IN VITRO CULTURES FOR PRODUCTION OF ZERUMBONE AND MASS PROPAGATION IN *Zingiber zerumbet* Smith.

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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ABSTRACT

Lempoyang (*Zingiber zerumbet* Smith.) is an important plant which has been propagated because of its medicinal values. Lempoyang is widely used as a folk medicine in Asian countries such as Malaysia, Indonesia, India and Taiwan. Zerumbone, a cyclic sesqueterpene found to be the major and main bioactive compound that gives great potential in medicinal purposes especially in the treatment of chronic diseases based on recent reports. Recently, it was found that zerumbone inhibited the growth of human leukemia cell line, HL-60 cell. Even though there are so many great values contain in the lempoyang but it is hard to obtain rhizome as raw material due to its exposure to pest and fungal infection through conventional propagation. Therefore, tissue culture was used to overcome pest and fungal infection faced through conventional propagation. *In vitro* plant was established in culture and acclimatized before field planting to produce rhizomes, extracted and the amount of zerumbone was compared to *in vivo* rhizomes. The amount was significantly higher in the *in vitro* derived rhizomes than *in vivo* rhizomes from three types of extraction methods used in this research. In addition, another promising alternative for micropropagation that could drive the efficiency of secondary metabolites production through the establishment of regenerable embryogenic cell suspension cultures. Cell suspension established in shake flask was also used as a source of zerumbone in this study. Zerumbone was found excreted in the liquid media. Even though the amount is not comparable from the *in vitro* derived rhizomes, but the presence can be enhanced by manipulating the production of zerumbone in future research.

ABSTRAK

Nilai perubatan yang tinggi menjadikan lempoyang atau *Zingiber zerumbet* Smith. salah satu daripada tumbuhan herba yang ditanam secara meluas. Lempoyang telah digunakan secara tradisional oleh penduduk dari negara di sekitar Asia seperti Malaysia, Indonesia, India dan Taiwan. Bahan bioaktif paling banyak di dalam lempoyang ialah zerumbon, sejenis 'cyclic sesqueterpene' yang mempunyai potensi yang baik dari segi perubatan terutamanya penyembuhan bagi penyakit kronik berdasarkan laporan sebelum ini. Terkini, dilaporkan bahawa zerumbon di dapati membantutkan pertumbuhan sel leukimia manusia, iaitu sel HL-60. Walaupun banyak kebaikan yang terkandung di dalam tumbuhan ini namun untuk mendapatkan rizom adalah sulit kerana tumbuhan ini berdepan dengan jangkitan kulat serta serangan serangga. Sebagai penyelesaian, kultur tisu merupakan satu cara untuk mengelakkan daripada jangkitan kulat serta serta serangan serangga dan penyakit. Tumbuhan kultur tisu yang diperolehi, diaklimatisasi sebelum dipindahkan ke ladang untuk menghasilkan rizom yang kemudiannya diekstrak dan dibandingkan amaun dengan rizom yang diperolehi secara *in vivo*. Hasil zerumbon yang diekstrak menunjukan '*in vitro* derived rhizomes' memberi hasil yang signifikan berbanding rizom *in vivo* daripada ketiga-tiga jenis cara pengekstrakan yang digunakan di dalam kajian ini. Ampaian sel merupakan salah satu kaedah kultur tisu yang dapat menjanjikan hasil tanpa perlu menunggu rizom matang untuk mendapatkan bahan bioaktif yang dikehandaki. Keputusan menunjukkan zerumbon hadir dalam kuantiti yang rendah serta tidak dapat menandingi hasil dari '*in vitro* derived rhizomes'

namun dengan memanipulasi sel ampaian, kadar penghasilan zerumbon boleh ditingkatkan dalam kajian yang akan datang.

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In the name of Allah

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
2,4-D	2,4-dichlorophenoxyacetic acid
B5	Gamborg, Miller and Ojima's, (1968) medium
BAP	Benzylaminopurene
cm	Centimetre
e.g.	For example
EDTA	Ethelenediaminetetraacetic acid
et. al.	Latin: et alii or English: and others
g	gram
GC	Gas chromatography
GCMS	Gas chromatography and Mass spectrometry
IAA	Indole-3 acetic acid
L or l	Litre
mg	Milligram
mg	Milligram
min	Minute
ml	millilitre
MS	Murashige and Skoog, (1962) medium
N6	Chu, (1978) medium
NAA	Naphtalic acetic acid
NaCl	Sodium Chloride
°C	Degree celcius
PGR	Plant growth regulator

pH	The negative logarithm of the hydrogen ion concentration
rpm	Revolution per minute
SCV	Settle cell volume
spp.	Species
TLC	Thin Layer Chromatography
uv	Ultra violet
v/v	Volume per volume
viz.	Latin: Vidalicet or English: namely
w/w	Weight per weight

CHAPTER 1

GENERAL INTRODUCTION

1.1. *Zingiber zerumbet* (L.) Sm

1.1.1. Classification

Zingiber zerumbet or lempoyang is one of the Zingiberaceae species, the largest families of plant kingdom. Most of the plants were used as raw materials in traditional medicine formulations that are commonly found in the market (Perry, 1980). Zingiberaceae was known to have over 50 genera and 1,300 species. Zingibereae is the tribe from zingiberacea where genus *Zingiber* is classified with ninety species around the world based to the IUPAC, 1999. This plant was believed originated from India and Malay Peninsula and being introduced throughout the pacific by Polynesian settlers, distributed by migrating people (Krauss, 1993).

The genus *Zingiber* is one of the important member of Zingiberaceae distributed in tropical and subtropical Asia and Far East Asia and consists of 150 species with medicinal, economic and horticulture significance (Ravinderan *et al.,* 2005). Besides ginger, this family includes turmeric, cardamom, large cardamom, grain of paradise, and others.

The genus *Zingiber* contains the true ginger, a group of plants with both modern and traditional medicinal values besides of culinary value that being

used for dishes preparing in many parts of the world. Table 1.1.1a gives the details of scientific classification of *Zingiber zerumbet* (Wagner *et al.,* 1990).

Table 1.1.1a Scientific classification

Scientific classification
Kingdom: Plantae
Division: Magnoliophyta
Class: Liliopsida
Order: Zingiberales
Family: Zingiberaceae
Tribe: Zingibereae
Genus: *Zingiber*
Species: *Z. zerumbet*
Binomial name *Zingiber zerumbet*

1.1.2.Synonyms and common names

There are many scientific names as well as common names for *Zingiber zerumbet* (L) J. E. Smith. which was better known as *Zingiber zerumbet* Smith. It was commonly known as wild ginger or 'Lempoyang' in the Malay language (Holtum, 1950). It is also known as 'Awapuhi kuwahiwi' in Hawai, 'Krathue' in Thailand, 'Bitter ginger' in North American. Sometimes it is known as 'Pinecone' because of the shape of its flower and 'Shampoo ginger' for the creamy liquid substance in the cones (used as shampoo) in Asia and Hawaii (Nalawade *et al.,* 2003; Sabu, 2003). For summary, table 1.1.2a and 1.1.2b gives the information of the synonyms in English and other language where this plant is grown (CAB International, 2007).

Table 1.1.2a Synonyms of *Zingiber zerumbet* (L.) Sm.

Language	Name
English	wild ginger , zerumbet ginger, shampoo plant
French	zerumbet , gingembre fou, gingembre blanc
Brazil	jenjibre-amargo
Hawaii	Awapuhi kuwahiwi
Indonesia	lampuyang , lempuyang, lampojang
Cambodia	khnhei phtu, prateal vong prenh atit
Laos	Hva nz ph'ai chai hluang
Malaysia	Lempoyang
Philippines	Barik, langkawas
Thailand	Kathue, kathue-pa, kawaen

Table 1.1.2b Other scientific names

Year	Name
1753	*Amomum zerumbet* L.
1918	*Zingiber aromaticum* Valeton
1918	*Zingiber littorale* Valeton

1.1.3. Plant description

The maximum height of lempoyang to grow reached until seven feet tall or at about 2 meter. The leaves are long and narrow assemble oppositely along the stem. The inflorescence of the plant will grow on separate stalk; usually it grows in the middle or late summer. The inflorescence started to grow with green cone shape and turn into complete red-orange colour indicates the maturation of the flower after couple of weeks. This flower is also known as pinecone. Small creamy flower will appear at the side of the pinecone.

Because of the inflorescence shape, lempoyang also called as 'pinecone ginger' or 'shampoo ginger' because of the creamy liquid inside the pinecone that being used as shampoo (Holtum, 1950; Sabu, 2003).

Traditionally, lempoyang is cultivated by vegetative propagation. From single rhizome, it can make a large clump in a couple of years. There are some requirements for the plant to grow easily such as sufficient moisture and fertile, organic soil, and full sun to partial shade. Usually the soil is fairly wet with regular moisture. Propagation is by division of clumps or pieces of rhizome at new part of soil (Chane *et al.,* 2003)(Fig. 1.1.3a and Fig. 1.1.3b).

Figure 1.1.3a *Zingiber zerumbet* plant

Figure 1.1.3b Green flower of Z. Zerumbet

1.1.4. Common usage

Zingiber zerumbet widely known as wild ginger or 'shampoo ginger' has so many traditional uses by people around the world. Native women between the Himalayas southward to Sri Lanka and eastward through the Malaysian-Indonesian archipelago have squeezed the viscous, aromatic liquid out of the flower heads and strained it through natural fibers for use as a natural shampoo and conditioner (Nalawade *et al.,* 2003). The gingery liquid could be used to quench thirst and was often added to massage oils as well (Neal, 1965).

The leaves of the plants have traditionally been used to flavour meats and fish when baked, to provide a layer of moist protection from the high temperature of stove, so the prepared dishes would cook without burning. Women sliced, dried and pulverized the roots, using the powder in medicinal preparations for a variety of ailments, including indigestion and motion sickness.

An infusion of the underground stems of wild ginger mixed with water was used for stomach ache (Burkill, 1966). A similar infusion mixed with salt was applied when there was an injury from a bump to the head. The same infusion-salt mixture was applied to skin for ring worm in children (Hasnah, 1991). Ashes from the leaves were mixed with ashes of bamboo and applied to cuts and skin sores. Sprains were treated with a mixture of several herbs and the rhizome of this wild ginger. Biting down on a piece of heated ginger root helped to ease the ache of a lost tooth (Nagata, 1970).

For native people in Thailand, wild ginger is used as a tonic, diuretic and promotes milk flow among women for breast-feeding. It is also popular among women in Malaysia and Indonesia to mix the part of rhizome or root to make a traditional 'jamu' believed to care their health and beauty of women, especially women after giving birth (Burkill, 1966).

1.1.5. Medicinal properties of zerumbone

Zingiberaceae has a rich source of compounds of phytomedical interest. Plants from this family have been reported to have anti inflammatory, antiulcer, antioxidant and antimicrobial properties. Few scientific researches by recent scientists towards lempoyang proved that it contained a novel product which has suppressive effect known as zerumbone, a bioactive compound in lempoyang. It has been found to act as antitumor activity (Kinghorn *et al.*, 1997 and Koshimizu *et al.*, 1988), anti-inflammatory effects and anti-proliferative potential in a variety of cell culture (Murakami *et al.*, 2002). Zerumbone also found to have great potential in chemoprevention, where it was found to inhibit Epstein-Bar virus (EBV), a herpes virus latently effecting human B-lymphocytes is believed to contribute in some human cancers (Murakami *et al.*, 1999). Zerumbone also induced the apoptosis in hepatocarcinoma cells, carried out *in vitro* using a well-differentiated transformed cell line, HepG2 cells which have been widely used and considered to be a good model for liver cancer research (Sharifah *et al.,* 2007).

1.2. Propagation of *Zingiber zerumbet* Smith.

1.2.1. Conventional propagation

Traditional propagation was carried out usually by using rhizomes as starter plant materials. Rhizomes were half buried in the loose soil in combination with little portion of sand and were left to grow. Even though the propagation is very simple and easy but still the production rate of vigorous plants as well as

rhizomes were low. This is because rhizomes cannot be stored for long time because it is susceptible to fungal diseases that will affect the quality of the rhizomes (Nalawade *et al.*, 2003). Tissue culture or micropropagation is one of the alternative ways to achieve high productivity, homogeneity, and good quality rhizomes because pathogen-free planting material is very crucial.

1.2.2. Micropropagation

Micropropagation or plant tissue culture refers to growth and multiplication of cells, tissues and organs on formulated semisolid or liquid media under aseptic and controlled environment. The explants itself must undergo sterilization process to make sure there is no unwanted microorganism.

Plant tissue culture technology is being widely used for large-scale plant multiplication. The commercial technology is primarily based on micropropagation, which produced rapid proliferation. This can be achieved by culturing the plants from tiny stem cuttings, axillary buds, and from somatic embryos, cell clumps in suspension cultures and bioreactors.

Plant tissue cultures are initiated from tiny pieces, called explants, taken from any part of a plant. Practically all parts of a plant have been used successfully as a source of explants. The explants was excised from original plant, surface sterilized and placed in suitable nutrient medium to initiate the sterile plant tissue culture or also called as mother culture. Repeated subculture helps to produce the plant in mass volume. In commercial micropropagation, the following parts were frequently used (Table 1.2.2a) (Ahloowalia *et al.,* 2004).

Table 1.2.2a Frequently used explants for commercial purpose

No	Part of explants	Note	Part/size used
1	Shoot tips and meristem culture	To free culture from viruses	Shoot tips and meristem size used 100 to 1000µm
2	Nodal and axillary bud culture	Easier to handle because it is larger than meristem	Stem with axillary bud with or without shoot
3	Floral meristem and bud culture	Not frequently used, but can generate complete plant	Floral meristem and bud
4	Other sources of explants	This part were taken in some plant to initiate culture	leaf discs, intercalary meristems from nodes, small pieces of stems, immature zygotic embryos and nucellus

There are few reasons why micropropagation techniques are preferred over the conventional asexual propagation because: (a) only small amount of tissue is required in micropropagation to generate millions of clonal plants., (b) a method to develop resistance in many species., (c) not dependent on season and quickly *in vitro* stock could be proliferated anytime., (d) long term storage of valuable germplasm is possible.

The micropropagation method involved four steps: (a) initiation of culture from explants like shoot tip on a suitable medium, (b) multiple shoot formation from

the cultured explants, (c) rooting of *in vitro* developed shoots and (d) transplantation to the field after acclimatization.

There are a few reports on *in vitro* multiplication of zingiberaceae species such as *Zingiber officinale* (Hosoki and Sagawa, 1997), *Alpinia galanga* (Borthakur *et al.,* 1998) and *Kaempferia galanga* (Chithra *et al.,* 2005). This paper reports on the protocol for the establishment of regenerable cell suspension of *Zingiber zerumbet* and subsequently the rhizome yield and zerumbone content of the transplanted regenerants. Other than that, Balachandran *et al.,* (1990) has reported on rapid clonal multiplication and short term conservation of *Curcuma domestica* Direct regeneration of shoots from immature inflorescence of *Curcuma domestica* has also been reported (Salvi *et al.,* 2000). Table 1.2.2b list some of the species from zingiberaceae family which have been successfully propagated by micropropagation.

Table 1.2.2b Explant frequently used for micropropagation of zingiberaceae species

No	Species	Explants used	References
1	*Zingiber zerumbet* (L) Sm.	Shoot tip	Hsu *et al.,* 1991
2	*Zingiber officinale* Roscoe	Rhizome bud	Hosoki and Sagawa *et al.,*1977
3	*Kaempferia galanga*	Rhizome bud	Vincent *et al.,* 1992; Chithra *et al.,* 2005
4	*Curcuma longa* Linn.	Rhizome bud	Nadgauda *et al.,* 1978; Shirgurkar *et al.,* 2001;

			Salvi *et al.,* 2002
5	*Curcuma domestica*	Immature inflorescence	Salvi *et al.,* 2000
6	*Alpinia galangal* Willd.	Rhizome bud	Borthakur *et al.*, 1998

1.2.3. Somatic embryogenesis

Plant parts such as leaf discs, meristems, stem-pieces, immature embryos, anthers, pollen, microspores and ovules have been cultured to initiate callus. A callus is a mass of unorganized cells. If the calluses were embryogenic callus, which have translucent glassy character and friable, upon transfer to suitable medium, is capable to grow into shoot-buds and somatic embryos, which then form complete plants.

The friable callus in liquid media will be placed on shakers for initiating cell suspensions. Liquid suspension cultures maintained on mechanical shakers in flask achieve fast and excellent multiplication rates. Cells are kept in Erlenmeyer flask or bioreactor for large scale production with suitable revolution per minute (rpm) and temperature.

There are several advantages of somatic embryogenesis. Unlimited numbers of somatic plantlets can be produced under controlled conditions. It is easy to scale up regeneration via somatic embryogenesis in liquid medium bioreactors (Jain *et al.,* 1995). Somatic embryos can be cryopreserved, with or without encapsulation (Blakesley *et al.,* 1996; Cyr *et al.,* 1994). Another common

application of somatic embryos is in genetic transformation using biolistics or *Agrobacterium.*

1.3. Objective of the study

This present study is divided into two sections, namely tissue culture and biochemistry. For tissue culture, it was mainly to investigate the potential of micropropagation of lempoyang via direct regeneration from *in vitro* plantlet to field transplantation until maturation was reached before rhizomes harvesting. Another objective is to study the establishment of embryogenic cell suspension cultures for micropropagation and production of secondary metabolites.

The objective in the biochemistry section is to study the three traditional methods of extraction which includes soaking, hydrodistillation and Soxhlet. Source of extraction are from *in vitro* derived rhizomes (originated from direct regeneration plants), *in vivo* rhizomes (from conventional cultivated plants) and cells suspension for both cell and liquid media culture. All extracts from three different sources will then be analyzed using Thin Layer Chromatography (TLC), Gas Chromatography (GC) and Gas Chromatography/Mass Spectrometry (GCMS). The results from GC will indicate the accumulated amount of zerumbone from all the sources. Finally, comparisons of the amount of zerumbone will be profiled.

1.4. General methodology

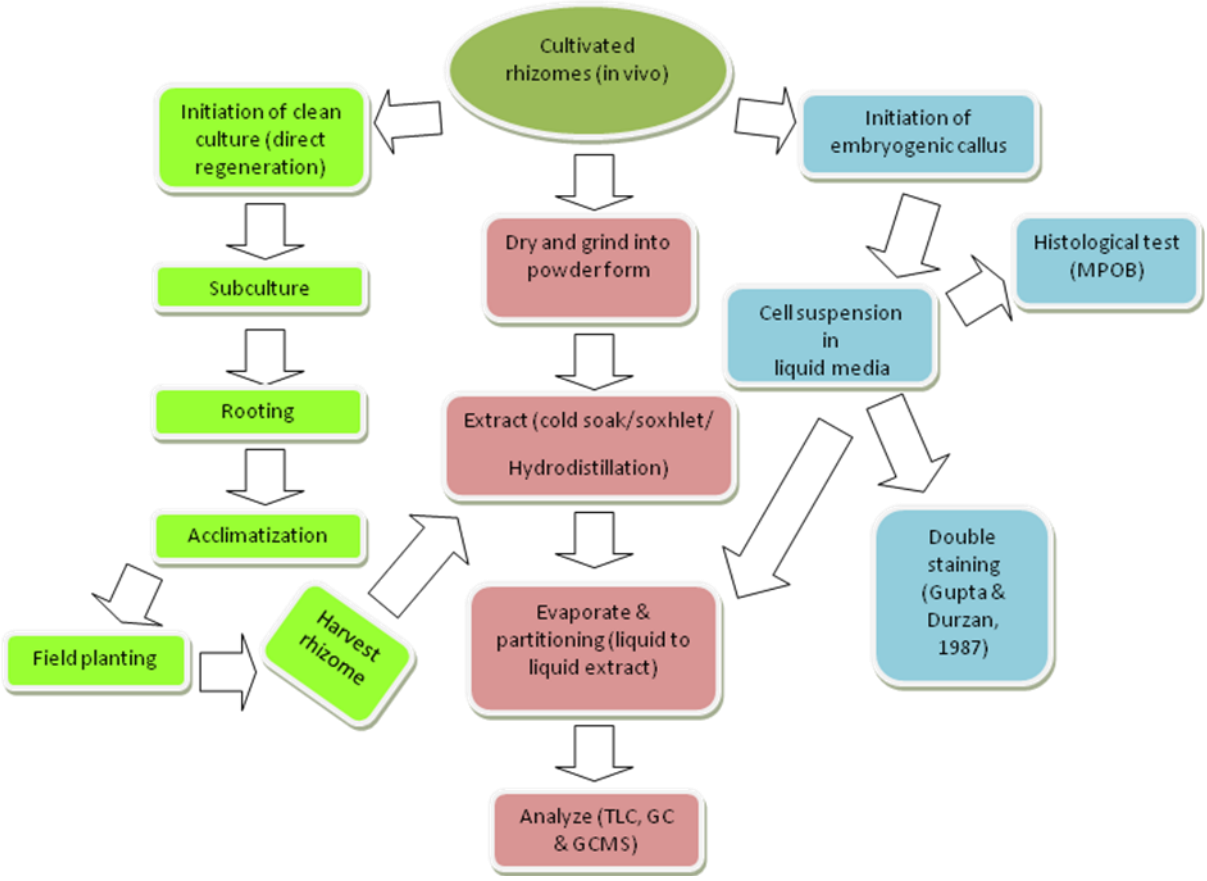


Figure 1.3a Scheme of overall methodology

CHAPTER 2

MICROPROPAGATION OF *Zingiber zerumbet* (L.) Sm : DIRECT AND INDIRECT REGENERATION

Objectives: **(i) To establish direct regeneration protocol from cultivated rhizomes until maturation in the field to provide *in vitro* derived rhizomes.**

(ii) To establish a protocol for embryogenic callus initiation to produce regenerable cell suspension cultures.

2.1. Micropropagation of lempoyang

Micropropagation has been used widely and extensively especially in medicinal herbs, for mass propagation, conservation of germplasm, study and production of bioactive compounds, and for genetic improvement (Nalawade and Tsay, 2007). Large-scale production from tissue culture technique is an alternative way to the traditional propagation for commercial approach by offering controlled condition and independent from season's factor for plant regeneration.

Micropropagation refers to growth and multiplication of cells, tissues and organs on formulated semisolid or liquid media under aseptic and controlled environment. The explant itself must undergo sterilization process to make sure there is no unwanted microorganism.

Plant tissue culture technology is being widely used for large-scale plant multiplication. This is because, important condition such as suitable temperature, nutrients and supplements were determined and applied to the cultured plant that will ensure the survival rate and healthy plants produced. Furthermore, the cultured plants were monitored closely in the growth room to make sure no contaminants occurred.

For lempoyang tissue culture, Hsu *et al.,* (1991) has successfully directly micropropagated this herb using shoot tip to obtain direct plant. For indirect micropropagation (callus initiation), Mahanom *et al.,* (2008) used shoot bud as the explants. In this present study both direct shoot micropropagation and indirect embryogenic callus were initiated from rhizome shoot bud.

2.2. Materials and methods

2.2.1. Plant material

Lempoyang was bought from Chow Kit Market was verified as *Zingiber zerumbet* (L) Sm. comparison with Herbarium species. Basal shoot explants used in this experiment were obtained from 'force growth rhizome'. 'Force growth rhizome' is where the rhizomes were forced to grow shoots with help of water instead of using soil. Water was used sparingly to avoid the rhizomes being too wet causing fungal contamination which can cause rot. Shoots were grown under lab condition.

After about two to three weeks, the small-green shoot bud will sprout, and with 1.5 cm to 2.0 cm height the shoot bud from the rhizomes is ready to be used for initiation of sterile culture.

2.2.2. Sterilization procedure

The shoot buds obtained from 'forced growth rhizome' were surface sterilized by using modified protocols for shoot buds. Segments were washed under tap water to remove dirt and soil on the surface of the shoot buds and excessive roots and leaves were trimmed. For thirty minutes, the shoot buds were then soaked in soapy water by adding two or three drops of Tween 20 to break or reduce surface tension. Shoot buds were then rinsed under running tap water until clean and subsequently submerged into 50% (v/v) Clorox (5.25% of sodium hypochlorite) solution with a few drops of Tween 20.

After fifteen minutes, the solution was removed and the shoot bud was placed in 0.5% (v/v) of mercury chloride dissolved in 70% (v/v) ethanol for one minute aseptically. Subsequently, the shoot buds were removed after one minute and rinsed again with sterile distilled water three times and air-dried on filter paper in the laminar flow chamber. Then, the shoot buds were cut into about 1.0 cm size on a cutting plate for subsequent tissue culture procedures.

2.2.3. Sterilization of media and apparatus

The shoot buds were cultured in sterile plant tissue culture media. Plant Tissue Culture media is generally sterilized in autoclave machine with 121˚C and

fifteen psi for twenty minutes. The time required for autoclaving culture media, forceps, scalpels, cutting plates, distilled water, and other equipment needed to be in sterile condition, usually were sterile in twenty to thirty minutes. The indicator tape were used to assist in determination of sterilization process was completed.

2.2.4. Preparation of Laminar flow signature

UV lights was used for fifteen to thirty minutes to sterile the laminar flow chamber before starting the tissue culture work. Blower was switched on to avoid outside air from entering the chamber. The spirit lamp or hot beads was used to sterilise the forceps and scalpel before and after the tissue culture work to minimize the chance of contamination.

2.2.5. Medium Composition

There are three basic components composed in *in vitro* plant tissue culture media. The three basic components are mineral ions as the essential elements, organic supplements that include vitamins and amino acid, and carbon source usually by adding sucrose. These three components were grouped in three different categories as macronutrient, micronutrient, and iron source.

For *Zingiber zerumbet*, there were several variations of the MS (Murashige and Skoog., 1962) (Appendix 1) media made. Direct regeneration required initiation, elongation and rooting medium (Appendix 1). For indirect regeneration, callus initiated on a M1 medium (Appendix 1) and maintenance

media for cell suspension is in liquid M2D medium (Appendix 1). Whole plant regeneration from cell suspension, the same procedure as direct regeneration stage was followed.

The basic compositions of MS media were modified to optimize the developmental stages for the regeneration of *Zingiber zerumbet*.

Besides MS media, other basal media used in this work for comparison were Chu (N6) (Chu, 1978) and Gamborg's B5 (Gamborg, *et al.,* 1968). The composition is shown in Appendix 1.

2.3. Direct regeneration

2.3.1. Preparation of explants

After surface sterilization, the shoot buds were left to dry on sterile filter paper before trimming. The dry explants were then trimmed to about 1.0 cm in size before culturing onto MS media without hormone. The cultures were then placed in the growth room at 23± 2˚C, sixth-teen hours light and eight hours dark condition for about one and half month. Subsequently, sub culturing was done for either direct shoot regeneration or callus initiation for cell suspension. This sub culturing was performed once in five weeks.

2.3.2. Media for shoot multiplication

A combination of 6-benzilaminopurine (BAP) and 1-naphtylacetic acid (NAA) were added to MS, Chu (N6) and Gamborg's B5 basal media for shoot multiplication. All media were adjusted to pH 5.8 with sodium hydroxide and chloride acid before autoclaving.

Both hormones were combined and added in MS basal media, Chu (N6) basal salt media and Gamborg's B5 basal salt media with nine combinations as shown as in the table 2.3.2a.

Table 2.3.2a Hormone combinations for shoot multiplication

	NAA (mg/L)								
	MS			Chu N6			Gamborg B5		
BAP 1 mg/l	0	2	4	1	3	5	1	3	5
BAP 2 mg/l	0	2	4	1	3	5	1	3	5
BAP 3 mg/l	0	2	4	1	3	5	1	3	5

Plants were sub cultured by separating the individual shoots into individual shoot before placing into various culture mediums. Cultures were placed in the growth room at 23± 2˚C, sixth-teen hours light and eight hours dark condition for about one and half month.

Observations were made after a month and a half and further analyzed by using multiple ANOVA (SPSS version 16) to determine the best media for shoot multiplication significantly.

2.3.3. Rooting media

Development of well-rooted plant is very crucial to be observed before transfer in order to ensure high survival rate. Therefore, MS media without hormone was supplemented with charcoal (1g/L) used for rooting medium. Plantlets were left for about a month in rooting medium for root development stage.

Individually, plants were separated and cut at about 1cm length without shoots and roots and cultured on rooting medium. The excised plants were kept in the growth room at 25± 2˚C, sixth-teen hours light and eight hours dark condition for a month before acclimatization.

2.3.4. Acclimatization

Complete plants were washed thoroughly under running tap water to make sure the roots were clean from the culture medium. This is important to avoid fungal and bacterial contamination in soil. The survival rates of roots and plants could also be increased.

Before trans-planting into soil, the plants firstly were transferred into jars containing water for about one week under laboratory condition. Plantlets were covered with transparent and holed plastic bag and sprayed with water twice a day. This procedure helped to ensure that the plants produced good functional roots.

Combination of dark soil or soil with compost fertilizer (humus) and sand were combined in the ratio 1:2 and prepared in small polyethylene bag. Plants were covered with transparent plastic to control the lost of humidity and sprayed with water twice a day. These plants were left in the green house for about one month before transplanting to the field. Every two weeks, small portion of fertilizer was added to give the plants supplement and nutrients.

2.4. Somatic Embryogenesis

2.4.1. Preparation of explants

Starter culture for callus initiation was obtained from aseptic plantlets cultures obtained through direct regeneration. Repeated subcultures were carried out to obtain sufficient material for starter culture.

2.4.2. Media

MS media was supplemented with 1.0 mg/L d-biotin which is vitamin H, 2.0 mg/L 2,4-D, 1 mg/L for both IAA and NAA. This media is called M1 media (App. A). IAA is very sensitive to heat and light, therefore this hormone was filter sterilized into the M1 media after media has been autoclaved.

To induce callus, combinations of auxin and cytokinin were used. In this M1 media, cytokinin was used as the main hormones, whereas variable concentrations of auxin were adopted. There were three types of auxins used - Dicamba, 2,4-D and Kinetin(Kn). The basic media MS media, M1 and Chu (N6) were augmented with this Plant Growth Regulator (PGR).

Osmotic stress in the media was provided by adding different concentration of sucrose in the media formulation. 30 g/L sucrose was used as a control. Modification to the media is shown in table 2.4.2a.

Table 2.4.2a PGR combinations for callus initiation

Media	Hormone (mg/L)		Sucrose
	Dicamba		
MS	0.1		30
			45
	0.3		30
			45
	0.5		30
			45
MS	2,4-D		
	1.0		30
			45
	2.0		30
			45
	3.0		30
			45
M1	2,4-D		
	1.0		30
			45
	2.0		30
			45
	3.0		30
			45
N6	2,4-D	Kinetin	

	0.1	0.5	30
			45
	0.3	0.5	30
			45
	0.5	0.5	30
			45

The data was collected after three and a half months and analyzed to determine the best media formulation.

2.4.3. Initiation of embryogenic callus

Plantlets from direct regeneration were used for embryogenic callus initiation. Plantlets were separated individually and roots from the plantlets were removed with sterile scalpel. The basal of the plantlets were sliced thinly until it reached its stem. Sliced explants were placed on MS, M1, and N6 medium containing several combination of PGR prepared in petri dishes. They were then kept in the growth room at 25±1˚C with dark condition for about three to three and a half months.

2.4.4. Development and maintenance of cell suspension culture

After about three to three and half months, explants responded to the media where swelling was observed before globular shaped callus were formed. For cell suspension initiation only yellowish friable callus or mixed callus (friable and non-friable in one clump) were used. Each of this callus clump was transferred into 250 ml Erlenmeyer flask containing 50 ml of MS modified liquid medium M2D (Dhed'a *et al.,* 1991). The cultures were then placed on an orbital shaker at a speed of 78 rpm/minutes in the growth room.

The modification (M2D) involved the use of half strength macronutrients and MS Fe EDTA and 10 mg/ml Dhed'a (Dhed'a *et al.,* 1991) vitamin. Besides that, 10 ml/L ascorbic acid (vitamin c) and 250 µl/L Zeatin were also added to the M2D media. Zeatin is very sensitive to heat and light; therefore this hormone was filter sterilized and was added after media has been autoclaved.

Cell suspensions were sub cultured into fresh M2D media once in two weeks. Sub culturing is important to bulk up the cells and to ensure enough oxygen and nutrients supply for the cells. Determination of the embryogenic callus was done by using Double Staining (Gupta and Durzan, 1987) technique and further confirmed by the histology of the cells. Growth curve of cell suspension were done after four subcultures to define the stability of cell.

2.4.5. Histo-chemical studies for somatic embryogenesis

For determination between embryogenic and non-embryogenic cell suspension, established double-staining protocol of Gupta and Durzan (1987) was used. Cell suspensions were placed in 1.0 ml micro tube and the media of the cells were drained. The cells were rinsed for three times to make sure media were completely drained from the cells. 1 % (w/v) of acetocarmine solution was added to submerge the cells before pipetted onto microscope slide. For better infiltration, the slide was heated above the Bunsen flame for a short while without boiling. Then, the excess acetocarmine was rinsed off thrice with distilled water. After all the excess of acetocarmine was drained off, the cells was stained with 0.1 %(w/v) Evan's Blue solution for two to three minutes before thrice rinsed with distilled water to remove excess Evan's Blue solution.

The double-stained sample was allowed to drain dry and then re suspended in distilled water before viewing under light microscope for better optical clarity. Those that are stained red with acetocarmine revealed the presence of somatic embryogenesis.

2.4.6. Histology study

Histology sections using resin were done to study the structure of cell suspension in liquid M2D medium. Samples for histology were first sectioned to the size according to the available mould and then fixed for twenty-four to forty-eight hours at room temperature in a Glutaraldehyde-Para formaldehyde-Caffeine (GPC) (Sigma Chemical Co., USA) fixative (50 ml 0.2 M Phosphate buffer, pH

7.2; 20 ml 10% (v/v) Paraformaldehyde; 4 ml 25% (v/v) Glutaraldehyde; 1 g Caffeine and distilled water to a total volume of 100 ml). Subsequently samples were dehydrated in ascending ethanol series (v/v): 30 % for 30 min; 50 % 45 min; 70 % 45 min; 80 % 60 min; 90 % 60 min; 95 % 60 min and twice in absolute ethanol for 60 min. During the dehydration process, extensive exposure to absolute ethanol was avoided to prevent hardening of tissues which may cause difficulty during sectioning. A vacuum was applied at low pressure gradually for approximately ten to twenty minutes to ensure complete infiltration of the solution in the samples. For recalcitrant tissues an extended dehydration procedure was carried out, three times with 100 % (v/v) butanol with a minimum of twenty-four hours treatment each to soften the tissue (Schwendiman *et al.,* 1988). The tissues were then ready for filtration with basic resin (Leica Historesin Embedding Kit, Germany) for twenty-four to forty-eight hours at 4˚C under slight vacuum. Larger tissues needed a longer infiltration period (one week or more). Infiltration was completed when the specimen appeared slight translucent and sunken to the bottom of the vessel. Following this, the specimens were embedded in a mould and once fully polymerised, holders were attached and 3 μm sections of each specimen were cut using microtome. An optional 0.5% (w/v) toluidine blue (Sigma, USA) (0.5 g tolouidine blue in 100 ml 0.2 M sodium acetate buffer, pH 4.6) stain was used to check these sections before proceeding further staining procedure. Good sections were then stained with 1 % (w/v) periodic acid for five minutes and then rinsed four times with distilled water at pH 4.5. After which, they were submerged in Schiff's reagent (1 g basic fuchsin, 2 g disodium metabisulfite in 1 N HCL, 0.5 g neutralized activated charcoal) for twenty minutes in the dark. The slides were then rinsed again with distilled water (pH 4.5) four times. Finally they were

counter stained with Naphthol Blue Black (Sigma, USA) [1 g Naphthol Blue Black in 100 ml 7 % (v/v) acetic acid] at 60˚C for five minutes followed by rinsing under running tap water. Periodic acid Schiff specifically stains polysaccharide (starch reserves and walls). Blue-black naphthol specifically stains soluble or reserve protein blue-black (Fisher, 1968). Samples were dried before they were viewed under the light microscope. For preservation, the slides were mounted with mounting medium and allowed to dry thoroughly before examinations were carried out. The prepared slides were examined through Zeiss Germany inverted microscope. All the images were photographed using Nikon digital camera.

2.4.7. Growth curve

Stability of cell suspensions is defined by sigmoid pattern of growth curve. The determination was done by measuring the settle cell volume (SCV) of cell suspension culture. SCV of cell suspension was measured for eight intervals in thirty-two days.

For growth curve determination, sterile polypropylene tube was used to measure cell suspension of lempoyang. 1 ml SCV of cell suspension (from one line) was used and placed in 250 ml Erlenmeyer flask containing 50 ml M2D medium. Every four days SCV of the cell was measured until it reached constant reading that indicates that the cell was in stationary phase. All the measurement was done in sterile condition. The media used remained unchanged until the experiment end. The data were collected and growth curve was plotted.

2.4.8. Regeneration and acclimatization of cell suspension derived plant

For plant regeneration, 250 µl of settle cell volume (SCV) from cell suspension was transferred onto sterile filter paper plated in a petri dish. This petri dish contained medium without any PGR. The filter paper was used to ease sub culturing process.

These cultures were placed in a growth room at 23˚C under dark condition for two to three months until the white primordial shoots were formed on the apex of the embryos. Subsequently, the cells were sub cultured by lifting the filter paper and placed onto new fresh MS medium containing 1 mg/l BAP in a petri dish to promote shoot formation where white primordial shoots observed turned into green shoots. At this stage, the cultures were kept in a growth room at 23± 2˚C, sixth-teen hours light and eight hours dark condition to let the shoots form completely.

After about a month and a half, the shoots were ready to be transferred into culture bottles containing multiplication medium same as formulated for direct regeneration for development into plantlets. Similar procedure and media were applied to these cell suspension derived plants for rooting and acclimatisation as described for direct regeneration.

2.5. Results

2.5.1. Direct regeneration-Effect of different media

Shoot buds from rhizome were collected and surface sterilised before trimming and culturing onto MS, N6 and B5 medium. Each medium was supplemented with combinations of different concentrations of PGR where the concentration as list in the (Appendix 1). For each combination, five replicates were used and the results of shoots multiplication were recorded after two months and shown in figures 2.5.1a, 2.5.1b and 2.5.1c. Results were then analyze and compared using multiple ANOVA (SPSS version 16).

Table 2.5.1a Shoots formation using different media with PGR combinations

Media	Hormone (mg/l)		Number of replicates	Number of shoots formation	Average number of shoots
	BAP	NAA			
MS	1	0	5	10	2.0
		2	5	9	1.8
		4	5	8	1.6
	2	0	5	19	3.8
		2	5	10	2.0
		4	5	9	1.8
	3	0	5	13	2.6
		2	5	12	2.4
		4	5	8	1.6
N6	1	1	5	7	1.4

		3	5	6	1.2
		5	5	7	1.4
	2	1	5	7	1.4
		3	5	4	0.8
		5	5	5	1.0
	3	1	5	4	0.8
		3	5	3	0.6
		5	5	6	1.2
B5	1	1	5	4	0.8
		3	5	6	1.2
		5	5	4	0.8
	2	1	5	3	0.6
		3	5	3	0.6
		5	5	3	0.6
	3	1	5	7	1.4
		3	5	6	1.2
		5	5	7	1.4

Among various combinations of PGR used in MS medium, 2 mg/L BAP supplemented media was significantly ($p < 0.05$) higher number of shoot multiplication (Figure 2.5.1a and Table 2.5.1c). From Figure 2.5.1b and 2.5.1c, where N6 and B5 media were used as basal medium, the best concentration of PGR could not be determined because of irregularity in explants response.

For ANOVA analyzed and comparison, the samples firstly were tested using Levene's Test of Homogeneity of Variance were this test is to prove the groups

have approximately equal variance on the dependent variable (Table 2.5.1b). Significant value from the Levene's Test of Homogeneity of Variance is 0.421 bigger than 0.05 prove that samples used in this test have no difference. Based on the result of ANOVA (Table 2.5.1c) on number of shoots produced using MS media is significantly different, F (8, 36) = 3.206, p<0.05.

Table 2.5.1b Levene's Test of Homogeneity of Variance

Test of Homogeneity of Variances

no. of shoots

Levene Statistic	df1	df2	Sig.
1.046	8	36	.421

Table 2.5.1c ANOVA analysis on effect of MS supplemented with BAP and NAA

ANOVA

no. of shoots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19.378	8	2.422	3.206	.007
Within Groups	27.200	36	.756		
Total	46.578	44			

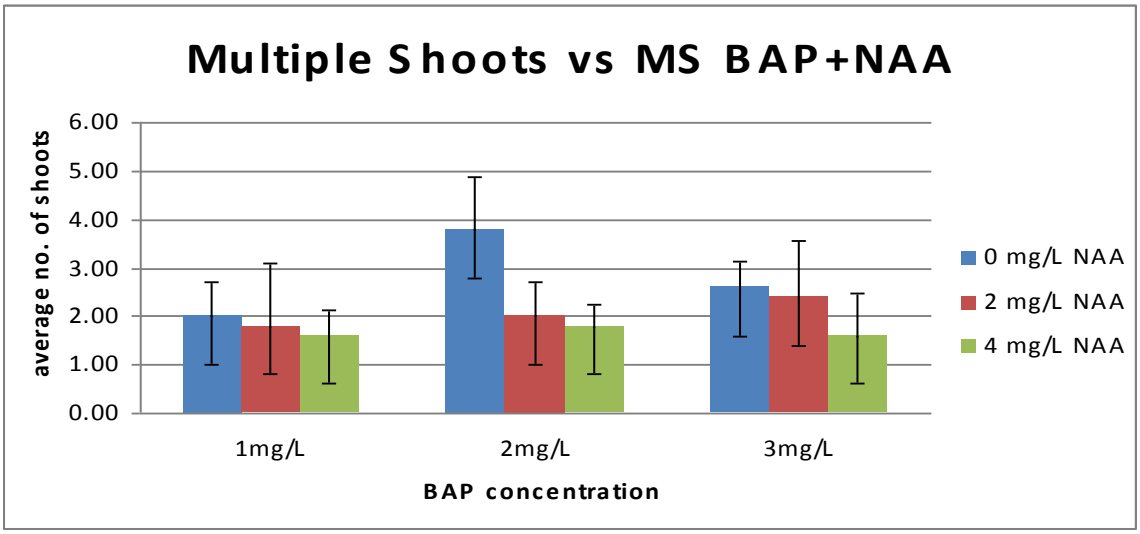


Figure 2.5.1a Multiple shoots formation using MS media supplemented with BAP and NAA

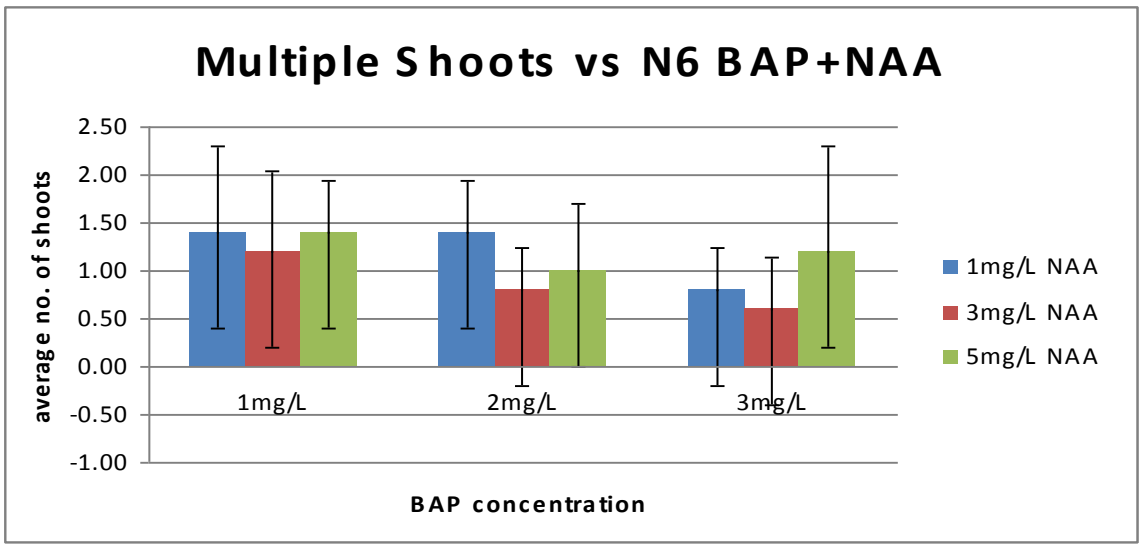


Figure 2.5.1b Multiple shoots formation using N6 media supplemented with BAP and NAA

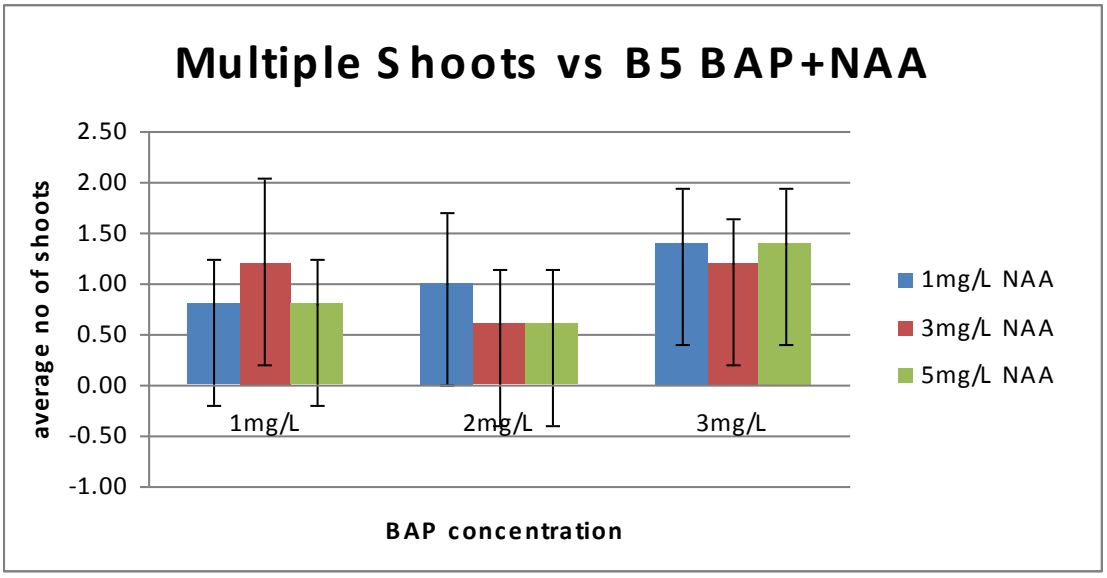


Figure 2.5.1c Multiple shoots formation using B5 media supplemented with BAP and NAA

Micropropagation stages involving shoots bud initiation to field planting are shown in figure 2.5.1d. Shoot bud explants were initiated on soilless media (figure 2.5.1d (i)). Excised shoot buds explants placed on the culture medium initially produced a single shoot after about three to four weeks Figure 2.5.1d (ii). For shoot multiplication, the single shoot was cut and subculture into MS media with 2 mg/L BAP (Figure 2.5.1d (ii) and (iii)). Shoot multiplication occurred after four to six weeks. Plantlets formed were then placed in rooting medium in order to enable plantlets to form complete roots. Plantlets were formed in MS without PGR supplemented with 1 mg/L activated charcoal after four to six weeks. Well rooted plants were then acclimatized for three weeks and followed by field planting (Figure 2.5.1d (vi) and (vii)).

Plants were acclimatized in combination of black soil or soil with compost fertilizer (humus) and sand with 1:2 ratios. For both acclimatizing and field

planting, plants were watered twice daily. Fertilizer was added after two weeks of acclimatization and once a month after field planting.

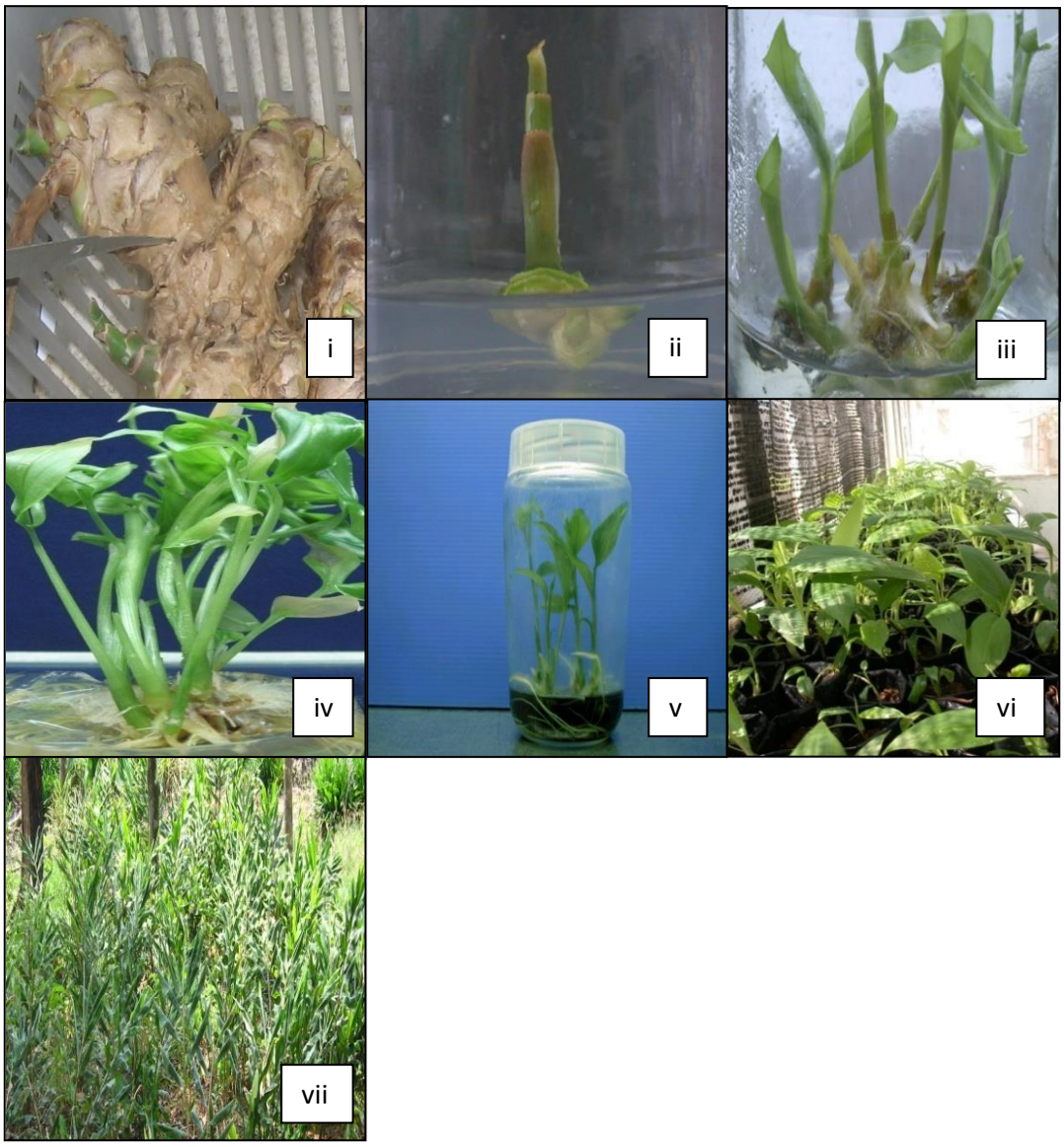


Figure 2.5.1d Micropropagation stages: i) Shoot bud; ii) Shoot initiation; iii) Early shoot multiplication; iv) Late shoot multiplication; v) Rooting stage; vi) Acclimatization; vii) Field planting

Among the three basal media used, MS media was shown to be the best media compared to B5 and N6 media. In MS media supplemented with 2 mg/L BAP, the mean number of shoot produced was 3.8 ±1.1 labels in red (Table 2.5.1d). The value was significantly higher among MS media supplemented with BAP and NAA (Table 2.5.1c). Whereas the highest score for mean number of shoots recorded for both N6 and B5 was 1.1±0.7 and 1.0±0.6. The difference in the number shoot multiplications for each types of media (MS, N6, B5) was also found to be significant ($p<0.05$).

Table 2.5.1d Effect of shoot multiplication using MS media supplemented with BAP and NAA

PGR Combination	Replicates (n)	Mean
1 BAP 0 NAA	5	2.00±0.7
1 BAP 2 NAA	5	1.80±1.3
1 BAP 4 NAA	5	1.60±0.6
2 BAP 0 NAA	5	3.80±1.1
2 BAP 2 NAA	5	2.00±0.7
2 BAP 4 NAA	5	1.80±0.5
3 BAP 0 NAA	5	2.60±0.6
3 BAP 2 NAA	5	2.40±1.1
3 BAP 4 NAA	5	1.60±0.9
Total		2.18±1.0

Table 2.5.1e Mean number of shoots multiplication from different medium

Type of media	Mean
MS	2.2±1.0
N6	1.1±0.7
B5	1.0±0.6

Table 2.5.1f ANOVA analyses for MS, N6 and B5 medium supplemented with BAP and NAA

ANOVA

number of shoots

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	38.711	2	19.356	30.336	.000
Within Groups	84.222	132	.638		
Total	122.933	134			

2.5.1.1. Field Evaluation

Before plantlets from tissue culture could be transplanted into field, firstly the plantlets have to adapt with new environment via acclimatizing to make sure the plantlets can survive in the field which the environment condition is not controlled. Acclimatization is compulsory for tissue culture derived plant.

Growth parameters used for assessment of plant derived from tissue culture materials planted in the field in comparison to *in vivo* plants (cultivated plant),

were shoots and leaf length, leaf width, tillers per plant and rhizomes weight. The measurements were taken after twelve months transplanting to the field.

Parameters such as tillers per plant, shoot length and rhizome weight were compared between tissue culture derived plants and cultivated plants (control), *in vitro* derived plants were found to be more than twice in shoot length compared to *in vivo* plants showed in (table 2.5.1.1a). The numbers of tillers per plant were also about more than twice in plant tissue culture derived material. After twelve months the *in vitro* derived plants produced 352 kg rhizomes whereas control plants produced 117 kg (Table 2.5.1.1a and Figure 2.5.1.1a (i-ii)). All comparisons were made and analyzed using Simple Independent T-Test and found to be significantly ($p < 0.05$) different for every parameter used (Table 2.5.1.1b, 2.5.1.1c, 2.5.1.1d).

Table 2.5.1.1a Comparison between *in vitro* derived plant and *in vivo* plant

Parameter (average)	***In vivo* (control)**	***In vitro* derived plant**
Tillers per plant	3.8±1.1	**9.80±1.9**
Shoot length (cm)	31.5±2.0	**81.8±2.0**
Rhizomes weight (g)	**117.3±16.7**	**352±20.9**

Figure 2.5.1.1a Comparison size of length between i) *in vitro* derived rhizomes and ii) *in vivo* rhizomes

Table 2.5.1.1b Independent Sample T-test for tillers per plant

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
tillers per plant	Equal variances assumed	2.017	.162	-13.416	48	.000	-6.000	.447	-6.899	-5.101
	Equal variances not assumed			-13.416	38.400	.000	-6.000	.447	-6.905	-5.095

Table 2.5.1.1c Independent Sample T-test for leaf length

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
shoot (cm)	length Equal variances assumed	.403	.529	-80.213	48	.000	-50.3000	.6271	-51.5608	-49.0392
	Equal variances not assumed			-80.213	46.383	.000	-50.3000	.6271	-51.5620	-49.0380

Table 2.5.1.1d Independent Sample T-test for weight of rhizomes

Independent Samples Test

	Levene's Test for Equality of Variances		t-test for Equality of Means						
								95% Confidence Interval of the Difference	
	F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper

rhizome weight (g)	Equal variances assumed	5.684	.021	-43.803	48	.000	-234.7000	5.3581	-245.4732	-223.9268
	Equal variances not assumed			-43.803	45.777	.000	-234.7000	5.3581	-245.4867	-223.9133

2.5.2. Somatic Embryogenesis

2.5.2.1. Effect of different media on callus initiation

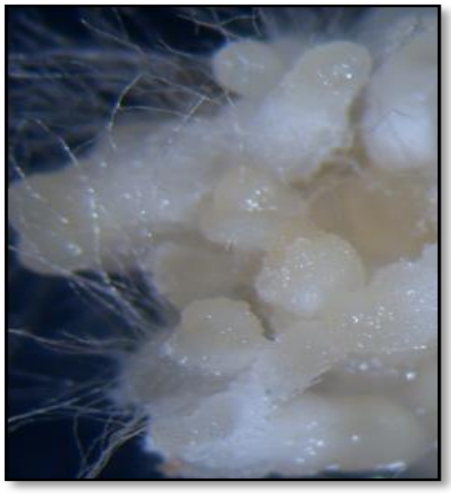
The initiation of embryogenic callus was experimented using three types of media (MS, N6 and B5) with various combinations of PGR and different concentrations of sucrose as shown in table 2.4.2a. Different types of response were seen including callus formation, root formation and direct shoot formation. From observation, for all concentration of 2,4-D augmented in M1 media showed that morphogenic calluses were formed. M1 media (Appendix 1) supplemented with 1 mg/l 2,4-D produced the highest percentage of producing callus where more than 50 % of the explants cultured produced calluses. From callus initiated, 5.4 % were observed as embryogenic callus.

Callus was first observed after three months. Two types of callus were formed, embryogenic and morphogenic callus. Morphogenic callus was observed as compact, watery and whitish callus (Figure 2.5.2.1a (ii)), while embryogenic callus was observed as dry, friable and yellowish callus (Figure 2.5.2.1a (i)). Embryogenic callus or friable observed callus for lempoyang usually initiated on the morphogenic or compact callus. Callus from lempoyang rhizome usually

initiated along with roots which could be observed from the two figures. Roots were easily initiated with almost of the explants which have response will produce roots either along with the callus formation or root alone.

For MS supplemented with various concentrations of 2,4-D, besides of callus formation, either morphogenic or embryogenic there was also adventitious root formation. From table 2.4.2a we can see that almost all concentration of 2,4-D used in MS media formed adventitious roots (Figure 2.5.2.1a (iii)) with highest data was recorded for 2 mg/L 2,4-D and 45g/L sucrose was at 19 %.

(i) Embryogenic callus



(ii) Morphogenic callus

(iii) Adventitious root formation

Figure 2.5.2.1a Effect of different media in callus initiation, (i) Embryogenic callus, (ii) Morphogenic callus, (iii) Adventitious root formation

From the callus initiated, 5.4 % were found to be embryogenic callus and the rest were vice verse (Figure 2.5.2.1c). The embryogenic producing media was labelled in red in table 2.5.2.1a. All of the explants didn't give any response for N6 supplemented with various combination of 2,4-D and Kinetin while for MS supplemented with various concentration of 2,4-D, mostly the explants produced direct roots.

The friable embryogenic calluses were then transferred into M2D liquid medium (Dhed'a *et al.,* 1991). For lempoyang, usually friable callus was formed on compact callus. This type of callus is also known as mixed callus. Each clump of mixed callus transferred into M2D medium and the friable callus dispersed in the media. Compact callus was removed upon sub culturing. Friable callus which have been transferred into M2D medium propagated and form single cells in liquid medium.

The embryogenic nature of the cells was determined by Double Staining method (Gupta and Durzan, 1987). This method using a combination of two different types of staining, Acetocarmine and Evan's Blue. Acetocarmine is positive stain and Evan's Blue is negative stain. From the double staining method, embryogenic cell was stained red (positive stain) and morphogenic callus was stained blue (negative stain) (Figure 2.5.2.1b).

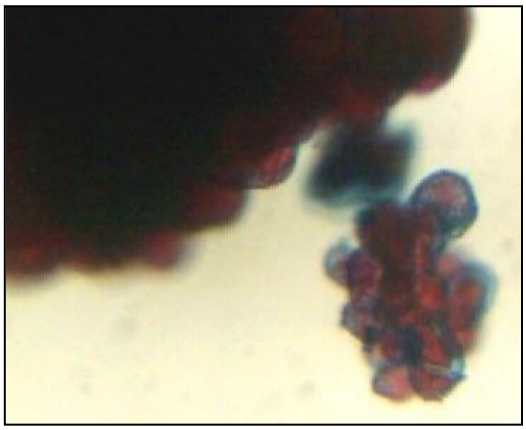


Figure 2.5.2.1b Double Staining of cell suspension. Red stained showed embryogenic characteristic of cell, and blue stained showed non-embryogenic characteristic cell.

Table 2.5.2.1a Response of explants in different media and hormone combination

Media	PGR (mg/l)		Amount of sugar (g/l)	No of explants	Number of responsive explants			Number of callus formation	
					Root production	Shoot production	No response	Morphogenic callus (%)	Embryogenic callus (%)
MS	2,4-D								
	1		30	40	4	3	32		

			45	34	3	-	30	2.94	
	2		30	46	5	7	34		
			45	46	9	-	37		
	3		30	47	-	-	47		
			45	50	-	-	50		
MS	Dicamba								
	1		30	43	8	-	35		
			45	55	-	-	55		
	2		30	50	-	-	48	4.72	
			45	50	-	-	50		
	3		30	47	-	-	47		
			45	50	-	-	50		
M1	2,4-D								
	1		**30**	**81**	**-**	**-**	**31**	**63.28**	**5.4**
			45	50	-	-	48	6.16	
	2		30	47	4	-	42	12.22	
			45	51	-	-	48	5.45	
	3		30	42	-	-	40	6.66	
			45	47	-	-	46	2.22	
N6	2,4-D	Kinetin							
	0.1	0.5	30	43	-	-	43		
			45	52	-	-	52		
	0.3	0.5	30	44	-	-	44		
			45	34	-	-	34		
	0.5	0.5	30	33	-	-	33		
			45	39	-	-	39		

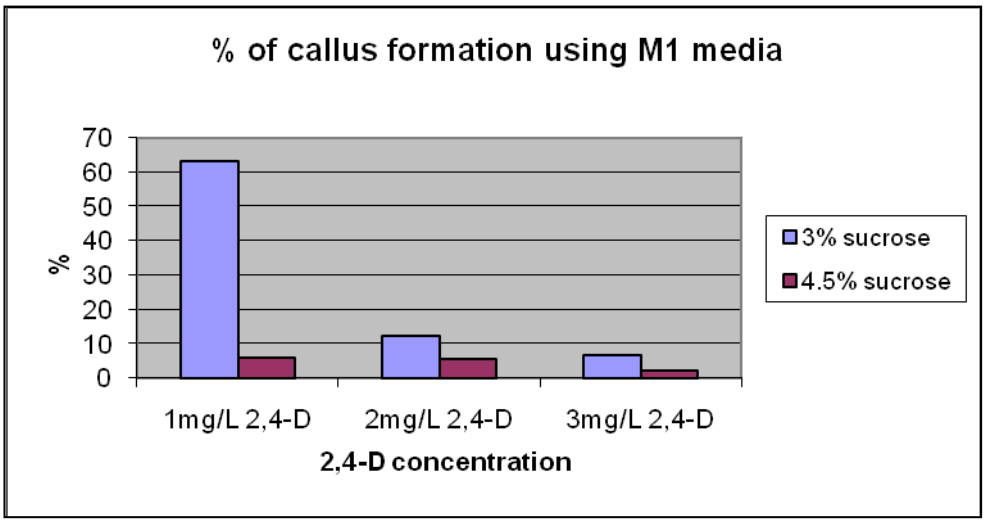


Figure 2.5.2.1c Percentage of callus formation on M1 media supplemented with various concentration of 2,4-D and sucrose

2.5.2.2. Histology of cells suspension

Well established cell suspension culture consists of cytoplasmic cell and absences of elongated and vacuolated cells. In this study, cell suspensions were viewed under inverted microscope (Zeiss Germany) Figure 2.5.2.2a (i) showed the yellow dense cytoplasm of cell suspension under inverted microscope at 20x magnifications while Figure 2.5.2.2a (ii) showed the histology profile of cell suspension under 200x magnifications. The figure, it showed that the cell suspension in M2D medium have complete structures of cells including the cell wall (**red arrow**), membrane (**blue arrow**) , nucleus and dense cytoplasm (**brown arrow**). No vacuolated cells or debris were found. Existing of vacuolated cells or debris will affect the growth of cells where propagation of cell will drop and propagation of debris will dominate the culture, so without debris, maximum growth of cell was observed. This was further proven through histological study where the embryogenic cell aggregates of cell suspension

cultures observed with dense cytoplasm with prominent nuclei and rich in protein. This is as a result of blue black staining that stained the cells. Periodic acid Schiff (red stain), specifically stains polysaccharide (starch reserves and walls). Blue black naphtol specifically stains soluble or reserved protein (blue black stain).

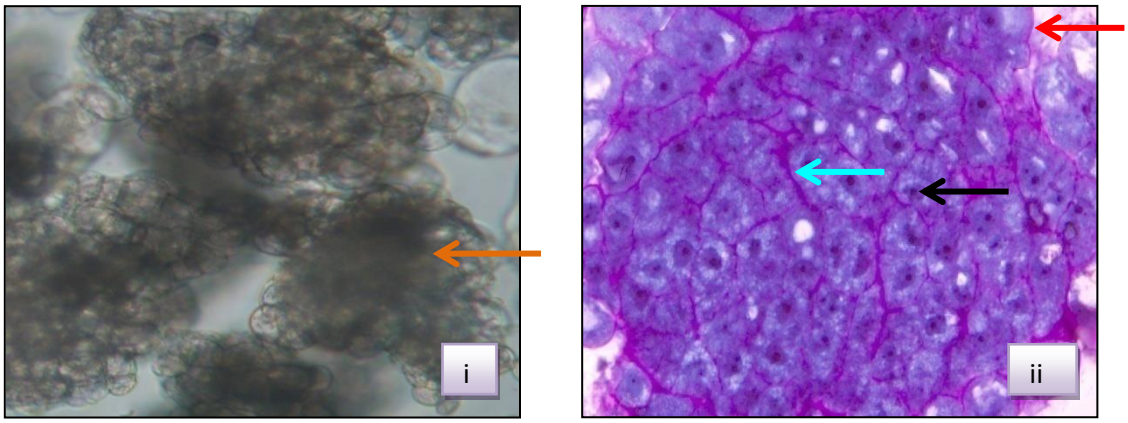


Figure 2.5.2.2a (i) Microscopic view of dense cytoplasm (20x) (ii) Histology of cell suspension from lempoyang. (L) (200x).

2.5.2.3. Determination of Growth Curve of cells suspension

For maintenance, cells were sub cultured every four-teen days. During subculture, 20 ml of the media was removed and replaced with fresh media. These procedures were repeated for about three months and the growth curved was determined to ensure the stability growth of the cell (Figure 2.5.2.3a). To establish the cell suspension growth curve, 1ml of settle cell volume (SCV) were cultured in 50 ml liquid media and SCV were measured for every four days.

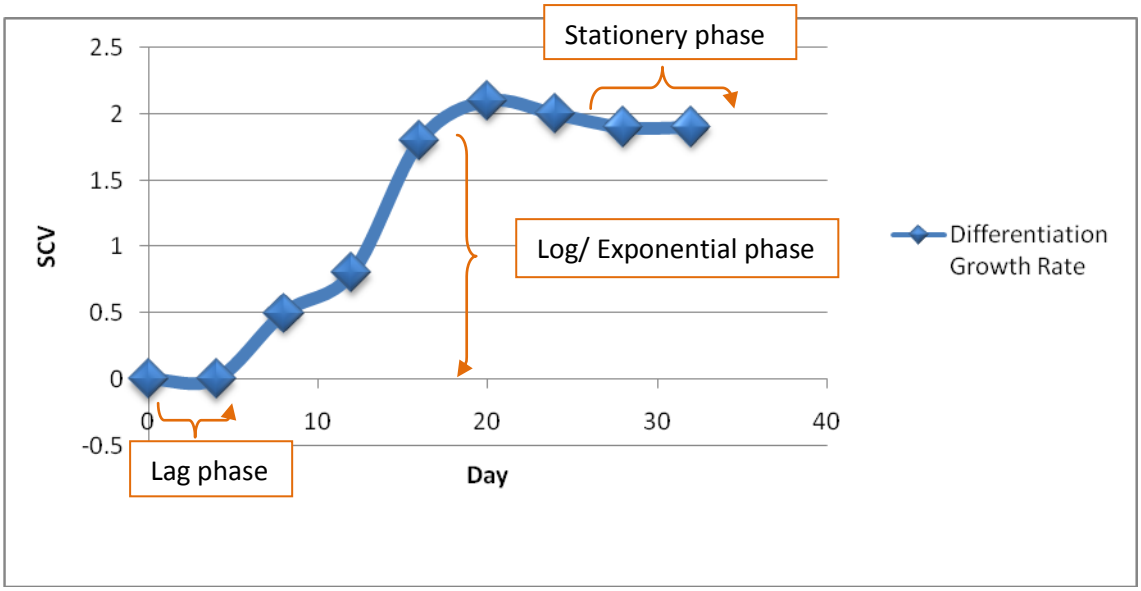


Figure 2.5.2.3a Growth curve of lempoyang cell suspension

From figure (2.5.2.3a), three clear stages for growth curve was obtained, namely lag phase, log phase or exponential phase and stationery phase. Lag phase took about three days before the cells started to multiply and cell mass increased until day 20. After day 20, the growth of cells decreased and cells started to become necrotic. The growth of cells completely stopped between day 28 to day 30.

2.5.2.4. Plant regeneration

Developmental stages of plant from cell suspension were shown in Figure 2.5.2.4a where observed were embryogenic cells growing into complete plantlet. This cell suspension can be regenerate back into whole plant by plating the cells onto MS medium without any PGR in dark condition. The single cell will develope (Figure 2.5.2.4a (iii)- 2.5.2.4a (viii)) into globular embryo, coleoptiles and scutellar stage and lastly whole plant. The morphological characteristics of plant regenerated from cell suspension observed to be the

same with direct regeneration plant before transferred into soil for acclimatization and field planting stage.

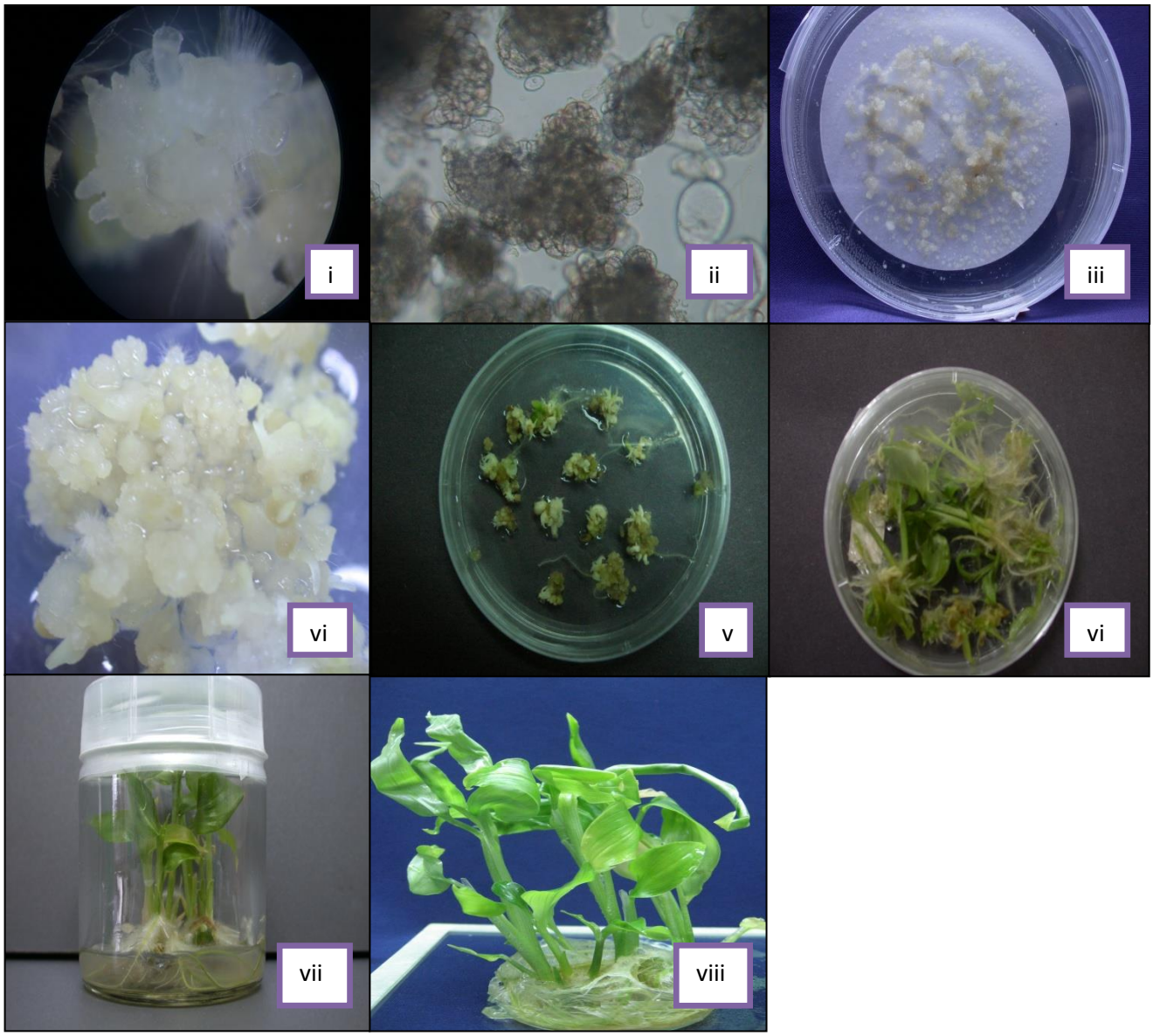


Figure 2.5.2.4a Developmental stage of cell suspension culture; i) Embryogenic callus; ii) Cell suspension; iii) Cells on regeneration media; iv) Cells randomly developing into various stages of embryogenesis; v) Formation of shoot bud structures; vi) Formation of complete plantlets; vii) Elongation of plantlets; viii) mature plants ready for potting

2.6. Discussion

2.6.1. Effect of different media and PGR

For tissue culture, *in vitro* shoot multiplication is very crucial for mass production of clonal lempoyang plants. One of the objectives is to establish micropropagation for lempoyang in mass production. The main problem in conventional propagation is low yield of plant production.

In this study, three types of basal media were experimented mainly MS, B5 and N6. From this three medium, MS is the richest media compared to B5 and N6. B5 medium lacked nitrate and potassium since macronutrient was devoid of ammonium nitrate and potassium phosphate monobasic (Gamborg *et al.,* 1968). As for N6 basal medium (Chu, 1978) ammonium nitrate was replaced with ammonium sulphate as a nitrogen source.

For micronutrient, N6 medium lacked cobalt chloride anhydrous, cupric sulphate anhydrous and molybdic acid sodium salt-$2H_2O$. In contrast to B5 medium, this media contained the same micronutrient components as MS basal medium but differed in the concentrations used.

As for vitamins, MS medium comprised of glycine, nicotinic acid, pyridoxine-HCl, thiamine-HCl and myoinositol whereas N6 medium lacked myoinositol while B5 lacked glycine.

Myo inositol is classified as one of the vitamin B, also known as vitamin B8. It is important in cell signalling, membrane, biogenesis and also participate in responses to salinity in plants (Nelson *et al.,* 1999). Glycine is a non-essential, neutral and genetically coded acid amino used as building block to numerous natural products. The richest vitamin components were in MS media because of the highest concentration used compared to N6 and B5 medium, while the lowest concentration used for every component was in B5 media.

Based on macro and micro nutrients and also vitamins, MS has the richest composition comparatively. This was significantly attributed to the best shoot multiplication and vigour of the regenerated plants.

Among the combinations of PGR used supplemented in MS media, shoots multiplication had been achieved successfully by culturing plantlets in MS media supplemented with 2 mg/L BAP without NAA in a month with a minimum of 3.8 auxiliary shoots produced from each plantlet cultured. For other combinations of PGR that have been used in MS basal media, there were comparatively lower multiplication of shoots observed. In this research, BAP was used in low concentration to produce complete plants and minimal probability of getting stunted or abnormal *in vitro* and field grown lempoyang plants. Stanly and Chan, (2007) also have proven that higher concentration of PGR at about 6-10 mg/L BA induced abnormality in lempoyang and for *C. zedoaria* as low as 2 mg/L and above simultaneously produce stunted roots and shoots with callus formation at the base of the shoot. For both lempoyang and *C. zedoaria* , only BA as low as 0.5 mg/L in combination with 0.5 mg/L IBA was used since there was no significant difference in shoot number formation

as compared to MS media supplemented with 6 mg/L BA. Balachandran *et al.,* (1990) also reported that, high concentration of kinetin was not suitable for *Zingiber officinale*. Instead of using BA alone, Hsu *et al.,* (1991) used the same concentration of BA (2 mg/L) but in combination with 0.5 mg/L NAA to produce lempoyang plants within one month. However, 4 mg/L BA alone used for shoots multiplication produced 4.78 shoots in a period of six weeks. In comparison with the results obtained from this research, less number of shoots was produced (3.8) within shorter period (four weeks) than that observed in Hsu's *et al.,* (1991) experiment. Nalawade *et al.,* (2003) adopted similar media to Hsu's *et al.,* (1991) in lempoyang cultures by using 4 mg/L BA in MS media. Besides, in *C. zedoaria* culture, other researcher used BA alone or in combinations with other PGR. Loc *et al.,* (2005) had reported that MS medium supplemented with 20 % (v/v) coconut water, 3 mg/L BA in combination with 0.5 mg/L IBA in *C. zedoaria*. With this combination, they reported that they could induce the formation of 5.6 shoots per explant. Bharalee *et al.,* (2005) reported that MS medium supplemented with 4 mg/L BAP and 1.5 mg/L NAA was the best medium for shoot multiplication of *C. caesia* producing 3.5 shoots per explant cultured. For *C. zedoaria* 1 mg/L BAP and 0.5 mg/L NAA and produced 4.5 shoots per explants (Bharalee *et al.,* 2005). Balachandran *et al.,* (1990) used one media formulation, MS media augmented with 3 mg/L BAP for *C. domestica, C. caesia and C. aeruginosa* which was in the same genus. Shoots obtained were 3.4 shoots per explants from *C. domestica*, 2.8 shoots per explants from *C. caesia* and 2.7 shoots per explants from *C. aeruginosa*. MS supplemented with 5 mg/L BAP was most effective media for *C. aromatica* producing an average of 3.3 shoots per explant (Nayak, 2000).

Swapna *et al.,* (2004) used combination of IAA at 0.5 mg/L and 2.5 mg/L BAP in micropropagating *Kaempferia galanga* Linn which is also in Zingiberaceae family. In *Zingiber petiolatum*, Prathanturarug *et al.,* (2004) used 4 mg/L BA but without other PGR added and showed an increment in number of shoots multiplication compared to other work carried out. This concentration of BA also been used in *Curcuma longa* L. with similar observation was made (Prathanturarug *et al.,* 2003). However, even though the number of shoots was increased, abnormality was recorded where the shoots length was retarded. Retarded plantlets were normalized in MS media without any PGR. For *Z. spectabile* Griff lower concentration of PGR was used than in *Z. petiolatum* and *C. longa* culture media where 2.25 mg/L BA and 0.9 mg/L IAA was used to produce the highest shoot multiplication (Faria and Illg, 1995).

Generally it was indicated that, high concentration of BAP resulted in abnormality in plant propagation. Since the application of micropropagation is for the efficient production, clonal plant abnormality must be avoided. In this study, MS supplemented with low PGR (2 mg/L BAP) was sufficient for the production of multiple shoots in lempoyang which were normal and better than traditionally propagated lempoyang.

2.6.2. Acclimatization and field evaluation

For acclimatization, complete plants were initially washed thoroughly under running tap water to make sure the roots were clean from the culture medium. This is important to avoid fungal and bacterial contamination in soil. The survival rates of roots and plants could also be increased. Before trans-planting

into soil, the plants firstly were transferred into jars containing water for about one week under laboratory condition. Plantlets were covered with transparent and holed plastic bag and sprayed with water twice a day. This procedure helped to ensure that the plants produced good functional roots.

Combination of dark soil or soil with compost fertilizer (humus) and sand were combined in the ratio 1:2 and prepared in small polyethylene bag. Plants were covered with transparent plastic to control the lost of humidity and sprayed with water twice a day. These plants were left in the green house for about one month before transplanting to the field. Every two weeks, small portion of fertilizer was added to give the plants supplement and nutrients.

After acclimatization for about four weeks, lempoyang plants with height of about 30.0 cm were then ready to be transplanted. For control experiment, field planted lempoyang were watered twice a day and fertilized once to twice a month. The plants were initially fertilized lightly and the volume of fertilizer was increased following age and requirement. Plants were grown to maturation for about ten to twelve months. These transplanted plants were measured and compared with the traditionally propagated plant (*in vivo*). After twelve months, parameters such as shoots and leaf length, leaf width, tillers per plant and rhizomes weight were compared to *in vitro* derived plants.

The difference between *in vitro* derived plants and *in vivo* plants was the source of planting material. *In vitro* derived plant originated from disease-free plants as initial planting material that was obtained from tissue culture technique. Mother plant or explants used also were selected from good breed

of lempoyang. From the results obtained, the differences in the rate of growth and yield produced showed that *in vitro* derived plant gave better results compared to *in vivo* plant. For instance, *in vivo* plants showed shoots length at 31.5 cm, 3.8 tillers per plant and 117.3 g of rhizomes weight per clump compared to *in vitro* derived plants where the average shoot length was 81.8 cm with 9.8 tillers per plant and 352 g of rhizomes per clump of plants. Similar results were obtained by Salvi *et al.,* (2003) in turmeric plants.

These findings showed that *in vitro* derived plants provide healthy and vigorous planting materials for mass propagation leading to high yield production in suitable plant species. The *in vitro* derived plants were observed to be morphologically identical with the mother plant (*in vivo* plant). Sharma and Sigh, (1997) also reported that more than 95 % of *in vitro* derived *Zingiber officinale* Rosc performed well under field conditions. They also observed that the plants were morphologically identical to the mother plants and were free from ginger yellows (*Fusarium oxysporum* f. sp. zingeberi). It was also shown that, well developed rhizomes obtained from tissue culture plants did not rot during storage until six months. Good keeping quality was also obtained from 'Gala' and 'Royal Gala' apples (Mcmeans *et al.,* 1998). In contrast, *in vitro* derived soybean plants showed no significant differences in seed quality, seed weight and yield compared to *in vivo* or mother plants (Stephens *et al.,* 1991).

However, other researchers reported that, usually *in vivo* plants showed better performance than *in vitro* derived plants in ginger (*Zingiber officinale* Roscoe) where leaves, shoots and roots of the first generation of *in vitro* plants were

smaller compared to *in vivo* plants (Smith and Hamill, 1996). Similar results were reported in turmeric (Smith and Hamill, 1996).

2.6.3. Embryogenic callus and cell suspension

Somatic embryogenesis is an asexual form of plant propagation in nature that mimics many of the events of sexual reproduction. Also, this process may be reproduced artificially by the manipulation of tissues and cells through tissue culture technique or *in vitro*. With the right selection of media and PGR, a successful initiation of somatic embryo could be obtained. *In vitro* somatic embryogenesis is important for the use of many biotechnological tools for genetic improvement, as well as for mass propagation.

Somatic embryogenesis is an efficient pathway in plant regeneration which produces mass plant propagation within a reasonable time. This method can produce clonal plants suitable as planting material. In this study, embryogenic cell suspension was produced to enable better production of plants compared to direct regeneration compared to shoot buds.

In this study, modified MS medium (M1) supplemented with 1 mg/l 2-4,D produced embryogenic callus from shoot bud explants in about three months. Other concentrations of 2-4,D in the same media did not produce embryogenic callus. N6 and MS medium supplemented with combination of 2,4-D and Dicamba also did not produce any embryogenic callus. 2-4,D is commonly used to induce embryogenic callus including other zingiberaceae species such as *Boensenbergia rotunda* (Tan *et al.,* 2005) with 1 mg/L

supplemented in MS media. For *C. amada*, MS media was supplemented with 2 mg/L 2,4-D and obtained semi-friable callus using leaf sheath as explants (Prakash *et al.,* 2004). In *Elettaria cardamomum*, Srinivasa *et al.,* (1982) used a combination of coconut water 18 % (v/v), 2 mg/L 2,4-D and 0.5 BAP added in MS medium for callus initiation and growth. Mohanty *et al.,* (2008) reported that callus formation could be initiated using either 2 mg/L 2,4-D or 0.5 mg/L kinetin in *C. aromatic* Salisb.

Some callus cultures were initiated and maintained in the same media. Embryogenic callus and embryogenic cell suspension of *Coriandrum sativum* was initiated and maintained by using similar media which was MS supplemented with 1 mg/L 2,4-D (Kim *et al.,* 1996). Callus (75%) was initiated in solid medium and upon transfer into liquid medium with same media formulation; they successfully obtained embryogenic cell suspension culture. However, in this research, cell suspension was maintained in M2D medium (Côte *et al.,* 1996) which was a different media used to initiate embryogenic callus.

Even though, 2,4-D is commonly used to initiate callus cultures, some researchers used other types of PGR. Salvi *et al.,* (2001) showed that 2 mg/L dicamba and picloram supplemented in MS medium induced callus in turmeric cultures. In domestic ginger, Kackar *et al.,* (1993) used MS media supplemented with 0.6 mg/L dicamba for producing callus culture. It was claimed that this was the effective media for inducing and maintaining callus culture in domestic ginger. Callogenesis of *C. zedoaria* was obtained by culturing root segments in MS media added with 1 mg/L NAA. In *Boesenbergia*

rotunda, it was shown that 2,4-D was not critical for callus initiation (Tan *et al.,* 2005).

Although MS medium was commonly used as basic medium, N6 medium was successfully adopted for barley culture. Low concentration of 2-4,D was supplemented in N6 medium (Bednarek *et al.,* 2007). N6 medium with various PGR has been attempted for *lempoyang* in this study, but there was no callus obtained. In this study, MS medium supplemented with 2,4-D produced adventitious roots instead of callus. Highest number of roots was obtained from MS supplemented with 2 mg/L 2,4-D.

According to Collins and Edwards (1998), the concentrations for all PGR used in the tissue culture medium are crucial to determine callus initiation and development. The combinations of media components will produce either friable, fast growing callus or a green chlorophyllous callus or embryo or root and shoot formation.

2.6.4. Verification and stability of embryogenic cell suspension

Mixed callus obtained from M1 medium supplemented with 1 mg/L 2,4-D was transferred into M2D liquid medium. The cells were suspended in growth room at 25± 2˚C, six-teen hours light and eight hours dark conditions on orbital shaker with speed of rotation at 78 rpm. The cells were subsequently subcultured in fresh M2D medium every four-teen days. For verification of embryogenic cell suspension culture, double staining method by Gupta and Durzan (1987) was used.

Embryogenic cells absorbed acetocarmine and cells were stained red intensely whereas non embryogenic cells were stained blue via Evan's blue stained. Cytoplasm and nuclei are known to exhibit affinity for the red acetocarmine. The result from double staining was found to be consistent with histological evidence where cell suspensions were clear to have dense cytoplasm with prominent nuclei and rich in protein with blue black stained. The same observation of embryogenic cells were made by other researchers (Mahanom *et al.*, 2008, Cote *et al.,* 1996, Grapin *et al.,* 2000, Ganapathi *et al.,* 2001, Khalil *et al.,* 2002 and Greoget *et al.,*2000).

Besides, the stability of cell suspensions was also established upon growth curve determination. From the results obtained, it was proven that the cell suspension established was stable with prominent log, lag and stationary phases over duration of one month. During log phase, cells were actively dividing for a period of fifteen days. Cells were transferred to regeneration media during early stationery phase.

2.7 Conclusion

Micropropagation of *Zingiber zerumbet (L) Sm.*Smith or lempoyang has been successfully obtained from direct and indirect regeneration. From the results, it was shown that higher yield of healthy rhizomes were obtained from *in vitro* derived rhizomes compared to conventionally propagated rhizomes. Besides that, cell suspensions were initiated, maintained and abled to regenerate into whole plants regeneration efficient of 210 plants per 1ml SCV.

CHAPTER 3

ISOLATION, IDENTIFICATION AND COMPARISON OF ZERUMBONE FROM DIFFERENT SOURCES

Objectives: **(i) To isolate zerumbone from *in vivo*, *in vitro,* and cell suspensions.**

(ii) To determine the most efficient method between soaking, hydro-distillation and Soxhlet for zerumbone extraction.

3.1. Bioactive compounds distribution in *Zingiber zerumbet* Smith.

Zingiberaceae has a rich source of compounds of phytomedical interest. Plants from this family have been reported to have anti inflammatory, antiulcer, antioxidant and antimicrobial properties. In *Zingiber zerumbet*, bioactive compounds are normally found in essential oil. Compounds in *Z. zerumbet* can be extracted from rhizomes, leaves and flowers (Chane *et al.,* 2003) and sixty-nine compounds were found in all different part of lempoyang plant.

There are three major compounds in essential oils obtained from rhizomes, zerumbone (37 %), alpha-humulene (14.4 %) and camphene (13.8 %). From leaves and flowers different types of compound were found. The oils present a large amount of (E)-nerolidol (21.4 % and 34.9 %), beta-caryophyllene (6.9 % and 10.2 %), and linalool (7.7 % and 17.1 %). The oils of leaves differ from others by the presence of a-pinenes and s-pinenes in high amount (10.3 % and 31.4%). The major compound of interest in this study is zerumbone (Chane *et al.,* 2003).

3.1.1. Zerumbone: General introduction

Sesquiterpene or sesquiterpenoid is a class of terpenes that consist of three isoprene units and have the molecular formula $C_{15}H_{24}$. Like monoterpenes, sesquiterpenes may be acyclic or contain rings, including many unique combinations. Biochemical modifications such as oxidation or rearrangement produce the related sesquiterpenoids. Zerumbone is a sesquiterpene with monocyclic or has one ring with fifteen carbons and 220 hydrogen ($C_{15}H_{220}$).

Zerumbone with molecular weight 218.340 is a construct of fifteen carbons with one double bond oxygen at r-chain ($C_{15}H_{22}O$). Figure 3.1.1a illustrates the structure of zerumbone. There are a few IUPAC name that refers to zerumbone such as (E,E,E)-2,6,9,9-Tetramethyl-2,6,10-cycloundecatrien-1-one, 2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, and (E,E,E)- 471-05-6. This compound is an intermediate between polar and non polar compound.

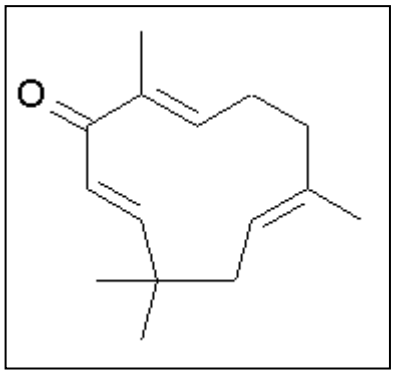


Figure 3.1.1a Chemical structure of zerumbone (Dai *et al.,* 1997)

To obtain the extract of zerumbone, a simple steam distillation and recrystalization could be performed. The estimated yield produced from this method is about 0.3 % to 0.4 % of zerumbone (Kitayama and Okamoto, 1999).

Zerumbone, compound isolated from *Zingiber zerumbet* Smith, like other plants in Zingiberaceae family, is thought to have a number of medical properties, *e.g.* inhibiting cancer, tumour, and even HIV. Recently, research has shown that zerumbone exhibited HIV-inhibitory and cytotoxic activities (Dai *et al.*, 1997). In addition, zerumbone has also been reported to suppress cancer-cell proliferation (Hoffman *et al.,* 2002) and has been implicated as one of the promising chemopreventive agents against colon and skin cancer (Nakamura *et al.,* 2004). Szabolcs *et al.,* (2007) discovered the beneficial effect of pre treating cholecytokinin octapeptide (CCK-8) with zerumbone. CCK-8 is known to induce acute pancreatitis in rat.

In Malaysia, research by Sharifah *et al.,* (2007) showed zerumbone to possess an anti proliferative activity towards HepG2 cells. Zerumbone was also found to inhibit the proliferation of non-malignant Chang Liver and MDBK cell lines.

3.2. Materials and methods

Bioactive compound of interest in this research is zerumbone. Zerumbone is a compound mainly found in the rhizome. Therefore, rhizomes from *in vivo* and *in vitro* sources were used as raw materials. There are several techniques that can be used to extract secondary metabolite such as cold soak, soxhlet

extraction, hydro-distillation, Super Critical Fluid (SCF) extraction, Simultaneous Distillation-Extraction (SDE), Microwave-Assisted Extraction (MAE) and others. There are advantages and disadvantages associated with each technique. The ideal technique should be reliable, economical, faster, and relatively simple. Three techniques that were tested for zerumbone extraction in this study was cold soak, soxhlet and hydro-distillation methods.

To extract zerumbone, which is a non polar compound, dichloromethane (DCM) (AR Grade, Merck) was used as a solvent because it can dissolve non-polar compound. DCM is also known as universal solvent because of its ability to extract polar and non polar compound.

3.2.1. Plant Materials: *in vivo* rhizomes, *in vitro* derived rhizomes, cell suspensions

Four different sources of raw materials were used in this study. *In vivo* rhizomes or cultivated rhizomes were obtained from local market, planted in green house until it reached maturity about ten months. After ten months, the rhizomes were harvested, washed, sliced, dried and ground into powder form. *In vivo* rhizomes were extracted to obtain baseline volume for zerumbone amount to compare with the *in vitro* sources.

In vitro derived rhizomes were rhizomes from tissue culture plant which have been planted in green house. Both *in vivo* and *in vitro* derived rhizomes were planted in the same green house under similar conditions. The rhizomes from *in vitro* derived plants were also harvested after ten months upon maturation. The

rhizomes were treated in similar manner as *in vivo* rhizomes before undergoing extraction process.

For cell suspension, the extraction was done for both cell mass and spent liquid medium from cell cultivation. Both cell suspensions and liquid media were collected when the cells population reached stationary phase after about four-teen days in liquid media culture. After separation, the cells were dried in an oven at 34˚C for a day and liquid media was kept chilled at 5˚C. Dried cells were then ground into powder form.

3.2.2. Cold soak and partitioning method

The term 'cold soak' was used because this extraction technique involved soaking the sample at room temperature. The samples (rhizomes or cells), were sliced (for rhizomes), dried and ground into powder before being placed into a glass vessel. A solvent was then poured into the container and the samples were allowed to soak for five days to obtain crude extract.

Approximately, 150 grams rhizomes from both *in vivo* and *in vitro* sources were washed thoroughly under tap water after harvesting to remove all the dirt and soils. The rhizomes were then sliced as fine as possible, labelled and dried in the oven at 40˚C to 45˚C until it reached constant weight to reduce the water content.

The dried sliced rhizomes were then ground into powdered form and placed in a clean glass container. DCM was added in twice the volume of the sample.

This is to make sure all the desired compounds were extracted out into the solvent. Samples were left in the solvent for five days. The mixtures were shaken regularly.

After five days, the samples were filtered using filter paper to separate the extract and the sample. The solvent was evaporated using rotary evaporator leaving behind a crude extract. The crude extract was subjected to thin layer chromatography (TLC) to identify the presence of zerumbone.

For zerumbone extraction from cell suspension, about 0.75 g of dry cell was used and undergoes the same treatments for rhizomes. For liquid extraction, partitioning or liquid-liquid extraction was used to obtain the crude extract from spent liquid medium of cell suspension.

Distilled water added with sodium chloride (NaCl) and DCM AR grade was used in partitioning to obtain zerumbone extract. NaCl was used to polarize the distilled water. Spent medium of cells suspension were measured at 100 ml and poured into 1L separating funnel. About 500 ml distilled water with NaCl and 500 ml DCM were also poured into the funnel.

The mixture of spent liquid medium, distilled water and DCM were then shaken vigorously to make sure the polar and non polar compounds were well-separated. Shaking will cause pressure increase inside the separating funnel. The pressure was released by opening the stop cock with the funnel in inverted position. After several repetitions, the sample was left to separate into two

layers. Lower part was DCM with non polar compounds, while upper layer was distilled water with polar compounds.

The lower layer was collected and the same procedure was repeated twice. The upper aqueous layer was discarded. Lastly, the DCM dissolved extract was kept overnight in sodium nitrate anhydrous to absorb residual water in the extract.

The extract was then poured into a round flask and evaporated using rotary evaporator. The dried extract was then kept in 10 ml DCM stock.

Aluminium plate was used for the TLC analysis. Combination of diethyl ether and petroleum ether at a ratio of 1:9 was used as the solvent system. The spots were detected under ultra-violet light at 254/365 nm. Further analyses were conducted using gas chromatography (GC FID, by Shimadzu) and gas chromatography/mass spectrometry (GCMS by Agilent Technology).

3.2.3. Soxhlet method

A Soxhlet extractor is a piece of laboratory apparatus invented in 1879 by Franz von Soxhlet (Soxhlet, 1879). It was originally designed for the extraction of a lipid from a solid material. However, a Soxhlet extractor is not limited to the extraction of lipids; it can also be used for other chemical compounds which remain stable during temperature changes.

By using Soxhlet extractor, extraction of desired compound that has only a limited solubility in a solvent can be increased by applying heat, and if there is impurity that is insoluble in the solvent, the impurity can easily be separated from the extract. Furthermore, the extraction time could be shorten to only a few hours compared to cold soak that took more than a day to ensure majority of the compounds have been extracted.

Since, heat will promote the higher solubility along with a relatively low boiling point of dichloromethane 39.8 ˚C; this technique is suitable for the extraction of zerumbone which is stable at this temperature.

Soxhlet extractor includes the condenser, thimble with thick filter paper, heating instrument and round flask where the extract was collected. The dried sample about 0.75 g and dichloromethane was placed in thick filter paper inside the thimble. The soxhlet extractor was set up with low temperature at 40˚C for four to six hours. After 6 hours, the dissolved compounds collected in round bottom flasks were evaporated in the rotary evaporator as in method 3.2.2.

3.2.4. Hydro-distillation method

Hydro-distillation is one of the methods that can be used to produce essential oil. During the hydro-distillation, essential oil components yield an azeotropic mixture with water. *Azeotropic mixture* is a solution that forms a vapour with the same concentration as the solution, distilling without a change in concentration.

Most of the essential oils do not mix well with water in the liquid phase, so after condensation, they are separated by decantation (separation of mixture by sucking, pumping or just pouring without disturbing the sediment or precipitate in the bottom of the container). The distillation period can take about ten to fifteen minutes or longer period.

This method can be carried out with two different ways, Clevenger distillation and steam distillation. For Clevenger distillation, the material to be extracted is immersed in water, which is then boiled, while steam distillation, steam passes through a bed of the material to be extracted.

For both methods, the vapours of the volatile components are carried by the steam to a condenser. On condensation oil-rich and water-rich layers are formed. These are separated by decantation.

However, both hydro-distillation methods will expose the sample to high temperature, nearly 100˚C. High temperature could possibly cause destruction of some compounds that are heat labile when exposed for a long period of time. Higher temperature also leads to pressure increase and the danger of decomposition of the compound. Thus, hydro-distillation at reduced pressure is preferable due to lower temperature range used.

Since zerumbone was found in the extract of the essential oil, hydro-distillation technique can be used for its extraction. The components of hydro-distillation extractor are condenser, dean stark, heating instrument and round flask.

For the use of this technique, the rhizomes used were fresh rhizomes. The clean rhizomes (150 grams) were chopped finely before being placed in the round flask. Water was added into the round flask slightly more than the samples to make sure the rhizomes were submerged before starting the process.

The essential oil was brought by the steams that condenses and collected in the dean stark pipe. Sometimes, if the volume of the zerumbone was high, the crystalline structure can be seen along the pipe. The essential oil can then be collected.

3.2.5. Establishment of standard calibration of zerumbone using gas chromatography (GC)

Pure zerumbone stock (crystal structure) was obtained from the Universiti Teknologi Malaysia (UTM) Skudai, Johor. Pure zerumbone extract was used in the standard calibration. A known amount stock was weighed and dissolved in dichloromethane.

The standard stock solution was then diluted into 0, 50, 100, 200, 300, 400, 500, 600 μg/ml concentrations. Each concentration was injected into the GC in triplicates. The average peak area for each concentration was determined and plotted against the concentrations to obtain a standard calibration.

3.2.6. Zerumbone identification: TLC

Thin layer chromatography (TLC) is a simple, quick and inexpensive procedure that will give the qualitative information regarding the number of components in the mixture or extract. To identify a particular compound in the mixture, R_f number of known compound and unknown compound is compared under the same condition using the same TLC plate.

TLC plate is a sheet of glass, metal or plastic coated with thin layer or solid adsorbent usually silica or alumina. This TLC plate is used as stationary phase where the small amount of mixture will be applied as a concentrated spot at the bottom of the TLC plate. The plate with the spot will then be placed vertically in solvent at a depth below to that of the spot. The solvent will acts as a mobile phase. In this study, the mobile phases were a combination of petroleum ether and diethyl ether at a ratio of 9:1. The solvent will slowly rise up the TLC plate due to capillary action.

As the solvent rise up, spots will appear on the TLC plate. If the compound is coloured, it can be observed with naked eyes. Otherwise, a UV lamp or colour development agent is used to see the spot that is colourless. In this study, anisaldehyde reagent was used. The preparation is state as follows:

P-anisaldehyde 0.5 ml
MeOH 85 ml
H_2SO_4 10 ml
Acetic acid 15 ml

The anisaldehyde reagent was prepared and used in fume cabinet and handle with highly precaution. The TLC plate was sprayed with the anisaldehyde reagent and dried on heating plate. Standard zerumbone was run together in the same plate with crude extract for ease of comparison.

3.2.7. Zerumbone identification: GC and GCMS

Gas chromatography (GC) is a type of chromatography in which the mobile phase is a carrier gas, usually an inert gas in normal condition such as helium, and the stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside glass or metal tubing, called a column.

Gas Chromatography is different from other forms of chromatography detection, such as HPLC, TLC and others, because the solutions travel through the column in a gas state. The interactions of these gaseous analysts with the walls of the column (coated by different stationary phases) causes different compounds to elute at different times called retention time. The comparison of these retention times is the analytical part of GC. This makes it very similar to HPLC.

Mass spectrometry (MS) is an analytical technique that identifies the chemical composition of a compound or sample on the basis of the mass-to-charge ratio of charged particles. The method relies on chemical fragmentation of a sample into charged particles or ions and measurements of two properties,

charge and mass. The fragmented particles will be passed through electric and magnetic fields in a mass spectrometer.

There are three main parts of mass spectrometry *viz.* an ion source, which transforms the molecules in a sample into ionized fragments; a mass analyzer, which sorts the ions by their masses by applying electric and magnetic fields; and a detector, which measures the value of some indicator quantity and provides data for calculating the abundances for each ion fragment present.

The technique has both qualitative and quantitative uses e.g. in identifying unknown compounds, determining the isotopic composition of elements in a compound, determining the structure of a compound by observing its fragmentation, quantifying the amount of a compound in a sample using carefully designed methods such as by comparison with known quantities of heavy isotopes, studying the fundamentals of gas phase in chemistry (the chemistry of ions and neutrals in vacuum), and determining other physical, chemical, or biological properties of compounds.

Thus, GCMS is a combination of gas chromatography and mass spectrometry in one construct with both functions.

In this study, gas chromatography (GC FID) from Shimadzu and gas chromatography/mass spectrometry (GCMS) from Agilent Technology was used. The crude extracts were stored in DCM before being diluted and injected into the machines.

For both GC and GCMS, identical operating parameters were set. The initial temperature was set at 100˚C with 10˚C ramping in a minute. For zerumbone determination, there was no holding time and final temperature was set 250˚C. This process took about fifteen minutes to complete. A sample was mixed with DCM in 0.5 ml vial. Then, 3.0 µl was injected into the GC using an auto injector. All results were further analyzed using analysis of variance, ANOVA (SPSS 16).

3.3. Results

3.3.1. Determination of standard calibrations of zerumbone using GC

Zerumbone standard was run through GC and retention time as well as GCMS profile was determined. GC detected the retention time for zerumbone was at twelve minutes and GCMS was used to confirm the standard identify as 2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl from its stored database.

Figure 3.3.1a showed the zerumbone standard calibration with linear equation relating zerumbone concentration (mg/ml) = 1/5883 x peak area and correlation coefficient, $R^2 = 0.997$.

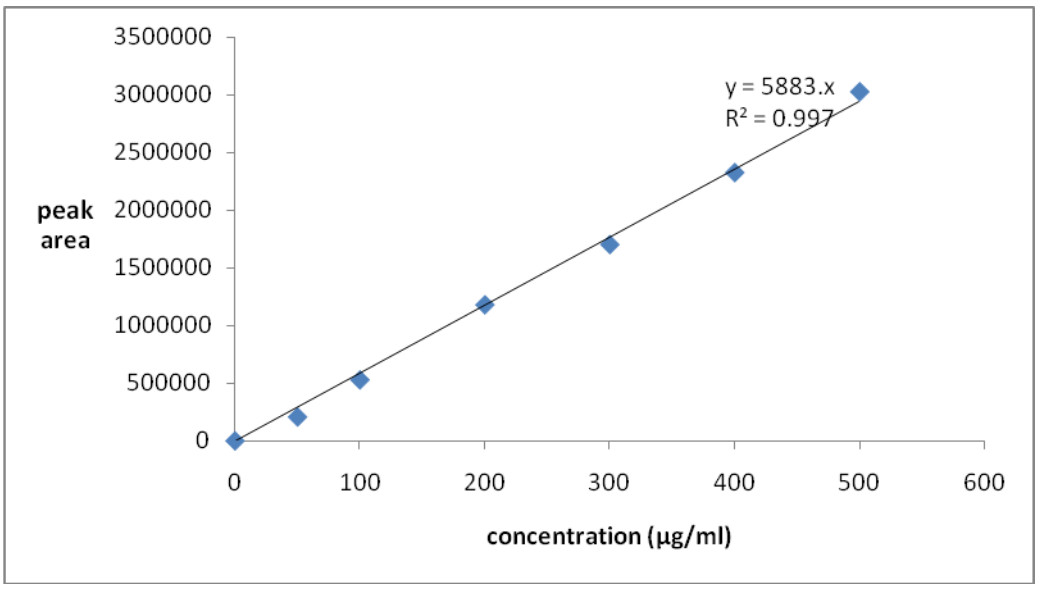


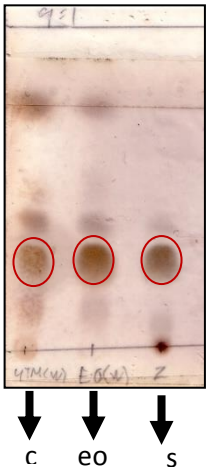
Figure 3.3.1a Standard calibration for zerumbone

3.3.2. Qualitative and quantitative analyses

3.3.2.1 Qualitative: TLC

TLC was carried out to detect the presence of zerumbone in the extract. Crude extract from *in vitro* derived rhizomes was tested along with zerumbone standard and essential oil from *in vitro* derived rhizomes. Figure 3.3.2.1a showed the TLC plate after being sprayed by anisaldehyde reagent. The figure clearly showed the spots which was identified as zerumbone for all three extractions (red circle).

Petroleum ether and diethyl ether were used as mobile phase in a ratio of 9:1. Essential oil and crude extract showed similar chromatographic pattern as the standard for the presence of zerumbone (Figure 3.3).



key:

◯ : zerumbone

S : standard

eo : essential oil

c : crude

Figure 3.3.2.1a Zerumbone detection on TLC plate

3.3.2.2. Quantitative: GC and GCMS for zerumbone

There were three extraction methods used namely cold soak, soxhlet and hydro-distillation. From the results obtained from the three different methods, *in vitro* derived rhizomes gave the higher yield compared to *in vivo* rhizomes.

For instance, results from cold soak method clearly showed that, zerumbone yield from *in vitro* derived rhizomes was greater than from *in vivo* rhizomes. From table 3.3.2.2a, 2.6±0.8 mg/g zerumbone for *in vitro* derived rhizomes and 1.7±0.7 mg/g zerumbone for *in vivo* rhizomes. The difference was significant at p=0.05

Table 3.3.2.2a Mean of zerumbone yield from *in vivo* and *in vitro* derived rhizomes (cold soak extraction)

Source of rhizomes	**zerumbone (mg/g)**
In vivo rhizomes	1.740±0.724
In vitro derived rhizomes	2.599±0.815

Figure 3.3.2.2a showed the peak of zerumbone ( ) extracted from *in vivo* rhizomes. From GC reading, zerumbone was the highest peak which indicates that this particular compound is the major component extracted from *Zingiber zerumbet*. Extract from *in vivo* was used as a control to compare with *in vitro* derived rhizomes. GC chromatograms for *in vivo* and *in vitro* sources were shown in Figures 3.3.2.2a and 3.3.2.2b.

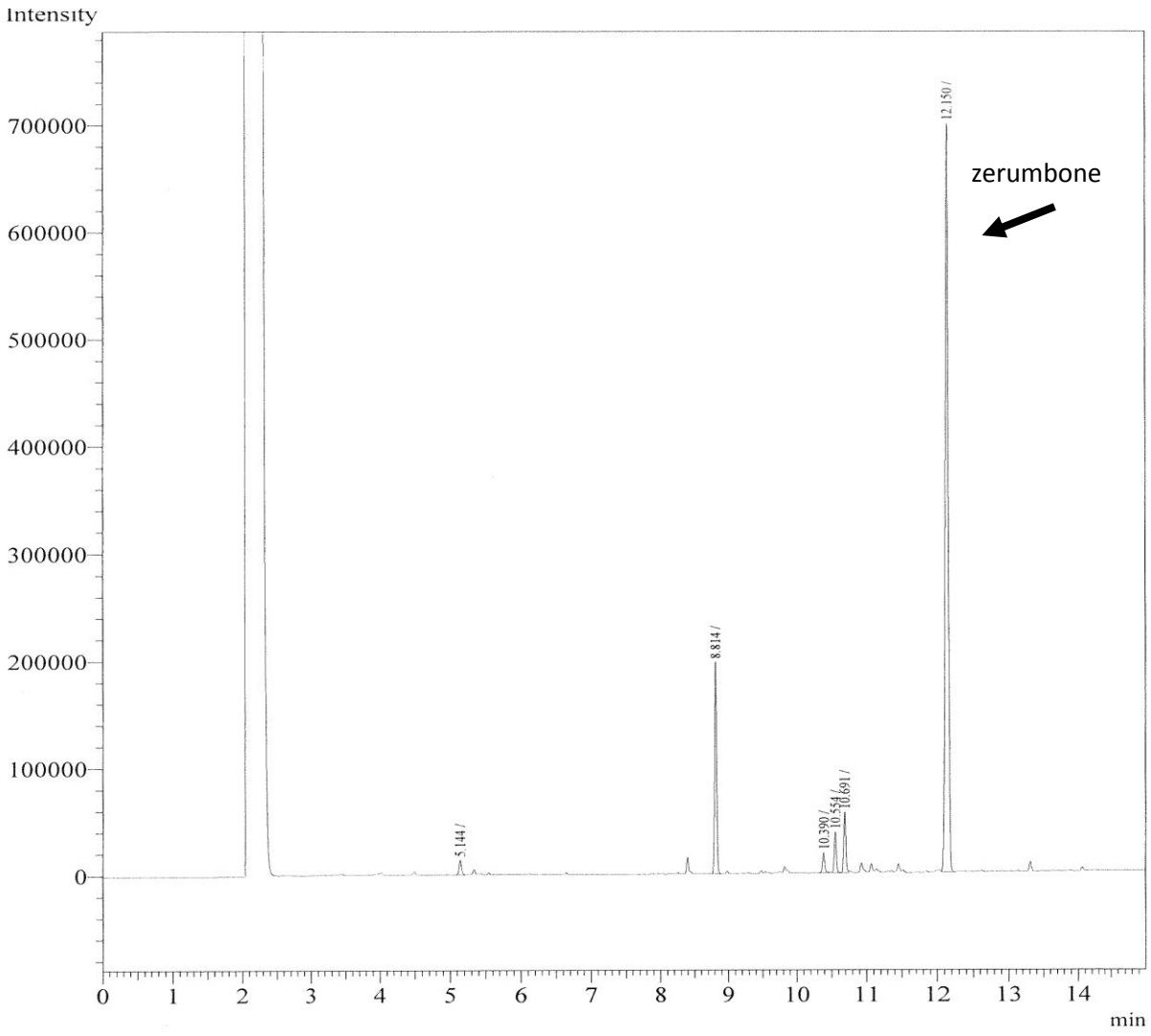


Figure 3.3.2.2a GC chromatogram of zerumbone from *in vivo* rhizomes using cold soak extraction method

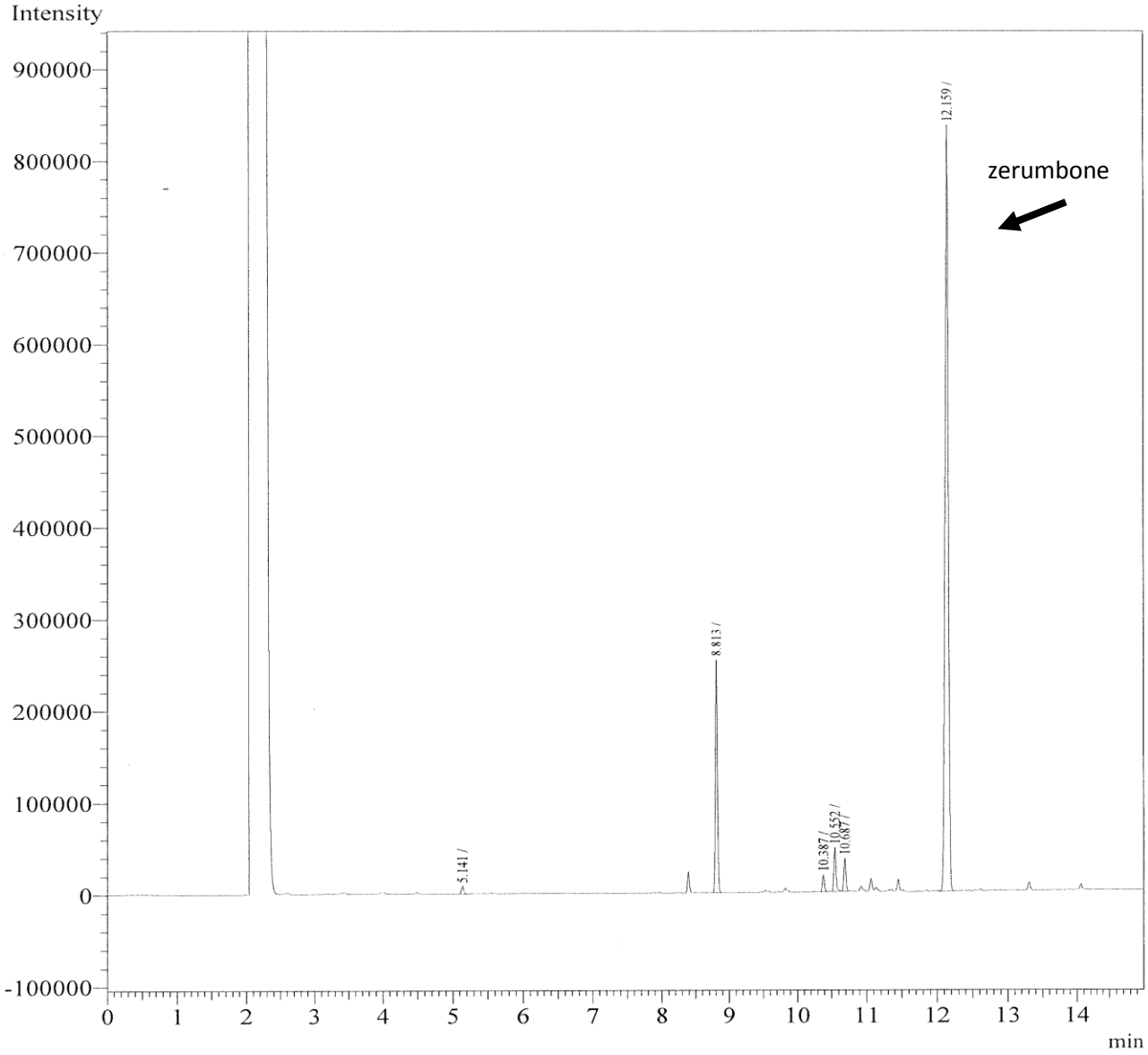


Figure 3.3.2.2b GC chromatogram of zerumbone from *in vitro* derived rhizomes using cold soak extraction method

Determination of amount of zerumbone was as follows: (mg zerumbone per gram fresh rhizomes)

Peak area = 19647552 (obtained from GC abundance axes)

Zerumbone concentration (x axis) = 1/5883 X peak area.

Thus x = 19647552/ 5883.1

= 333.966 μg/ml

Since the injection volume is 3 μl, hence the mass of zerumbone in 3 μl

3 μl x 333.966 μg/ 1000 μl = 1μg zerumbone

Total amount of zerumbone in 520 μl extract in DCM solution in injection vial is (1 μg x 520 μl) / 3 μl

= 173.7 μg, and in 1μl mixture of extract and DCM solution, zerumbone concentration is 173.7 μg/520 μl = 0.33 μg/μl or 0.33 g/L.

Since *in vivo* rhizomes extract was kept in 20 ml stock, correcting for 26x dilution factor on 0.02 ml that was taken from the stock for injection into GC, the total amount of zerumbone per 150 g fresh rhizomes was:

0.33 g /L x 26 = 8.7 g/L

8.7 g / 1000 ml x 0.02 ml = 0.000174 g ⇨ mg = 0.174 mg

0.174 mg / 0.02 ml x 20 ml /150 g = **1.16 mg/g**

The concentrations of zerumbone in other plant material sources were calculated using similar method.

GCMS chromatogram (Figure 3.3.2.2c (i-ii), 3.3.2.2d (i-ii)) was done for both sources of extract to confirm the compound was zerumbone. All extraction from *in vivo* and *in vitro* showed similar typical chromatogram in all methods

used. GC and GCMS chromatogram for both soxhlet and hydro-distilation were referred in Appendix 2.

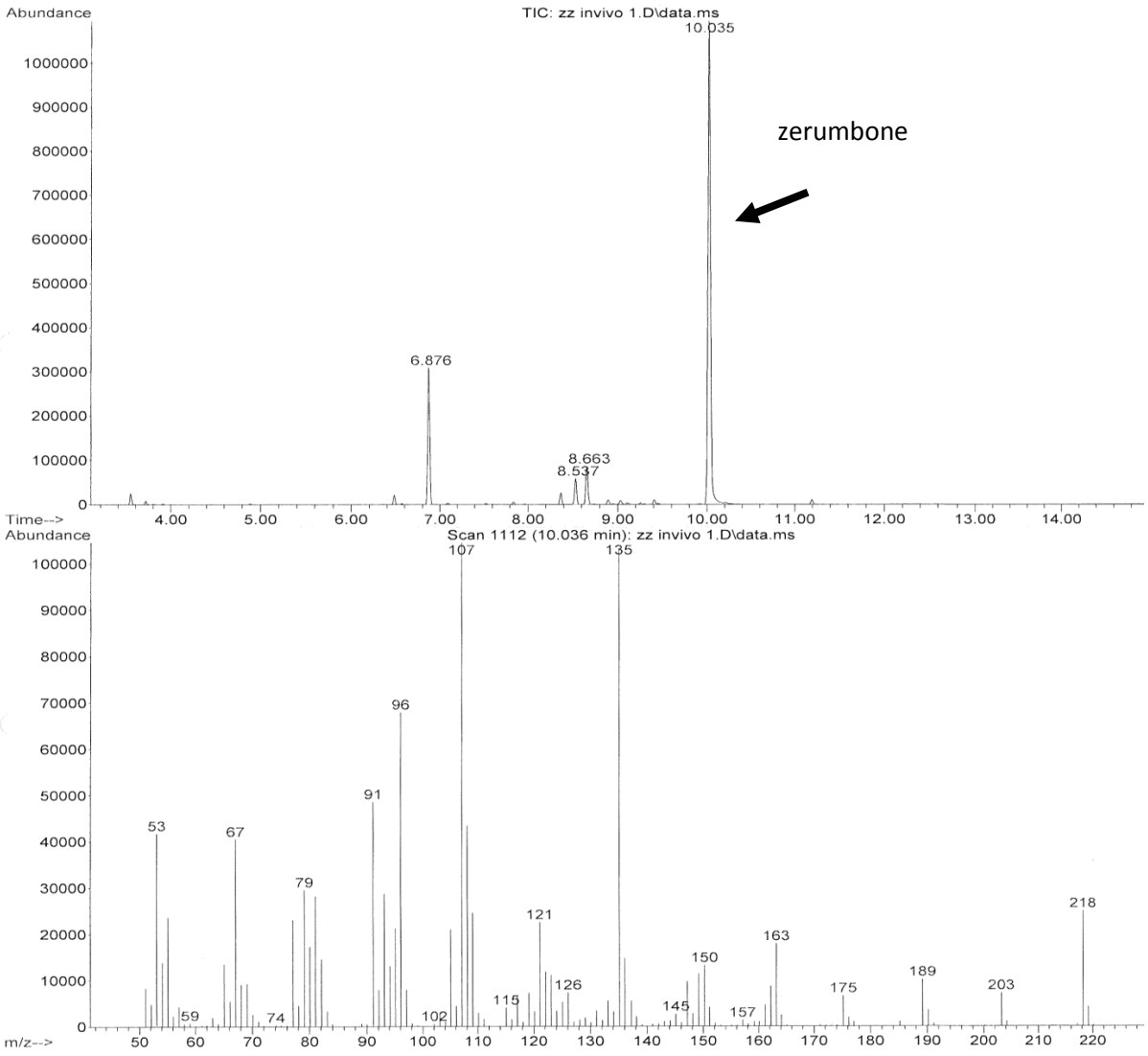


Figure 3.3.2.2c (i) GCMS chromatogram of zerumbone from *in vivo* derived rhizomes using cold soak extraction method

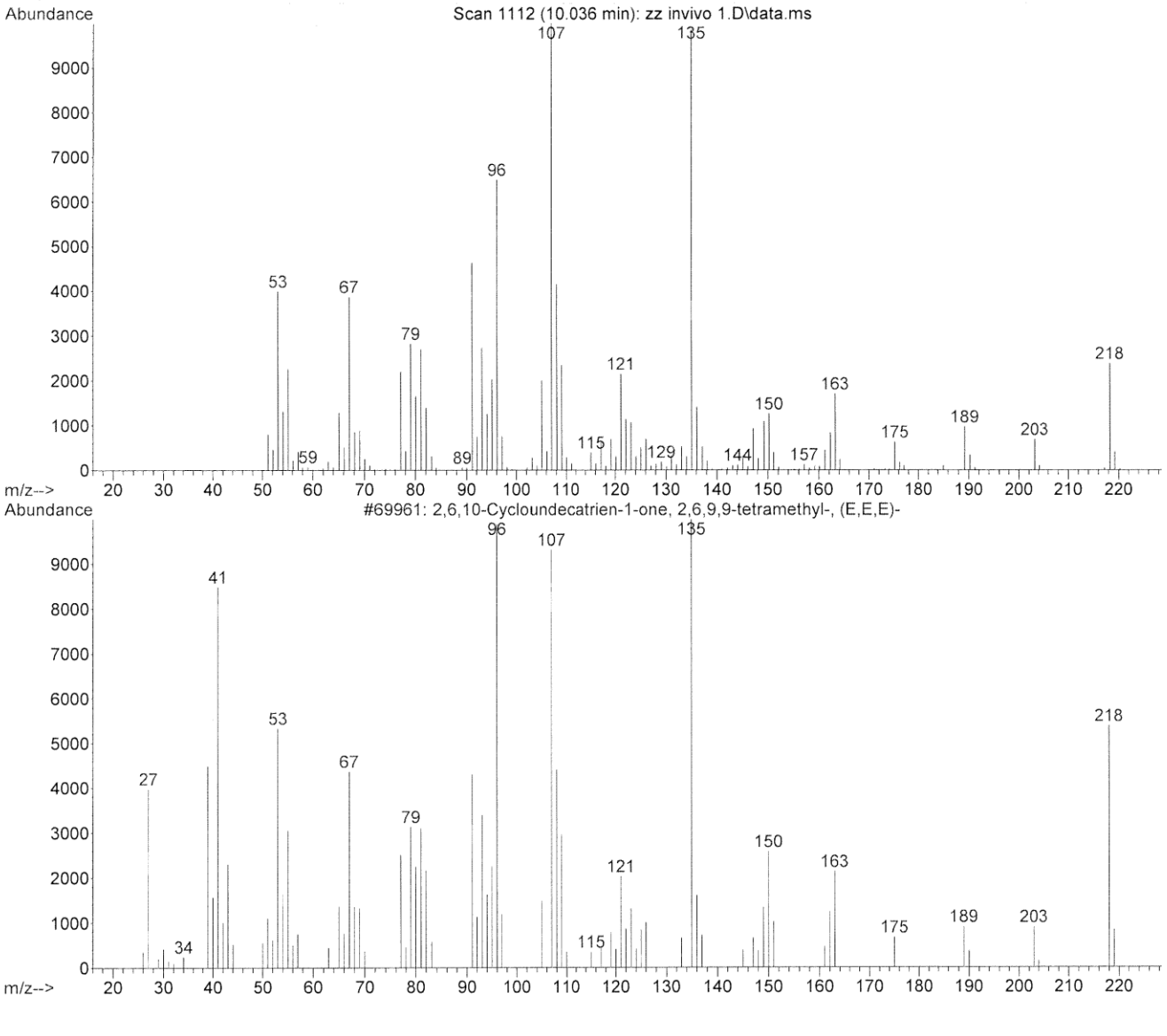


Figure 3.3.2.2c (ii)Cont. GCMS chromatogram of zerumbone from *in vivo* rhizomes using cold soak extraction method

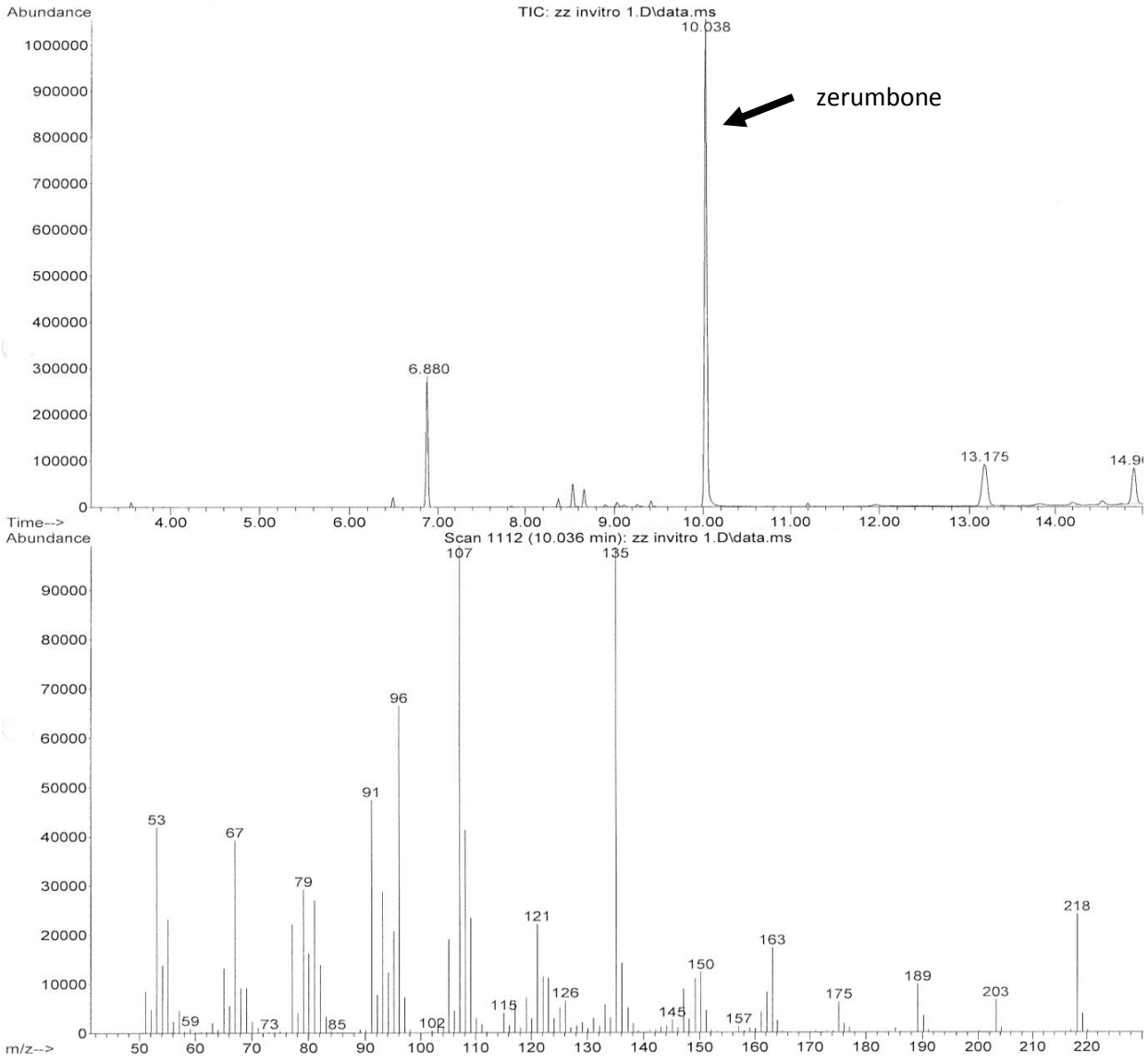


Figure 3.3.2.2d (i) GCMS chromatogram of zerumbone from *in vitro* derived rhizomes using cold soak extraction method

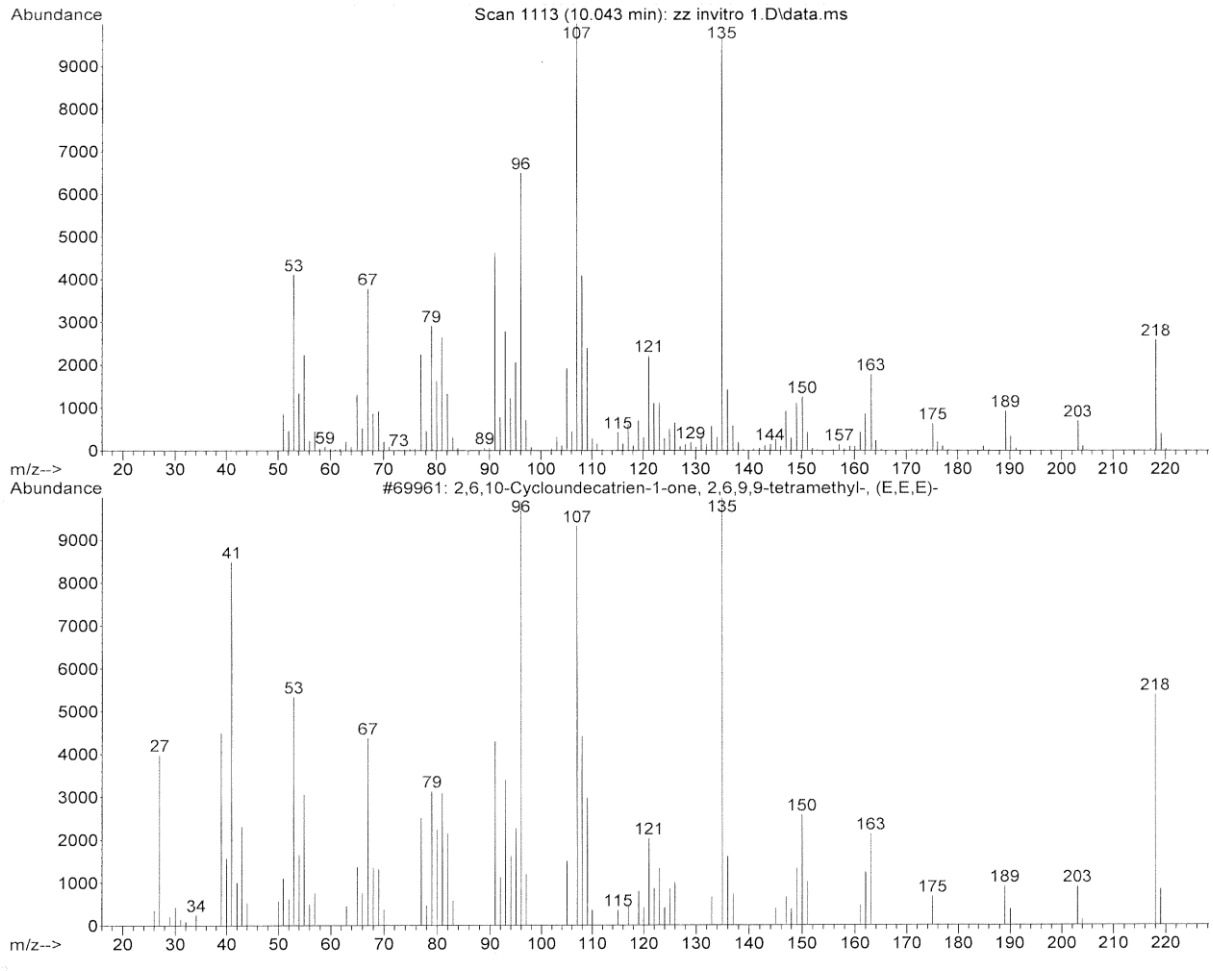


Figure 3.3.2.2d (ii) Cont. GCMS chromatogram of zerumbone from *in vitro* derived rhizomes using cold soak extraction method

Another extraction method used in this study was soxhlet extraction. The same pattern as cold soak extraction was found where *in vitro* derived rhizomes gave the higher yield of zerumbone as compared to *in vivo* rhizomes. Table 3.3.2.2b showed clearly the difference between both sources. The difference was significant at *p*=0.05. *In vitro* derived rhizomes gave higher yield at 4.35±0.2 mg/g than *in vivo* rhizomes with 3.27±0.01 mg/g yield.

Table 3.3.2.2b Mean of zerumbone yield from *in vivo* and *in vitro* derived rhizomes (soxhlet extraction)

Source of rhizomes	**zerumbone (mg/g)**
In vivo rhizomes	3.27±0.1
In vitro derived rhizomes	4.35±0.2

Similar result was obtained from the third extraction method used which was hydro-distillation method. Table 3.3.2.2c showed that *in vitro* derived rhizomes gave higher yield of 2.21±0.2 mg/g than *in vivo* rhizomes with 1.64±0.2 mg/g yield. The difference for both sources were significantly different (*p*=0.05).

Table 3.3.2.2c Mean of zerumbone yield from *in vivo* and *in vitro* derived rhizomes (hydro-distillation)

Source of rhizomes	**zerumbone (mg/g)**
In vivo rhizomes	1.64±0.2
In vitro derived rhizomes	2.21±0.2

All extraction methods showed that *in vitro* derived rhizomes produced the higher yield of zerumbone from *Zingiber zerumbet*.

Cell suspensions and spent liquid media were thought as other sources of zerumbone. From the cell, there was no detection of zerumbone, but approximately 0.04% (w/w) of zerumbone was found in the spent liquid media. The spent liquid media was extracted using partitioning liquid to liquid media and zerumbone was detected using GC and GCMS.

This zerumbone concentration was far lesser from zerumbone obtained from *in vivo* rhizomes and *in vitro* derived rhizomes at thirty-three folds.

3.3.3. Comparison of zerumbone yield from *in vivo* and *in vitro* derived rhizomes with three different extraction methods

From the results obtained using *in vivo* and *in vitro* derived rhizomes, it was observed that *in vitro* derived rhizomes produced higher yield of zerumbone. For instance, from cold soak extraction, *in vitro* derived rhizomes obtained 2.60±0.8 mg/g compared to *in vivo* that only produced 1.74±0.7. Similar pattern were observed from soxhlet and hydro-distillation methods (Figure 3.3.3a).

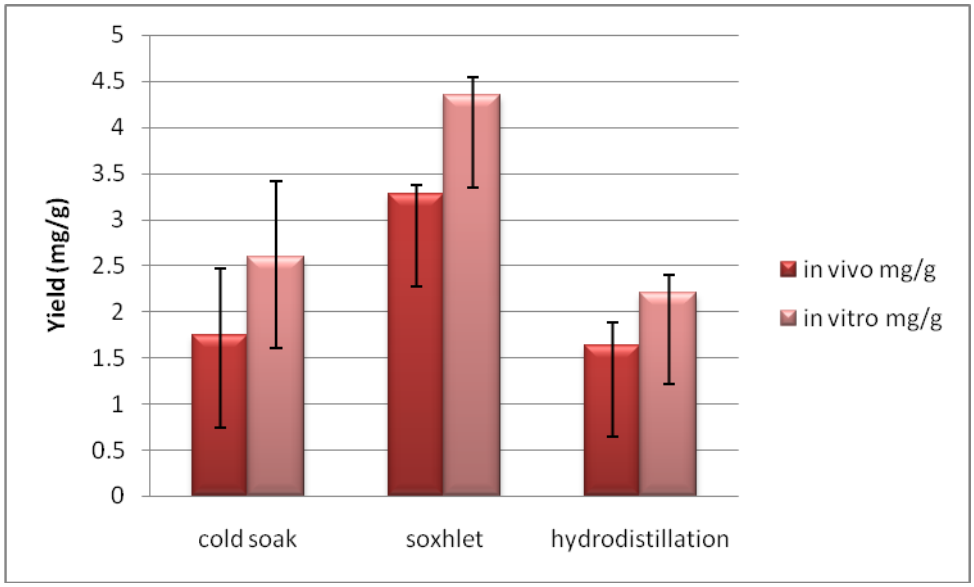


Figure 3.3.3a Comparison of yield using different extraction methods for *in vivo* and *in vitro* derived rhizomes from *Zingiber zerumbet* Smith.

When using raw materials from the same sources, soxhlet extraction gave the highest yield i.e. 4.35±0.2 mg/g compared to two other extraction methods viz. 2.60±0.8 mg/g from cold soak and 2.21±0.2 from hydro-distillation. The data was proven to be significant difference at p=0.005.

3.4. Discussion and conclusion

In vitro plant was known could produce important chemicals such as secondary metabolites. In this study, *in vitro* plants were successfully initiated from tissue culture technique and the zerumbone amount was assessed.

The crude extracts were obtained from *in vivo* rhizomes and *in vitro* derived rhizomes of *lempoyang* using three different methods which are cold soak,

soxhlet and hydro-distillation and their respective yields obtained were then compared. In comparison, the best extraction method for zerumbone was soxhlet with high extraction yield, ease of handling and least time consuming.

It was shown that the yield of zerumbone, a sesquiterpene in *in vitro* derived rhizomes was higher than in *in vivo* rhizomes. Similar results were found for all three extraction methods used in this study. Similar observation was made by Kumari *et al.,* (2007), where secondary metabolites of interest namely esculin and inulin were higher in *in vitro* derived plant, where they used root material for source of extraction. High accumulation of zerumbone in *in vitro* derived plants could be attributed to the fact that the plant materials from tissue culture grew in sterile environment, controlled condition with no interference from contaminants. However, that also could have led to low production of secondary metabolites production in other plants (Lila, 2005).

A sesquiterpene, secondary metabolite called parthenolide was also significantly accumulated in *in vitro* derived plant as compared to open field herbs (Rateb *et al.,* 2007). Junaid *et al., (*2009) found that vincristine, which is an important secondary metabolite for cancer treatment, was accumulated higher in *in vitro* leaves of *Catharanthus roseus.* Overall, *in vitro* plants will give the higher amount of secondary metabolites compared to *in vivo* plant, where it only differ by which plant part was used for the source of extraction (e.g. shoots, leaves, roots, rhizomes etc).

In this study, cell suspension culture was one of the *in vitro* sources (which were initiated from embryogenic callus) examined for zerumbone. Zerumbone was

found present in the spent medium and none in the dried cell mass from the cell suspension culture. Similar observations were made in celery (*Apium graveolens*) where the terpenoids compound namely limonene were found in the media (Watts *et al.,* 1984). Essential oils of *M. Piperita* were also found in the spent liquid medium (Kim *et al.,* 1996).

Although it could be suspected that cells lyses were causing the zerumbone to exist in the spent liquid medium, but in the other hand, secretion of terpenoids such as essential oil was confirmed by Evert, (2006). Since zerumbone is one of the terpenoids and also present in essential oil form, zerumbone could have been excreted also into the liquid medium.

The concentration of zerumbone in the liquid medium was low and was not comparable to the amount found in the *in vitro* derived rhizomes. In many cases plant cells suspension yield of secondary metabolites are quite low. This could be due to cells which were initiated from callus is undifferentiated entities (Kim *et al.,* 1996, Pande *et al.,* 2002, Hiraoka *et al.,* 2004, Ma *et al.,* 2006, and Zayeda *et al.,* 2006). Kumari *et al.,* (2007) reported that secondary metabolites like inulin and esculin were found in low level when extracted from callus culture. Chawla, (2004) also stated that lack of tissues and organ differentiations in most callus and cell suspension caused the lower yield of secondary metabolites. Low production of secondary metabolites could also be due to high hormone concentration (1 or 2 mg/L), which promotes non-productive callus since all the cells are mitotic rather than producing secondary metabolites n (Rateb *et al.,* 2007).

As a conclusion, tissue culture sources which were *in vitro* derived rhizomes and suspension cultures could be alternative sources of zerumbone instead of using traditionally propagated rhizomes. Significantly, accumulation of zerumbone higher in *in vitro* derived rhizomes proved the relevance of tissue culture derived materials to be used in plant mass propagation. Modification of culture conditions, hormonal manipulation and addition of suitable precursors to suspension cultures could enhance the production of zerumbone and thus a promising method.

CHAPTER 4

GENERAL CONCLUSION

In this study, direct and indirect plant regeneration of *Zingiber zerumbet (L) Sm.*Smith or lempoyang have been successfully carried out. Field assessment results showed that *in vitro* derived rhizomes were more vigorous and healthier than conventionally propagated rhizomes. In addition, a more efficient source of plant mass propagation through regenerable cell suspensions were developed producing 210 plants per ml settled cell volume.

Both *in vitro* derived rhizomes and suspension cultures could be used as alternative sources of zerumbone instead of traditionally propagated rhizomes. In comparison, zerumbone yield was twice higher from *in vitro* derived rhizomes compared to rhizomes conventional propagation. Cell suspension cultures also produced zerumbone extracellular. This is advantageous since this will ease compound extraction. Although the amount is not comparable to the field grown rhizomes, this allows further work on the enhancement of this compound through physical methods and metabolic engineering.

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APPENDIX 1

Media formulation

MS (Murashige and Skoog, 1962) basal media

Essential element	**Concentration in stock solution (mg/l)**	**Concentration in medium (mg/l)**
1. Macro nutrient		
$CaCl_2.2H_2O$	4400	440
NH_4NO_3	16500	1650
KNO_3	19000	1900
KH_2PO_4	1700	170
$MgSO_4.7H_2O$	3700	370
2. Micro nutrient		
KI	8.3	0.83
$CoCl_2.6H_2O$	0.25	0.025
H_3BO_3	62.0	6.2
Na_2MoO_4	2.5	0.25
$MnSO_4.H_2O$	223.0	22.3
$CuSO_4.H_2O$	0.25	0.025
$ZnSO_4.7H_2O$	86.0	8.6
3. Iron source		
$FeSO_4.7H_2O$	278.5	27.85
Na_2EDTA	372.5	37.25
4. Organic supplement		
Glycine	20	2.0

Nicotinic acid Pyridoxine- HCl Thiamine- HCl Myoinositol	5 5 1 1000	0.5 0.5 0.1 100
5. Carbon source Sucrose		30g/l
6. Solidify agent Phytagel		2g/l

Chu (N6) basal media

Essential element	**Concentration in stock solution (mg/l)**	**Concentration in medium (mg/l)**
1. Macro nutrient $CaCl_2.2H_2O$ NH_4NO_3 KNO_3 KH_2PO_4 $MgSO_4.7H_2O$	 1253.3 - 28300 4000 903.7	 125.33 - 2830 400 90.37
2. Micro nutrient KI $CoCl_2.6H_2O$ H_3BO_3 Na_2MoO_4 $MnSO_4.H_2O$ $CuSO_4.H_2O$ $ZnSO_4.7H_2O$	 8.0 - 16 - 33.3 - 15	 0.80 - 1.6 - 3.33 - 1.5

3. Iron source		
$FeSO_4.7H_2O$	278.5	27.85
Na_2EDTA	372.5	37.25
4. Organic supplement		
Glycine	20	2.0
Nicotinic acid	5	0.5
Pyridoxine- HCl	5	0.5
Thiamine- HCl	1	0.1
Myoinositol	-	-
5. Carbon source		
Sucrose		30g/l
6. Solidify agent		
Phytagel		2g/l

Gamborg's B-5 basal media

Essential element	**Concentration in stock solution (mg/l)**	**Concentration in medium (mg/l)**
1. Macro nutrient		
$CaCl_2.2H_2O$	1123.4	112.34
NH_4NO_3	-	-
NH_4PO_4	1340	134
KNO_3	25000	2500
KH_2PO_4	-	-
$MgSO_4.7H_2O$	1220.9	122.09

2. Micro nutrient		
KI	7.5	0.75
$CoCl_2.6H_2O$	0.25	0.025
H_3BO_3	3.0	0.3
Na_2MoO_4	2.5	0.25
$MnSO_4.H_2O$	100.0	10.0
$CuSO_4.H_2O$	0.25	0.025
$ZnSO_4.7H_2O$	20.0	2.0
3. Iron source		
$FeSO_4.7H_2O$	278.0	27.8
Na_2EDTA	373.0	37.3
4. Organic supplement		
Glycine	-	-
Nicotinic acid	10.0	1.0
Pyridoxine- HCl	10.0	1.0
Thiamine- HCl	100.0	10.0
Myoinositol	1000	100
5. Carbon source		
Sucrose		30g/l
6. Solidify agent		
Phytagel		2g/l

Callus induction media (M1) Cote *et al.,* (1987)

Essential element	**Concentration in stock solution (mg/l)**	**Concentration in medium (mg/l)**
1. Macro nutrient		
$CaCl_2.2H_2O$	4400	440

NH_4NO_3	16500	1650
KNO_3	19000	1900
KH_2PO_4	1700	170
$MgSO_4.7H_2O$	3700	370
2. Micro nutrient		
KI	8.3	0.83
$CoCl_2.6H_2O$	0.25	0.025
H_3BO_3	62.0	6.2
Na_2MoO_4	2.5	0.25
$MnSO_4.H_2O$	223.0	22.3
$CuSO_4.H_2O$	0.25	0.025
$ZnSO_4.7H_2O$	86.0	8.6
3. Iron source		
$FeSO_4.7H_2O$	278.5	27.85
Na_2EDTA	372.5	37.25
4. Organic supplement		
Glycine	20	2.0
Nicotinic acid	5	0.5
Pyridoxine- HCl	5	0.5
Thiamine- HCl	1	0.1
Myoinositol	1000	100
5. Carbon source		
Sucrose		30g/l

6. Solidify agent Phytagel		2g/l
7. Others D biotin 2,4 D IAA NAA		1mg/L 1mg/L 1mg/L 1mg/L

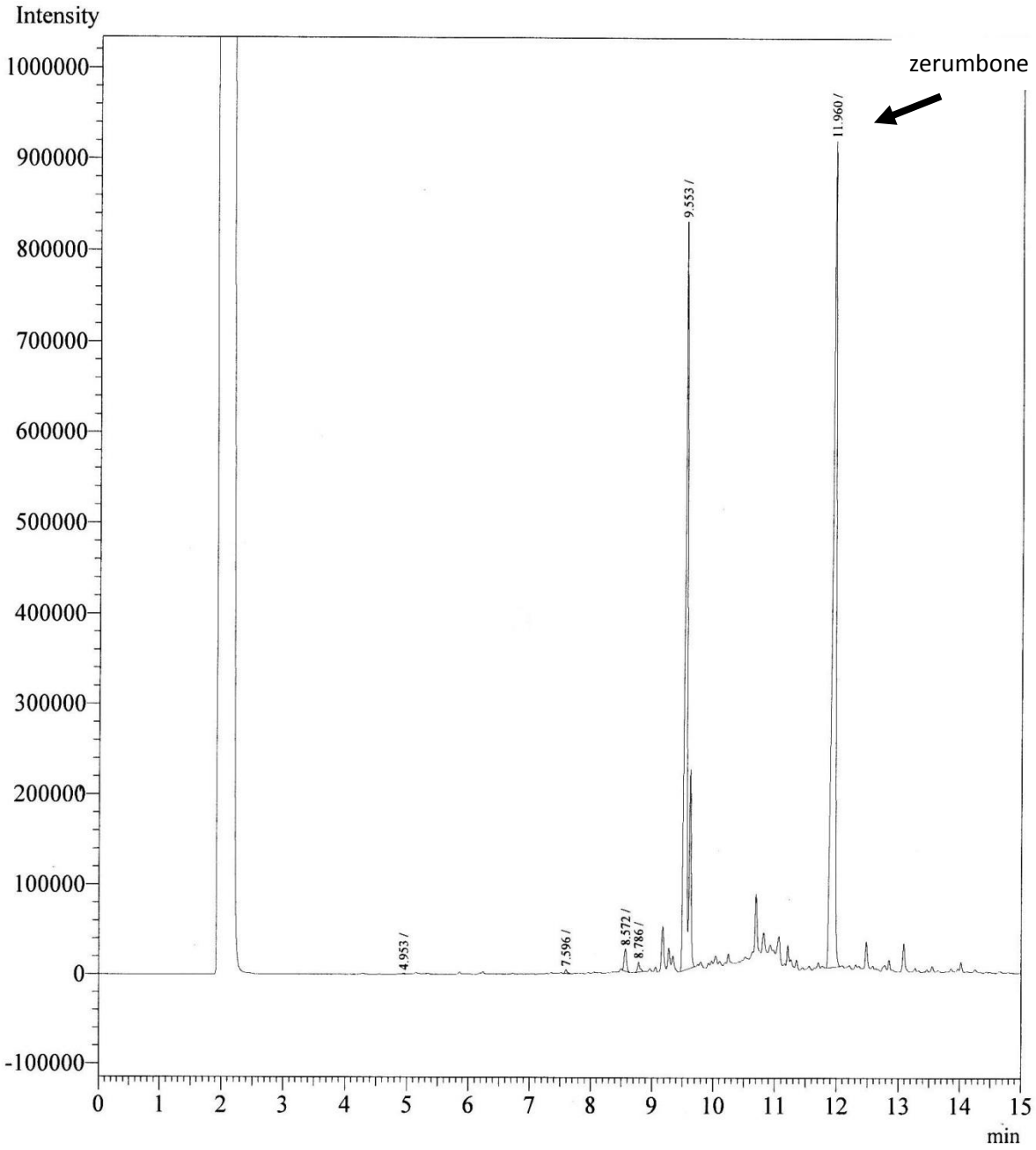
Cell suspension maintenance media (M2) Dhead'a *et al.,* 1967

Essential element	**Concentration in stock solution (mg/l)**	**Concentration in medium (mg/l)**
1. Macro nutrient $CaCl_2.2H_2O$ NH_4NO_3 KNO_3 KH_2PO_4 $MgSO_4.7H_2O$	 4400 16500 19000 1700 3700	 Half strength
2. Micro nutrient KI $CoCl_2.6H_2O$ H_3BO_3 Na_2MoO_4 $MnSO_4.H_2O$ $CuSO_4.H_2O$ $ZnSO_4.7H_2O$	 8.3 0.25 62.0 2.5 223.0 0.25 86.0	 0.83 0.025 6.2 0.25 22.3 0.025 8.6

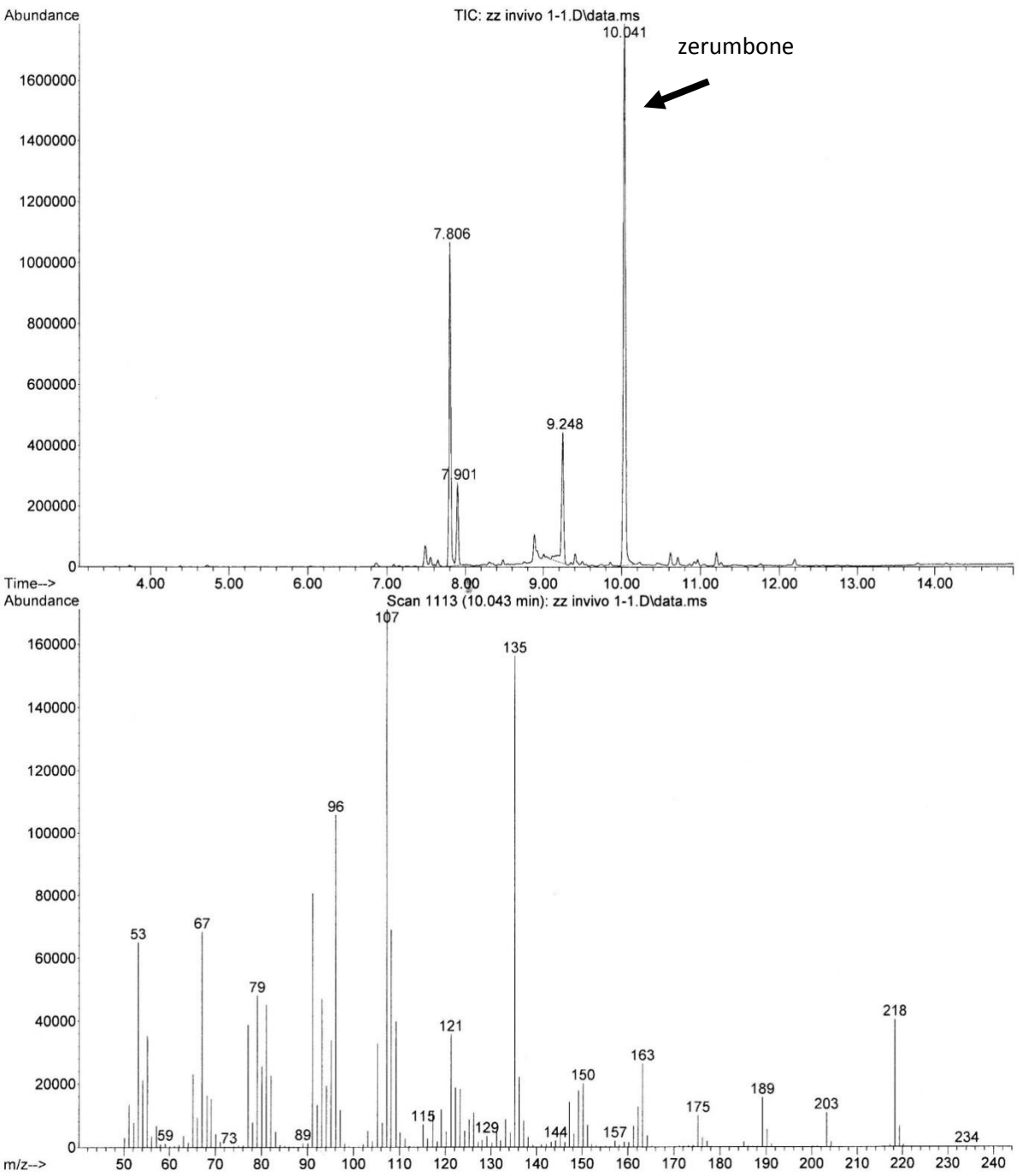
3. Iron source $FeSO_4.7H_2O$ Na_2EDTA	 278.5 372.5	 27.85 37.25
4. Dhed'a vitamin Glycine Nicotinic acid Pyridoxine- HCl Thiamine- HCl	 200 500 500 400	 2.0 0.5 0.5 0.4
5. Carbon source Sucrose		 30g/l
6. Solidify agent Phytagel		 2g/l
7. Others Ascorbic acid Zeatin	 1 1	 10 ml 0.25

APPENDIX 2

CHROMATOGRAM



GC chromatogram of zerumbone from *in vivo* rhizomes using soxhlet extraction



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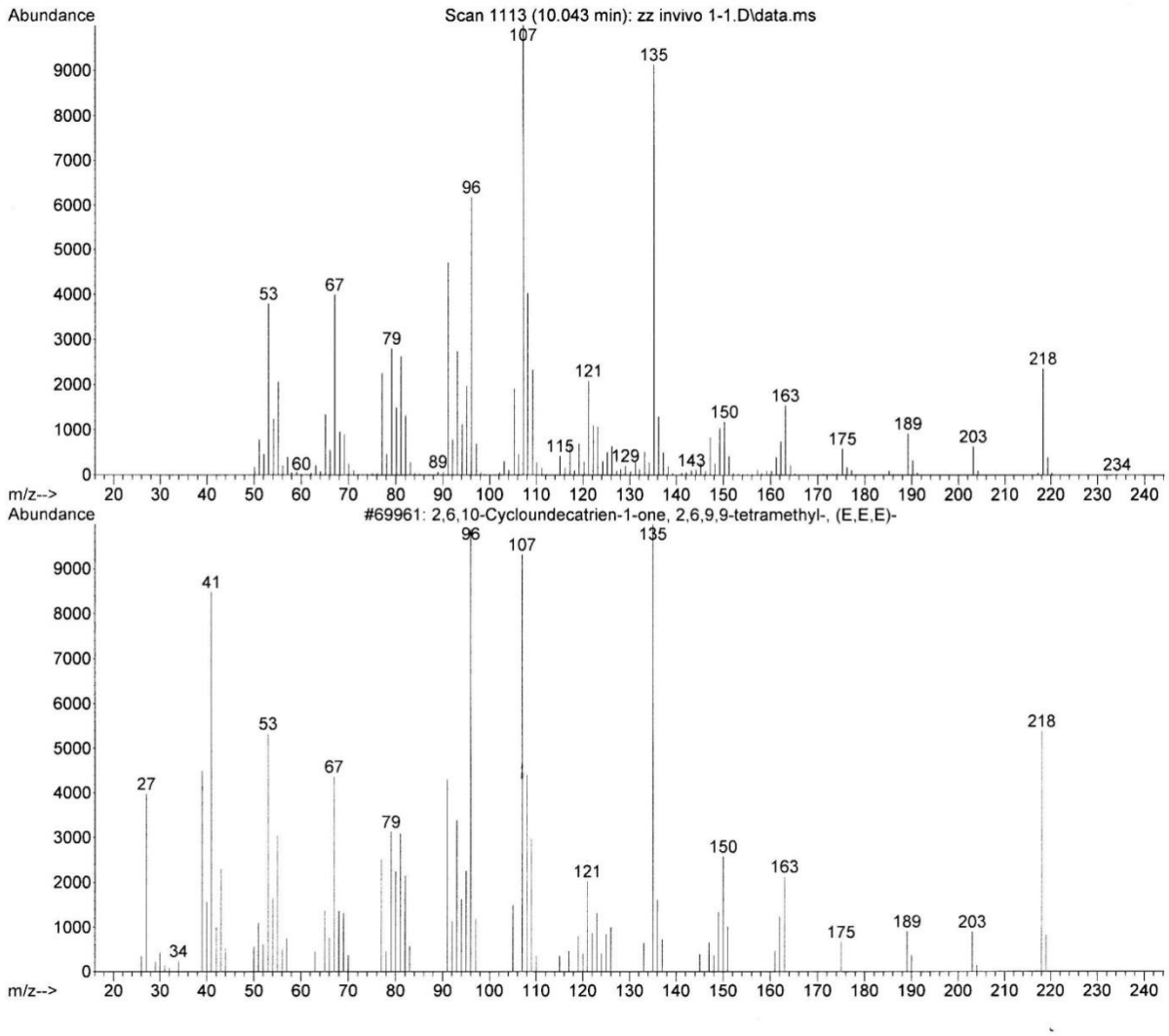
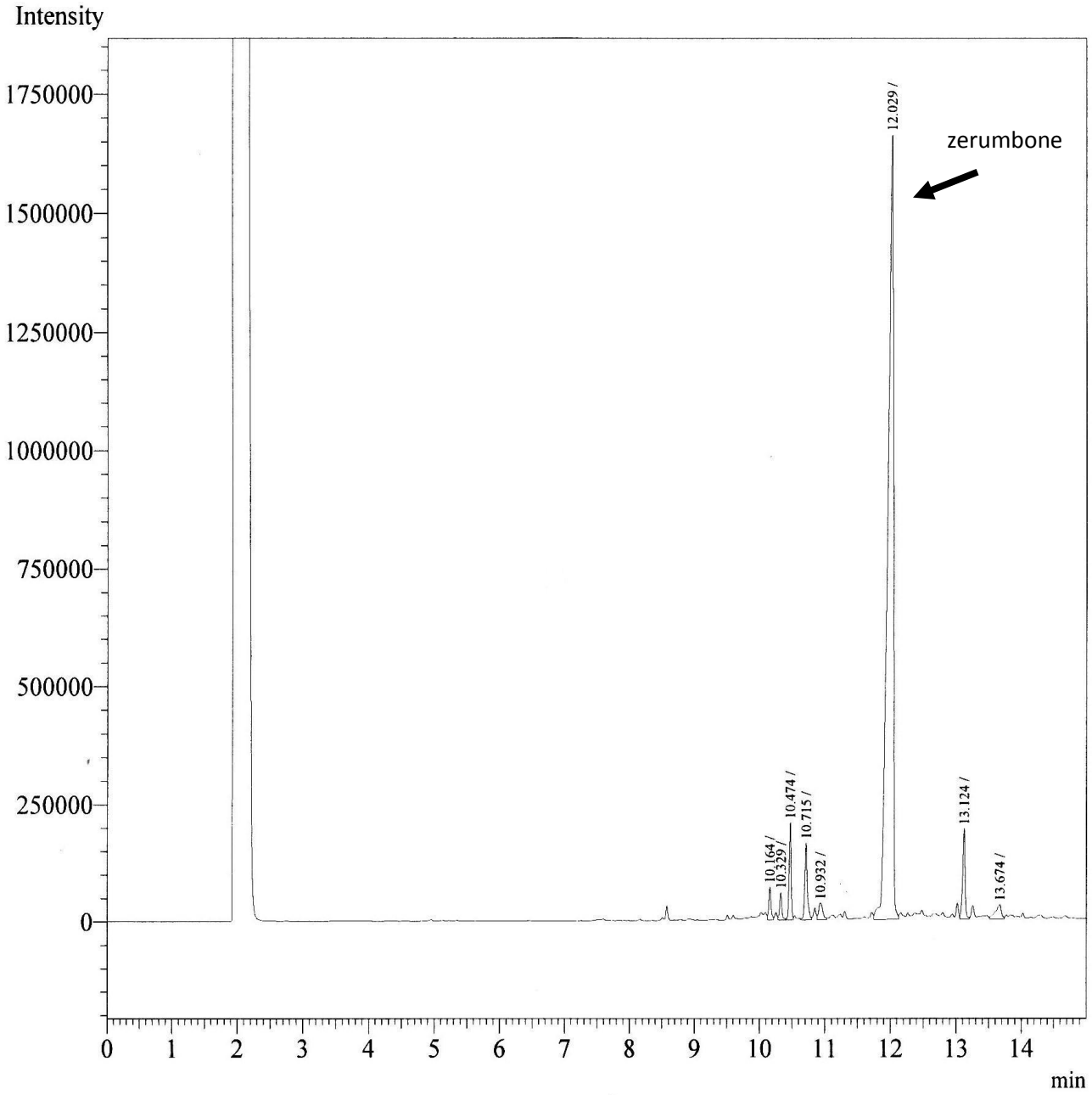
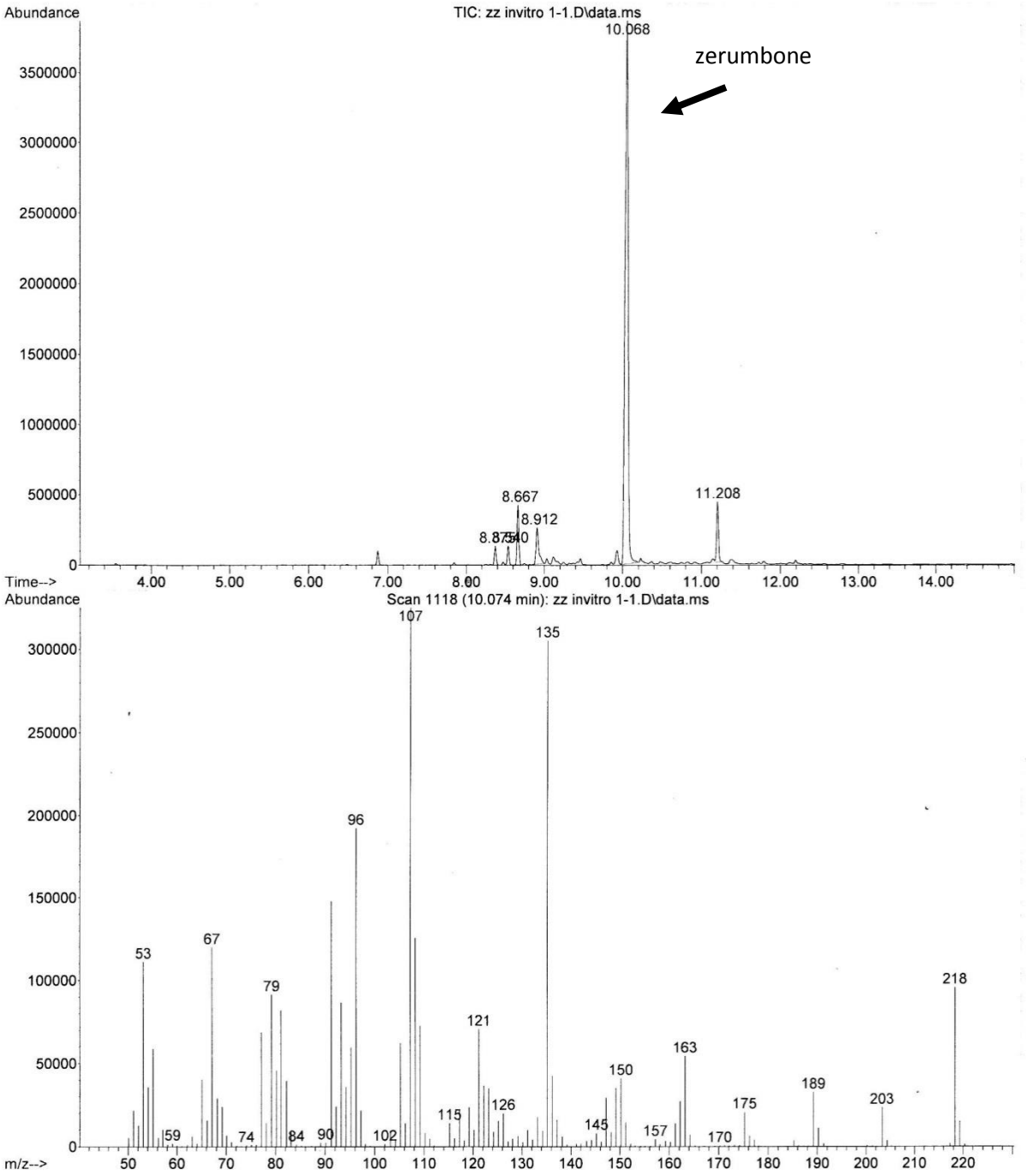


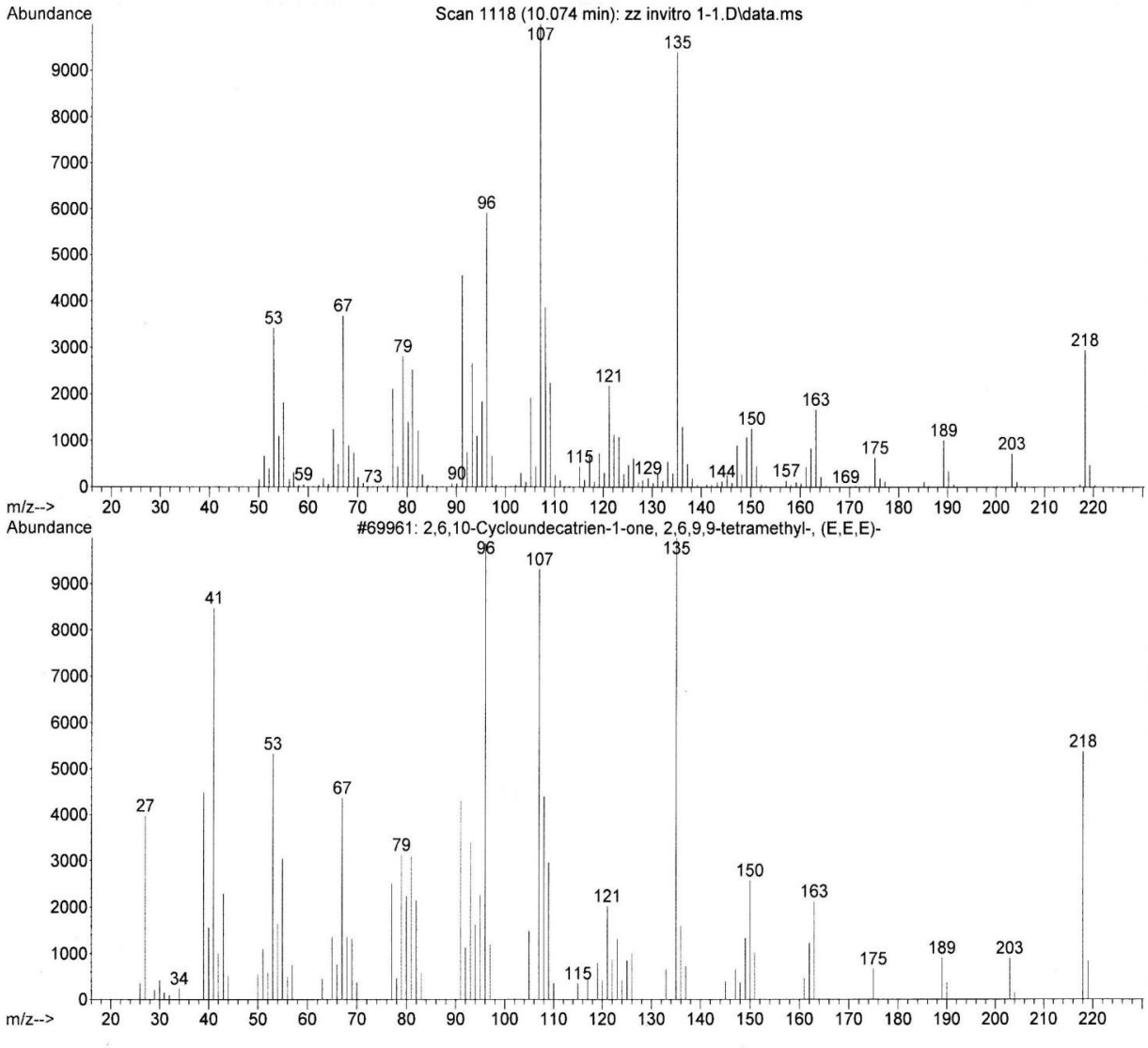
GCMS chromatogram of zerumbone from *in vivo* rhizomes using soxhlet extraction



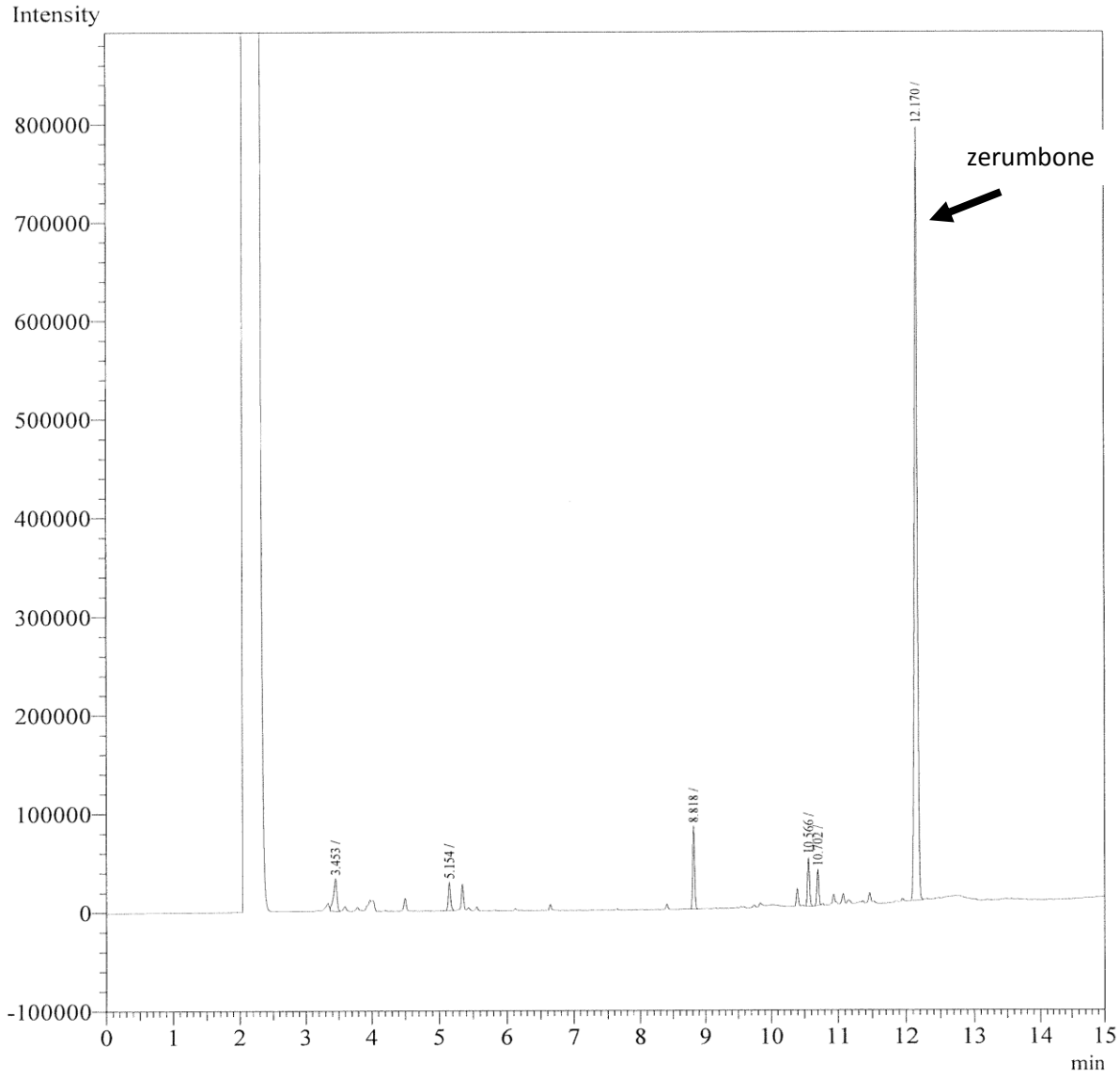
GC chromatogram of zerumbone from *in vitro* derived rhizomes using soxhlet extraction



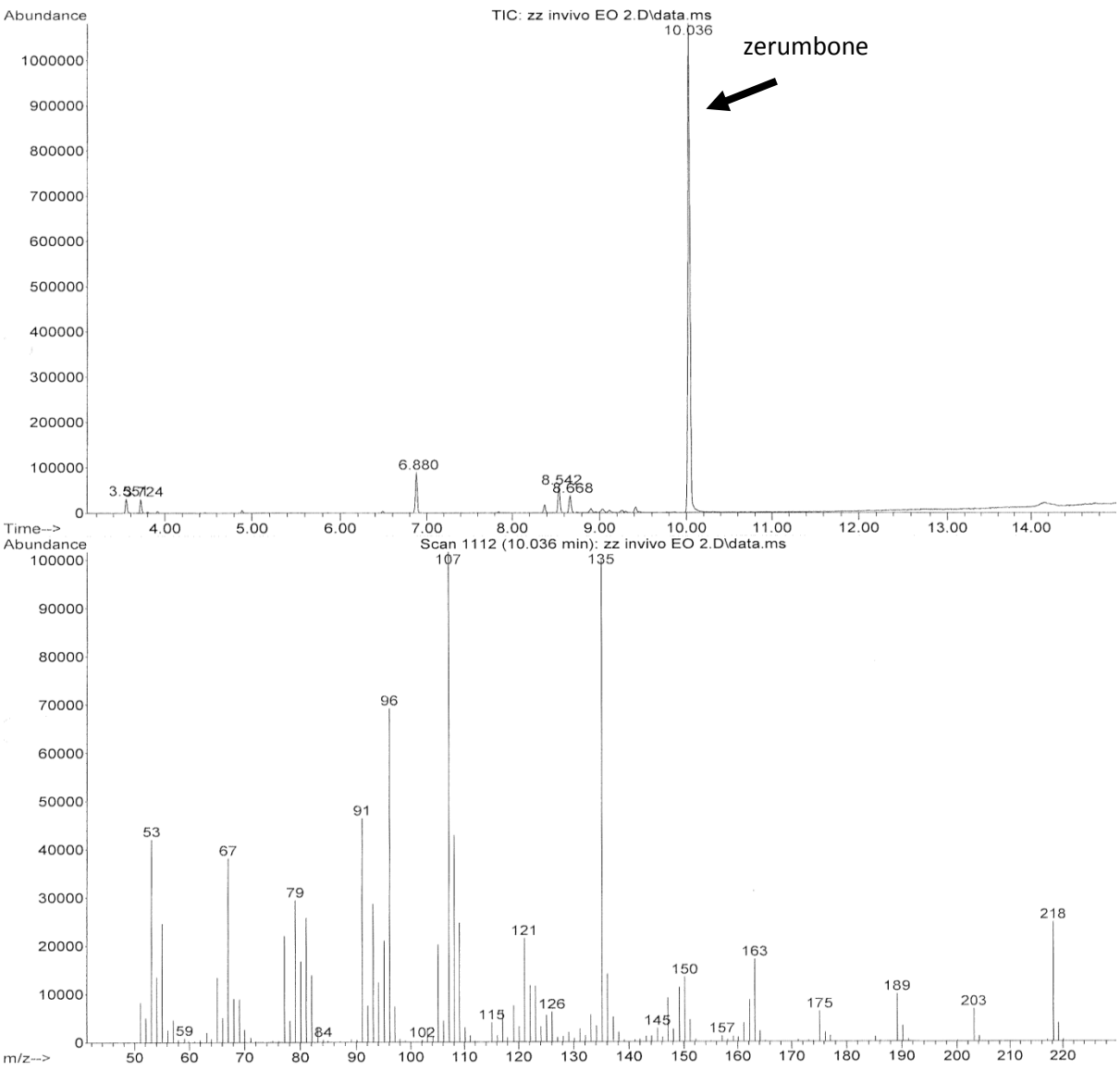
GCMS chromatogram of zerumbone from *in vitro* derived rhizomes using soxhlet extraction



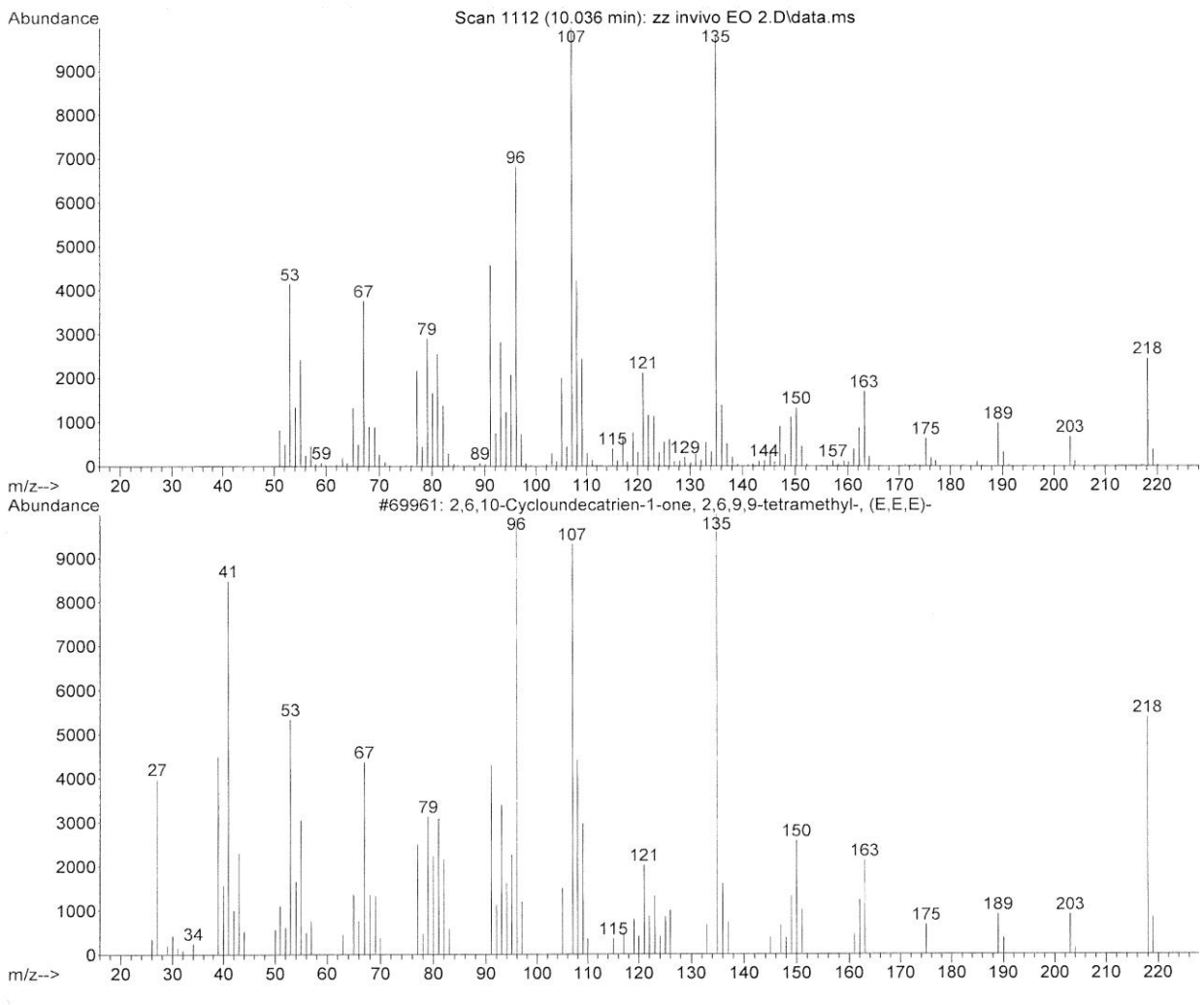
GCMS chromatogram of zerumbone from *in vitro* derived rhizomes using soxhlet extraction



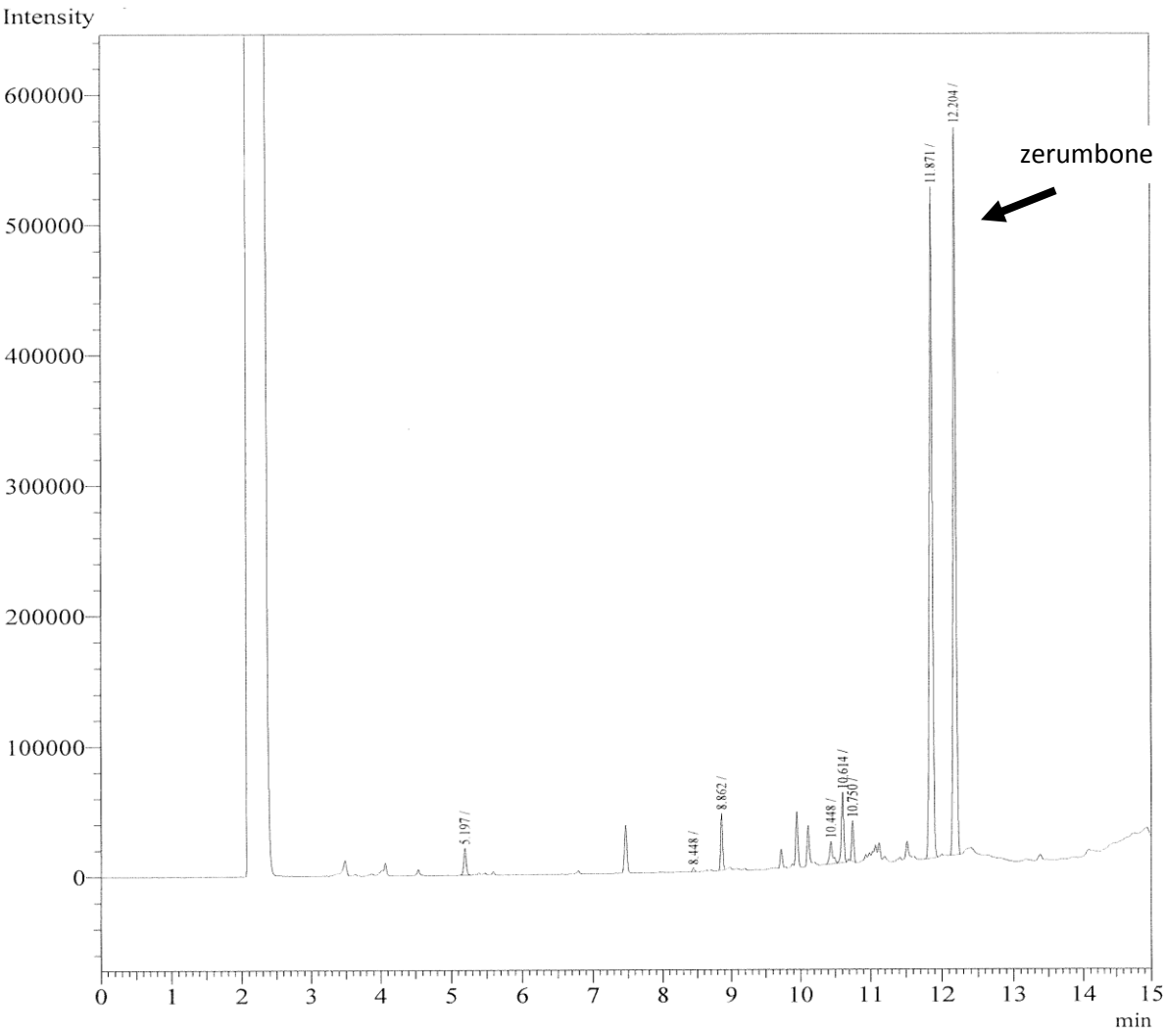
GC chromatogram of zerumbone from *in vivo* rhizomes using hydro-distillation method



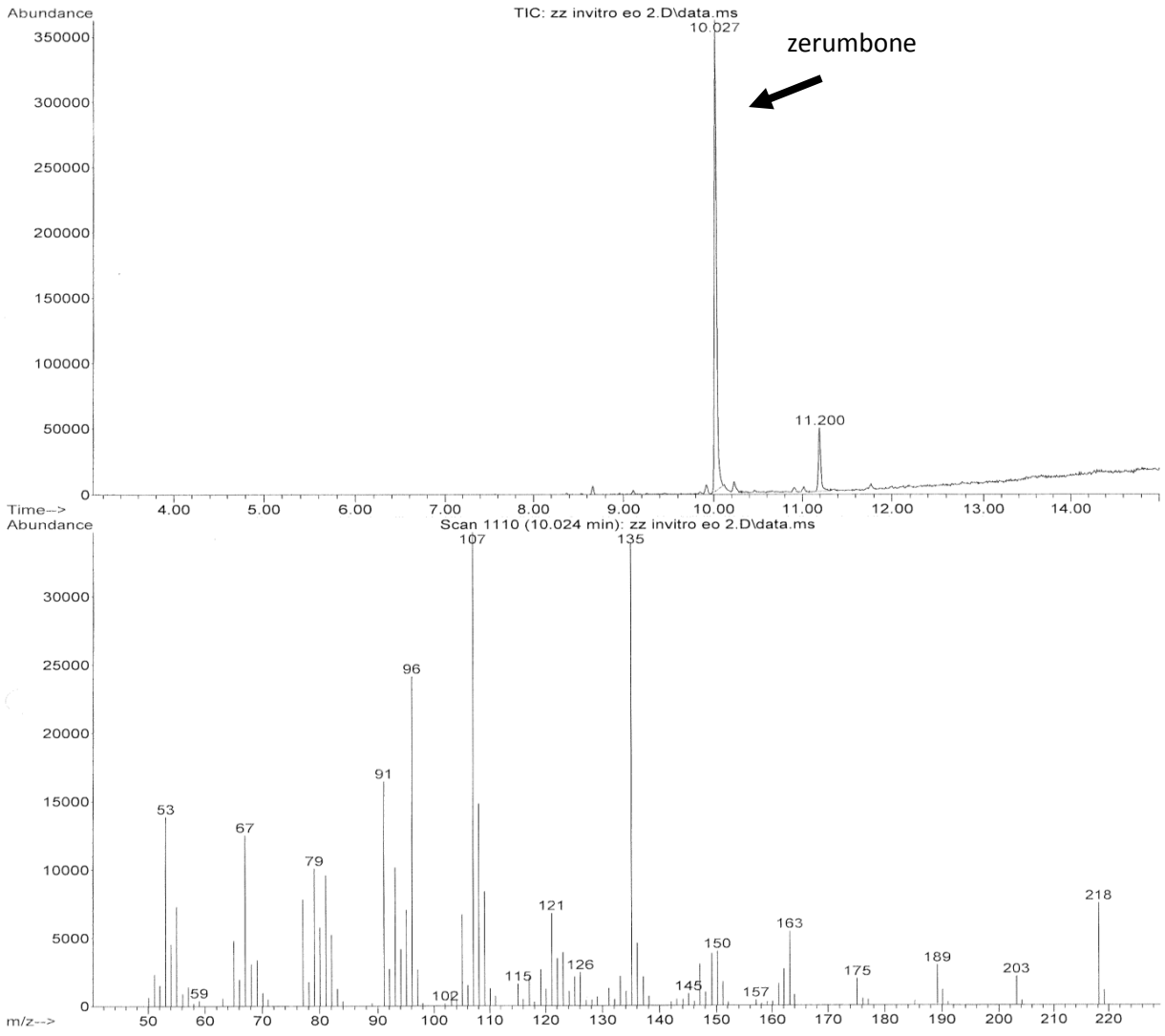
GCMS chromatogram of zerumbone from *in vivo* rhizomes using hydro-distillation method



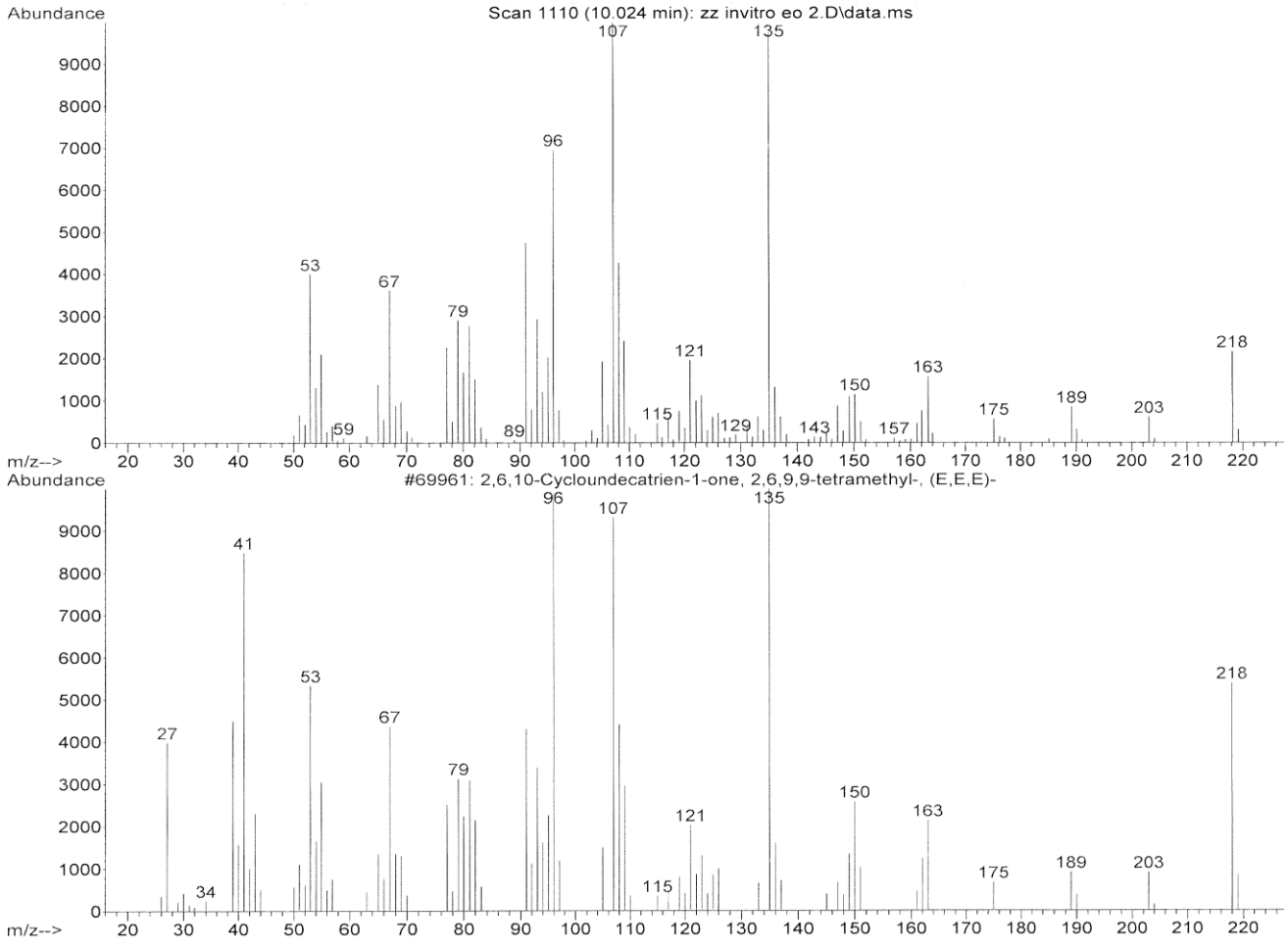
GCMS chromatogram of zerumbone from *in vivo* rhizomes using hydro-distillation method



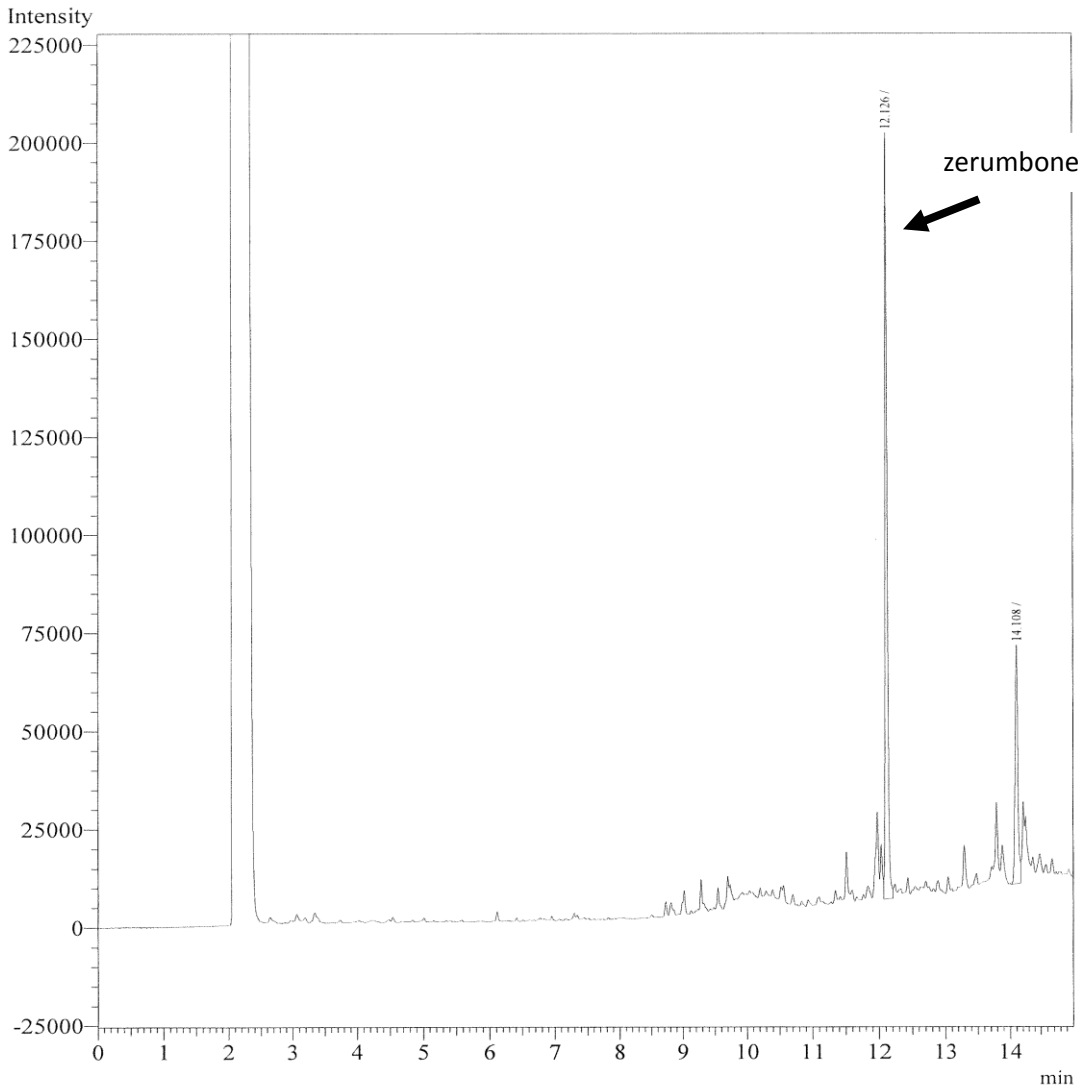
GC chromatogram of zerumbone from *in vitro* derived rhizomes using hydro-distillation method



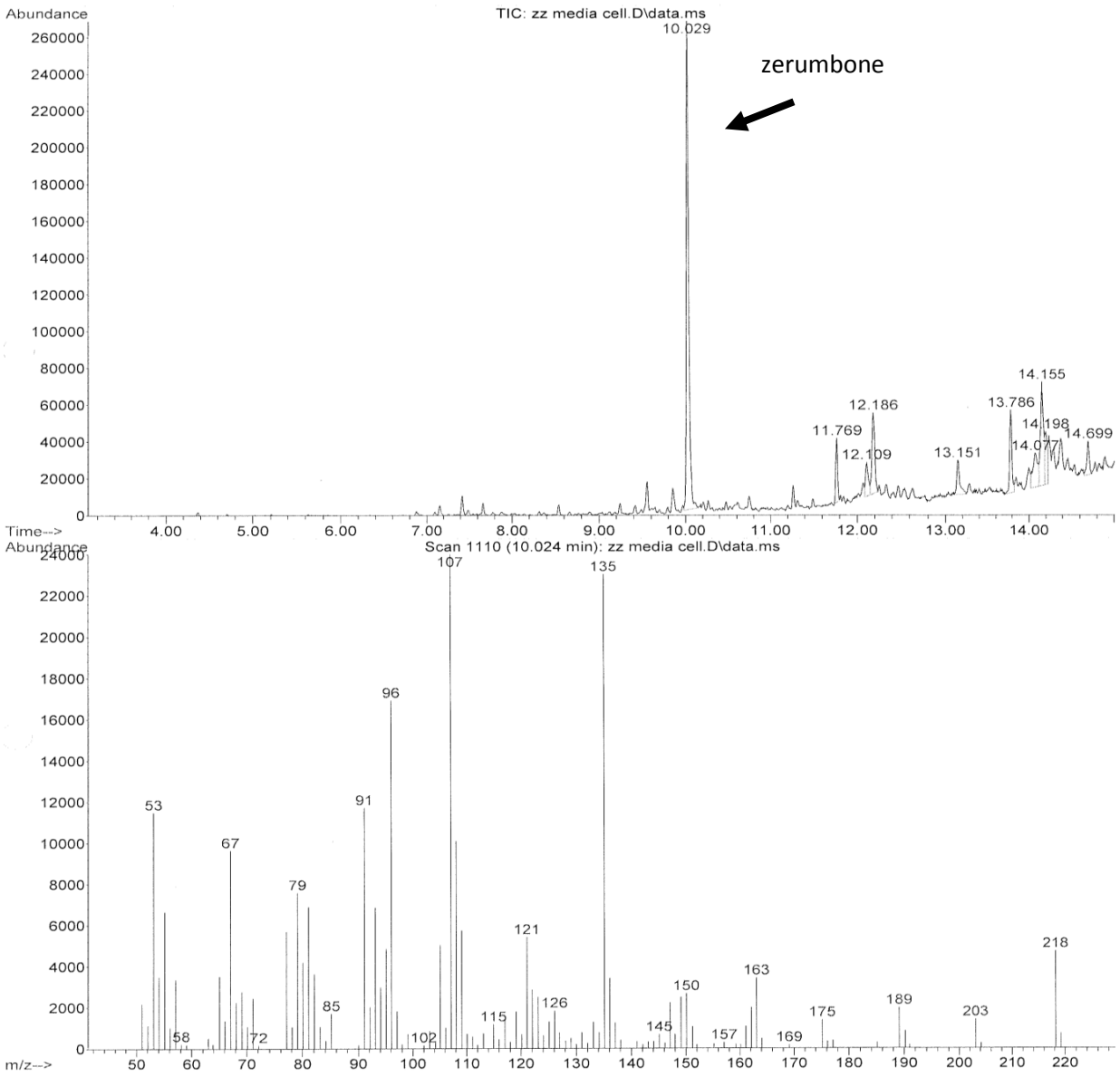
GCMS chromatogram of zerumbone from *in vitro* derived rhizomes using hydro-distillation method



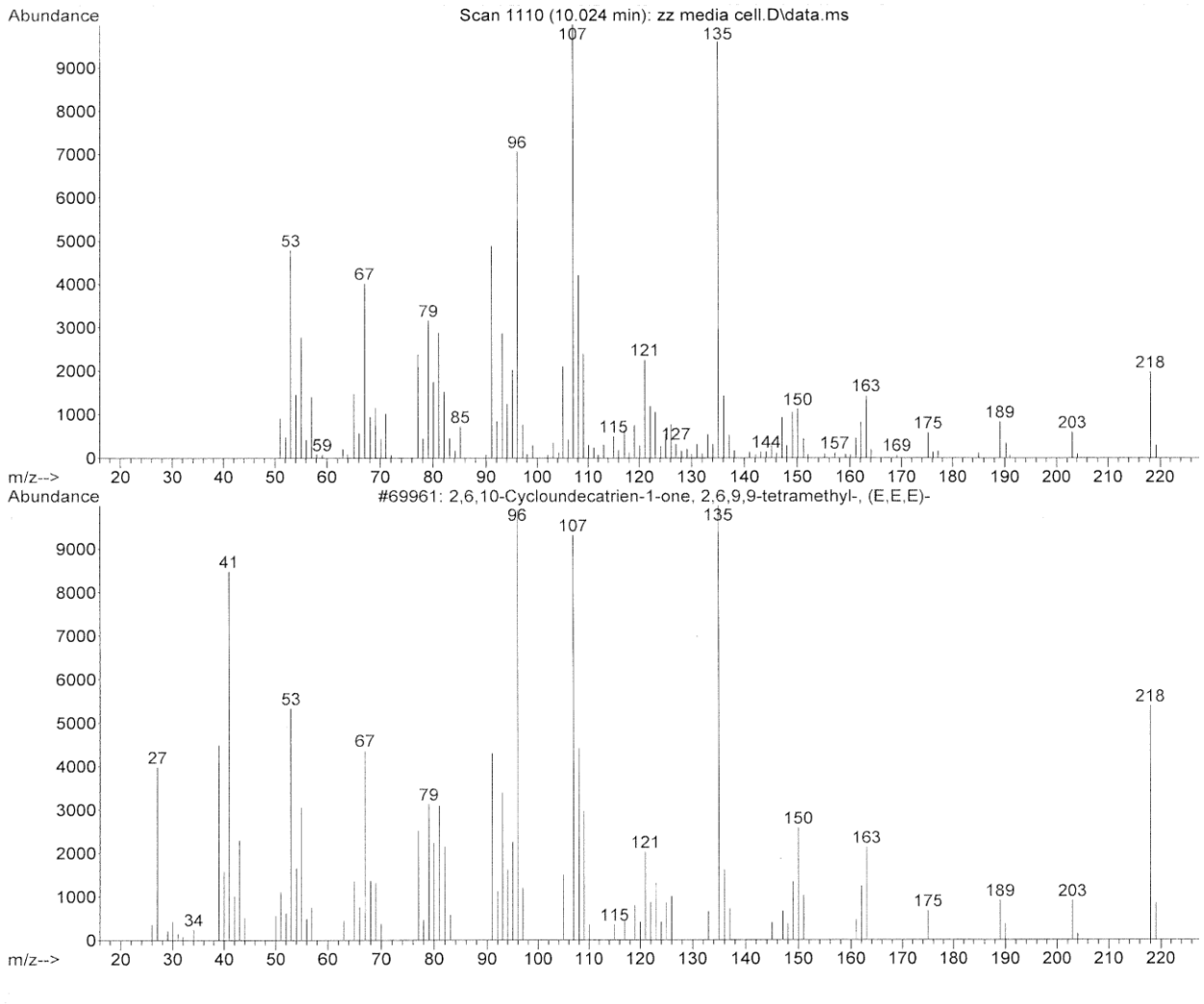
GCMS chromatogram of zerumbone from *in vitro* derived rhizomes using hydro-distillation method



GC chromatogram of zerumbone from media of cell suspension culture using hydro-distillation method



GCMS chromatogram of zerumbone from media of cell suspension culture using hydro-distillation method



GCMS chromatogram of zerumbone from media of cell suspension culture using hydro-distillation method