity.

### **Cell lines and culture condition**

The human breast carcinoma (MCF-7) and normal bovine kidney (MDBK) cell lines were obtained from American Type Culture Collection (ATCC), Maryland, USA. They were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) in 75 cm<sup>2</sup> flasks at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

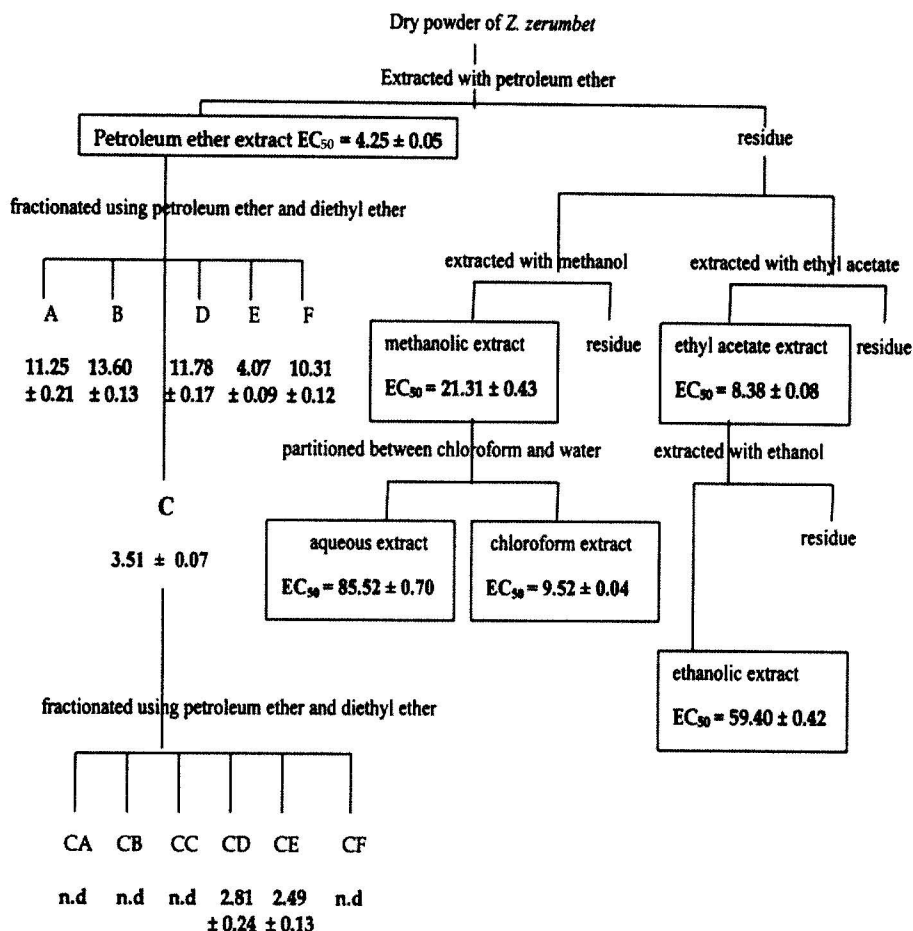


Fig. 1: Bioassay-guided fractionation of dried and ground rhizome of *Z. zerumbet*.  $EC_{50}$  values were expressed as the mean  $\pm$  s.e.m ( $\mu\text{g/mL}$ );  $n = 5$   
 \*n.d = no  $EC_{50}$  value determined

## Cell proliferation assay

Cells were seeded in 96 wells plate ( $5 \times 10^4$ – $5 \times 10^5$  cells/mL) containing DMEM, supplemented with 5% FBS. The cells were treated with extracts and fractions at increasing concentrations ranging from 0.38–9.99  $\mu\text{g/mL}$ . The assay was done using the method previously described (Lin and Hwang 1991). Briefly, after 3 days of treatment, glutaraldehyde at final concentration of 2.5% was added to fix the viable cells. The plate was agitated for 15 mins to accelerate the fixation. Then, the media containing

glutaraldehyde was discarded and washed with 0.15 M NaCl in order to remove dead cells. To stain the fixed cells, 100  $\mu$ L of 0.05% methylene blue was added to each well and agitated for 15 mins, mainly to stain the fixed cells. Excess dye was washed away and 200  $\mu$ L of 0.33 M HCl was added to remove methylene blue. Absorbance was read at 660 nm using Dynatech MR5000 microplate reader. Cells also treated with tamoxifen (the same concentration of extract), for the positive control. Dimethyl sulfoxide (DMSO) (final concentration of 1%) was used as the negative control. Percentage of viable cells was obtained using the following formula:

$$\frac{\text{Absorbance of extract}}{\text{Absorbance of control}} \times 100\%$$

The concentration of each extract required to inhibit 50% of cell proliferation,  $EC_{50}$ , was determined. Each experiment was carried out in 5 replications and the results were expressed as their mean  $\pm$  S.E.M.

## RESULTS AND DISCUSSION

Results of the activity-guided fractionation of *Z. zerumbet* extracts and fractions on MCF-7 cell lines were summarised in Figure 1. Petroleum ether extract was the most effective crude extract ( $EC_{50}$  value of  $4.25 \pm 0.05$   $\mu$ g/mL) in reducing cell proliferation of MCF-7. Both ethyl acetate and chloroform extracts showed reasonable inhibitory effects on MCF-7,  $EC_{50}$  values of  $8.38 \pm 0.08$  and  $9.52 \pm 0.04$   $\mu$ g/mL, respectively. Methanolic and ethanolic extracts showed low antiproliferative effects,  $EC_{50}$  values of  $21.31 \pm 0.43$  and  $59.40 \pm 0.42$   $\mu$ g/mL, respectively.

Therefore, the petroleum ether crude extract was further fractionated, yielding fraction C (yellowish crystals). Fraction C possessed  $EC_{50}$  value of  $3.51 \pm 0.07$   $\mu$ g/mL. As such, this fraction was further purified using the same column and condition, yielding 6 major subfractions. From the cell proliferation assay, subfractions CD and CE were the only active subfractions with  $EC_{50}$  values of  $2.81 \pm 0.24$  and  $2.49 \pm 0.13$   $\mu$ g/mL, respectively. Others, subfractions CA, CB, CC and CF, did not possess any antiproliferative effect on MCF-7 cell lines.

TLC profile of fraction C, subfractions CD and CE, showed that these two subfractions consist fewer components compared to fraction C. Furthermore, both subfractions CD and CE contained zerumbone (Fig. 2).

Figure 3 shows the gas chromatography-mass spectrometry (GC-MS) chromatogram of the most active subfraction, CE. Compound zerumbone (2,6,10-cycloundecatrien-1-one, 2,6,9,9-tetramethyl) detected in this subfraction and was found to be 94% similar to zerumbone in GC-MS library.

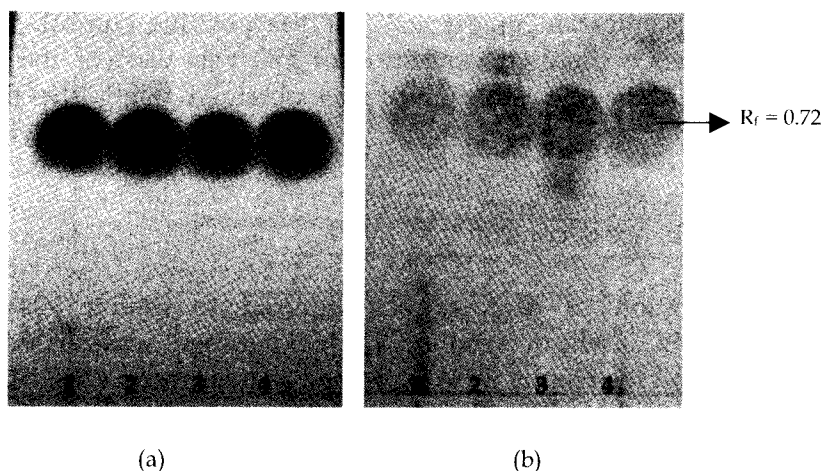


Fig. 2: TLC profiles of *Z. zerumbet* fractions a) under UV 254 nm and b) after react with reagent vanilin. Fraction C (lane 1), subfraction CD (lane 2), subfraction CE (lane 3) and zerumbone standard (lane 4) at same concentration show obviously consist of zerumbone with  $R_f$  of 0.72.

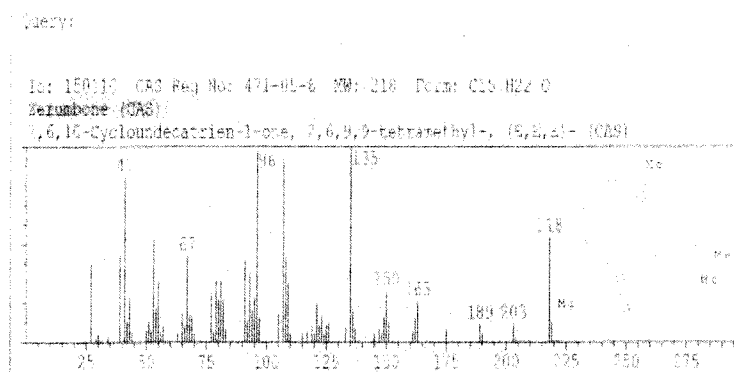


Fig. 3: GC-MS chromatogram of subfraction CE. Subfraction CE was proved to contain a major component with the chemical name 2,6,10-cycloundecatrien-1-one, 2,6,9,9-tetramethyl. This component was believed as zerumbone based on the chemical structure and the similarity index (SI) of 94%.

**Table 1:** EC<sub>50</sub> (µg/mL) Values of Subfractions CD and CE against MCF-7 and MDBK Cell Lines Using Methylene Blue Assay

	MCF-7	MDBK
Subfraction CD	2.81 ± 0.24	2.88 ± 0.39
Subfraction CE	2.49 ± 0.13	7.20 ± 0.32
Tamoxifen	4.39 ± 0.21	2.56 ± 0.59

Table 1 shows the antiproliferative effects of subfractions CD and CE, and tamoxifen on both MCF-7 and MDBK cell lines. Tamoxifen exhibited EC<sub>50</sub> values of 4.39 ± 0.21 and 2.56 ± 0.59 µg/mL on MCF-7 and MDBK cell lines, respectively. Subfraction CE, which contains zerumbone proved to be the most effective constituent with EC<sub>50</sub> value of 2.49 ± 0.13 µg/mL against MCF-7, but less cytotoxic to MDBK with EC<sub>50</sub> value of 7.20 ± 0.32 µg/mL.

## CONCLUSION

The above data suggested that bioassay-guided fractionation of *Z. zerumbet* results in active subfractions containing zerumbone. Therefore, further study is suggested to investigate the possible mechanisms of action for this activity.

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