CHARACTERIZATION OF COMPOUNDS AND ESSENTIAL OILS FROM *Curcuma zedoaria*, AND EVALUATION OF THEIR CYTOTOXIC AND APOPTOTIC PROPERTIES

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CHARACTERIZATION OF COMPOUNDS AND ESSENTIAL OILS FROM *Curcuma zedoaria*, AND EVALUATION OF THEIR CYTOTOXIC AND APOPTOTIC PROPERTIES

ABSTRACT

The phytochemical and cytotoxic studies were carried out on the rhizome of *Curcuma zedoaria* (Christm) Rosc. known as white turmeric or zedoary locally known as *temu putih*. *C. zedoaria* is an aromatic perennial herb, belonging to the family Zingiberaceae. It has been used in traditional and alternative medicine to treat various ailments including cancer in Asian countries. The phytochemical studies of the Indonesian *C. zedoaria* crude extracts were done by bioassay-guided isolation. The ethanol extract, hexane soluble fraction, dichloromethane soluble fraction and the residue were tested for their cytotoxic activity against selected cancer cell lines. Hexane soluble-fraction was selected to be further fractionated on lung cancer cell line, A549. Four sesquiterpene compounds namely spathulenol (1), *β*-eudesmol (2), dehydrocurdione (3) and curcumenone (4) were characterized from the active fraction H1.2. spathulenol (1), *β*-eudesmol (2) and dehydrocurdione (3) exhibited cytotoxic activity towards lung cancer cell lines, A549 and SK-LU-1 with IC_{50} values ranging from 11.0 µg/mL to 22.9 µg/mL with spathulenol being the most potent. The rhizome essential oils of *C. zedoaria* from Malaysia and Indonesia were obtained by hydro distillation and analysed by GC-FID, GC/MS and Kovat indices. Comparison of the main chemical constituents of both essential oils revealed camphor (17.6% and 19.7%, respectively), zerumbone (17.1% and 12.1%) and curzerenone (10.2% and 7.4%). These essential oils exhibited dose dependent cytotoxic activity (MTT assay) against cancer cell lines: breast (MCF-7 and MDA-MB-231), lung (A549 and SK-LU-1) and cervical

(HeLa S3 and SiHa) with IC_{50} values in the range of 6.4 µg/mL to 22.0 µg/mL. Overall, Malaysian *C. zedoaria* essential oil showed higher potent cytotoxic activity as compared to the Indonesian essential oil towards cancer cell lines tested in particular the cervical cancer cell lines, HeLa S3 and SiHa. The IC_{50} values of Malaysian *C. zedoaria* oil are 6.4 µg/mL for HeLa S3 and 9.8 µg/mL for SiHa while 21.6 µg/mL and 11.5 µg/mL, respectively for the Indonesian oil. The cell death of cervical cancer cell lines, HeLa S3 and SiHa was confirmed by live dead viability assay. Apoptosis was detected by Annexin V-FITC/PI assay and caspase-3/7 activity assay.

Keywords: *Curcuma zedoaria*, bioassay-guided isolation, essential oils, cytotoxic activity, apoptosis.

PENCIRIAN SEBATIAN SEMULAJADI DAN MINYAK PATI *Curcuma zedoaria* DAN PENILAIAN SITOTOKSIK DAN APOPTOTIK

ABSTRAK

Ujikaji fitokimia dan sitotoksik telah dijalankan keatas rizom *Curcuma zedoaria* (Christm) Rosc. yang dikenali dengan nama tempatan sebagai *temu putih*. *C. zedoaria* adalah sejenis tumbuhan herba aromatik dari famili Zingiberaceae. Tumbuhan ini telah digunakan di dalam perubatan tradisional dan sebagai rawatan alternatif untuk mengubati pelbagai penyakit termasuk barah di negara-negara Asia. Kajian fitokimia ke atas rizom *C. zedoaria* dari Indonesia telah dijalankan melalui pemecilan berpandu bio-esei. Aktiviti sitotoksik ekstrak etanol, fraksi larut dalam heksana, fraksi larut dalam diklorometana dan bakinya telah diuji keatas sel-sel barah terpilih. Fraksi larut dalam heksana telah dipilih untuk fraksinasi seterusnya terhadap sel barah paru-paru, A549. Empat sebatian seskuiterpena telah dikenalpasti daripada fraksi H1.2 yang aktif iaitu spatulenol (**1**), *β*-eudesmol (**2**), dihidrokurdion (**3**) dan kurkumenon (**4**). Spatulenol (**1**), *β*-eudesmol (**2**) dan dihidrokurdion (**3**) menunjukkan aktiviti sitotoksik terhadap A549 dan SK-LU-1 dengan nilai perencatan 50% (IC_{50}) di antara 11.0 µg/mL ke 22.9 µg/mL dan spathulenol adalah yang paling poten. Minyak pati rizom *C. zedoaria* dari Malaysia dan Indonesia diperolehi melalui penyulingan hidro dan dianalisis menggunakan GC-FID, GC/MS dan index Kovat. Perbandingan komposisi kimia utama di antara kedua-dua minyak pati tersebut menunjukkan kehadiran kamfor (masing-masing 17.6% dan 19.7%), zerumbon (17.1% dan 12.1%) dan curzerenon (10.2% dan 7.4%). Minyak pati ini menunjukkan aktiviti sitotoksik pergantungan dos terhadap sel-sel barah: payudara ((MCF-7 dan MDA-MB-231), paru-paru (A549 dan SK-LU-1) dan servik (HeLa S3 dan SiHa) dengan nilai perencatan 50% di antara 6.4 µg/mL ke 22.0 µg/mL. Secara

keseluruhan, minyak pati *C. zedoaria* dari Malaysia menunjukkan keputusan ujikaji sitotoksik yang lebih poten berbanding minyak pati *C. zedoaria* dari Indonesia terhadap sel-sel barah yang diuji terutamanya sel barah servik. Nilai IC_{50} bagi minyak pati *C. zedoaria* dari Malaysia adalah 6.4 µg/mL terhadap sel HeLa S3 dan 9.8 µg/mL terhadap sel SiHa berbanding masing-masing 21.6 µg/mL dan 11.5 µg/mL untuk minyak pati dari Indonesia. Kematian sel barah servik, HeLa S3 dan SiHa disahkan melalui ujian hidup dan mati sel. Apoptosis dikesan melalui esei Annexin V-FITC/PI dan esei kaspas 3/7.

Katakunci: *Curcuma zedoaria*, pemecilan berpandu bio-esei, minyak pati, aktiviti sitotoksik, apoptosis.

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LIST OF SYMBOLS AND ABBREVIATONS

%	:	Percentage
µg	:	Microgram
µL	:	Microliter
µM	:	Micromolar
^{13}C	:	Carbon with Number Atom 13
^{1}H	:	Hydrogen with Number Atom 1
g	:	Gram
IC_{50}	:	Inhibitory Concentration at 50%
J	:	Coupling Constant
kg	:	Kilogram
L	:	Litre
m	:	Meter
m/z	:	Mass to Charge Ratio
mg	:	Milligram
mL	:	Millilitre
mm	:	Millimetre
mM	:	Millimolar
ºC	:	Degree Celsius
sp.	:	Species
spp.		More than One Species
v/v	:	Volume Per Volume
w/v	:	Weight Per Volume
α	:	Alpha
β	:	Beta
δ	:	Chemical Shift
ATCC	:	American Type Culture Collection
CARIF	:	Cancer Research Initiative Foundation
CO_2	:	Carbon Dioxide
dH_2O	:	Distilled Water
DED	:	Death Effector Domains
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl Sulfoxide

EMEM	:	Eagle's Minimum Essential Medium
EtOH	:	Ethanol
FBS	:	Fetal Bovine Serum
FITC	:	Fluorescence Isothiocyanate
GC/MS	:	Gas Chromatography/ Mass Spectrometry
GC-FID	:	Gas Chromatography- Flame Ionization Detector
MeOH	:	Methanol
MF	:	Molecular Formula
Min	:	Minutes
MTT	:	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MW	:	Molecular Weight
NMR	:	Nuclear Magnetic Resonance
PBS	:	Phosphate Buffer Saline
PCD	:	Programmed Cell Death
PS	:	Phosphatidylserine
RPMI	:	Rosewell Park Memorial Institute
SD	:	Standard Deviation
TLC	:	Thin Layer Chromatography
WHO	:	World Health Organization
α-MEM	:	Minimum Essential Medium (MEM) Alpha Medium

CHAPTER 1: INTRODUCTION

Southeast Asia (SEA) region is rich with plenty of bioactive tropical plants and Malaysia being no exception, has a great potential to develop industries based on abundant natural resources such as herbal products and drug discovery from higher plants. Malaysia is host to more than 1300 medicinal plant species (Burkill, 2002). In recent years, the use of traditional medicine as alternative medicine has become very popular in treating various illnesses including cancer.

Alternative medicines are very popular among rural communities and in most developing countries as compared to the expensive modern medicine. According to the World Health Organization (WHO), about three-quarters of the world population relies upon traditional remedies (mainly herbs) for health care. They not only provided food but also served the humanity to cure different ailments.

In Malaysia and Indonesia, the use of traditional medicine in the treatment and prevention of maladies are still widely practised and utilized as an alternative to modern medicines. Different cultures in Malaysia have their own traditional remedies to treat ailments and to improve health. Multi races in Malaysia, that is, Malay, Chinese, Ayurveda (Indian) and aboriginal medicine (*orang asli / orang asal)* have their own traditional beliefs and practices in treating illness. In many cases, the people claim that there are many advantages and benefits of herbal products in healthcare, however, further research is needed to ensure the efficacy and safety of the practices and usage of medicinal plants.

Worldwide, traditional or natural medicine existed in one way or another in different cultures, such as Egyptians, Western, Chinese and others. It has been reported from The World Health Report in 2002 that 80% of the African people, 40% of Asian

and Chinese and 40% of the people from South America use medicinal plants as their primary care.

At present, modern treatment such as surgery, radiotherapy, chemotherapy, hormonal therapy and symptomatic and supportive therapy are applied for cancer patients (Lim, 2002). However, all those treatments have not been fully effective against the high disease incidence, undesired side effects and low survival rate of most cancer patient. Thus, the discovery and development of anti-cancer agents from plants or natural products have been practised since ancient time. In recent years, considerable attention has been focused on identifying natural substances which are able to combat the inhibition of multistage of cancer development with minimal side effects. Natural products are valuable source of novel bioactive secondary metabolites. The established plant derived compounds such as vincristine, vinblastine, paclitaxel (taxol), doxotaxel, topotecan, irinotecan, flavopiridol, acronyciline, bruceantian and thalicarpin were successfully employed in cancer treatment (da Rocha et al., 2001).

These alternative medicines are usually consumed in the form of containing one or more medicinal plant species. The combination of bioactive natural compounds in the decoction is believed to help in the treatment or prevention of diseases. Traditional remedies also act as alternative medicine to synthetic chemicals and become popular among rural communities and in developing countries.

Traditional medicine using plant sources is also used as chemopreventive agents. Chemopreventive agents are molecules, including dietary or herbal chemicals to prevent diseases, as opposed to chemotherapeutics, where chemicals, mostly synthetic, are used to remove or alleviate the symptoms of diseases (Gosslau & Chen, 2004).

Plants contain alkaloids, saponins, tannins and many other groups of compounds as well as essential oil. Essential oil is a volatile oil obtained by hydro distillation or steam distillation from any plant parts. The success of a medicament often depends on its

presentation in a pleasant and attractive form. Most pharmaceuticals are therefore formulated in a vehicle that contain flavour and odour additives. Examples of volatile oils commonly used as pharmaceutical flavouring agents include spices and herbs such as anise, caraway, cardamom, peppermint and spearmint.

The anti-cancer activities of essential oils from more than twenty plant families have been tested on various types of cancer in the last decade with the first publication published in 1960s (Bayala et al., 2014). The various cancer types include glioblastoma, melanoma, leukaemia, oral cancer, bone, breast, cervix, colon, kidney, liver, lung, ovary, pancreas, prostate and uterus. Currently ongoing research includes identifying the mode of action and specific targets. As mentioned before, essential oil or volatile oil contain more than one chemical constituents. The results of the activity tested may be due to the major compounds or the synergistic effect with other minor compounds present in the oil.

Rural communities in Malaysia and Indonesia commonly use herbs as food and traditional medicine. Thus, in this study, *Curcuma zedoaria* which is known locally as '*temu putih'*, belonging to the Zingiberaceae family was selected for such investigation. The rhizomes were collected from two different localities, Temerloh, Pahang, Malaysia and Tawangmangu, Solo, Indonesia. Figure 1.1 showed the leaves, flowers and the rhizome of *C. zedoaria*.

1.1 Research Objectives

Curcuma zedoaria has been widely used as herbal medicine and the phytochemical and biological activities have been extensively investigated. However, these studies were not exhaustive with respect to some disease, such as cancer. Hence this study is carried out with the following objectives:

Part A – Characterization of compounds from *Curcuma zedoaria* from Indonesia using bioassay-guided isolation

1. To investigate the cytotoxic activity of crude extracts and fractions of the rhizome and identify the active fraction and compounds
2. To investigate the cytotoxic effect of the compounds from the active fraction

Part B – Essential oils analysis of *Curcuma zedoaria* from Malaysia and Indonesia and characterize their cytotoxic and apoptosis abilities

3. To identify and compare the chemical constituents of the essential oils
4. To investigate the cytotoxic activity of the essential oils
5. To analyse the apoptotic effect of the most active oil.

1.2 Thesis Outline

This thesis contains six chapters. Chapter one describes the brief introduction of this study and the research objectives while chapter two describes the literature review on phytochemistry, ethnomedicinal and pharmacological properties of *Curcuma zedoaria* and the general aspects of cancer research. The third chapter is on the experimental investigations involved in this study. The results are divided into two parts based on the objectives and presented in chapter four. Chapter five covers the discussion, chapter six is the conclusion and followed by the references and appendices.

Figure 1.1: The leaves, flowers and rhizome of *Curcuma zedoaria*

CHAPTER 2: LITERATURE REVIEW

2.1 Zingiberaceae Family

Zingiberaceae is one of the largest family in the order Zingiberales and consists of about 50 genera and 1,600 species throughout tropical Africa, Asia and Americas with its greatest diversity in Southeast Asia (SEA) (Holttum, 1950; Xu & Chang, 2017). The center of distribution is in SEA. The greatest concentration for most of the genera and species is in the Malesian region (Indonesia, Malaysia, Singapore, Brunei, the Philippines and Papua New Guinea). Among the important genera include *Alpinia* (~225 spp.), *Globba* (~100 spp.), *Amomum* (~90 spp.), *Zingiber* (~80 sp.), *Renealmia* (~70 spp.), *Curcuma* (~54 spp.), *Boesenbergia* (~50 spp.) and *Hedichium* (~40 spp.) (Riswan & Setyowati, 1996; Hartati et al., 2014). Approximately more than 18 genera and more than 160 species of Zingiberaceae are found in Peninsular Malaysia, mostly growing naturally in damp, shaded parts of the lowland or hill slopes, as scattered plants or thickets (Larsen et al., 1999).

The family Zingiberaceae consist of many species which are important resources for food, spices, medicines, dyes and perfumes (Jantan et al., 2003). Interestingly, Zingiberaceae family has been used traditionally as food condiment. The species of *Alpinia, Curcuma*, *Kaempferia, Boesenbergia* and *Zingiber* are commonly used in cooking especially in the SEA region including Malaysia, Indonesia and Thailand. The examples of ingredients used in food are the rhizome of *Alpinia galanga* (locally known as *lengkuas*), *Curcuma domestica* (*kunyit*), *Curcuma xanthorhiza* (*temu lawak*), *Kaempferia galanga* (*cekur*), *Zingiber officinale* (*halia*) and the flower of *Etlingera elatior* (*bunga kantan*) and most of these species can be found easily in the market.

Zingiberaceae species also have a long history of medicinal uses and as herbal medicine all over the world including Malaysia. Other than those mentioned above,

Boesenbergia rotunda (*temu kunci*), *Curcuma zedoaria* (*temu putih*), *Curcuma aeruginosa* (*temu hitam*), *Curcuma manga* (*temu mangga*), *Zingiber officinale* var. *rubrum* (*halia bara / merah*) are used in traditional medicine of multi-racial community in Malaysia (Malay, Indian, Chinese and aborigines). Table 2.1 list the ethnomedicinal properties of several Zingiberaceae species that have reported in literature.

Other than being used in cooking and medicinal purposes, some species of Zingiberaceae family which have beautiful flowers were used as ornamentals in the garden and as cut flowers. Some examples are *Alpinia purpurata*, *Etlingera elatior, Zingiber spectabile, Curcuma* spp., *Globba* spp., *Hedychium* spp., *Roscoea* spp. and many others.

Table 2.1: Ethnomedicinal properties of several Zingiberaceae species

Scientific name	Local name	Traditional uses
Alpinia conchigera	*Lengkuas ranting*	• Food flavouring • Treatment for rheumatism, arthritis (Sirat & Nordin, 1995), skin problem, aches and pains (Ong & Nordiana, 1999) • As antifungal and anti-inflammatory agent (Sirat & Nordin, 1995)
Alpinia galanga	*Lengkuas*	• Food flavouring • Treatment for stomach ache, antibacterial, antifungal, anti-tumour, anti-ulcer, anti-allergic, antioxidant (Oonmetta-aree et al., 2006), diabetes mellitus (Jaju et al., 2009), diarrhoea (Ong & Nordiana, 1999)
Alpinia purpurata	-	• Ornamental • Treatment for tuberculosis • As anti-mycobacterial activity and anti-inflammatory agent (Villaflores et al., 2010)
Boesenbergia rotunda	*Temu kunci*	• Food flavouring • Treatment for rheumatism, muscle pain, febrifuge, gout, gastrointestinal disorders, flatulence, carminative, stomach ache, dyspepsia and peptic ulcer (Eng-Chong et al., 2012)
Curcuma aeruginosa	*Temu hitam*	• Treatment for rheumatic disorders (Hossain et al., 2015) • As anti-microbial agent

'Table 2.1, continued'

Scientific name	Local name	Traditional uses
Curcuma amada	-	• Used as an appetizer, antipyretic, aphrodisiac, diuretic, emollient, expectorant and laxative • Treatment for biliousness, itching, skin diseases, bronchitis, asthma, hiccough and inflammation due to injuries (Policegoudra et al., 2011)
Curcuma domestica	*Kunyit*	• Food flavouring (spice) and colouring • Treatment for wound healing and inflammatory disorders • Used as blood purifier
Curcuma mangga	*Temu mangga*	• Eaten raw as *ulam*
Curcuma xanthorrhiza	Temu lawak	• Treatment for stomach diseases, liver disorders, constipation, bloody diarrhoea, haemorrhoids, dysentery, children fever, and skin eruptions (Lin et al., 1996)
Curcuma zedoaria	*Temu putih*	• Treatment for inflammation, pain and wounds, menstrual irregularities and ulcers (Ullah et al., 2014)
Elettaria cardamomum	*Buah pelaga*	• Food flavouring (spice) • Treatment for gastrointestinal disorders (Jamal et al., 2006) • As antihypertensive and antioxidant (Verma et al., 2009)
Elettariopsis curtisii	-	• Food flavouring • Used as appetiser • As anti-microbial agent (Ibrahim et al., 2009)
Etlingera elatior	*Bunga kantan* (flower)	• Food flavouring
Zingiber cassumunar	*Bonglai*	• Treatment for joint, muscular pain and inflammation (Chaiwongsa et al., 2013)
Kaempferia galanga	*Cekur / kencur*	• Food flavouring (spice) • As ingredient in Malay traditional tonic (*jamu)* • Treatment for rheumatism, abdominal pain and toothache
Zingiber officinale	*Halia*	• Food flavouring (spice) • Treatment for stomach pain, motion sickness, nausea, vomiting, rheumatism and hypertension
Zingiber officinale var. *rubrum*	*Halia bara*	• Treatment for stomach discomfort, tumours, relieving rheumatic pains and as post-partum medicine
Zingiber spectabile	-	• Treatment for inflammation of the eyes burns, headaches, back pain • As food preservation agent • Ornamental

'Table 2.1, continued'

Scientific name	Local name	Traditional uses
Zingiber zerumbet	*Lempoyang*	• Food flavouring • Used as appetizer • Treatment for inflammatory and pain mediated diseases, worm infestation and diarrhoea (Yob et al., 2011)

2.2 The Genus *Curcuma*

Curcuma is one of the largest and important genera in the Zingiberaceae family. This genus comprises about 80 spp. widely distributed in the tropics of Asian region from India to South China, Southeast Asia, Papua New Guinea and Northern Australia (Larsen et al., 2005). The name *Curcuma* was derived from the Arabic word *kurkum* meaning yellow which is referring to the colour of the rhizome. However, the rhizomes of other species also contain pigments such as orange, yellow, citron, amber, blue, greenish-blue and violet-blue (Burkill, 2002).

The classification of Zingiberaceae listed in Table 2.2 proposed that there are four sub-families and six tribes. The genus *Curcuma* has been placed in the sub-families *Zingiberoideae* and the tribe of *Zingibereae* together with *Boesenbergia, Camptandra, Haniffia, Hedychium, Kaempferia, Roscoea* and *Zingiber* (Kress et al., 2002).

Many species of *Curcuma* has long history of uses, mostly are reported to be economically useful as herbal and in tribal medicine (Ravindran et al., 2007). As an example, the most common and most investigated species of *Curcuma* is *C. domestica*. *C. domestica* or turmeric locally known as *kunyit* is a tropical rhizomatous plant having its importance as a spice, flavouring agent, colourant and it is use in most of the system of medicine to treat various illnesses.

Table 2.2: The classification of the family Zingiberaceae, adapted from Kress et al., 2002

Family	**ZINGIBERACEAE**					
Sub-family	*Siphonochiloideae*	*Tamijioideae*	*Alpinioideae*		***Zingiberoideae***	
Tribe	*Siphonochileae*	*Tamijieae*	*Alpinieae*	*Riedeliaea*	***Zingibereae***	*Globbeae*
Genera	*Siphonochilus*	*Tamijia*	Examples:	Examples:	Examples:	Examples:
			Aframomum	*Burbidgea*	*Boesenbergia*	*Gagnepainia*
			Alpinia	*Pleuranthodium*	*Camptandra*	*Globba*
			Amomum	*Riedelia*	***Curcuma***	*Hemiorchis*
			Elettaria	*Siamanthus*	*Haniffia*	*Mantisia*
			Elettariopsis		*Hedychium*	
			Etlingera		*Kaempferia*	
			Geocharis		*Roscoea*	
			Hornstedtia		*Zingiber*	

2.2.1 Phytochemical Studies of the Genus *Curcuma*

Extensive research on the phytochemicals of the genus *Curcuma* over the past half century resulted in the isolation of various types of compounds. The compounds present can be classified into few major groups namely monoterpenoids, sesquiterpenoids, diterpenoids, diphenylheptanoids (curcuminoids), along with other minor constituents. The most common and most investigated species from the genus *Curcuma* is *C. domestica* (locally known as *kunyit*). The other most investigated species includes *C. longa, C. xanthorrhiza, C. aeruginosa, C. amada, C. aromatica* and *C. zedoaria*. Table 2.3 listed some of the compounds isolated from these species in addition with some less known species such as *C. comosa, C. ecalcarata, C. heyneana, C. phaeocaulis, C. soloensis* and *C. wenyujin.*

Table 2.3: Compounds isolated from the genus *Curcuma*

Compounds isolated	Resources	References
1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one	*C. mangga*	(Abas et al., 2005)
13-hydroxygermacrone	*C. zedoaria*	(Makabe et al., 2006)
9-oxoneoprocurcumenol	*C. aromatica*	(Madhu et al., 2010)
Aerugidiol	*C. comosa*	(Qu et al., 2009)
Alismol	*C. comosa*	(Qu et al., 2009)
Alismoxide	*C. comosa*	(Qu et al., 2009)
ar-Turmerone	*C. zedoaria*	(Hong et al., 2001)
Bisacumol	*C. longa*	(Ohshiro et al., 1990)
Bisacurone	*C. longa,* *C. soloensis*	(Ohshiro et al., 1990; Vitasari et al., 2016)
Bisdemethoxycurcumin	*C. mangga*	(Malek et al., 2011)
Calcaratarin A	*C. mangga*	(Abas et al., 2005)
Coronarin B	*C. amada*	(Alan & Nair, 2012)
Coronarin D	*C. amada*	(Alan & Nair, 2012)
Curculonol	*C. comosa*	(Qu et al., 2009)
Curcumadione	*C. comosa*	(Qu et al., 2009)

'Table 2.3, continued'

Compounds isolated	Resources	References
Curcumanggoside	*C. mangga*	(Abas et al., 2005)
Curcumanolide A	*C. heyneana*	(Firman et al., 1988)
Curcumanolide B	*C. heyneana*	(Firman et al., 1988)
Curcumenol	*C. zedoaria,* *C. longa,* *C. heyneana,* *C. aeruginosa*	(Firman et al., 1988; Ohshiro et al., 1990; Sukari et al., 2007; Lobo et al., 2009)
Curcumenone	*C. zedoaria,* *C. aeruginosa,* *C. aromatic,* *C. longa,* *C. comosa*	(Kuroyanagi et al., 1987; Ohshiro et al., 1990; Sirat et al., 1998; Makabe et al., 2006; Lobo et al., 2009; Qu et al., 2009)
Curcumin	*C. zedoaria,* *C. manga,* *C. aromatic,* *C. soloensis*	(Abas et al., 2005; Lobo et al., 2009; Malek et al., 2011; Bamba et al., 2011; Pant et al., 2013; Vitasari et al., 2016)
Curcuminol D	*C. wenyujin*	(Zhang et al., 2008)
Curcuminol E	*C. wenyujin*	(Zhang et al., 2008)
Curcuminol F	*C. wenyujin*	(Ma et al., 2009)
Curcuminol G	*C. wenyujin*	(Ma et al., 2009)
Curcumrinol A	*C. wenyujin*	(Huang et al., 2008)
Curcumrinol B	*C. wenyujin*	(Huang et al., 2008)
Curcumrinol C	*C. wenyujin*	(Huang et al., 2008)
Curdione	*C. zedoaria,* *C. aromatic,* *C. comosa*	(Kuroyanagi et al., 1987; Qu et al., 2009)
Curdionolide A	*C. wenyujin*	(Lou et al., 2009)
Curdionolide B	*C. wenyujin*	(Lou et al., 2009)
Curdionolide C	*C. wenyujin*	(Lou et al., 2009)
Curzeone	*C. zedoaria*	(Shiobara et al., 1986; Makabe et al., 2006)
Curzerene	*C. zedoaria*	(Lobo et al., 2009)
Curzerenone	*C. zedoaria,* *C. comosa*	(Makabe et al., 2006; Lobo et al., 2009; Qu et al., 2009)

'Table 2.3, continued'

Compounds isolated	Resources	References
Dehydrocurdione	*C. zedoaria, C. aeruginosa, C. aromatic, C. longa, C. comosa, C. heyneana*	(Kuroyanagi et al., 1987; Firman et al., 1988; Ohshiro et al., 1990; Makabe et al., 2006; Lobo et al., 2009; Qu et al., 2009)
Demethoxycurcumin	*C. manga, C. aromatica*	(Malek et al., 2011; Bamba et al., 2011; Pant et al., 2013)
Difurocumenonol	*C. amada*	(Policegoudra et al., 2007)
Epicurzerenone	*C. zedoaria*	(Lobo et al., 2009)
Epiprocurcumenol	*C. longa*	(Ohshiro et al., 1990)
Furanodiene	*C. zedoaria*	(Makabe et al., 2006; Lobo et al., 2009)
Furanodienone	*C. aeruginosa, C. comosa, C. zedoaria*	(Sirat et al., 1998; Makabe et al., 2006; Qu et al., 2009)
Germacrone	*C. aromatic, C. comosa, C. zedoaria*	(Kuroyanagi et al., 1987; Makabe et al., 2006; Qu et al., 2009)
Isocurcumenol	*C. zedoaria, C. aeruginosa*	(Sukari et al., 2007; Lakshmi et al., 2011)
Isofuradienone	*C. comosa*	(Qu et al., 2009)
Isofuranodiene	*C. aeruginosa*	(Sirat et al., 1998)
Isoprocurcumenol	*C. longa, C. comosa*	(Ohshiro et al., 1990; Qu et al., 2009)
Isozedoarondiol	*C. comosa*	(Qu et al., 2009)
Labda-8(17),12-diene-15,16-dial	*C. mangga*	(Abas et al., 2005)
Isocurcumenol	*C. zedoaria, C. aeruginosa*	(Sukari et al., 2007; Lakshmi et al., 2011)
Isofuradienone	*C. comosa*	(Qu et al., 2009)
Isofuranodiene	*C. aeruginosa*	(Sirat et al., 1998)
Isoprocurcumenol	*C. longa, C. comosa*	(Ohshiro et al., 1990; Qu et al., 2009)
Isozedoarondiol	*C. comosa*	(Qu et al., 2009)
Labda-8(17),12-diene-15,16-dial	*C. mangga*	(Abas et al., 2005)

'Table 2.3, continued'

Compounds isolated	Resources	References
Neocurdione	*C. zedoaria,* *C. aromatic,* *C. comosa*	(Kuroyanagi et al., 1987; Lobo et al., 2009; Qu et al., 2009)
Neoprocurcumenol	*C. aromatica*	(Madhu et al., 2010)
Phacadinane B	*C. phaeocaulis*	(Ma et al., 2015)
Phacadinane C	*C. phaeocaulis*	(Ma et al., 2015)
Isocurcumenol	*C. zedoaria,* *C. aeruginosa*	(Sukari et al., 2007; Lakshmi et al., 2011)
Isofuradienone	*C. comosa*	(Qu et al., 2009)
Isofuranodiene	*C. aeruginosa*	(Sirat et al., 1998)
Isoprocurcumenol	*C. longa, C. comosa*	(Ohshiro et al., 1990; Qu et al., 2009)
Isozedoarondiol	*C. comosa*	(Qu et al., 2009)
Labda-8(17),12-diene-15,16-dial	*C. mangga*	(Abas et al., 2005)
Neocurdione	*C. zedoaria,* *C. aromatic,* *C. comosa*	(Kuroyanagi et al., 1987; Lobo et al., 2009; Qu et al., 2009)
Neoprocurcumenol	*C. aromatica*	(Madhu et al., 2010)
Phacadinane B	*C. phaeocaulis*	(Ma et al., 2015)
Phacadinane C	*C. phaeocaulis*	(Ma et al., 2015)
Phacadinane D	*C. phaeocaulis*	(Ma et al., 2015)
p-Hydroxycinnamic acid	*C. mangga*	(Abas et al., 2005)
Pinocembrin	*C. ecalcarata*	(Rameshkumar et al., 2015)
Piperitenone	*C. ecalcarata*	(Rameshkumar et al., 2015)
Procurcumadiol	*C. longa*	(Ohshiro et al., 1990)
Procurcumenol	*C. aromatic,* *C. longa,* *C. comosa*	(Kuroyanagi et al., 1987; Ohshiro et al., 1990; Qu et al., 2009)
Scopoletin	*C. mangga*	(Abas et al., 2005)
Turmerone	*C. zedoaria*	(Lobo et al., 2009)
Zederone	*C. zedoaria,* *C. comosa,* *C. aromatica*	(Pant et al., 2001; Makabe et al., 2006; Lobo et al., 2009; Qu et al., 2009)

'Table 2.3, continued'

Compounds isolated	Resources	References
Zedoalactone A	*C. aeruginosa*	(Takano et al., 1995)
Zedoalactone B	*C. comosa,* *C. aeruginosa*	(Takano et al., 1995; Qu et al., 2009)
zedoarol	*C. aeruginosa,* *C. zedoaria*	(Shiobara et al., 1986; Sirat et al., 1998; Sukari et al., 2007)
Zedoarondiol	*C. aromatic,* *C. longa, C. comosa,* *C. zedoaria*	(Kuroyanagi et al., 1987; Ohshiro et al., 1990; Makabe et al., 2006; Qu et al., 2009)
Zerumbone	*C. zedoaria*	(Lobo et al., 2009)
Zerumin A	*C. manga,* *C. amada*	(Malek et al., 2011; Alan & Nair, 2012)
Zerumin B	*C. amada,* *C. mangga*	(Abas et al., 2005; Alan & Nair, 2012)
Zingiberene	*C. zedoaria*	(Lobo et al., 2009)
α-Phellandrene	*C. zedoaria*	(Lobo et al., 2009)
β-Eudesmol	*C. zedoaria*	(Lobo et al., 2009)
β-Sitosterol	*C. manga,* *C. ecalcarata*	(Malek et al., 2011; Rameshkumar et al., 2015)
β-sitosterol-3-*O*-β-d-glucopyranoside	*C. aromatica*	(Pant et al., 2013)

2.2.2 Essential Oils in *Curcuma* Species

The rhizome of *Curcuma* spp. is well known to be rich in essential oil. Analysis of essential oil constituents of *Curcuma* spp. are usually dominated by volatile compounds from terpene group; monoterpenes (hydrocarbon or oxygenated) and sesquiterpenes (hydrocarbon or oxygenated). Essential oils of *Curcuma* spp. have a pleasant odour and the fragrance may be due to the presence of the volatile compounds. Some species have also reported to be used in perfumery industries. Other information about essential oils was described in subdivision 2.4 (page 30).

Table 2.4 listed the major compounds in essential oils of some *Curcuma* spp. such as *C. aeruginosa, C. amada, C. aromatic, C. caesia, C. domestica, C. inodora, C. longa, C. manga* and *C. xanthorrhiza*. Meanwhile, the major compounds reported in essential oil for *C. zedoaria* were listed in Table 2.6 (page 27).

Table 2.4: Major compounds in several essential oils of *Curcuma* spp.

Scientific name (local name)	From	Part	Major compounds of essential oil	References
Curcuma aeruginosa (Temu hitam)	Malaysia	Rhizome	Curzerenone (24.6%), 1,8-cineol (11.0%), camphor (10.6%), zedoarol (6.3%), isocurcumenol (5.8%), curcumenol (5.6%), furanogermenone (5.5%).	(Sirat et al., 1998)
	Hulu Langat, Selangor, Malaysia	Rhizome	Curzerenone (30.4%), 1,8-cineole (25.2%), camphor (6.8%)	(Jantan et al., 1999)
	Kerala, India	Rhizome	Curcumenol (38.7%), β-Pinene (27.5%), β-eudesmol (3.6%).	(Angel et al., 2014)

'Table 2.4, continued'

Scientific name (local name)	From	Part	Major compounds of essential oil	References
Curcuma amada	North-eastern India	Rhizome	Myrcene (88.6%)	(Choudhury et al., 1996)
	India	Rhizome	Myrcene (80.54%)	(Singh et al., 2002)
	Uttarakhand, India	Leaves	*epi*-Curzerenone (10.76%), curzerenone (9.53%), curzerene (3.95%)	(Padalia et al., 2013)
	Uttarakhand, India,	Rhizomes	Myrcene (88.84%), *β*-pinene (3.74%), (*E*)-*β*-ocimene (2.61%)	(Padalia et al., 2013)
	Calcutta, India	Rhizome	(*Z*)-*β*-farnesene (21.9%), guaia-6,9-diene (19.8%), *α*-longipinene (14.8%), α-guaiene (14.5%)	(Mustafa et al., 2005)
	Madras, India	Rhizome	β-Curcumene (29.93%), ar-curcumene (22.10%), xanthorrhizol (16.20%)	(Hisashi et al., 1998)
	Kerala, India	Rhizome	Camphor (18.8%), camphene (10.2%), 1,8-cineole (10.1%), borneol (8.2%)	(Angel et al., 2014)
Curcuma caesia	India	Rhizome	Camphor (28.3%), *ar*-turmerone (12.3%), (*Z*)-*β*-ocimene (8.2%), *ar*-curcumene (6.8%), 1,8-cineole (5.3%)	(Pandey & Chowdhury, 2003)
Curcuma domestica	Kuala Selangor, Malaysia	Rhizome	ar-Turmerone (45.8%), curcumenol (18.2%)	(Jantan et al., 2012)
	Hulu Langat, Selangor, Malaysia	Rhizome	α-Tumerone (45.3%), linalool (14.9%), *β*-tumerone (13.5%)	(Jantan et al., 1999)
Curcuma domestica	Kuala Selangor, Malaysia	Rhizome	ar-Turmerone (45.8%), curcumenol (18.2%)	(Jantan et al., 2012)
	Hulu Langat, Selangor, Malaysia	Rhizome	α-Tumerone (45.3%), linalool (14.9%), *β*-tumerone (13.5%)	(Jantan et al., 1999)

'Table 2.4, continued'

Scientific name (local name)	From	Part	Major compounds of essential oil	References
Curcuma inodora	Perak, Malaysia	Rhizome	Curzerenone (20.8%), 1,8-cineole (5.3%), germacrone (11.1%), curdione (7.5%)	(Malek et al., 2006)
	Perak, Malaysia	Leaves	Curzerenone (16.9%), germacrone (7.5%), 1,8-cineole (5.8%), β -elemenone (5.3%)	(Malek et al., 2006)
Curcuma longa (Kunyit)	Brazil	Rhizome	ar-Turmerone (33.2%), *α*-Turmerone (23.5%), *β*-Turmerone (22.7%)	(Ferreira et al., 2013)
	India	Rhizome	ar-Turmerone (51.7%), ar-turmerol (11.9%), *β*-bisabolone (10.7%), zingiberene (10.2%)	(Singh et al., 2002)
	Bhutan	Rhizome	α-Turmerone (30–32%), ar-turmerone (17–26%), β-turmerone (15–18%)	(Sharma et al., 1997)
	Kerala, India	Rhizome	ar-Turmerone (49.8%), *α*-Turmerone (9.1%), *β*-Turmerone (7.9%)	(Angel et al., 2014)
	Bhutan	Leaves	α-Phellandrene (18.2%), 1,8-cineole (14.6%), *p*-cymene (13.3%)	(Sharma et al., 1997)
	Nigeria	Leaves	*α*-Phellandrene (47.7%), terpinolene (28.9%)	(Oguntimein et al., 1990)
Curcuma mangga (Temu pauh)	Penang, Malaysia	Rhizome	Myrcene (78.6%), (E)-*β*-ocimene (5.1%), β-pinene (3.7%), α-pinene (2.9%)	(Wong et al., 1999)
	Hulu Langat, Selangor, Malaysia	Rhizome	Myrcene (81.4%), *β*-pinene (10.4%)	(Jantan et al., 1999)
Curcuma xanthorrhiza (Temu lawak)	Kuala Selangor, Malaysia	Rhizome	Xanthorrhizol (31.9%), ar-curcumene (13.2%), *β*-curcumene (17.1%)	(Jantan et al., 2012)
	Hulu Langat, Selangor, Malaysia	Rhizome	Xanthorrhizol (44.5%), Zingiberene (10.2%), ar-curcumene (7.6%)	(Jantan et al., 1999)
Curcuma zedoaria (Temu putih)			Refer to Table 2.6 (page 27).	

2.2.3 Ethnomedicinal Properties of the Genus *Curcuma*

Curcuma spp. have been exploited as medicine, ornamentals, dye, cosmetic, food and spices since ancient time. Usually, the part most used purposes is the rhizome which contain pigments giving different colours for different species such as orange, yellow, citron, amber, blue, greenish blue and violet blue (Burkill, 2002). The species may be distinguished by the rhizome colour and fragrance.

In general, the rhizomes of *Curcuma* spp. are edible and most of them were utilised in Malay and Indian traditional food as food additive and colouring. The most common species for food is *C. domestica* (turmeric), locally known as *kunyit.* The rhizome of turmeric is dark yellow in colour and used as food colouring and flavouring in curry and utilised in many other foods. In addition, the rhizome of *C. mangga* also can be eaten raw as *ulam*.

In Malay traditional medicine, the rhizomes of *Curcuma* spp. are used as ingredient together with other herbs in preparing traditional tonic called *jamu*. *Jamu* can be consumed to freshen up and prevent body from ailment. It is also consumed during confinement among women in Southeast Asian region to improve health and to heal wounds. The most common of the *Curcuma* spp. used in *jamu* include *C. domestica, C. xanthorhiza* and *C. zedoaria.*

Other than the rhizome, the leaves of *C. domestica* are also used as flavour additive in curry. The young inflourescence of this species can be eaten raw as vegetable (*ulam*) or cook in coconut milk among Asia community.

Table 2.5 summaries the selected ethnomedicinal properties of the genus *Curcuma.*

Table 2.5: Selected ethnomedicinal properties of the genus *Curcuma* (Perry & Metzger, 1980; Burkill, 2002)

Scientific name	Local name	Parts	Traditional uses	Used by
C. aeruginosa	*Temu hitam*	Rhizome	Gastrointestinal remedies: treatment for diarrhoea, colic Postpartum care: uterine involution, treatment for uterine pain and uterine inflammation	Asia
C. domestica	*Kunyit*	Rhizome	Ingredient of *jamu*	Malays
			Food additive and colouring (Malay rice dye, frying fish, etc.)	
			Used in Hindu marriage ceremonies	Indian
			Medicine for elephant: Externally – poultice and lotion Internally – vermifuge	
			Arrow poison	
		Leaves	Food additive Fish wrap (grill and fry)	Malays
C. mangga	*Temu pauh*	Rhizome	Raw vegetable (*ulam*)	Malays
			Treatment for abdominal pain	Javanese
			Food seasoning	Javanese
C. purpuresence	*Temu tis*	Rhizome	Medicine	
C. xanthorhiza	*Temu lawak*	Rhizome	Dye	Malays
			Ingredient of *jamu*	Malays
C. zedoaria	*Temu putih*	Rhizome	Medicine	Malays
			Birth ceremony	Dayaks of Northern Borneo

2.2.4 Pharmacological Properties of the Genus *Curcuma*

Studies by various researchers on the *Curcuma* spp. revealed many biological activities, such as anti-microbial activity, anti-oxidant, anti-inflammatory, anti-cancer and many others. These studies also supported the traditional use of *Curcuma* spp. for medicinal purposes and to treat many diseases including cancer.

Petroleum ether, benzene, chloroform, methanol and aqueous extracts of *C. longa* inhibited the standard strain and clinical isolates of *Staphylococcus aureus* using disk diffusion method. The results showed a broad spectrum of anti-microbial potential with zone of inhibition ranging between 9 mm and 21 mm (Gupta et al., 2015).

The rhizome extracts of *C. zedoaria* and *C. malabarica* were tested against six bacterial and two fungal strains using agar well diffusion and broth dilution methods. Acetone and hexane extracts of both rhizomes showed comparable anti-microbial activity with minimum inhibitory concentration (MIC) values ranging from 0.01 to 0.06 mg/mL, however other extracts such as petroleum ether, chloroform and ethanol extracts of *C. malabarica* showed significantly lower activity than those of *C. zedoaria* (Wilson et al., 2005).

In another study, the essential oil of *C. longa* and curcumin were tested for the *in vitro* anti-fungal activity using MIC assay against fifteen (15) isolates of dermatophytes (*Tricophyton rubrum, Tricophyton mentagrophytes, Epidermophyton floccosum* and *Microsporum gypseum*). The oil inhibited dermatophytes at MIC values ranging from 114.9 – 919.2 µg/mL, but none were inhibited by curcumin (Apisariyakul et al., 1995).

Five *Curcuma* spp. namely *C. amada, C. aromatica, C. longa, C. zeodaria* and *C. caesia* have been tested for anti-oxidant activity using Fenton's reaction. The leaf extracts of the five *Curcuma* spp. displayed immune-modulation activity in different concentration and was found to increase the phagocytic activity of macrophages against yeast cells (Bhardwaj et al., 2011).

Cytotoxic investigation of the crude extracts (methanol, hexane and ethyl acetate) of *C. mangga* against six human cancer cell lines, namely, breast (MCF-7), nasopharyngeal epidermoid (KB), lung (A549), cervical (CaSki), colon (HCT 116 and HT-29) and non-cancer human fibroblast cell line (MRC-5) was performed using an *in vitro* neutral red cytotoxicity assay (Malek et al., 2011). It was reported that methanol extracts of *C. mangga* and their fractions (hexane and ethyl acetate fractions) possess good cytotoxic effect against all cancer cell lines without affecting normal cells (MRC-5).

The methanol extract of the leaves and the rhizome of *C. amada* exhibited strong cytotoxic activity towards breast cancer cell lines, MCF-7 and MDA-MB-231. Interestingly, the extracts showed less cytotoxicity towards non-cancerous breast cell line HBL-100 (Jambunathan et al., 2014).

2.3 *Curcuma zedoaria* (Christm.) Rosc.

Curcuma zedoaria (Christm.) Rosc. known as white turmeric or zedoary and locally known as *temu putih* is an aromatic medicinal herbs indigenous to India, Sri Lanka and Bangladesh and widely cultivated throughout Southeast Asia, China, Japan, Brazil and Nepal (Lobo et al., 2009). This plant grows in tropical and subtropical wet forest regions, largely utilised in Malay traditional medicine and oriental medicine (Carvalho et al., 2010). This plant has been called by various names by the natives such as *temu putih* (Malaysia and Indonesia), *kachur* (Hindi), *gajitsu* (Japan), *tamahiba* (Philippine), *phet buri* (Thailand), *nga truat* (Vietnam) and *er-jyur* (China).

C. zedoaria can grow up to 1.2 m in height. The leaves have dark purple strips along the midrib on both surfaces. The edible rhizome is very light yellow on the outside and bright yellow on the inside, however the taste is quite bitter, therefore it is less frequently used as a spice compared to turmeric (Pemberton, 2006; Sirirugsa et al., 2007).

2.3.1 Phytochemical Studies of *Curcuma zedoaria*

The rhizome of *Curcuma zedoaria* are believed to contain many bioactive compounds which are responsible for various biological activities. The chemical investigation of the rhizome of *C. zedoaria* from many different countries have been conducted. Previous phytochemical studies on this plant led to the isolation of many sesquiterpenoids, flavonoids and curcuminoids such as zedoarol, germacrone, curdione, *β*-elemene and curzeone (Shiobara et al., 1985; Shiobara et al., 1986). Numerous phytochemical analyses were carried out by several groups of researchers on this species. Some compounds which were successfully isolated are *ar*-turmerone and *β*-turmerone (Hong et al., 2001), curcudezerone and naringenin (Eun et al., 2010), curcuzedoalide, curcuminol D and indole-3-aldehyde (Park et al., 2012), curdione, alismol, zederone, dehydrocurdione and dihydroalismol (Rahman et al., 2013). Hamdi et al., 2014 reported the presence of 19 compounds namely labda-8(17), 12 diene-15, 16dial, dehydrocurdione, curcumenone, comosone II, curcumenol, procurcumenol, germacrone, zerumbone epoxide, zederone, 9-isopropylidene-2,6-dimethyl-11-oxatricyclo [6.2.1.0] undec-6-en-8-ol, furanodien, germacrone-4,5-epoxide, carcaratarin A, isoprocurcumenol, germacrone-1,10-epoxide, zerumin A, curcumanolide A, curcuzedoalide and gweicurculactone. Other compounds reported to be present in the rhizome of *C. zedoaria* is listed in Table 2.3. Figure 2.1 displayed the structure of selected compounds reported in *C. zedoaria*.

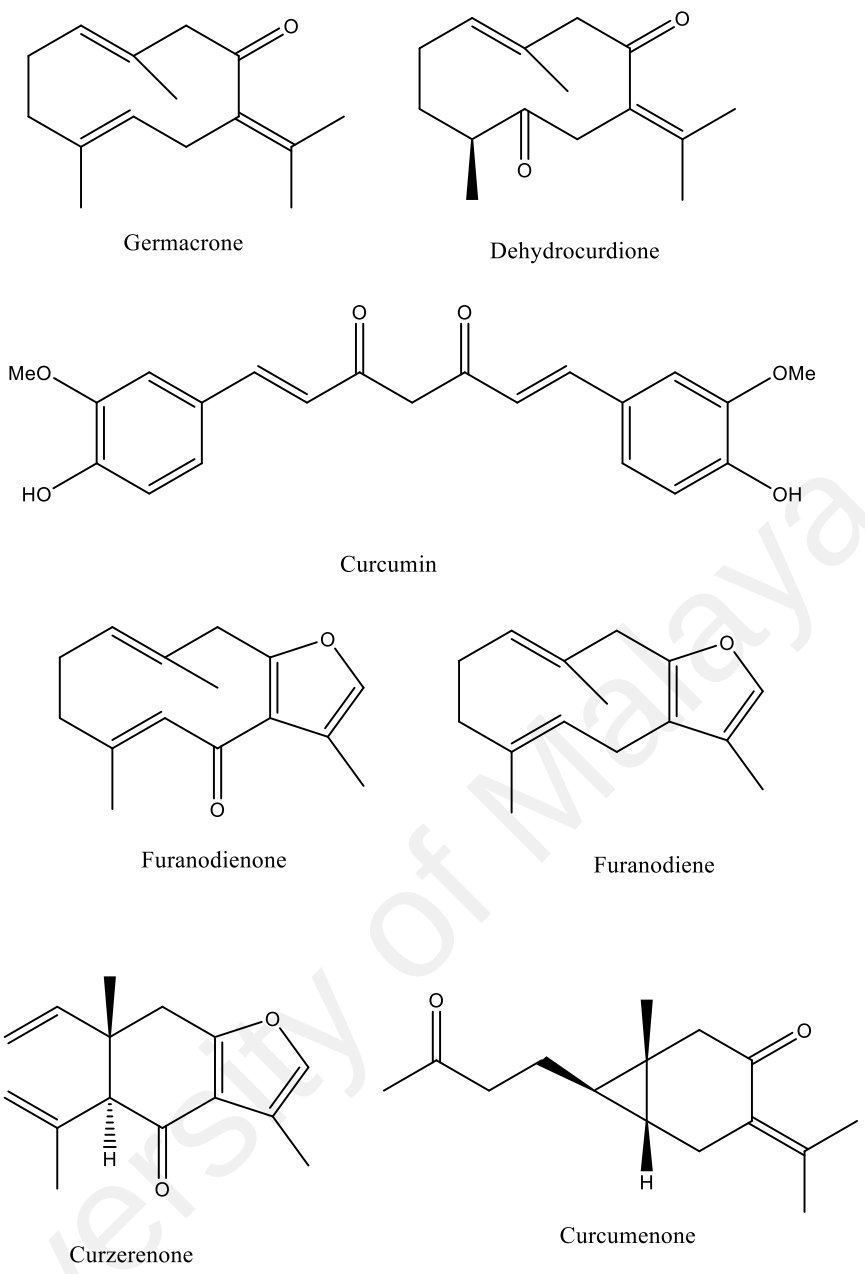
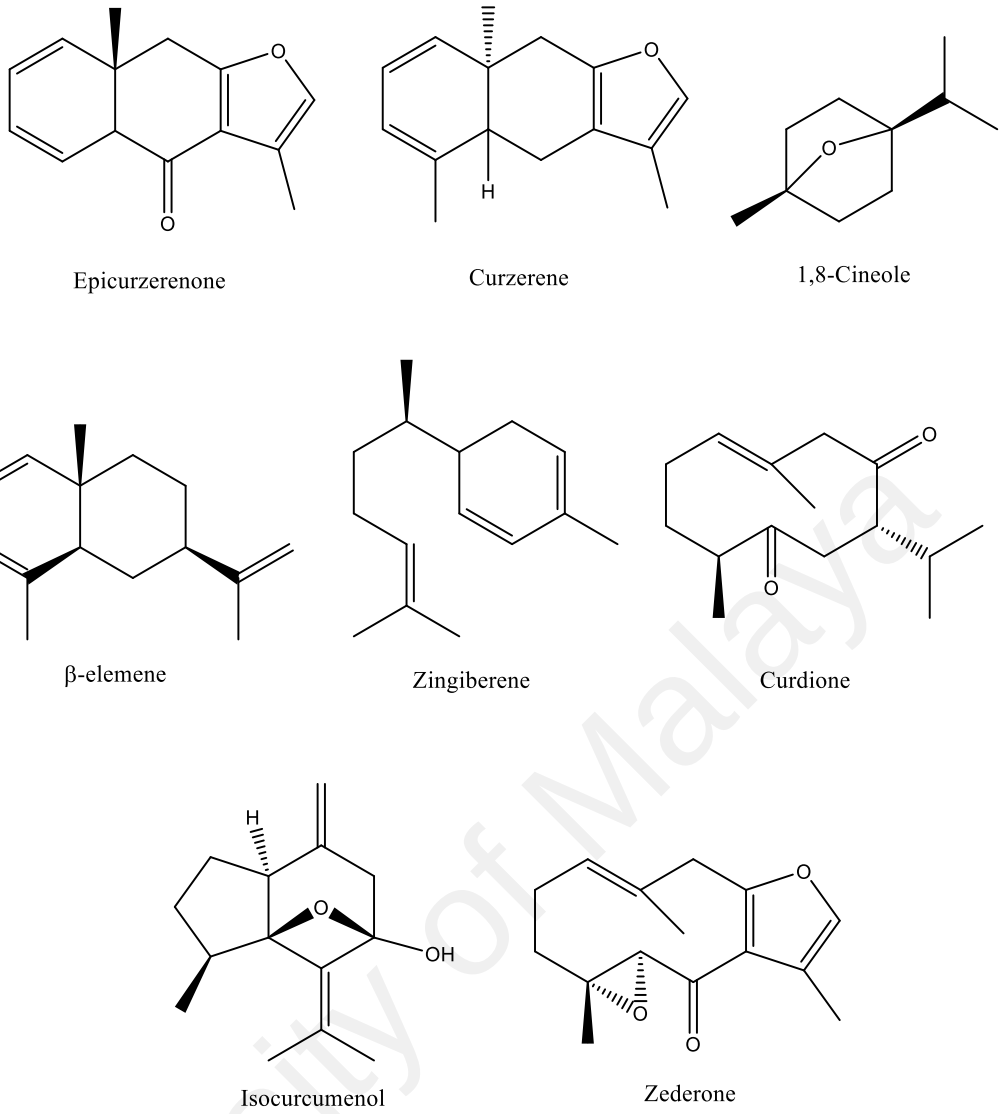


Figure 2.1: Structure of selected compounds reported in *Curcuma zedoaria*



'Figure 2.1, continued'

2.3.2 Essential Oils of *Curcuma zedoaria*

The rhizome oil of *Curcuma zedoaria* is mainly dominated with monoterpene and sesquiterpene. Mau et al., (2003) revealed epicurzerenone (24.1%), curdione (7.0%), 5-isopropylidene-3, 8-dimethyl-1(5H)-azulenone (4.3%) and isocurcumenol (3.0%) as the major compounds in the essential oil of *C. zedoaria* from China. Another research group from China, Lai et al., 2004 also reported that epicurzerenone (46.6%) as the major compound followed by curdione, 5-isopropylidene-3, 8-dimethyl-1(5H)-azulenone, *β*-elemene, curcumol, camphor, *α*-terpineol and 1, 8-cineole. The *C. zedoaria* oil from Nepal was reported to have 1,8-cineole (15.8%), *β*-eudesmol (10.6%), *p*-cymene (7.0%) (Yonzon et al., 2005) while the oil from India contain curzerenone (22.3%), 1,8-cineole (15.9%), germacrone (9.0%) (Purkayastha et al., 2006), 1,8-cineole (18.5%), cymene (18.42%), *α*-phellandrene (14.9%) (Singh et al., 2002). Among the constituents of *C. zedoaria* from Indonesia include camphor (49.5%) and isobornyl alcohol (12.7%). Other less significant compounds present in less than 5% namely borneol, furanodiene, furadienone, 1, 8-cineole, camphene, *β*-pinene, 2-nonanon and germacrene-D (Retnowati et al., 2014). Other research from India revealed the major compounds in the rhizome oil of *C. zedoaria* are epi-curzerenone (19.0%), ar-curcumene (12.1%) and zingiberene (12.0%) (Angel et al., 2014), meanwhile 1,8-cineole (20.1%), curzerenone (16.3%) and furanogermenone (13.7%) were reported as the major compounds in rhizome oil *C. zedoaria* from Malaysia (Abdullah et al., 2002). Selina-4(15),7(11)-dien-8-one (9.4%) and dehydrocurdione (9%), on the other hand were reported as main constituents in the leaf oil of *C. zedoaria* from India (Garg et al., 2005). The chemical constituents identified in *C. zedoaria* oil from different region is displayed in Table 2.6.

Table 2.6: Chemical constituents of *Curcuma zedoaria* essential oils from different regions

Origin	Part	Major compounds	References
China	Rhizome	Epicurzerenone (24.1%), curdione (7.0%), 5-isopropylidene-3, 8-dimethyl-1(5H) azulenone (4.3%) and isocurcumenol (3.0%)	(Mau et al., 2003)
China	Rhizome	Epicurzerenone (46.6%), curdione (13.7%), 5-isopropylidene-3, 8-dimethyl-1(5H)-azulenone (9.2%), β –elemene (1.9%), curcumol (1.9%), camphor (1.5%), α-terpineol (1.5%), 1, 8-cineole (1.4%)	(Lai et al., 2004)
India	Rhizome	1, 8-cineole (18.5%), cymene (18.4%), α-phellandrene (14.9%)	(Singh et al., 2002)
India	Rhizome	Curzerenone (22.3%), 1, 8-cineole (15.9%), germacrone (9.0%)	(Purkayastha et al., 2006)
Indonesia	Rhizome	Camphor (49.5%), isobornyl alcohol (12.7%), borneol (4.2%), furanodiene (3.6%), furanodienone (3.5%), 1, 8-cineole (3.4%), camphene (2.3%), β-pinene (1.8%), 2-nonanon (0.8%) and germacrene-D (1.2%)	(Retnowati et al., 2014)
Kerala, India	Rhizome	Epi-curzerenone (19.0%), ar-curcumene (12.1%), zingiberene (12.0%)	(Angel et al., 2014)
Malaysia	Rhizome	1, 8-cineole (20.1%), curzerenone (16.3%), furanogermenone (13.7%), camphor (7.6%)	(Abdullah et al., 2002)
Nepal	Rhizome	1, 8-cineole (15.8%), β-eudesmol (10.6%), *p*-cymene (7.0%)	(Yonzon et al., 2005)
India	Leaf	Selina-4(15),7(11)-dien-8-one (9.4%), dehydrocurdione (9%), α-Terpinyl acetate (8.4%), isoborneol (7%)	(Garg et al., 2005)

2.3.3 Ethnomedicinal Properties of *Curcuma zedoaria*

The rhizome of *Curcuma zedoaria* has a long history of medicinal uses in various ethnic traditional medicine of Malaysia particularly in Malay traditional medicine. In Malay traditional medicine, this plant can be consumed on its own or as a mixture with other herbs to improve health as well as a postpartum medicine (Hamdi et al., 2014). In addition to this, the rhizome was reported to be used in the treatment of menstrual disorder, vomiting, dyspepsia, cancer, cold, cough, fever and many other ailments (Burkill, 2002; Lobo et al., 2009). Decoctions containing the rhizome of *C. zedoaria*, prepared as a tonic by Malays is good for digestion. In Japanese and Chinese traditional medicines, the rhizome of *C. zedoaria* was reported to relieves flatulence, to treat wounds, diarrhoea, ulcers, skin disorder (Matsuda et al., 2001), hepatitis and in the treatment for lack of appetite (Roosita et al., 2008). *C. zedoaria* has reported as an alternative medicine for cancer treatment such as cancer of abdomen, cervical, uterus, breast, testicles, liver and pancreas (Garg et al., 2005). *C. zedoaria* has been reported to have anti-bacterial activity due to the claim that the rhizomes have been used for the treatment of bacterial and fungal infections (Wilson et al., 2005).

In Europe, the essential oils from the rhizome and roots of *C. zedoaria* were extracted by steam distillation and used in perfumes, soaps, oils and others. The scent is described as similar to mango, camphor or ginger-oil (Burkill, 2002).

2.3.4 Pharmacological Properties of *Curcuma zedoaria*

Recent research suggests that the rhizome of *C. zedoaria* possesses anti-cancer properties. The anti-tumour effect of isocurcumenol, a compound from *C. zedoaria*, has been conducted by Lakshmi et al., 2011. This compound significantly inhibited the cell proliferation in human lung, leukaemia, nasopharyngeal carcinoma and murine lymphoma cells. Another compound, α-curcumene also possess cytotoxic effect on the

growth of human ovarian cancer, SiHa cell line (Shin & Lee, 2013). Hamdi et al., (2014) has reported the cytotoxic activity of the crude extracts (hexane, dichloromethane, ethyl acetate and methanol) of *C. zedoaria* against MCF-7 and CaSki cancer cell lines. The best activity was shown by hexane extract against MCF-7 and CaSki without affecting normal cell, HUVEC. The cytotoxic activity of 19 compounds isolated from the hexane and dichloromethane extracts were performed towards MCF-7, CaSki, PC-3, HT-29 and HUVEC. Amongst these, two compounds, namely curcumenone and curcumenol which are present in the hexane extract were able to induce apoptosis in MCF-7 cell line by inhibiting the proliferation of the cancer cells (Hamdi et al., 2014). Petroleum ether extracts of *C. zedoaria* have been tested on the proliferation of human triple negative breast cancer cell line MDA-MB-231. The results showed that MDA-MB-231 cells were inhibited by petroleum ether extracts of *C. zedoaria*. The combination and synergistic effect of all compounds in the extracts may be responsible for the above activity (Gao et al., 2014).

Besides anti-cancer, other bioactivities of *C. zedoaria* include anti-oxidant (Paramapojn & Gritsanapan, 2009; Cho & Kim, 2012; Sumathi et al., 2013), anti-inflammatory (Kaushik & Jalalpure, 2011; Ullah et al., 2014), tumour progression and immuno-modulation (Carvalho et al., 2010), analgesic and anti-microbial activity (Wilson et al., 2005; Das & Rahman, 2012) and anti-fungal against *Candida* spp. (Shinobu-Mesquita et al., 2011).

2.4 Essential Oil

Essential oil is a concentrated hydrophobic liquid, also known as volatile oil containing volatile, aromatic and organic constituents obtained from aromatic plant resources. The scents vary according to the plant species and content of the constituents in the oil. Essential oil can be extracted from various plant parts such as leaves, bark, seeds, flower, fruit, root and rhizome using several technique such as hydro distillation, steam distillation, solvent extraction and expression under pressure, supercritical fluid and subcritical water extractions (Edris, 2007). However, hydro distillation and steam distillation are the most common (Bauer, 2001; Bowles, 2003). Plant rich in essential oils are mainly found in species from the families Apiaceae, Asteraceae, Cupressaceae, Lamiaceae, Lauraceae, Myrtaceae, Pinaceae, Piperaceae, Rutaceae, Santalaceae and Zingiberaceae.

Essential oil is chemically a complex mixture, often containing more than hundreds of individual components. Essential oil is made up of compounds such terpenes (monoterpene and sesquiterpenes), aldehydes, esters, ketones, phenols and alcohols. Most of the oils have one to several major components which impart the characteristic flavour and aroma such as sweet and spicy. However, there are also many minor constituents which also play their part in producing the final product (Waterman, 1993). Essential oil has been used widely as perfumes, flavours for foods and beverages, or to heal both body and mind for thousands of years (Wei & Shibamoto, 2010). Nowadays, essential oil is incorporated in pharmaceutical, cosmeceutical, nutraceutical and many other products.

2.4.1 Terpenes in Essential Oils

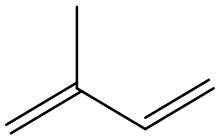


Figure 2.2: Isoprene unit

Table 2.7: Classification of terpenes based on isoprene unit

Number of isoprene units (C_5H_8) n	Types of terpenes
$C_{10}H_{16}$	Monoterpenes
$C_{15}H_{24}$	Sesquiterpenes
$C_{20}H_{32}$	Diterpenes
$C_{25}H_{40}$	Sesterterpenes
$C_{30}H_{48}$	Triterpenes
$C_{40}H_{64}$	Tetraterpenes

Terpene compounds are a coalition of several isoprene units. Isoprene unit is defined as five carbon unit with molecular formula C_5H_8 (Figure 2.2). Most essential oils from plants consist of monoterpenes and sesquiterpenes. Monoterpene compounds consist of two isoprene units and sesquiterpene compounds consist of three isoprene units. Essential oils are commonly classified into two principle constituents that is hydrocarbon with their structure based on the isoprene unit and oxygenated compounds with the compounds containing oxygen atoms including alcohols, esters, aldehydes, ketones, lactones, coumarins, ethers, oxide and others (Leland et al., 2006). Both represent a large class of natural products with a wide range of biological activities. Each functional group which attach to the main skeleton also play a role in the bioactivities. Table 2.7 display the classification of terpenes based on isoprene unit.

Functionalized group (Leland et al., 2006):

I. **Aldehyde** – any class of compounds characterized by the presence of a carbonyl group (C=O group) in which the carbon atom is bonded to at least one hydrogen atom.

II. **Ketones** – compounds where the carbon atom of the carbonyl group is bonded to two other carbon atoms.

III. **Alcohols** – any class of compounds characterized by the presence of a hydroxyl group (-OH group) bonded to saturated carbon atom.

IV. **Esters** – ester are any class of compounds structurally related to carboxylic acid but in which the hydrogen atom in the carboxyl group (-COOH group) was replaced by a hydrogen group, resulting in a –COOR structure where R is the hydrocarbon.

V. **Phenols** – phenols constitute a large class of compounds in which a hydroxyl group (-OH group) is bound to an aromatic ring.

2.4.2 Monoterpene

Monoterpene is a class of terpene that consists of two isoprene units and ten carbon atoms with molecular formula $C_{10}H_{16}$. Example of monoterpenes commonly present in essential oils are menthol, terpinen-4-ol, α-terpineol, carvacrol, linalool, myrcene, citronellol, citronellal, sabinene, thujane, β-pinene and α-pinene. The structure of selected monoterpene compounds is displayed in Figure 2.3.

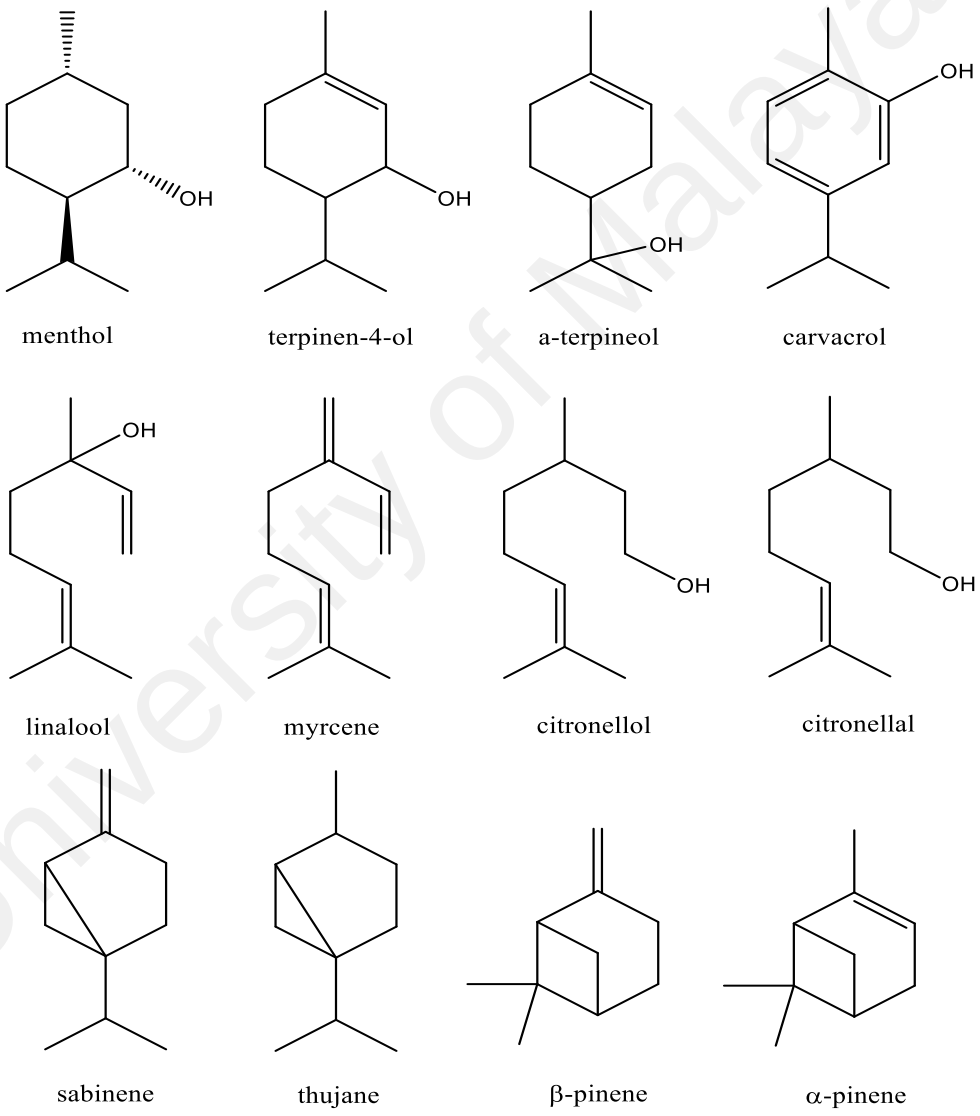


Figure 2.3: Structure of selected monoterpene compounds presence commonly in plant essential oils

2.4.3 Sesquiterpene

Sesquiterpene consists of three isoprene units with molecular formula $C_{15}H_{24}$. Figure 2.4 displayed the structure of selected sesquiterpene compounds in essential oil such as ***E, E***-*α*-farnesene, *α*-zingiberene, *β*-bisabolene, *β*-curcumene, *α*-bisabolol, *β*-eudesmol, caryophyllene oxide, spathulenol and *α*-cadinol.

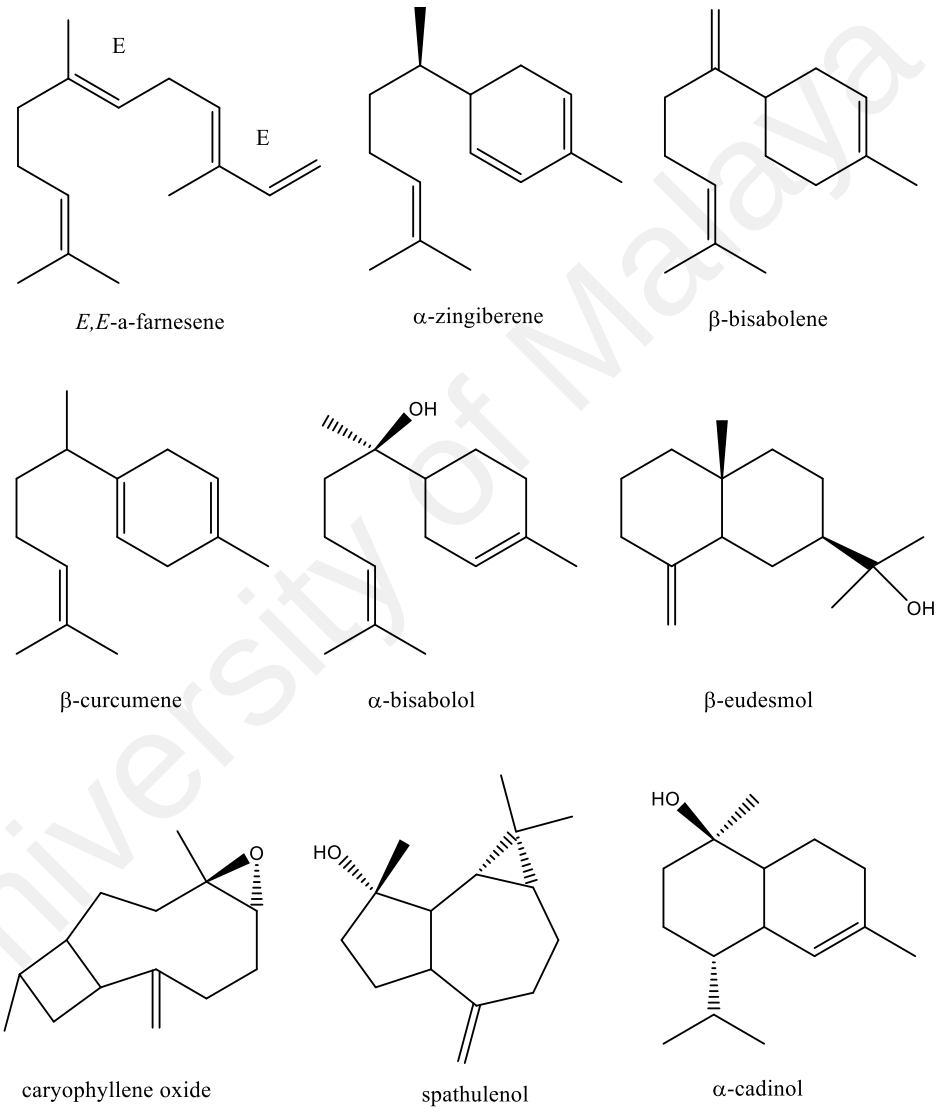


Figure 2.4: Structure of selected sesquiterpene compounds presence commonly in plant essential oils

2.5 Overview of Cancer

Cancer can be defined as an uncontrolled growth and invasion of the abnormal cells (usually derived from a single abnormal cell) and leading to the formation of a tumour. In recent decades, cancer is one of the major health problems and leading cause of death in the world either in developed or developing country. Many cancers are in the form of solid tumours, which are masses of tissues. Tumours in the body might be malignant or benign. Cancerous tumours are malignant, which means they can spread into, or invade to nearby tissues. Generally, in many cases, cancer cells can break off and migrate to other places in the body (metastasize) through the blood or the lymph system and can form new tumours away from the original tumours. On the contrary, benign tumours are not dangerous and do not migrate or invade to other sites.

In Malaysia, the number of cancer patients increase every year. In 1998, lung cancer (20.90%) was the leading cause of death among cancer patients followed by liver (9.60%), breast (7.60%), leukaemia (6.90%), stomach (5.90%), colon (5.30%), nasopharynx (4.80%), cervical (4.0%), lymphoid tissue (3.60%) and ovarian cancer (2.70%) (Lim, 2002). In 2007, the statistics of cancer patients in Malaysia changed, with the most frequent cancers being breast followed by colorectal, tracheae, bronchus and lung, nasopharynx, cervix uteri, lymphoma, leukaemia, ovary, stomach and liver (Figure 2.5) (Malaysia National Cancer Registry Report, 2007). In 2012, it was estimated about 14.1 million new cancer cases and 8.2 million deaths occurred due to cancer worldwide (Torre et al., 2015).

Generally, majority of patients are diagnosed at a late stage of the disease. The increasing number of cancer patients may be attributed due to change in lifestyle and environmental pollution such as change in food consumption (poor diet), smoking behaviour, alcohol consumption, physical inactivity or being overweight, chronic infections, exposure to harmful radiations and chemicals.

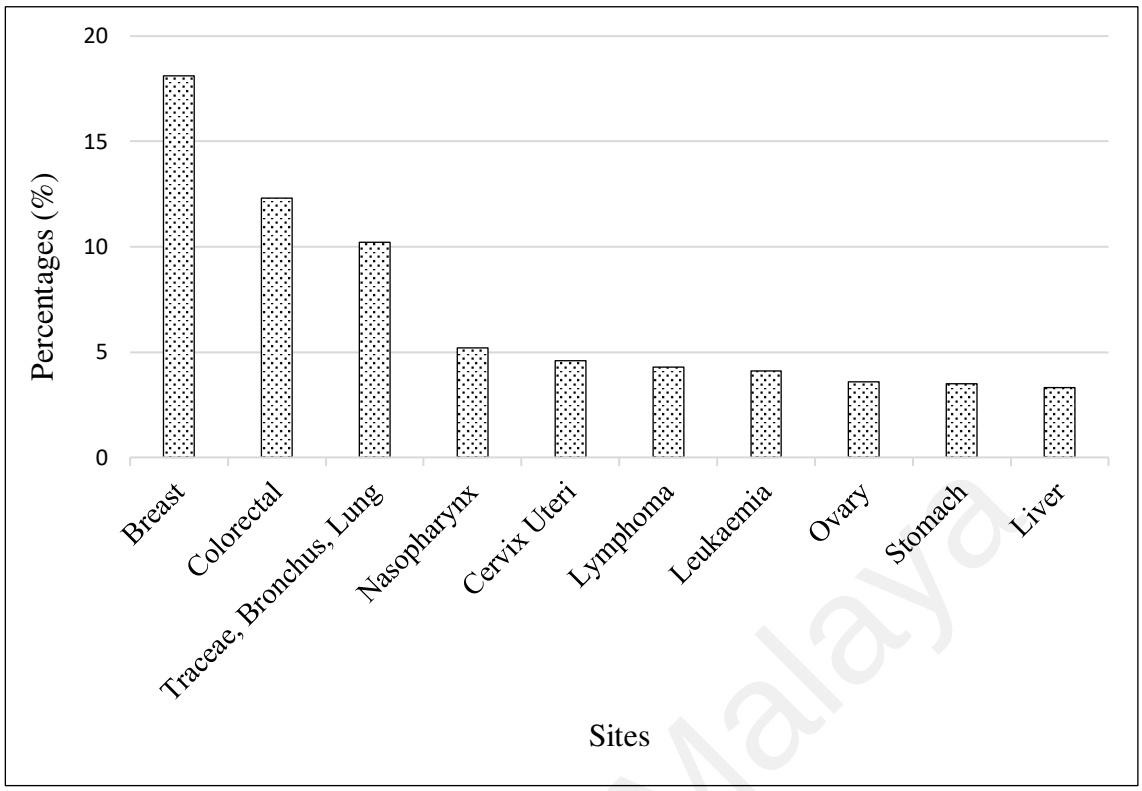


Figure 2.5: The ten most common cancers in Malaysia in 2007 (Malaysia National Cancer Registry, 2007)

2.6 Human Cancer and Normal Cell Lines

Cancer cell lines namely breast adenocarcinoma (MCF-7 and MDA-MB-231), cervical adenocarcinoma (HeLa S3 and SiHa), lung carcinoma (A549 and SK-LU-1), hepatocellular carcinoma (HepG2), oral (tongue) squamous cell carcinoma (HSC-4) and prostate epithelial (PC-3) and normal fetal lung fibroblast cell line (MRC-5) were used in this study.

2.6.1 Breast Cancer

Breast cancer is a complex and heterogeneous disease and it is the most common cancer among women worldwide with 231,840 (29.0%) estimated new cases with about 40,290 (15.0%) estimated death as compared to other cancer type in 2015 (American Cancer Society). Breast cancer is the most common cancer type afflicting women in

Malaysia. According to Malaysian Oncology Society, about one in 19 women in this country are at risk, compared to one in eight in Europe and the United States.

Breast cancer occurs when the cells in the lobules (milk producing glands) or the ducts become abnormal and divide uncontrollably. These abnormal cells begin to invade the surrounding breast tissue and may eventually spread via blood vessels and lymphatic channels to the lymph nodes, lungs, bones, brain and liver. Breast cancer can also occur among men, but the cases are quite rare and estimated about 100 times less as compared to women. In this study, we used two type of cancer cell lines; MCF-7 and MDA-MB-231.

2.6.1.1 MCF-7 - Luminal A Breast Cancer Cell Line

MCF-7 is a human breast adenocarcinoma cell lines and established in 1973 at the Michigan Cancer Foundation (Soule et al., 1973). MCF-7 is the most commonly used for studies in breast cancer research and its popularity is largely due to its exquisite hormone sensitivity through expression of oestrogen receptor (ER), making it an ideal model to study hormone response (Levenson & Jordan, 1997).

2.6.1.2 MDA-MB-231- Triple Negative Metastatic Breast Cancer Cell Line

MDA-MB-231 is an epithelial type cell line and derived from a 51 years old Caucasian lady. Other than MCF-7, this cell is also the most commonly used metastatic breast cancer cell lines in cancer research.

2.6.2 Cervical Cancer

Cervical cancer is listed as the third in the list of estimated new cases among women in 2008 after breast and colon cancer worldwide (Jemal et al., 2011). Cervical cancer is a potentially preventable disease, however, cervical cancer is still the second

most common cancer that strikes Malaysian women (Zaridah, 2014). It is occurring uncommonly before the age of 30 years old, the risk of developing it increases with age, with a peak incidence at ages 60 to 69 years and declining thereafter. In this present study, cervical cancer cell lines used are HeLa S3 and SiHa.

2.6.2.1 HeLa S3 – Mutant Strain of HeLa Cervical Adenocarcinoma Cell Line

HeLa S3 is a clonal derivative of the parent HeLa cell line that requires less human serum for growth, isolated in 1955. The HeLa S3 clone has been very useful in the clonal analysis of mammalian cell populations relating to chromosomal variation, cell nutrition, and plaque-forming ability (ATCC). HeLa S3 cells have an epithelial morphology. It harbours human papilloma virus-18.

2.6.2.2 SiHa – Cervical Squamous Carcinoma Cell Line

SiHa is a grade II squamous cell carcinoma derived from a 55 years old Asian female. SiHa cells displays an epithelial morphology and growth in adherent culture. The cell line is derived from HPV-16 positive cancer tissue in cervix uteri.

2.6.3 Lung Cancer

Lung cancer also known as lung carcinoma is the second most common cancer affecting men and the third most common cancer with 2,100 Malaysians diagnosed each year and the number of patients increase every year. Generally, the main contributor to lung cancer is smoking. Lung cancer could be avoided by eliminating smoking initiation and increasing smoking cessation among current smokers. A549 and SK-LU-1 cell lines were the selected cell lines used in this present study.

2.6.3.1 A549 - Alveolar Epithelial Carcinoma Cell Line

A549 is a human lung carcinoma cells obtained through the removal and culturing of cancerous lung tissue in the explanted tumour from a 58 years old Caucasian male. These cells are routinely used as *in vitro* and *in vivo* models by researchers. These cells are human alveolar basal epithelial cells and grow adherently as a monolayer *in vitro*.

2.6.3.2 SK-LU-1 – Lung Adenocarcinoma Cell Line

SK-LU-1 was established in 1969 from a 60 years old Caucasian female with adenocarcinoma of the lung. SK-LU-1 displays an epithelial-like morphology and growth in adherent culture.

2.6.4 Liver Cancer: HepG2 – Liver Carcinoma Cell Line

In Malaysia, liver cancer was ranked as the ten most common cancer type among Malaysians (Malaysia National Cancer Registry, 2007). HepG2 is a human liver carcinoma cell line derived from a 15 years old Caucasian male which has been widely used in cancer research. HepG2 cells are suitable for *in vitro* model system for the study of polarized human hepatocytes. During the year 1979 to 2009, more than 9,000 HepG2 references have been used in the scientific literature (López et al., 2009).

2.6.5 Oral Cancer: HSC-4 – Oral Squamous Cell Line

Oral cavity cancer is amongst the most prevalent cancers worldwide and incidence rates are higher in men than women. Oral cancer appears as a growth or sore in the mouth that does not go away. Oral cancer, which includes cancers of the lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx (throat), can be life threatening if not diagnosed and treated early. HSC-4 is a human oral cancer cell lines

originating from a 64 years old Japanese male diagnosed with squamous carcinoma of the tongue. HSC-4 have an epithelial-like morphology.

2.6.6 Prostate Cancer: PC-3- Prostate Adenocarcinoma Cell Line

Prostate cancer is adenocarcinoma of the prostate. PC-3 was derived from a 62 years old Caucasian male. PC-3 stand for prohormone convertase-3. PC-3 is the epithelial cell line used in this present study representing prostate cancer. Most cases of prostate cancer are diagnosed in men over 50 years of age. The development of cancer in the prostate involves a gland in the male reproductive system. It is located at the base of the bladder surrounding the first part of the urethra, which carries urine from the bladder. In Malaysia, it is the sixth most frequent cancer and it accounts for 5.7% of cancer cases in males. The incidence of prostate cancer in Malaysia is still considered low as compared to the western countries (Sothilingam et al., 2010).

2.6.7 Normal cell: MRC-5 - Fetal Lung Fibroblast Cell Line

MRC-5 stand for Medical Research Council cell strain 5. The MRC-5 cell line is commonly utilized in *in vitro* cytotoxicity testing. This cell was derived from the human lung tissue of a 14-week old Caucasian male fetus aborted from a 27-year-old woman in September 1966. MRC-5 cells are used to produce several vaccines such as MMR (rubella), Varivax (chickenpox), Imovax (rabies), Havrix and Vagta (hepatitis A), Acambis1000 (smallpox) and Pentacel (polio).

2.7 Cell Death - Cytotoxic Activity

The anti-cancer properties of the plant extracts can be detected using many assays which measure the ability of the plant products (extracts, fractions or pure compounds) to kill cancer cells. There are many different approaches to access the cytotoxicity effect of plant extracts or compounds. These include MTT 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide test (Awang et al., 2010; Hasima et al., 2010; Subramaniam et al., 2016), neutral red uptake test (Repetto et al., 2008; Rahman et al., 2013), trypan blue exclusion test, resazurin test or Hoechst 33342 and propidium iodide test. However, in this study, MTT assay was selected for the screening of cytotoxic activity in Part A while in Part B, MTT assay and live death assay were applied against essential oils.

2.7.1 MTT Assay

MTT assay has been extensively reported in many anti-cancer researches as the *in vitro* basic screening and the quantitative method of cell death. In the literature, MTT assay is commonly used for various type of samples such as extracts, fractions, compounds and essential oils. Previous papers have reported the ability of natural compounds or essential oils from plant sources in killing cancer cells by using MTT assay. Some recent examples are acetoxycarvicol acetate and 1′S-1′-acetoxyeugenol acetate from *Alpinia conchigera* (Awang et al., 2010; Hasima et al., 2010), natural compounds from *C. zedoaria* (Hamdi et al., 2014) and geranylated 4-phenylcoumarins (Suparji et al., 2016). Some examples of MTT assay on essential oils are from *Curcuma wenyujin* (Xiao et al., 2008), *Rosmarinus officinalis* (Hussain et al., 2010), *Eucalyptus benthamii* (Döll-Boscardin et al., 2012) .

2.7.2 Live Dead Cell Viability Assay

Other than MTT assay, live dead assay is also used to exhibit cytotoxic activity. Live dead assay is a quick and easy two-colour assay to determine viability of cells in a population based on plasma membrane integrity and esterase activity. Some examples from literature which reported live dead assay of extracts and essential oils in cytotoxic activity are from *Chenopodium ambrosiodes* (Ya-Nan et al., 2015), *Garcinia mangostana* (Yostawonkul et al., 2017) .

2.8 Programmed Cell Death (PCD)

Initially, two main forms of cell death had been identified, apoptosis and necrosis (Kerr et al., 1972). Programmed cell death (PCD), is crucial for all multicellular organism. In the past decades, the mechanism which referred to apoptosis and programmed necrosis, is proposed to be death of a cell in any pathological format. Usually, cell death can be classified according to its morphological appearance such as apoptotic, necrotic, autophagy or associated with mitosis.

Other alternative models of PCD have therefore been proposed including autophagy (self-digestion by a cell through the action of enzymes originating within the same cell), cornification (specialized cell death of keratinocytes), paraptosis (apoptosis in the absence of cellular fragmentation), mitotic catastrophe (cell death that occurs during mitosis due to deficient cell-cycle checkpoints that lead to DNA damage), anoikis (apoptosis triggered by loss of contact with extracellular matrix), excitotoxicity (damage and death of neuron mediated by receptors for excitatory neurotransmitters), Wallerian degeneration (a form of degeneration occurring in nerve fibres as a results of their division) and the descriptive model of apoptosis-like and necrosis like PCD (Okada & Mak, 2004; Melino et al., 2005; Lorenzo & Susin, 2007; Moretti et al., 2007; Boujrad et al., 2007).

2.8.1 Apoptosis

Apoptosis is the best-studied form of the programmed cell death. The term apoptosis had been coined to describe the morphological processes leading to controlled cellular self-destruction and was first introduced in a publication by Kerr et al., 1972 and has been used since. Current cancer therapies, such as chemotherapy, γ-irradiation, immunotherapy or suicide gene therapy, were primarily applied for their anti-tumour effect by triggering apoptosis in cancer cells (Makin & Dive, 2001; Fulda & Debatin, 2004; Fulda & Debatin, 2006). Apoptosis is described by its morphological characteristics such as cell shrinkage, plasma membrane blebbing, organelle relocalisation and compaction, phosphatidylserine exposure, chromatin condensation in the nucleus, DNA fragmentation and production of membrane-enclosed particles containing intracellular material known as apoptotic bodies (Kerr et al., 1972; Bold et al., 1997; Strasser et al., 2000; Kaufmann & Hengartner, 2001).

Apoptosis occur normally during development and aging, and as a homeostatic mechanism to maintain cell population in the tissue. In most cases, the process of apoptosis involves the degradation of cellular constituents by a family of cysteine proteases called caspases that act as common death effector molecules in various forms of cell death (Degterev et al., 2003). Apoptosis pathways can be initiated through different entry sites. There are two main apoptotic signalling pathways in the regulation of apoptosis: the death-receptor induced extrinsic pathway and the mitochondrial apoptosome mediated apoptotic intrinsic pathway (Figure 2.6). Extrinsic (receptor mediated) pathway involves death receptors that are members of the tumour necrosis factor (TNF) receptor gene superfamily (Locksley et al., 2001). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signalling pathway. Caspase-8 is a critical initiator of the extrinsic apoptosis pathway

(Fulda, 2009). Procaspase-8 binds to the death effector domains (DED) of the adaptor molecules and followed by oligomerization and activation of caspase-8.

In the intrinsic or mitochondrial pathway of apoptosis, caspase activation is closely linked to permeabilisation of the outer mitochondrial membrane by pro-apoptotic members of the Bcl-2 family (Green & Kroemer, 2004). The other stimuli involved in death process incudes radiation, toxins, hypoxia, hyperthermia, viral infections, free radicals and others. The loss of the mitochondrial transmembrane potential is due to an opening of the mitochondrial permeability transition (MPT) pore, release of pro-apoptotic proteins which consists cytochrome *c* from the intermembrane space into the cytosol (Saelens et al., 2004). These proteins activate the caspase-dependent mitochondrial pathway. Cytochrome *c* binds and activates Apaf-1 as well as procaspase-9, forming an apoptosome (Chinnaiyan, 1999; Hill et al., 2004). Caspase-8 or caspase-9 then either directly activates caspase-3 and -7 or activates the cytosolic protein Bid which translocate to the mitochondria and causes the release of cytochrome *c* (Hengartner, 2000).

Overall, both the intrinsic and extrinsic apoptotic pathways activate caspases which are responsible for the execution of cell death. Even though apoptosis was considered as synonymous with programmed cell death the latter is currently sub-categorized into caspase-dependent PCD (classical apoptosis) and caspase-independent PCD.

2.8.2 Necrosis

Necrosis is identified as cell death in a negative fashion and lacking the feature of apoptosis or autophagy (Golstein & Kroemer, 2007). In contrast to apoptosis, some of the major morphological changes in necrosis include cellular swelling, formation of cytoplasmic vacuoles, distended endoplasmic reticulum, formation of cytoplasmic blebs, condensed, swollen or ruptured mitochondria, disaggregation and detachment of

ribosomes, disrupted organelle membranes, swollen and ruptured lysosomes and eventually disruption of the cell membrane (Kerr et al., 1972; Majno & Joris, 1995). Necrosis occurs when a cell is damaged by an external force, such as poison, a bodily injury, an infection or getting cut off from the blood supply (which might occur during a heart attack or stroke). When cells die from necrosis, it's a rather messy affair. The death causes inflammation that can cause further distress or injury within the body. Figure 2.6 showed the hallmarks of the apoptotic and necrotic cell death process.

Determination of whether a cell dies by necrosis or apoptosis depends on the nature of the cell death signal, the tissue type, the developmental stage of the tissue and physiologic surrounding (Elmore, 2007). However, both processes can occur independently, sequentially, as well as simultaneously. In some case it's the time of stimuli and or the degree of stimuli that determines if cells die by apoptosis or necrosis. For example, at low doses, a variety of injurious stimuli such as heat, radiation, hypoxia and cytotoxic anti-cancer drugs induces apoptosis but these same stimuli at higher doses can instead result in necrosis (Elmore, 2007). In addition, Table 2.8 showed the comparison of cellular changes associated with apoptosis and necrosis (Bold et al., 1997).

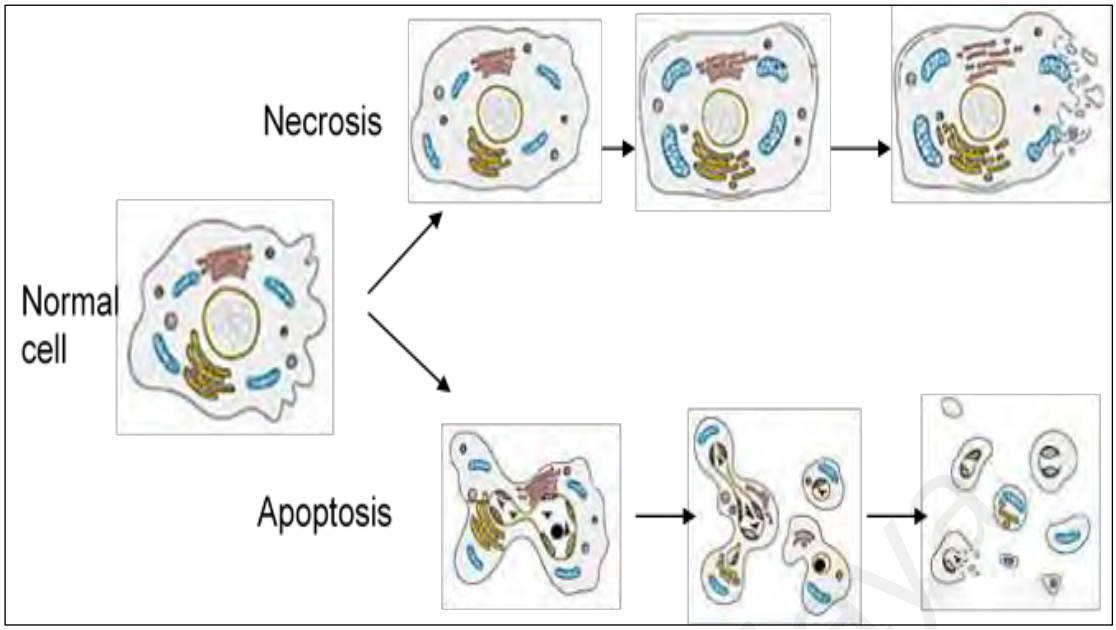


Figure 2.6: Hallmarks of the apoptotic and necrotic cell death process. *Source: http://thebrain.mcgill.ca/flash/capsules/outil_bleu17.html*

Table 2.8: Comparison of cellular changes associated with apoptosis and necrosis (Bold et al., 1997)

Apoptosis	Necrosis
➢ Physiologic, regulated	➢ Pathologic, unregulated
➢ Cell shrinkage	➢ Cell swelling
➢ Chromatin condensation	➢ Irregular chromatin clumping
➢ Preservation of intracellular organelles	➢ Dysfunction and destruction of organelles
➢ Membrane blebbing, apoptotic bodies	➢ Disruption of cellular membranes
➢ Organized chromatin digestion to small fragments (DNA ladders)	➢ Non-specific and random degradation of DNA (DNA smears)

2.9 Related Anti-Cancer Research on *Curcuma zedoaria*

Anti-cancer effects on the compounds from *C. zedoaria* including their essential oil have been extensively reported. All these findings supported the uses of *C. zedoaria* rhizomes in traditional medicine (Tuna et al., 2011; Yin et al., 2013) for the treatment of cancer-related diseases. The followings are some studies of *C. zedoaria* on anti-cancer research.

α-Curcumene which was present in the essential oil studied by Shin et al. (2013) also isolated from *C. zedoaria* showed a hallmark features of apoptosis such as characteristic nucleosomal DNA fragmentation pattern and the percentage of sub-diploid cells increased in a concentration manner on ovarian cancer cells, SiHa. Activation of caspases have showed by the release of mitochondrial cytochrome *c* and from *in vitro* caspase-3 assay (Shin & Lee, 2013). Another study in 2013 has reported that curzerenone, which also presented in the *C. zedoaria* essential oil showed induction of apoptosis through the activation of caspase-3. It can thus be suggested that curzerenone are modulated by apoptosis via caspase-3 signalling pathway (Rahman et al., 2013).

Essential oil of *C. zedoaria* have been reported to induce apoptosis-mediated cell death against non-small cell lung carcinoma cells (NSCLC), H1299 by *in vitro* and *in vivo*. This oil induced cleavage and activation of caspase and affected the production of reactive oxygen species (ROS). From western blot analysis, the *C. zedoaria* oil showed expression of the *bcl-2* family, released of cytochrome *c*, Endo G and AIF from mitochondria into the cytosol, slightly inhibited the phosphorylation of ERKI/2 but enhances the phosphorylation of p38 and JNK1/2 and inhibited phosphorylation of AKT and $I_K B\alpha$. For the *in vivo* anti-tumour activity, the results exhibited that administration of *C. zedoaria* essential oil induced a dose-dependent inhibition of H1299 tumour volume and a reduction in tumour weight (Chen et al., 2013).

CHAPTER 3: MATERIALS AND METHODS

Part A: Characterization of compounds from *Curcuma zedoaria* from Indonesia using bioassay-guided isolation

3.1 Plant Materials

The rhizomes of *Curcuma zedoaria* (Christm.) Roscoe was collected from Tawangmangu, Solo, Indonesia (S 7º 36' 58.9", E 111º 02' 58"), registered with herbarium number, KLU49447. The voucher specimen has been deposited at the herbarium of Rimba Ilmu Botanical Garden, University of Malaya.

3.2 Bioassay-guided Isolation of *Curcuma zedoaria*

In this present study, all crude extracts from the rhizome of *Curcuma zedoaria* were screened for cytotoxic activity (MTT assay) against selected cancer cell lines. This was followed by fractionation and MTT assay on the most potent fractions against selected cancer cell lines. Further, active compounds were isolated from the active fraction and tested for their cytotoxic effects.

3.2.1 Solvents and Chemicals

Organic solvent used in this research for extraction and fractionation are industrial grade (distilled) such as hexane (C_6H_{14}), dichloromethane (CH_2Cl_2) and ethanol (C_2H_6O). Dimethyl-sulfoxide (DMSO) was purchased from Sigma-Aldrich, St. Louis, Mo, USA and MTT (3-(4, 5-diamethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) reagents was purchased from Calbiochem, USA. Spathulenol (colourless oil, >90% purity) and β-eudesmol (white powder, >98% purity) were purchased from Biopurify Phytochemicals Ltd. (Chengdu, Sichuan, China).

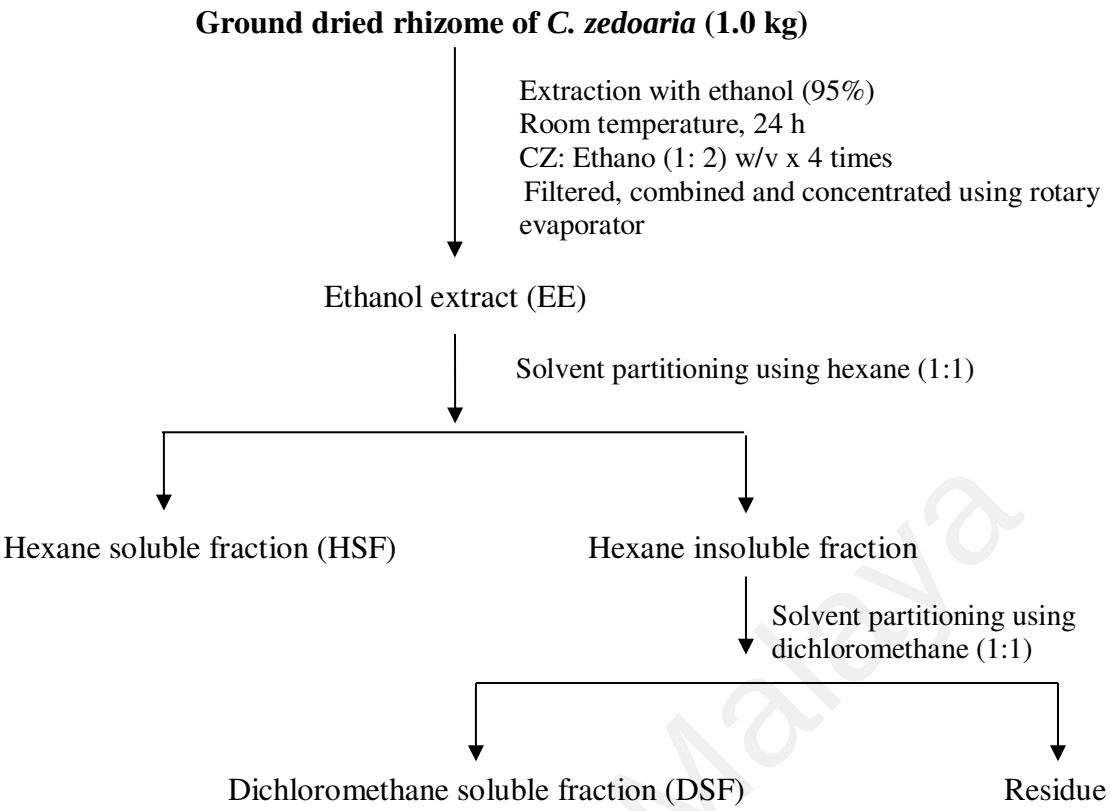
3.2.2 Extraction Procedure

The fresh rhizomes of *Curcuma zedoaria* from Indonesia were washed and air dried, then sliced. Next, the sliced rhizomes were dried in hot air oven (± 45 ºC) for three days consecutively until dried. The air-dried rhizomes were grounded into powder. 1.0 kg of the powdered rhizomes of *C. zedoaria* was extracted for four days with 95% ethanol at room temperature (± 27 ºC). After 24 h, the plant material and solvent were separated. New batch of ethanol was added to the plant material and replaced every day. The ethanol extracts were combined, concentrated using rotary-evaporator and labelled as ethanol extract (EE). EE was subjected to solvent partitioned with hexane to get hexane soluble fraction (HSF) and hexane insoluble fraction. Next, dichloromethane was added to hexane insoluble fraction, separated and concentrated to get dichloromethane soluble fraction (DSF) and the residue. The flow of extraction process is shown in Scheme 3.1. The information of the yield such as weight, percentage of yield and description of the crude extracts are shown in Table 3.1.

3.2.3 Chromatography Techniques

3.2.3.1 Thin Layer Chromatography (TLC)

Aluminium supported silica gel 60 F_{254} plates (Merck) was used to observe the spots of the compounds in the fractions. Thin layer chromatography (TLC) was developed using the optimum solvent system. UV light model UVGL-58 mineralight lamp 230V, 50/60 Hz was used to examine spots or band using short and long wavelength. Vanillin solution was used routinely as reagent to detect the terpene spot on the TLC. 1.0 g of vanillin was added in 10 mL of concentrated H_2SO_4 and 90 mL of ethanol before ready to be sprayed onto TLC. The developed TLC was heated at 50 ºC until colours had been observed. The purple, blue, red and brown spots indicated the presence of terpene compounds.



Scheme 3.1: Schematic extraction of *Curcuma zedoaria*

Table 3.1: The yield of *Curcuma zedoaria* crude extracts

Raw materials: 1.0 kg of dried rhizome of *C. zedoaria*			
Samples	Yield (g)	Percentages (%)	Description
Ethanol extract (EE)	100.9	10.1	Oily, sticky
Solvent partitioning using 59.0 g of ethanol extracts			
Hexane soluble fraction (HSF)	11.2	18.9	Oily, sticky
Dichloromethane soluble fraction (DSF)	21.6	36.6	Dark brown powder
Residue	6.7	11.4	sticky

3.2.3.2 Column Chromatography (CC)

Silica gel 60, 230-400 mesh ASTM (Merck) was used for column chromatography (approximately 30:1 silica gel to sample ratio). All solvents used in this experiment (ethanol, hexane and dichloromethane) are distilled from industrial grade.

3.2.3.3 Gas Chromatography- Flame Ionization Detector (GC-FID)

Analyses of compounds present in *Curcuma zedoaria* were carried out by gas chromatography (GC) equipped with flame ionized detector (FID) and gas chromatography / mass spectrometry (GC/MS). Initially, the samples were dissolved in pentane (C_5H_{12}) at a concentration of 3.0 mg/mL. It was followed by GC-FID analysis using 7890A gas chromatography instrument (Agilent Technologies, Santa Clara, CA, USA) with HP-5 capillary column HP-5 (30 m x 0.25 mm, film thickness 0.25 µm). The oven temperature was set as: initial temperature 70 ºC for 10 min, 70 ºC to 250 ºC (4 ºC / min) and subsequently isothermal at 250 ºC for 15 min. The total run time was 70 min.

3.2.3.4 Gas Chromatography / Mass Spectrometry (GC/MS)

GC/MS analysis was performed using a Shimadzu GC-2010 system coupled to a quadruple mass spectrometer, MS-QP2010 Plus. Column and the oven parameters were set up same as with the conditions of GC-FID.

3.2.3.5 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) spectra of 1D (^{1}H and ^{13}C) were obtained using Bruker (400 MHz). The ^{1}H and ^{13}C NMR spectra were recorded using deuterated chloroform ($CDCl_3$) with the residual solvent peaks as reference (7.27 ppm for ^{1}H and 77.2 ppm for ^{13}C NMR). The chemical shifts (δ) were expressed in ppm and the coupling constants (*J*) are given in Hz.

3.2.4 Fractionation and Purification of Compounds by Bioassay-guided Isolation

Each of these extracts namely ethanol extracts (EE), hexane soluble fraction (HSF), dichloromethane soluble fraction (DSF) and the residue were tested for cytotoxic activity on selected cancer cell lines. The most active fraction was selected and subjected to MTT assay against cancer cell line. This procedure was continued until the bioactive compounds were successfully identified and isolated.

3.3 Cell Preparation for Cytotoxic Activity

In general, prior to cytotoxic activity or cell viability assays, some preparation such as thawing of cryopreserved cells from liquid N_2 tank, cultivation of cell lines and cell counting were carried out.

3.3.1 Thawing of Cryopreserved Cells

Cryopreserved cells were removed from liquid N_2 tank and thawed immediately in a water bath at 37 ºC for 2 min. Every 1 mL of thawed cell suspension was diluted 10 x in 10 mL of growth medium containing 10% (v/v) FBS (Cambrex, USA) and centrifuged at 1,500 rpm for 5 min. The supernatant containing the cryoprotective agent DMSO (Merck, Germany) was discarded and the pellet was re-suspended in fresh media containing 10% (v/v) FBS (Cambrex, USA). Re-suspended cells were split into T-75 cm^2 flasks and incubated at 37 ºC in a 5% CO_2 and 95% humidity level atmosphere.

3.3.2 Cultivation of Cell Lines

All cells used were split (sub-cultured) every three to four days, or when 80-90% confluence was attained on the culture flask surface. Media was removed, and cells were washed with 1x PBS (Lonza, USA) to remove any residual serum that could inactivate trypsin activity. The PBS was removed and 2 mL of 0.25% (v/v) Trypsin (Lonza, USA)-

EDTA (Gibco, USA) solution was added. Cells were then incubated at 37 ºC for 10 min to completely detach cells from the T-75cm^2 flask surface. Equal volumes of media containing 10% (v/v) FBS (Cambrex, USA) was added to inactive trypsin activity, and transferred into a 15-mL centrifuge tube. Trypsinized cell were then centrifuged at 1,500 rpm for 5 min and the supernatant was discarded. The cells pellet was re-suspended in fresh media and split for further usage. Passage numbers was recorded in every maintaining process.

3.3.3 Cell Counting

A dye exclusion viability assay using a haemocytometer was used to determine the number of cells present in a specific population. Monolayer of cells were detached by trypsinization, centrifuged and re-suspended in media. Approximately 20 µL of cell suspension was mixed with 20 µL of 0.04% (v/v) trypan blue (Merck, Germany) dye solution. The solution was then transferred to a haemacytometer counting chamber and spread evenly by capillary action. Using an inverted microscope (Labscope, Taiwan) at 40 x magnification, the number of stained viable cells in each of the four-square grid corners was counted and the average number of cells was obtained. Each square grid represents a 0.1 mm^3 or 10^4 volumes and the concentration of cells were determined (refer to Equation 1) with a dilution factor of two. Using Equation 1 and Equation 2, the concentration of cells needed was prepared for the assays. The haemacytometer slide and glass cover slip was immediately rinsed and clean with 70% ethanol (Merck, Germany) between samples and after use.

[Equation 1]

Cell concentration = n x D x 10^4

Where,

n = Average number of cells counted

D = Dilution factor (=2)

[Equation 2]

M_1V_1 = M_2V_2

Where,

M_1 = C (cell concentration from equation 1)

V_1 = Volume needed from cell suspension

M_2 = Concentration needed to prepare

V_2 = Volume needed to prepare

3.3.4 Preparation of Frozen Stocks

The frozen stocks of unused cell lines need to be prepared for long-term storage. Confluent cells were washed with 1 x PBS (Lonza, USA) and detached from the flask surface by adding 2 mL of 0.25% (v/v) Trypsin (Lonza, USA) – EDTA (Gibco, USA) solution at 37 ºC for 10 min. Equal volumes of media supplemented with 10% (v/v) FBS (Cambrex, USA) was added to the detached cells to inactivate and prevent prolonged trypsin activity. Cell suspension was then pipetted into 15.0 mL tubes and centrifuged at 1,500 rpm for 5 min. The supernatant was discarded, and the cell pellet was re-suspended in fresh media containing 20% (v/v) FBS and 10% (v/v) DMSO as the cryoprotecting agent. Several stocks of 1 mL aliquots were prepared in 2.0 mL cryovial, frozen gradually at -20 ºC for 3 h and finally stored in liquid Nitrogen (N_2) at -80 ºC.

3.4 Cytotoxic Activity

Cytotoxic activity of the crude extracts (ethanol extract, hexane soluble fraction, dichloromethane soluble fraction and the residue), their fractions and compounds were carried out using the MTT assay.

3.4.1 Cell Lines Used in the Cytotoxic Activity of Crude Extracts

The human cancer cell lines namely breast (MCF-7), lung (A549), prostate (PC3), oral (HSC-4), liver (HepG2) and cervical (CaSki) were used in bioassay-guided isolation process. Normal lung cell line (MRC-5*)* was used as control. MCF-7, PC-3 and A549 cell were cultured as monolayers in Rosewell Park Memorial Institute (RPMI-1640), while CaSki, HepG2 and HSC-4 were cultured in Dulbecco's Modified Eagle Medium (DMEM). MRC-5 was cultured in Eagle's Minimum Essential Medium (EMEM). All media was supplemented with 10% (v/v) fetal bovine serum (FBS) and for MRC-5 cell line, 1% of sodium pyruvate (Sigma) was added to the media. All cultured cells were maintained at 37 ºC in 5% CO_2 and 95% air.

3.4.2 Preparation of MTT Reagent

MTT (3-(4,5-diamethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reagents was prepared by adding 60 mg of MTT (Calbiochem, USA) to 12 mL of 1 x PBS (Lonza, USA). The reagent was vigorously shaken and vortexed to ensure that the MTT granules were completely dissolved. The final concentration of the MTT working solution was 5 mg/mL. The MTT working solutions were kept in the refrigerator (4ºC) while the MTT stock was kept in the dark at room temperature (25ºC).

Table 3.2: Human cancer and normal cell lines used in bioassay-guided isolation

Human cancer cell lines	Type	Source	Culture Media + 10% FBS
A549	Lung adenocarcinoma epithelial cells	ATCC	RPMI 1640
HepG2	Liver hepatocytes carcinoma cells	Faculty of Medicine, UM	DMEM
HSC-4	Oral squamous carcinoma cells	CARIF	DMEM
MCF-7	Breast adenocarcinoma cells	CARIF	RPMI 1640
PC3	Prostate cancer cells	ATCC	RPMI 1640
SiHa	Cervical epidermoid carcinoma cells	Faculty of Medicine, UM	DMEM
MRC-5	Normal lung cells	ATCC	EMEM + 1% sodium pyruvate

3.4.3 MTT Assay

Cytotoxic activity of all extracts on six cancer cell lines and normal cell line were investigated by MTT assay. All extracts were dissolved in DMSO to obtain a stock concentration of 10 mg/mL and were filtered through 0.45 µM nylon filter (Waters) for further uses. Initially, 1 x 10^4 cells per l00 µL/well were seeded in 96's well plate. The cells were plated in triplicate. After overnight incubation for adherence, the cells were treated with the samples at various concentrations (20, 40, 60, 80, 100 and 120 µg/mL) and incubated at 37 ºC for another 24 hours.

20 µL of MTT reagent (5 mg/mL) was added into each well and incubated for 45 min. After incubation, the reagent was removed and 200 µL of DMSO was added to dissolve purple formazan precipitates. Microtiter plate reader (Tecan Sunrise®, Switzerland) was used to detect absorbance / reference at 570 nm and 650 nm. The percentage of cell viability of the test samples were calculated according to the formula (Equation 3) whereas NC refers to number of cells obtained from the plate reader. The potency of extracts to inhibit the growth of cancer cells at 50% was expressed as IC_{50}

(inhibition concentration at 50%). All assays were performed in triplicate of independent experiments ($n = 3$). All the data were presented as mean ± standard deviation (SD). The same procedures were also applied to fractions and compounds.

The selectivity index (SI) of each sample was determined by the ratio between IC_{50} of the samples on normal cell line and IC_{50} of the samples on cancer cell lines (refer Equation 4). SI value indicates selectivity of the samples to the cell lines tested. Samples with an SI greater than 3.0 are considered to have high selectivity towards cancer cells.

[Equation 3]

$$\textbf{\% Cell viability} = NC_{untreated} - \frac{NC_{DMSO} - NC_{sample}}{NC_{untreated}} \times 100$$

[Equation 4]

$$\textbf{SI} = \frac{IC_{50} \text{ of the extracts or compounds on normal cell line}}{IC_{50} \text{ of the extracts or compounds on cancer cell lines}}$$

Part B – Essential oils analysis of *Curcuma zedoaria* from Malaysia and Indonesia and characterize their cytotoxic and apoptosis abilities

3.5 Plant Materials

The rhizomes of *Curcuma zedoaria* (Christm.) Roscoe were collected from Pahang, Malaysia (N 3° 16' 12.00", E 102° 15' 0.0") (herbarium number: KLU49446) and from Tawangmangu, Solo, Indonesia (S 7º 36' 58.9", E 111º 02' 58") (herbarium number: KLU49447). The voucher specimens have been deposited at the herbarium of Rimba Ilmu Botanical Garden, University of Malaya.

3.5.1 Extraction of Essential Oils

Dried rhizomes (200 g/replicate) of *C. zedoaria* collected from Malaysia and Indonesia were individually hydrodistilled for 4 h using Clevenger-type apparatus laboratory scale according to the standard procedure (Sivasothy et al., 2011). Each sample was extracted in triplicate ($n = 3$). Pentene was used as collecting solvent. The oils were separated from water layer and dried using drying agent, sodium sulphate (Na_2SO_4). Then, the solvent was carefully removed using a gentle stream of nitrogen gas.

3.5.2 Determination of Oil Yield

The yield of the oils was calculated based on the dried weight of the plant materials. Percentage yield based on dry weight of plant material was calculated using the formula as below:

[Equation 5]

$$\textbf{Percentage of yield (\%) (w/w)} = \frac{\text{Oil collected}}{\text{Weight of raw material}} \times 100$$

The mean of triplicate measurements was calculated ($n = 3$)

Figure 3.1: Extraction of essential oils using Clevenger apparatus

3.6 Essential Oil Analysis

The *Curcuma zedoaria* essential oils from Malaysia and Indonesia collected through hydro distillation were subjected to further chemical analysis which involved GC-FID, GC/MS and Kovat Index (KI) analysis. The following sub chapters discuss briefly the analysis of these oils.

3.6.1 Gas Chromatography – Flame Ionization Detector (GC-FID)

Analyses of essential oils from *Curcuma zedoaria* were carried out by GC equipped with flame ionization detector (FID) and gas chromatography/mass spectrometry (GC/MS). Initially, the oils were dissolved in pentane (C_5H_{12}) at concentration of 3 mg/mL. It was followed by GC-FID analysis using 7890A gas chromatography instrument (Agilent Technologies, Santa Clara, CA, USA) with HP-5 capillary column HP-5 (30 m x 0.25 mm, film thickness 0.25 µm). The carrier gas was nitrogen at 1.0 mL/min. constant flow, injection volume 0.2 µL, injector and inlet temperature were set at 250 ºC and a splitting ratio 1:20. The oven temperature was set as: initial temperature 70 ºC for 10 min, 70 ºC to 250 ºC (4 ºC / min.) and subsequently isothermal at 250 ºC for 15 min. The total run time was 70 min.

The standard mixture of $C_7 – C_{30}$ and other homologous series of *n*-alkanes used in the GC-FID analysis were purchased from Sigma Chemical Co., USA.

3.6.2 Gas Chromatography / Mass Spectrometry (GC/MS) Analysis for Essential Oils

GC/MS analysis was performed using a Shimadzu GC-2010 system coupled to a quadrupole mass spectrometer, MS-QP2010 Plus. Column and the oven parameters were set up same as the GC conditions as described above. Carrier gas used was helium. Mass spectra were performed at 70 eV over a scan range of 40-600 amu.

3.6.3 Kovat Retention Index (KI)

Kovat retention index (KI) were determined by using C_7 to C_{30} alkane series (Supelco, USA) standard hydrocarbon as references (refer Equation 6). Relative amounts of individual compounds were based on peak areas obtained without FID response correction factor. The constituents of the oils were identified by matching their mass spectral with those from GC/MS Libraries NIST 08 and comparing their KI value with data from the literature (Adams, 2001). KI value of each compound was calculated using the formula as below;

[Equation 6]

$$\mathbf{KI} = 100 \frac{[\text{Log}\,(Tx - Tm) - \text{Log}\,(Tn - Tmh)]}{[\text{Log}\,(Tn + 1 - Tmh) - \text{Log}\,(Tn - Tmh)]} + 100\,(N)$$

Where:

Tx = Sample component retention time

Tm = Void volume / void time

Tn = Standard hydrocarbon containing carbon retention time

Tmh = Mobile phase retention time for standard hydrocarbon

N = Lowest carbon value

3.6.4 Statistical Analysis for Essential Oil Components

The mean and standard deviation (SD) of each component present in the oils were performed by using Microsoft Excel (n = 3). SD was calculated based on three different GC-FID data of each component in the essential oils.

3.7 Cytotoxic Activity

The cytotoxic activity of essential oils of *C. zedoaria* from Malaysia and Indonesia were performed using the MTT assay. The cytotoxic activity of selected active oil was confirmed by using live dead assay against selected cancer cell lines.

3.7.1 Cell Lines Used in Cytotoxic Activity of Essential Oils

The human cancer cell lines used in cytotoxic activity of essential oils were breast (MCF-7 and MDA-MB-231), lung (A549 and SK-LU-1) and cervical (HeLa S3 and SiHa). Normal lung cell line (MRC-5*)* was used as control. MCF-7, MDA-MB-231 and A549 cell were cultured as monolayers in Rosewell Park Memorial Institute (RPMI-1640), SK-LU-1 was maintained in α-Minimum Essential Medium Eagle (α-MEM) while HeLa S3 and SiHa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). MRC-5 was cultured in Eagle's Minimum Essential Medium (EMEM). All media were supplemented with 10% (v/v) of FBS and for MRC-5 cell line, 1% of sodium pyruvate (Sigma) was added to the media. All cells cultured were maintained at 37 ºC in 5% CO_2 and 95% air.

Table 3.3: Human cancer and normal cell lines used in cytotoxic activity of essential oils

Human cancer cell lines	**Type**	**Source**	**Culture Media + 10% FBS**
MCF-7	Breast adenocarcinoma epithelial cell	CARIF	RPMI 1640
MDA-MB-231	Breast adenocarcinoma epithelial cell	ATCC	RPMI 1640
A549	Lung adenocarcinoma epithelial cells	ATCC	RPMI 1640
SK-LU-1	Lung adenocarcinoma epithelial cells	ATCC	α-MEM
HeLa S3	Cervical cancer cells	ATCC	DMEM
SiHa	Cervical cancer cells	ATCC	DMEM
MRC-5	Normal lung cells	ATCC	EMEM + 1% sodium pyruvate

3.7.2 Preparation of MTT Reagents

- Same as in subheading **3.4.2** (refer page 55)

3.7.3 MTT Assay

- Same as in subheading **3.4.3** (refer page 56)

3.7.4 Live Dead Cell Viability Assay

Assessment of cell viability upon treatment with Malaysian *C. zedoaria* oil was conducted using the LIVE / DEAD Viability / Cytotoxicity kit (Molecular Probes, Invitrogen, NY, USA). Cells were cultured on glass cover slip placed in 6-well plates for 20 h until the density of cells reach 70% – 80%. After the incubation period, cells were treated with Malaysian *C. zedoaria* oil at the IC_{50} values for 6 h. The spent media were aspirated, and the cells were washed with 1 x ice-cold PBS to remove serum esterase activity generally present in serum supplemented growth media, which could cause increase in extracellular fluorescence due to hydrolysed calcium AM.

A dual fluorescence staining system consisting of calcein AM (emits green fluorescence when cleaved by intracellular esterases) and ethidium homodimer (EthD) (emit red fluorescence upon entering non-viable cells and binding to nucleic acid) (Molecular Probes, Invitrogen, USA) were used for staining of viable versus non-viable cells. 150 µL of the combine Live/ Dead assay reagent containing calcein-AM (2.0 µM) and ethidium homodimer (EthD) (4 µM) were added (using optimized concentration) to the surface of 22 mm square cover slips containing cell treated with Malaysian *C. zedoaria* oil, DMSO treatment and untreated cells. The cover slips were then incubated for 30 min at room temperature and kept away from the light to ensure complete formation of fluorescent products within cells. The excess dyes were washed with 1 x ice cold PBS to a clean the microscope slides. Fine tipped forceps were used to carefully

invert and mount the wet cover slips on the microscope slide. The cover slip was sealed to the glass slide using fingernail polish to prevent evaporation and avoid damaging or shearing of the cells during the slide preparation.

Finally, excitation and emission wavelengths were set at 494/517 nm for calcein-AM and 528/617 nm for EthD respectively. Visualization of samples was done using a Nikon Eclipse TS-100 fluorescence microscope (Nikon, Japan) under 100 x magnification.

3.8 Apoptosis Assay

Apoptosis assay was conducted on the selected active essential oil of *C. zedoaria* to elucidate the effect of cell death capability through apoptosis. Thus, in this study, Annexin-V-FITC/PI binding assay (flow cytometry assay) and caspase-3/7 activity assay has been performed.

3.8.1 Annexin-V-FITC/PI Binding Assay

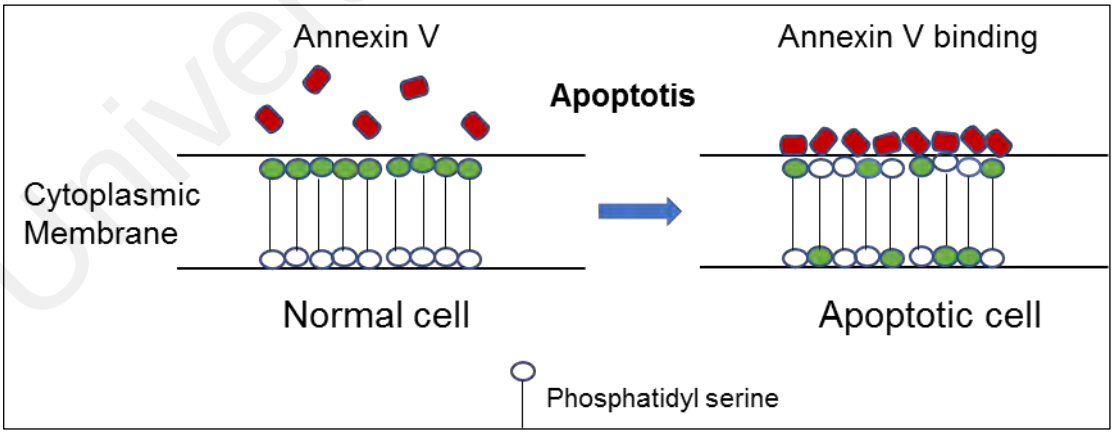


Figure 3.2: Annexin-V-FITC/PI binding assay. *Source: https://www.biocat.com/cell-biology/apoptosis/apoptosis-detection phosphatidylserine-annexin-based*

Detection and differentiation of various stages were conducted using the Annexin-V-FITC Apoptosis Detection Kit (Calbiochem, USA). Hela S3 and SiHa cell lines were treated with Malaysian essential oil at various concentrations for 24 h before harvesting. After treatment, the used media containing detached cells were collected in 15 mL tubes. The remaining adherent cells were trypsinized, neutralized by media containing 10% (v/v) of FBS and centrifuged at 1000 x g for 5 min. Supernatants were discarded and cell pellets were rinsed with 1 mL 1x ice-cold PBS and centrifuged at 1,000 x g for 5 min. All cells were resuspended and diluted in 6 mL of media to a final concentration of 1.0 x 10^5 cell/mL. Aliquots containing 500 µL of media (5.0 x 10^5 cell/mL) were transferred into microcentrifuge tubes and 10 µL of media binding reagent was added. Phosphatidyl serine (PS) externalization was detected by adding 1.25 µL of FITC-conjugated Annexin-V anticoagulant (200 µg/mL) into each tube and incubated for 15 min at room temperature in the dark. All tubes were then centrifuged at 500 x g for 5 min and the supernatant was discarded. The pellet was re-suspended in 500 µL of 1 x ice cold binding buffer, followed by the addition of 10 µL of PI (30 µg/mL). All tubes were kept on ice in the dark until analysed by a flow cytometer. All assays were carried out using the BD FACS Canto 11™ cytometer (Becton Dickenson, USA).

3.8.1.1 Data Analysis Using FASC Diva Software

Total populations of 1.0 x 10^4 cells were counted for each sample and a four-quadrant dot plot of untreated and treated samples were created using FSC-parameter and SSC-parameter based on gated populations. Sole FITC signals were detected at a 518-nm wavelength, while sole PI signals were detected at a 620-nm wavelength. Combination of both FITC and PI signals were detected at a 488-nm wavelength. Quantification and visualization of early apoptosis, late apoptosis and necrotic cell populations were defined

by quadrant boundaries that were created based on single dye-signal controls of each tumour cell line.

3.8.2 Caspase-3/7 Activity Assay

Caspase-3/7 activity was detected using Caspase-Glo 3/7 assay kit (Promega). HeLa S3 and SiHa cell lines were treated with either DMSO, 30, 40 and 50 µg/mL of Malaysian *C. zedoaria* oil for 5 h. The caspase 3/7 activity in each sample was then measured according to manufacturer's protocol after 1 h incubation in the dark at 25 ºC using the GloMax Multi Luminescence Multimode Reader.

3.8.3 Data Analysis

All assays were performed in triplicate of independent experiments ($n = 3$). Data from all experiments were presented as mean ± standard deviation (SD). Student *t*-test was used to determine whether the results are statistically significant at $p \leq 0.05$ and $p \leq 0.01$.

CHAPTER 4: RESULTS

This chapter consists of two parts; Part A and Part B. Part A is the characterization of cytotoxic compounds from the Indonesian *Curcuma zedoaria* using bioassay-guided isolation. This part mainly reports the cytotoxic activity of the extracts, fractions and pure compounds isolated from the rhizomes of *C. zedoaria.* Cytotoxic activity of the crude extracts namely ethanol extract, hexane soluble fraction (HSF), dichloromethane soluble fraction (DSF) and the residue have been performed against cancer cell lines; lung (A549), liver (HepG2), oral (HSC-4), breast (MCF-7), prostate (PC-3), cervical (SiHa) and lung normal cell line (MRC-7) using MTT assay method. This process was continued with the fractionation of selected active fractions and tested for their cytotoxic activity against selected cancer cell lines (lung cancer cell line, A549). Characterization of compounds from the active fraction have been performed by the GC-FID, GC/MS, Kovat retention index (KI) and NMR analysis.

Part B deals with the analysis of the rhizome essential oils of *C. zedoaria* from both Malaysia and Indonesia, followed by investigation of their cytotoxic and apoptotic properties. The dried rhizomes of *C. zedoaria*, were hydrodistilled and their chemical constituents were analysed by using GC-FID, GC/MS and Kovat retention index (KI). The chemical constituents were compared and both oils were subjected to cytotoxic activity (MTT assay) against cancer cell lines; breast (MCF-7 and MDA-MB-231), lung (A549 and SK-LU-1) and cervical (HeLa S3 and SiHa). The most potent oil was selected for further assays such as live dead assay on selected cancer cell lines followed by apoptotic investigation such as annexin-V flow cytometry assay and caspase-3/7 assay.

Part A: Characterization of compounds from *Curcuma zedoaria* from Indonesia using bioassay-guided isolation

The rhizome of *Curcuma zedoaria* from Indonesia was successfully extracted using ethanol to get ethanol extract (EE). Solvent partitioning was performed on the EE using hexane to get hexane soluble fraction (HSF). Solvent partitioning was performed on the hexane insoluble fraction using dichloromethane to get dichloromethane soluble fraction (DSF) and the residue. The description of the yield of extracts is shown in Table 3.1 (page 50).

4.1 Cytotoxic Activity of the Crude Extracts

All extracts of *Curcuma zedoaria*, EE, HSF and DSF and the residue were evaluated for their cytotoxic activity by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay against selected human cancer cell lines; lung (A549), liver (HepG2), oral (HSC-4), breast (MCF-7), prostate (PC-3) and cervical (SiHa). The cytotoxic effects also assessed the normal lung cell (MRC-5) as control to ensure that the crude extracts induce only minimal cytotoxicity on normal cells. The MTT assay was performed to determine the IC_{50} values based on the ability of the crude extracts to achieve 50% killing of the viable cells upon treatment. To evaluate whether the activity of the extracts is concentration dependent, several concentrations ranging from 20 to 120 µg/mL were used for 24 h of treatment and the IC_{50} of each extract were calculated. Furthermore, to ensure that the cytotoxicity was induced by the extracts instead of dimethyl sulfoxide (DMSO) as the control, a treatment of DMSO was also conducted at various concentration (20.0 – 120.0 µg/mL). The MTT data for DMSO was deducted from the calculation to get IC_{50} values (refer to Equation 3 on page 57) with the aim to get the net cytotoxic effect of the crude extracts tested.

The MTT assay on EE against A549, HepG2, HSC-4, MCF-7, PC-3 and SiHa cell lines displayed the concentration dependent cytotoxic effect with IC_{50} values in the range of 31.2 µg/mL to 81.5 µg/mL. The cytotoxic activity against SiHa and HepG2 cells were considered not active where the IC_{50} values showed more than the maximum concentration used in this assay (120.0 µg/mL). The results also revealed that EE did not inhibit MRC-5 cell growth at the maximum concentration (>120 µg/mL). From these results, selectivity index (SI) of EE on A549 cell was calculated and SI showed the highest value of more than 3.8. Selectivity index is an index that shows the selectivity effect of samples as compared to the normal cells. It is considered effective for values greater than 3.0. In certain cases such as for the crude extracts and essential oil, the values greater than or equal to 2.0 are also indicative of effective selective index (Oliveira et al., 2015).

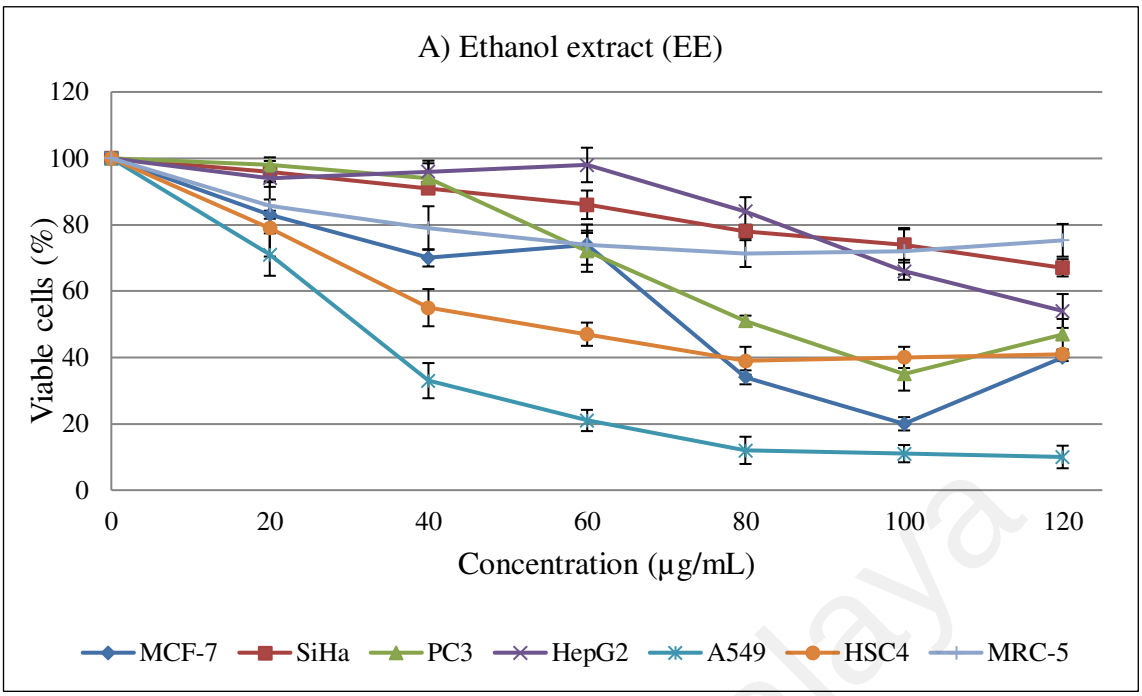
Thus overall, the best inhibitory effect of EE was exhibited on A549 cells (IC_{50}: 31.2 µg/mL) and based on this result, further MTT assay was focused on this lung cancer cell line. HSF and DSF which originated from EE by solvent partitioning technique showed the IC_{50} values of 44.1 µg/mL and 53.7 µg/mL, respectively, towards A549 cells. As compared to DSF, HSF was selected for further fractionation due to its greater activity on all tested cancer cells and showed less inhibition towards MRC-5 cells. HSF did not inhibit MRC-5 cell growth at the maximum concentration used in this study (>120.0 µg/mL), while DSF inhibited MRC-5 at the IC_{50} value of 47.3 µg/mL. However, the residue did not show any inhibition against all cancer cell lines including normal cell (MRC-5). Table 4.1 display the IC_{50} values on the cytotoxic effect of EE, HSF, DSF and the residue against various human cancer cell lines. The results were obtained by three different experiments and presented as the means ± standard deviation (n = 3). Comparison of total relative cell viability (%) between various cancer cell lines and

normal cell line after treatment with crude extracts are shown in Figure 4.1 (A to D) indicating the concentration dependent cytotoxic effects.

Table 4.1: MTT screening of ethanol extract (EE), hexane soluble fraction (HSF) and dichloromethane soluble fraction (DSF) and the residue on selected human cancer cell lines

Cell lines	IC_{50} (µg/mL) ± SD *						
	EE	SI	HSF	SI	DSF	SI	Residue
A549	31.2 ± 7.4	>3.8	44.1 ± 5.9	>2.7	53.7 ± 5.5	0.9	>120
HepG2	>120.0	1.0	69.8 ± 6.8	>1.7	82.7 ± 3.6	0.6	>120
HSC-4	52.3 ± 7.2	>2.3	93.6 ± 4.0	>1.3	43.6 ± 6.1	1.1	>120
MCF-7	72.3 ± 3.5	>1.7	37.6 ± 4.1	>3.2	57.6 ± 3.3	0.8	>120
PC-3	81.5 ± 6.4	>1.5	45.4 ± 7.6	>2.6	60.0 ± 6.2	0.8	>120
SiHa	>120.0	1.0	110.7 ± 5.3	>1.1	>120.0	-	>120
MRC-5	>120.0		>120.0		47.3 ± 7.3		>120

SI: Selectivity index
Data are expressed as the means ± standard deviation (n = 3)
* After 24 h of treatment



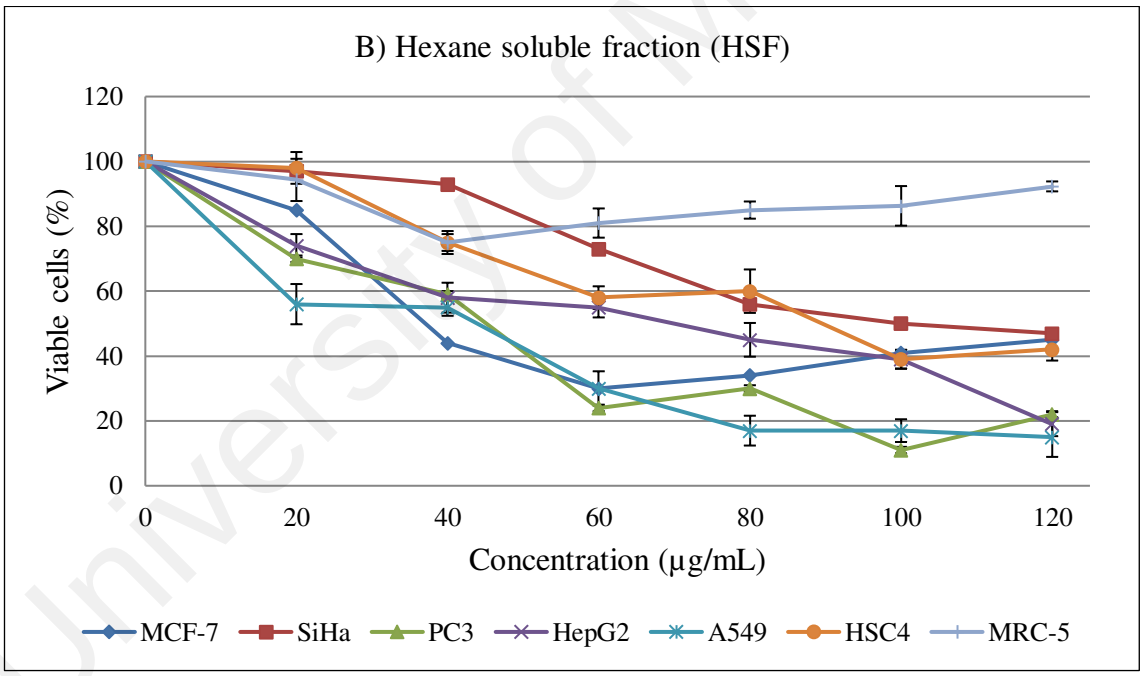
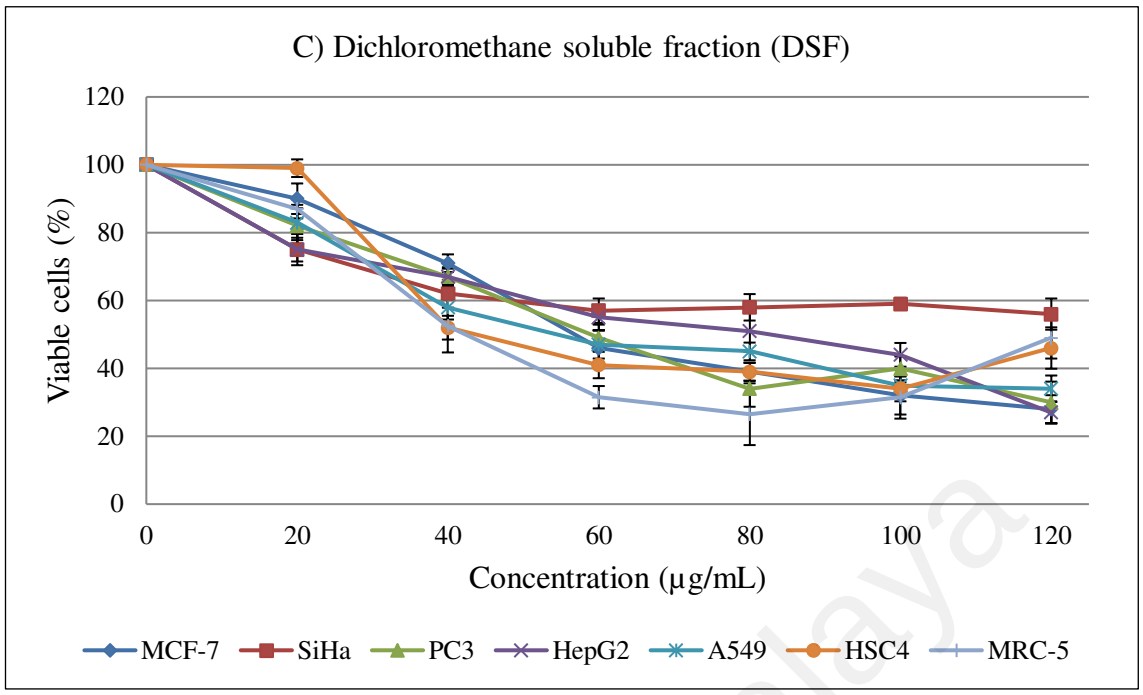
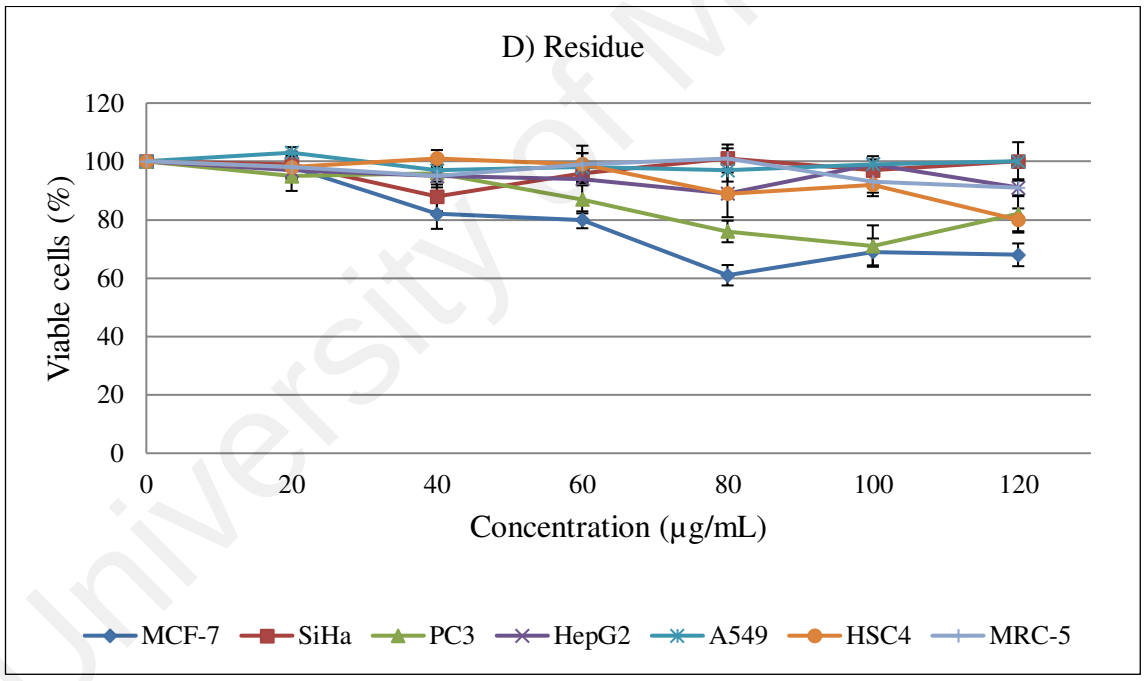


Figure 4.1: Comparison of total relative cell viability (%) between various cancer cell lines and normal cell line (MRC-5), after treatment with (A) ethanol extract (EE), (B) hexane soluble fraction (HSF), (C) dichloromethane soluble fraction (DSF) and (D) residue at different concentrations (0 to 120 µg/mL) at 24 h incubations, indicating concentration-dependent cytotoxicity. Results are expressed as total percentage of viable cells. Each value is the mean ± SD of three replicate (n =3)





'**Figure 4.1,** continued'

4.2 Chemical Profiling of Hexane Soluble Fraction (HSF) of *Curcuma zedoaria*

The results showed that among the extracts, hexane soluble fraction (HSF) was the most active fraction (Table 4.2). Thus, the chemical profiling using gas chromatography equipped with flame ionization detector (GC-FID) and gas chromatography / mass spectrometry (GC/MS) was performed to identify the chemical constituents in HSF. HP-5 fused silica capillary column of 5% phenyl 95% dimethylpolysiloxane (30 m x 0.32 mm id., 0.25 µm film thickness) was employed. The analysis of compounds was carried out by GC/MS analysis using the NIST08 mass spectral library. The Kovat retention index (KI) and the mass spectra were compared with data in the literature. For GC-FID and GC/MS analysis, the optimum parameter was set as; nitrogen as carrier gas at 1.0 mL/min constant flow, injection volume 1 µL, injector and inlet temperature were set at 250 ºC and a splitting ratio 1:20. The oven temperature was set as: initial temperature 70 ºC for 2 min, 70 ºC to 250 ºC (3 ºC/min) and subsequently isothermal at 250 ºC for 10 min. In overall, the total run time was 72.0 min.

The chemical profiling of HSF was done with the aim to identify the possible compounds that may be responsible for the cytotoxic activity. The results revealed the presence of 23 identified compounds totalling up to 93.0%. Curcumenol (20.6%), velleral (13.4%), dehydrocurdione (9.1%), germacrone (7.4%) and curcumenone (7.0%) were identified as the major compounds in this non-polar fraction. All the major compounds are sesquiterpenes. Most of the compounds in the HSF fraction were also found in many plant essential oils including other species in Zingiberaceae such as 1, 8- cineol, camphor, isoborneol, borneol, α-selinene, β-bisabolene, spathulenol, β-eudesmol and germacrone. Table 4.2 list the phytochemical constituents identified in HSF of the rhizome of *C. zedoaria*.

Table 4.2: Phytochemical constituents identified in the hexane soluble fraction (HSF) of the rhizome of *Curcuma zedoaria*

No.	Compound identified	Rt (min)	KI [a]	KI [b]	Area (%) ± SD
1	1, 8 – Cineol	6.60	1049	1031	0.3 ± 0.6
2	Camphor	10.05	1160	1146	4.9 ± 0.2
3	Isoborneol	10.48	1176	1162	2.0 ± 0.4
4	Borneol	10.82	1185	1169	1.1 ± 0.2
5	1,7- Octadien, 3-methylene	19.20	1398	-	0.9 ± 0.2
6	α-Selinene	22.74	1492	1498	1.6 ± 0.4
7	α-Bulnesene	23.10	1501	1510	1.4 ± 0.5
8	β-Bisabolene	23.68	1610	1506	1.8 ± 0.3
9	Curzerenone	27.35	1663	1606, c, e	3.0 ± 0.2
10	Spathulenol	28.10	1673	1578, d	3.2 ± 0.1
11	β-Eudesmol	28.77	1682	1651, d	1.4 ± 0.6
12	Germacrone	30.38	1702	1694, d	7.3 ± 0.4
13	Curcumenol	31.62	1742	1734, d	20.6 ± 0.4
14	Zerumbone	32.42	1766	1734	2.2 ± 0.4
15	Dehydrocurdione	33.39	1794	c, d	9.1 ± 0.3
16	Velleral	33.73	1804	-	13.4 ± 0.2
17	Curcumenone	35.34	1856	d	7.0 ± 0.4
18	ar-Turmerone	35.89	1873	-	1.5 ± 1.2
19	Unidentified	39.21	1979	-	1.8 ± 1.6
20	Unidentified	39.58	1991	-	2.7 ± 0.8
21	Isocurcumenol	39.99	2004	-	1.8 ± 0.6
22	3-ethyl-2-cyclopenten-1-one	45.41	2193	-	1.8 ± 0.5
23	3-bromo, cyclodecene	50.02	2366	-	2.0 ± 0.1
				Total	92.8 %

[a] Kovat indices (KI) relative to *n*-alkanes $C_7 – C_{40}$ on HP-5 capillary column ($n = 3$)
Identification by GC/MS NIST08 library, KI and comparing mass spectral data with literature:
[b]: (Adams, 2001), c: (Rahman et al., 2013), d: (Hamdi et al., 2015), e: (Sirat et al., 1998)

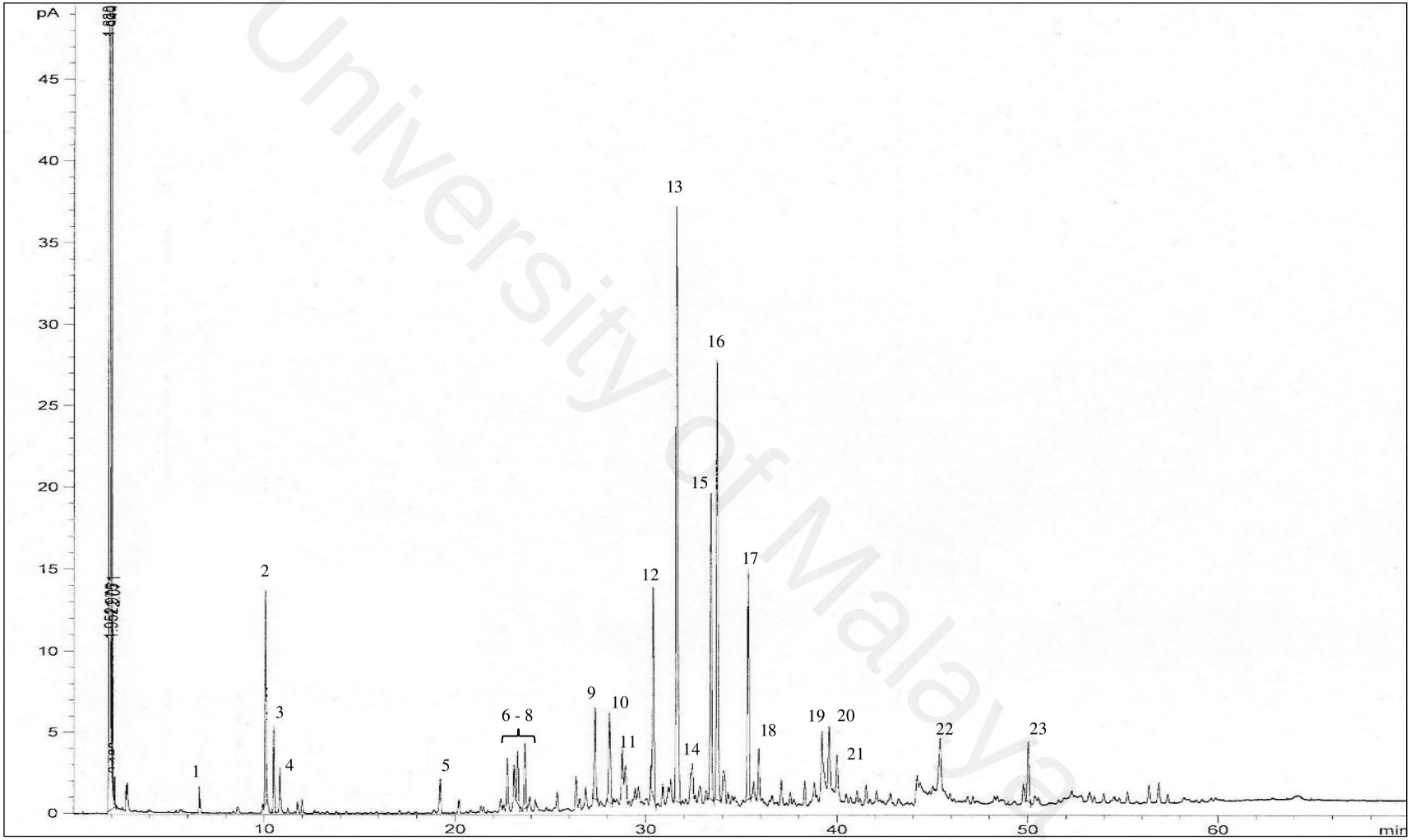


Figure 4.2: Gas chromatogram of hexane soluble fraction of *Curcuma zedoaria* by using GC-FID

4.3 Fractionation of Hexane Soluble Fraction (HSF) and Their Cytotoxic Activity

The hexane soluble fraction (HSF) then was subjected for further fractionation using bioassay guided isolation focusing on lung cancer cell line, A549. The column chromatography was performed by using silica gel column with increasing solvent polarity. The fractionation from 3.0 g of HSF produced six fractions and labelled as fraction H 1 to H 6. The fractions obtained are from different polarity of hexane and ethyl acetate (v/v): H 1 (100 hexane: 0 ethyl acetate), H 2 (60 hexane: 40 ethyl acetate), H 3 (40 hexane: 60 ethyl acetate), H 4 (20 hexane: 80 ethyl acetate), H 5 (0 hexane: 100 ethyl acetate), H 6 (90 ethyl acetate: 10 methanol). All fractions obtained were concentrated using rotary evaporator and tested for their cytotoxic effect on A549 cell using MTT assay technique for 24 h.

Table 4.3 list the information of the yield of HSF and the results on MTT assay against A549. Fraction H 1 displayed the most abundant yield with 53.9% while fraction H 4 showed the lowest percentage of the total yield (5.1%). However, both fractions H 1 and H 4 exhibited cytotoxic activity with IC_{50} values of 56.0 µg/mL ± 5.6 and 79.2 µg/mL ± 4.7, respectively. Meanwhile, fractions H 2, H 3, H 5 and H 6 exhibited weak activity with the IC_{50} values recorded as more than 120 µg/mL which was the maximum concentration used in this assay.

From Figure 4.3, fraction H1 showed the best activity and the highest yield, hence this fraction was selected for further fractionation. GC/MS profile of fraction H1 is shown in Figure 4.3. However, GC-FID and GC/MS analysis was not analysed because this was done previously.

Table 4.3: Yield and IC_{50} of hexane soluble fraction (HSF) against A549 cell line based on MTT assay

Fraction	Yield (mg)	Percentage (%)	A549 (IC_{50} - µg/mL)
H 1	**1,620.0**	**53.9**	**56.0 ± 5.6**
H 2	470.0	15.7	> 120.0
H 3	160.0	5.2	> 120.0
H 4	153.0	5.1	79.2 ± 4.7
H 5	220.0	7.4	> 120.0
H 6	210.0	6.9	> 120.0

* Results are expressed as mean ± SD (n = 3)

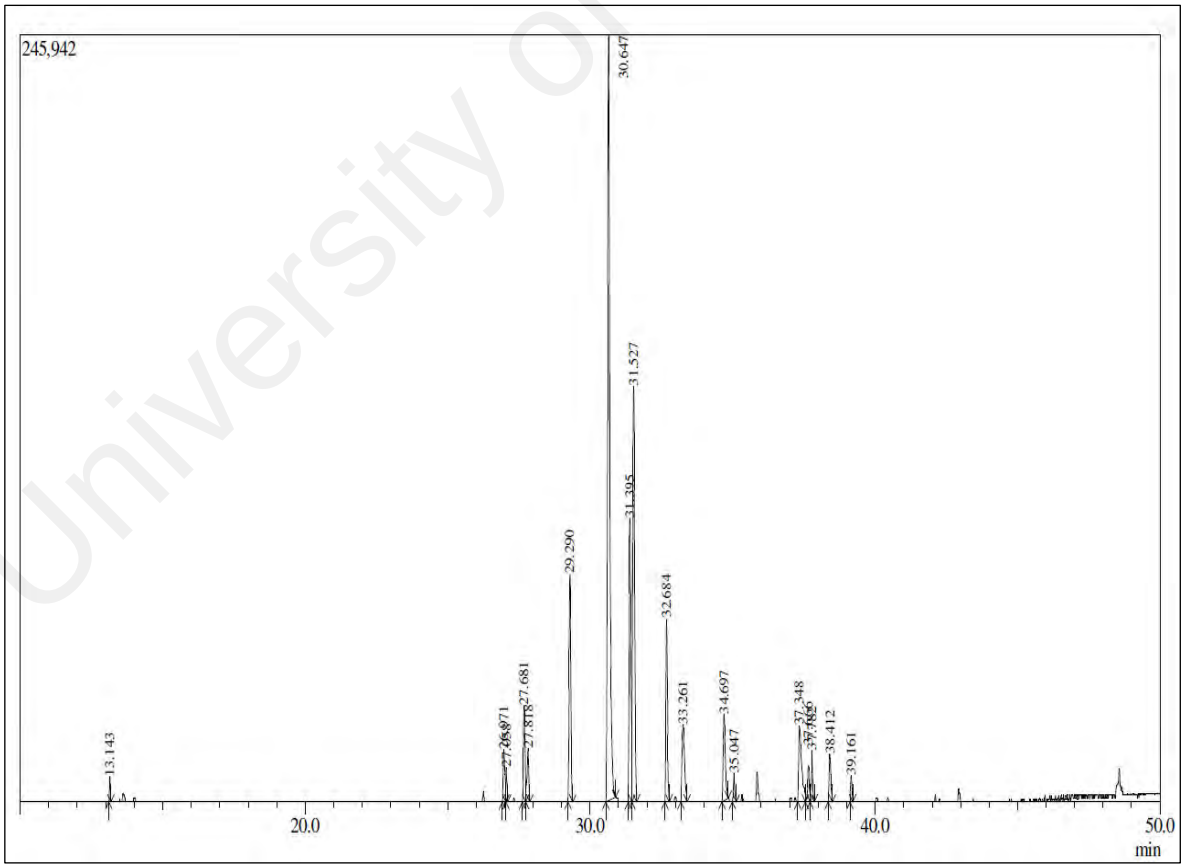


Figure 4.3: Gas chromatogram of fraction H 1

Due to the promising cytotoxic effects on A549 cells, fraction H 1 (150.0 mg) was further subjected to fractionation by using column chromatography. As a result, seven fractions using hexane and ethyl acetate with solvent system 9:1 ratio (v/v) were obtained from the thin layer chromatography (TLC). The fractions were labelled as H 1.1 to H 1.7. Subsequently, these seven fractions were evaluated for cytotoxic activity against A549 cells and the results are summarized in Table 4.4. Among the fractions tested, fraction H 1.2 (38 mg: 25.3%) exhibited the highest cytotoxic effect against A549 cell with IC_{50} value of 19.5 µg/mL ± 2.6. On the other hand, the other fractions only exhibited mild cytotoxic effect with IC_{50} values ranging from 29.4 µg/mL to 59.4 µg/mL.

GC-FID and GC/MS analysis of fraction H 1.2 was performed to identify the chemical components and the chromatogram is shown in Figure 4.4. Four major peaks have been detected in the chromatogram. The compounds were analysed by their mass spectral in GC/MS NIST08 library and Kovat retention indices. Next, the data was compared with those published data.

Hereafter, isolation of the components in fraction H 1.2 was performed by using TLC while hexane and ethyl acetate (9:1 ratio) are used as the solvent system. About 28.0 mg of fraction H 1.2 has been used for TLC development. Scheme 4.1 provided the full scheme of fractionation and isolation of cytotoxic compounds from the rhizome extracts of *C. zedoaria* using bioassay-guided fractionation.

Table 4.4: Yield and IC_{50} of fraction H1 against A549 cell line based on MTT assay

Fraction	Yield (mg)	Percentage (%)	A549 (IC_{50} - µg/mL)
H 1.1	44.8	29.9	46.5 ± 4.0
H 1.2	**38.0**	**25.3**	**19.5 ± 2.6**
H 1.3	6.9	4.6	48.1 ± 5.1
H 1.4	18.0	12.0	30.8 ± 6.6
H 1.5	3.3	2.2	33.5 ± 3.8
H 1.6	7.9	5.3	29.4 ± 4.1
H 1.7	5.9	3.9	59.4 ± 5.8

Results are expressed as mean ± SD (n = 3)
Loading amount = 150 mg

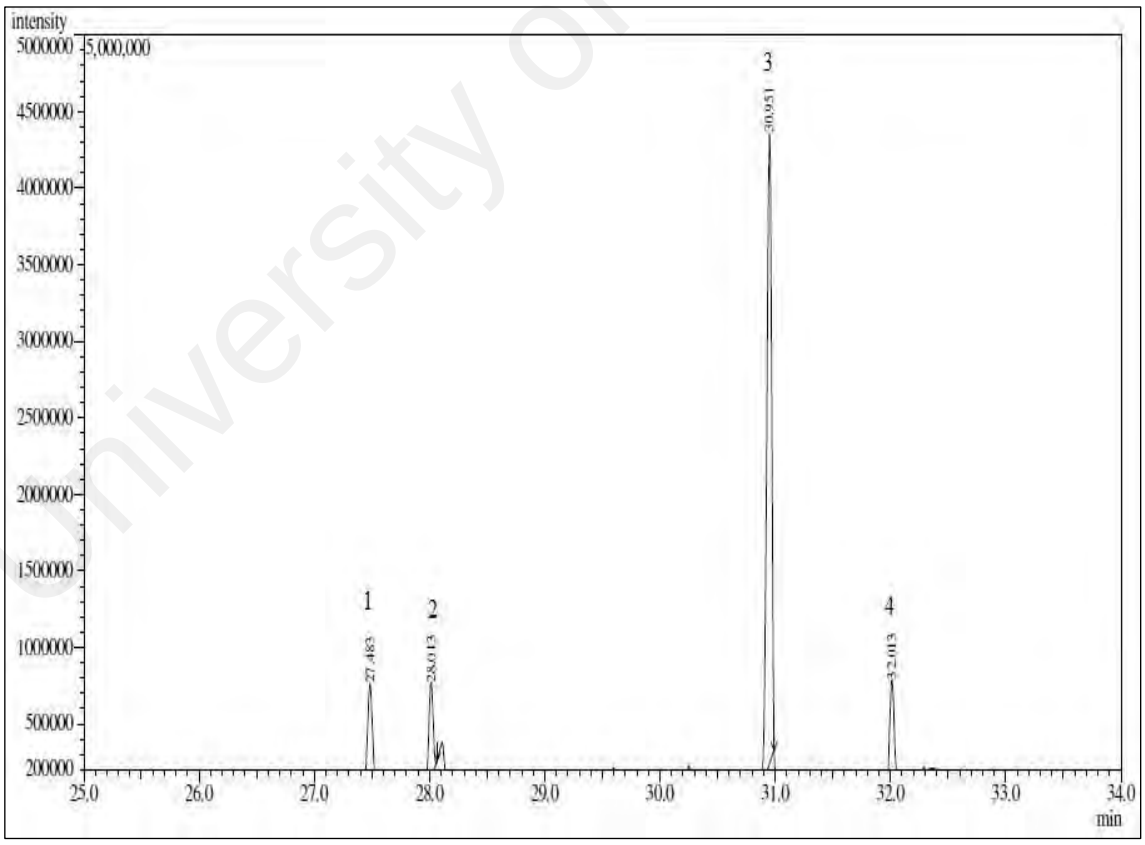
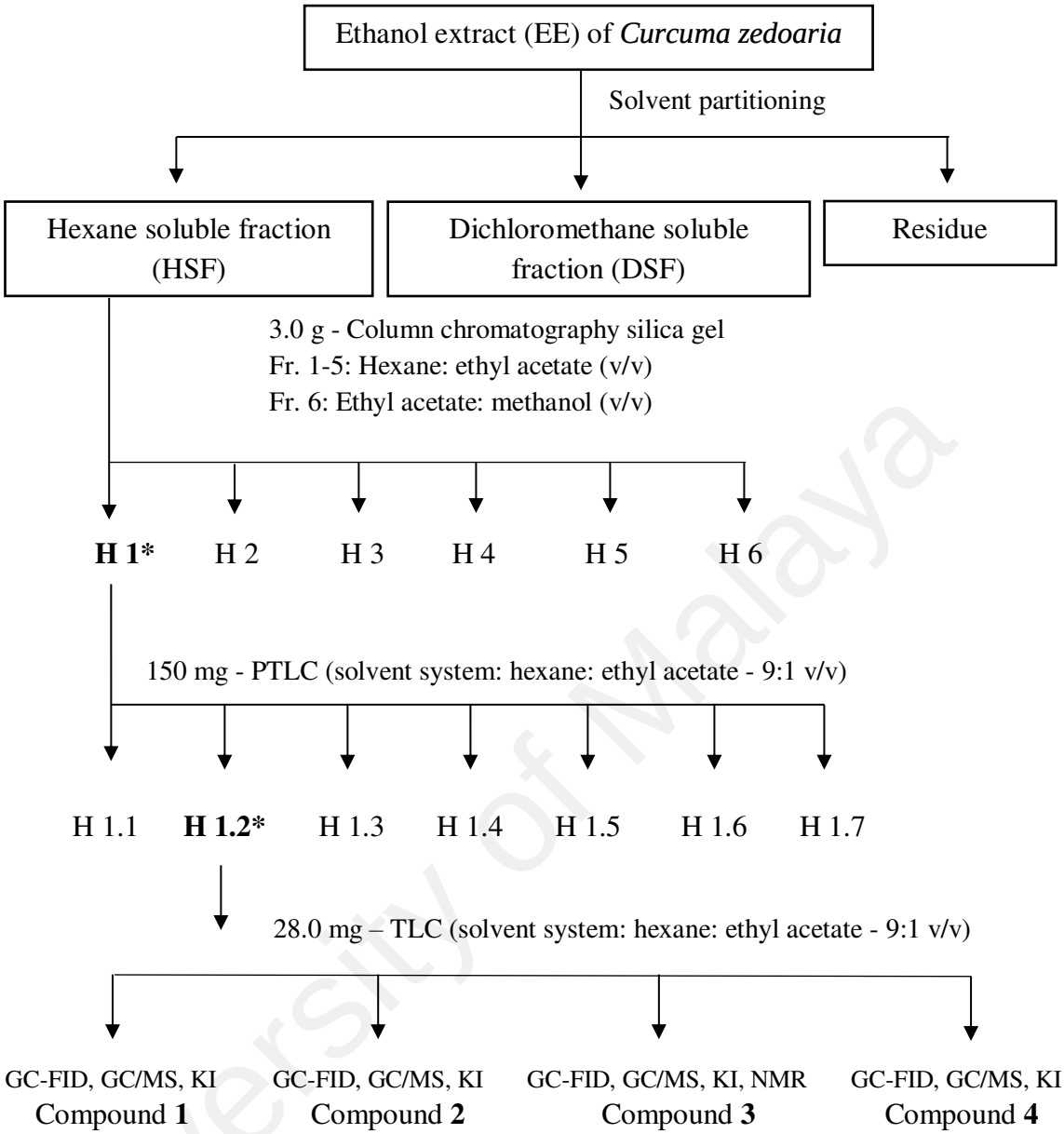


Figure 4.4: Gas chromatogram of fraction H 1.2



Scheme 4.1: Fractionation and isolation scheme of the bioactive compounds from ethanol extract of *Curcuma zedoaria*

4.4 Identification of Compounds 1-4 (Fraction H1.2)

The bioassay-guided isolation of the hexane soluble fraction (HSF) afforded four compounds labelled as compounds 1, 2, 3 and 4. Gas chromatography / mass spectrometry (GC/MS) chromatogram of fraction H1.2 (Figure 4.4) displayed four peaks of the main components in the fraction. From here, compounds 1, 2, 3 and 4 were identified by GC-FID, GC/MS, Kovat retention indices (KI) analysis and by comparing their mass spectral with those reported in the literature (Adams, 2001; Jirovetz et al., 2002; Rahman et al., 2013). These analyses were selected based on the fraction H1.2 which originated from non-polar fractions and the analysis is like essential oil's analysis. From the analysis and the data obtained, compounds 1, 2 and 4 were identified as spathulenol, *β*-eudesmol and curcumenone, respectively. Compound 3 was undetectable from the GC/MS NIST 08 library; however, the molecular weight was recorded at m/z 234.

Subsequently, the purification of compounds from the active fraction, H1.2 was performed by TLC chromatography using hexane and ethyl acetate (9:1, v/v) as the solvent system resulting in successful isolation of compound 3. Sufficient amount of compound 3 was isolated hence can be confirmed by ^{1}H-NMR and ^{13}C-NMR. Whereas the yield of compound 1 and 2 were too low and insufficient for NMR analysis. Due to the low yield of compound 1 and 2, both compounds were purchased from Chengdu Biopurify Phytochemicals, China for further cytotoxic activity test. Isolation of compound 4 was unsuccessful hence unavailable for cytotoxic activity. The mass spectral characterisation of compound 1 to compound 4, is as follows:

4.4.1 Compound 1: Spathulenol

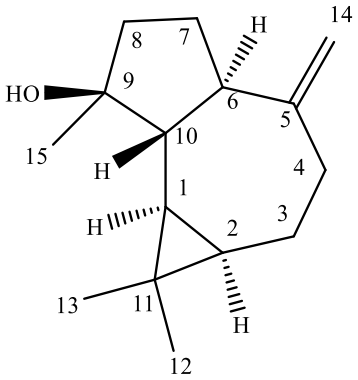
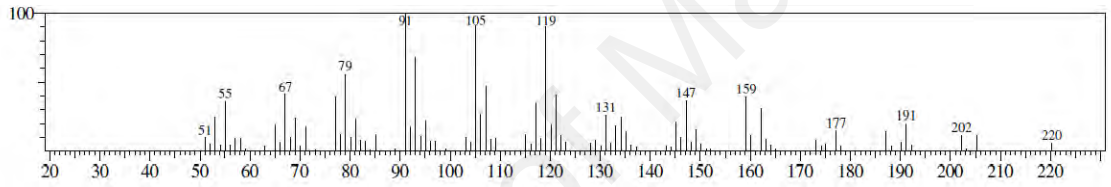


Figure 4.5: Compound 1: Spathulenol

Spathulenol (Figure 4.5), formed as a colourless oil was assigned the molecular formula of C_{15} H_{24} O. From GC-FID, retention time was recorded at 27.5 min, significant fragmentations revealed by the mass spectrum were *m/z* 220, 202, 191, 177, 159, 147, 131, 119, 105, 91, 79, 67, 55 and 51. The mass spectral data was consistent with previous published data (Adams, 2001; Wang et al., 2003). From the gas chromatography/ mass spectrometry (GC/MS) analysis and Kovat indices (KI) was calculated relatively to *n*-alkanes series C_7 – C_{40} using HP-5 capillary column. KI calculation revealed a value of 1632 and the data is acceptable as compared with the value of 1619 reported by Jirovetz et al., 2002. Table 4.5 describe the characteristics of spathulenol.

Table 4.5: Description of Compound 1: Spathulenol

Physical appearance	: Colourless oil
Molecular formula	: C_{15} H_{24} O
Molecular weight	: 220
Retention time (min)	: 27.5 min.
Kovat Indices [1]	: 1632
Kovat Indices (ref.)	: 1619 [a]
References	: Mass spectral [b, c]
EI/MS	: 220, 202, 191, 177, 159, 147, 131, 119, 105, 91, 79, 67, 55 and 51 *m/z*.



[1] Kovat indices (KI) relative to *n*-alkanes C_7 – C_{30} on HP-5 capillary column
[2] Identification by GC/MS NIST08 library, KI and comparing mass spectral data with literature: [a] (Jirovetz et al., 2002), [b] (Adams, 2001) and [c] (Wang et al., 2003)

4.4.2 Compound 2: *β*-Eudesmol

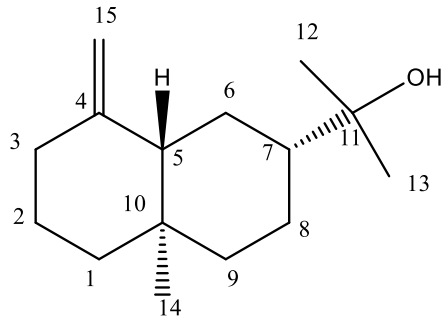
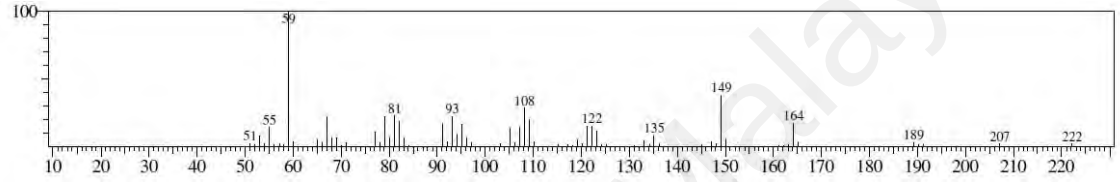


Figure 4.6: Compound 2: *β*-Eudesmol

β-Eudesmol is an oxygenated sesquiterpene. The mass spectrum obtained by GC/MS presented a molecular ion peak at *m/z* 222 which corresponded to the molecular formula of $C_{15}H_{26}O$. At retention time 28.0 min, significant fragmentations revealed by the mass spectrum were *m/z* 222, 207, 189, 164, 135, 122, 108, 93, 81, 59, 55 and 51. The mass spectral data was consistent with previous published data (Adams, 2001; Hamdi et al., 2015). Table 4.6 described the information of compound 2, *β*-eudesmol.

Table 4.6: Description of Compound 2: β-Eudesmol

Physical appearance	: White powder
Molecular formula	: C_{15} H_{26} O
Molecular weight	: 222
Retention time (min)	: 28.0 min.
Kovat Indices [1]	: 1651
Kovat Indices (ref)	: 1651 [a], mass spectral [b]
EI/MS	: 222, 207, 189, 164, 135, 122, 108, 93, 81, 59, 55 and 51 *m/z*.



[1] Kovat indices (KI) relative to *n*-alkanes C_7 – C_{30} on HP-5 capillary column
[2] Identification by GC/MS NIST08 library, KI and comparing mass spectral data with literature: [a] (Adams, 2001) [b] (Hamdi et al., 2015)

4.4.3 Compound 3: Dehydrocurdione

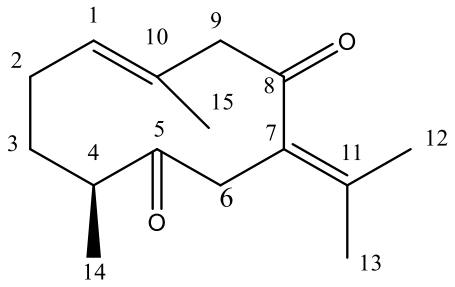


Figure 4.7: Compound 3: Dehydrocurdione

Dehydrocurdione was isolated as a yellow pale coloured oil. Dehydrocurdione is a sesquiterpene with germacrene skeleton. The mass spectrum of dehydrocurdione obtained by GC/MS presented a molecular ion peak at *m/z* 234 which correspond to the molecular formula of $C_{15}H_{22}O_2$. At retention time 30.9 min, significant fragmentations revealed by the mass spectrum were *m/z* 234, 198, 178, 164, 152, 121, 96, 79, 67, 53 and 50. The mass spectral data was consistent with previous published data (Rahman et al., 2013). Table 4.7 described the information of dehydrocurdione.

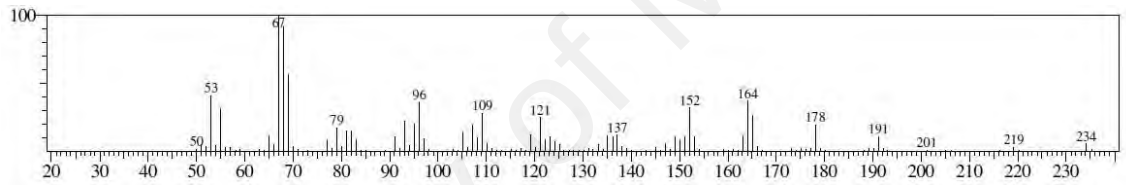
Table 4.8 showed the spectral data of ^{1}H and ^{13}C NMR of dehydrocurdione on 400 MHz in $CDCl_3$. The ^{1}H NMR spectrum (Figure 4.8) indicated the presence of four methyl protons (δ: 0.9 *d*, 1.6 *s*, 1.7 *s* and 1.7 *s*), four methylene protons (δ: 1.6-2.1 *m*, 2.1 *m*, 3.0-3.2 *dd* and 3.1-3.2 *dd,* a methine (2.3 *m*) proton and a vinylic proton at 5.0- 5.1 (*t*).

The ^{13}C NMR spectra (Figure 4.9) showed the presence of 15 carbons (ppm) compatible with proposed structure. There are four methyls, C-15, C-14, C-12 and C-13 at position (δ_c: 16.4, 18.4, 21.1 and 22.2) respectively, four methylene, at δ_c 29.7 (C-2), 34.2 (C-3), 43.5 (C-6) and 57.1 (C-9), a methine C-4 (δ_c: 46.5), a vinylic carbon C-1 (δ_c: 133.0), three quaternary carbons, C-7, C-10 and C-11 (δ_c: 129.2, 130.0 and 137.0) and the two signals are for the carbonyl carbons C-8 (δ_c: 207.2) and C-5 at position δ_c: 211.1).

Identity of compound **3** has been confirmed as dehydrocurdione with the NMR data as described in the literature (Diastuti et al., 2014; Hamdi et al., 2015).

Table 4.7: Description of Compound 3: Dehydrocurdione

Physical appearance	: Yellow pale oil
Molecular formula	: C_{15} H_{22} O_2
Molecular weight	: 234
Retention time (min)	: 30.9 min.
Kovat Indices [1]	: 1787
References	: Mass spectral [a, b]
EI/MS	: 234, 198, 178, 164, 152, 121, 96, 79, 67, 53 and 50 *m/z*.



[1] Kovat indices (KI) relative to *n*-alkanes C_7 – C_{30} on HP-5 capillary column
[2] Identification by GC/MS NIST08 library, KI and comparing mass spectral data with literature: [a] (Rahman et al., 2013), [b] (Hamdi et al., 2015)

Table 4.8: 1D-NMR (^{1}H and ^{13}C) [400 MHz, δ_H] spectral data of dehydrocurdione in $CDCl_3$

Position	δ_H (*J* in Hz)		δ_C (ppm)	
	^{1}H (Exp.)	^{1}H (500 MHz) (Diastuti et al., 2014)	^{13}C (Exp.)	^{13}C (125 MHz) (Diastuti et al., 2014)
1	5.0- 5.1 (*t*)	5.1 (*t*)	133.0	128.9
2	2.1 (*m*)	2.1 (*m*)	26.7	22.4
3	1.6 – 2.1 (*m*)	1.6-2.0 (*m*)	34.2	30.2
4	2.3 (*m*)	2.4 (*m*)	46.5	42.6
5 = O	-	-	211.1	207.1
6	3.1 - 3.2 (*dd*) (16.25)	3.2 (*dd*)	43.5	39.4
7	-	-	129.2	133.0
8 = O	-	-	207.2	203.1
9	3.0- 3.2 (*dd*) (11.32)	3.0-3.2 (*dd*)	57.1	53.0
10	-	-	130.0	125.3
11	-	-	137.0	125.9
12	1.7 (*s*)	1.7 (*s*)	21.1	17.0
13	1.7 (*s*)	1.7 (*s*)	22.2	18.1
14	0.9 (*d*)	0.9 (*d*)	18.4	14.4
15	1.6 (*s*)	1.6 (*s*)	16.4	12.3

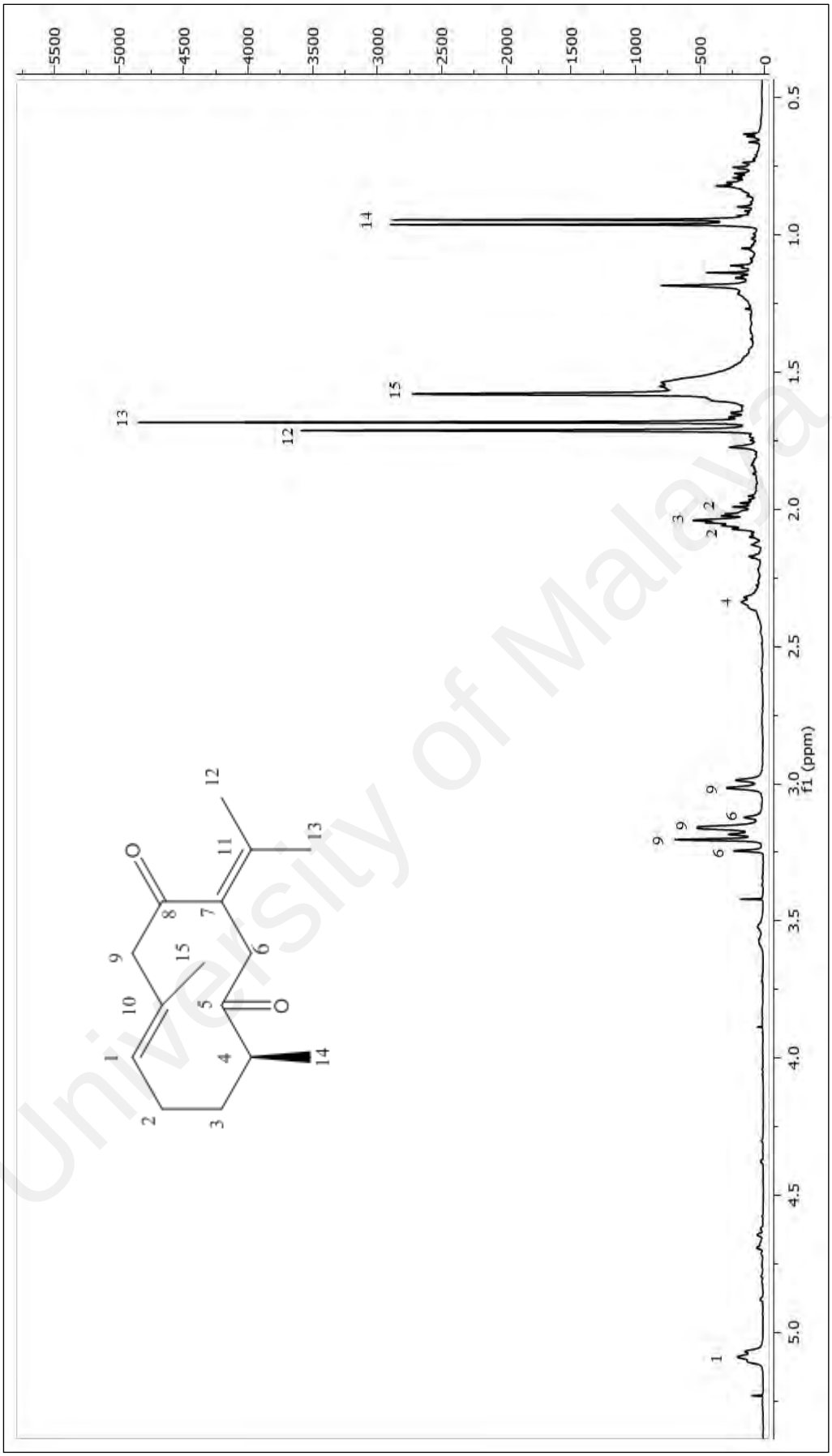


Figure 4.8: ^{1}H NMR spectrum of dehydrocurdione in $CDCl_3$

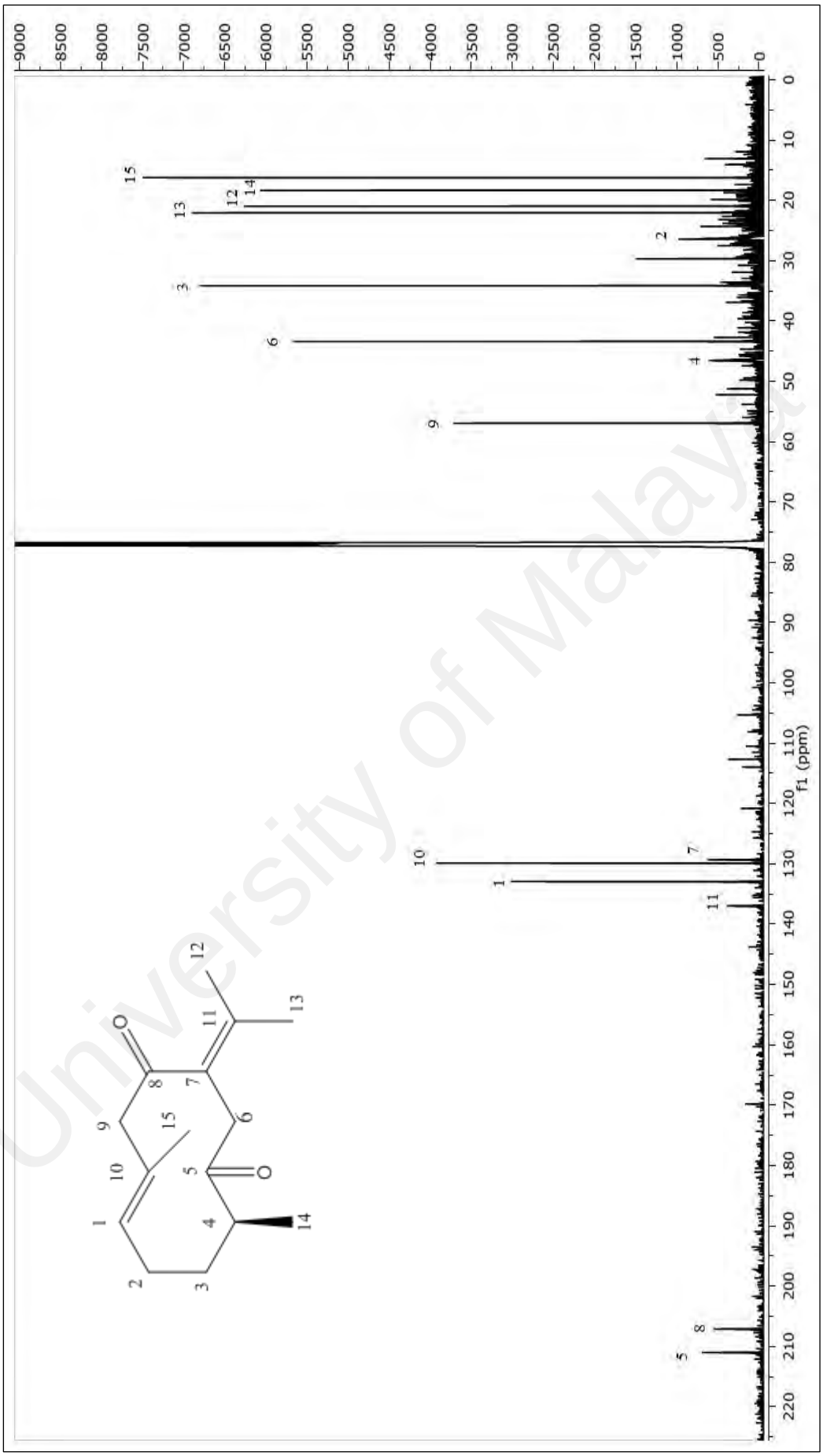


Figure 4.9: ^{13}C NMR spectrum of dehydrocurdione in $CDCl_3$

4.4.4 Compound 4: Curcumenone

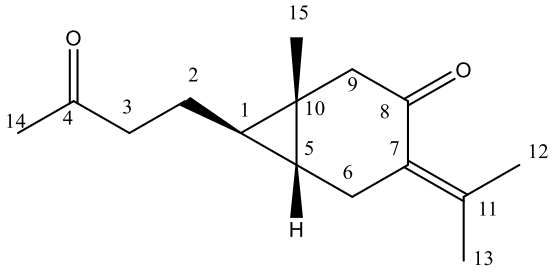
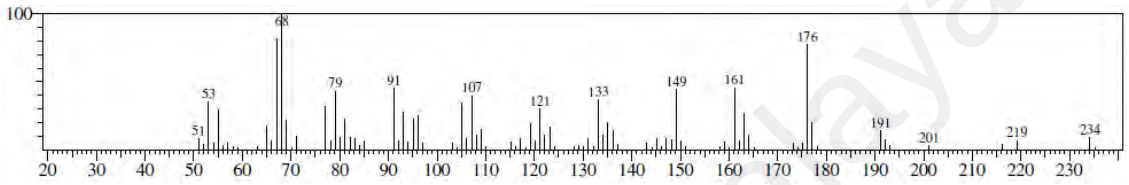


Figure 4.10: Compound 4: Curcumenone

Curcumenone is a sesquiterpene with carabrane skeleton. The mass spectrum obtained by GC/MS presented a molecular ion peak at *m/z* 234 which corresponding to the molecular formula of $C_{15}H_{22}O_2$. At retention time 34.3 min, significant fragmentations revealed by the mass spectrum were *m/z* 234, 219, 201, 191, 176, 161, 149, 133, 121, 107, 91, 79, 68, 53 and 51. The mass spectral data was consistent with previous published data (Yang et al., 2011; Rahman et al., 2013; Hamdi et al., 2015). Table 4.9 described the information of curcumenone.

Table 4.9: Description of Compound 4: Curcumenone

Molecular formula	: C_{15} H_{22} O_2
Molecular weight	: 234
Retention time (min)	: 34.3 min.
Kovat Indices [1]	: 1823
References	: Mass spectral [a, b, c]
EI/MS [2]	: 234, 219, 201, 191, 176, 161, 149, 133, 121, 107, 91, 79, 68, 53, 51.



[1] Kovat indices (KI) relative to *n*-alkanes C_7 – C_{30} on HP-5 capillary column.
[2] Identification by GC/MS NIST08 library, KI and comparing mass spectral data with literature: [a] (Rahman et al., 2013), [b] (Hamdi et al., 2015), [c] (Yang et al., 2011)

4.5 Cytotoxic Effect of Compounds

Further investigation was carried out to assess the cytotoxic activity of compounds 1, 2 and 3 from the rhizome of *Curcuma zedoaria*. The three compounds yielded from the cytotoxic active fraction H 1.2 which originated from the hexane soluble fraction namely spathulenol, *β*-eudesmol and dehydrocurdione were subjected to the cytotoxic activity using the MTT assay. The assay was conducted on two different lung cancer cell lines, A549 and SK-LU-1. The normal cell line, MRC-5 was used as control and doxorubicin was used as the drug control. The IC_{50} values were calculated based on MTT assay data and represented the concentration of compounds required to kill 50% of cancer cell population. Previously, the effect of DMSO as solvent control were deducted from the calculation to get IC_{50} values. The aim is to get the net cytotoxic effect of each compound tested. Selectivity index (SI) was calculated by comparing the effect of compounds on the cancer and normal cell lines.

Table 4.10 exhibited the cytotoxic effect of all compounds towards lung cancer cell lines (A549 and SK-LU-1) and normal cell line (MRC-5). In addition, the graph of MTT cell viability assay on spathulenol, *β*-eudesmol and dehydrocurdione against A549, SK-LU-1 and MRC-5 are shown in Figure 4.11.

Among these compounds, the IC_{50} values of spathulenol and *β*- eudesmol clearly indicated the highest cytotoxic effect against A549 cell at 11.0 µg/mL and 12.2 µg/mL, respectively. On the other hand, the cytotoxic effects of both compounds against SK-LU-1 was slightly lower as compared to A549 cell (22.3 µg/mL and 22.9 µg/mL). Both compounds inhibited MRC-5 at IC_{50} value of 32.4 µg/mL and 25.3 µg/mL, respectively, which showed less cytotoxic effect as compared to the lung cancer cell lines. The SI value of spathulenol and *β*-eudesmol on A549 towards MRC-5, were calculated as 2.9 and 2.1, meanwhile SI values of 1.5 and 1.1 on SK-LU-1, respectively. Dehydrocurdione however showed IC_{50} values of 54.6 µg/mL and 44.3 µg/mL on A549 and SK-LU-1 cell,

respectively. Interestingly, this compound inhibited MRC-5 at a concentration more than the maximum concentration used in this study that is 60.0 µg/mL (SI values of both compounds are calculated as >1.1 and >1.4, respectively).

Table 4.10: Cytotoxic effect of HSF and compounds against lung cancer cell lines

Fraction / Compounds	IC_{50} (µg/mL) ± SD				
	A549	SI	SK-LU-1	SI	MRC-5
Hexane soluble fraction (HSF)	44.1 ± 5.9	>2.7	UT	-	>120.0
Spathulenol	11.0 ± 3.3	2.9	22.3 ± 1.3	1.5	32.4 ± 3.4
β-Eudesmol	12.2 ± 4.5	2.1	22.9 ± 2.8	1.1	25.3 ± 7.5
Dehydrocurdione	54.6 ± 3.9	>1.1	44.3 ± 1.6	>1.4	> 60.0
Doxorubicin	9.3 ± 4.6	>6.5	51.5 ± 2.1	>1.2	> 60.0

Results are expressed as mean ± SD (n = 3)
SI: Selectivity index (IC_{50} of normal cells / IC_{50} of cancer cells)
Doxorubicin as a positive control
UT: Untreated

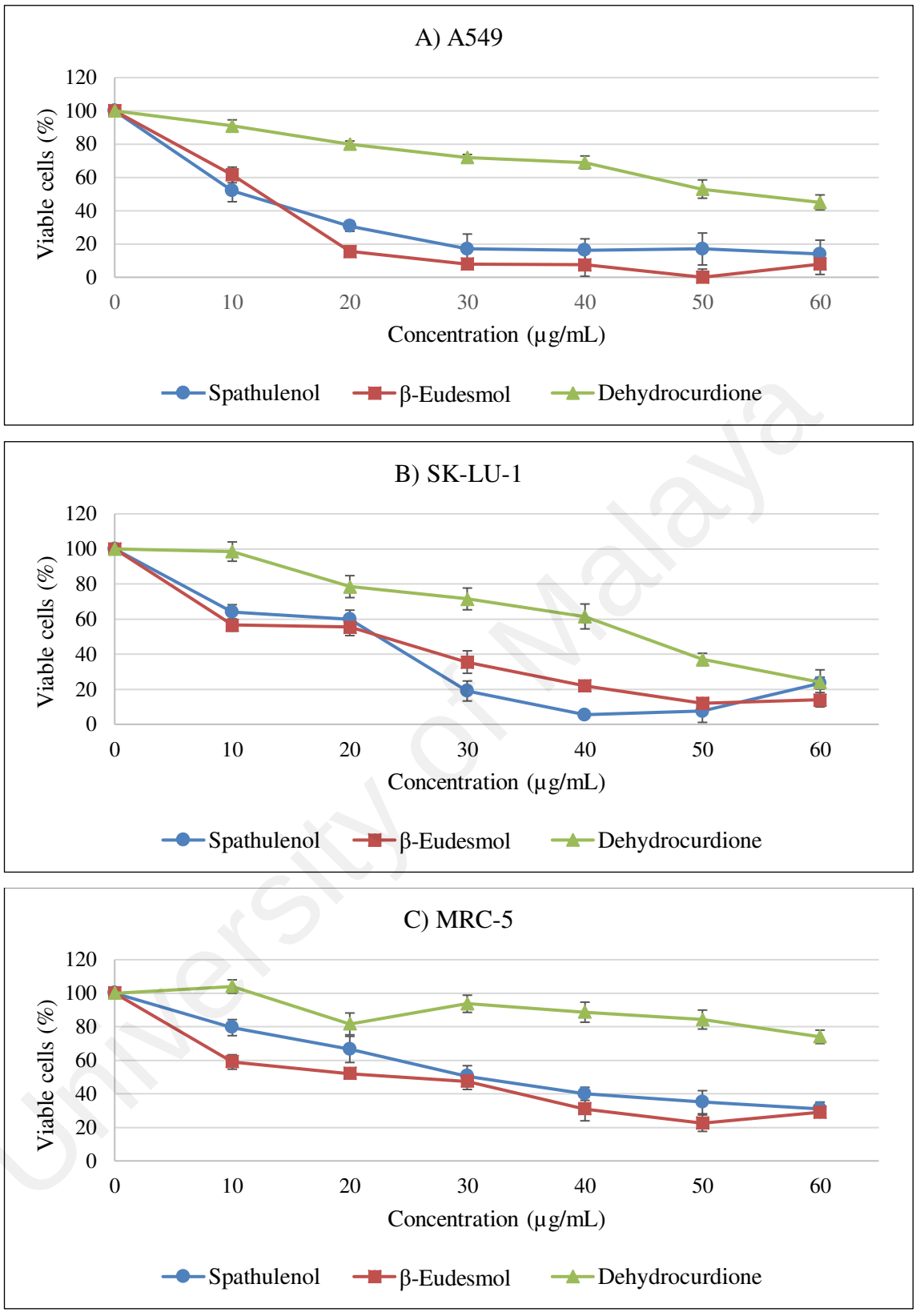


Figure 4.11: MTT cell viability (%) of spathulenol, *β*-eudesmol and dehydrocurdione at different concentrations (0 – 60.0 µg/mL) for 24 h against (A) A549, (B) SK-LU-1 and (C) MRC-5 ($n = 3$)

Part B – Essential oils analysis of *Curcuma zedoaria* from Malaysia and Indonesia and characterize their cytotoxic and apoptosis abilities

4.6 Chemical Composition of Essential Oil of *Curcuma zedoaria*

The essential oils from the dried and ground rhizomes of *Curcuma zedoaria* which were collected from Temerloh, Pahang, Malaysia and Tawangmangu, Solo, Indonesia were investigated for their chemical constituents. The rhizomes were extracted by hydro distillation using Clevenger apparatus in three replications (n = 3). The yields of the essential oils were calculated based on the dry weight of each sample. The percentages of the yield and description of the oils obtained are shown in Table 4.11 and Figure 4.12.

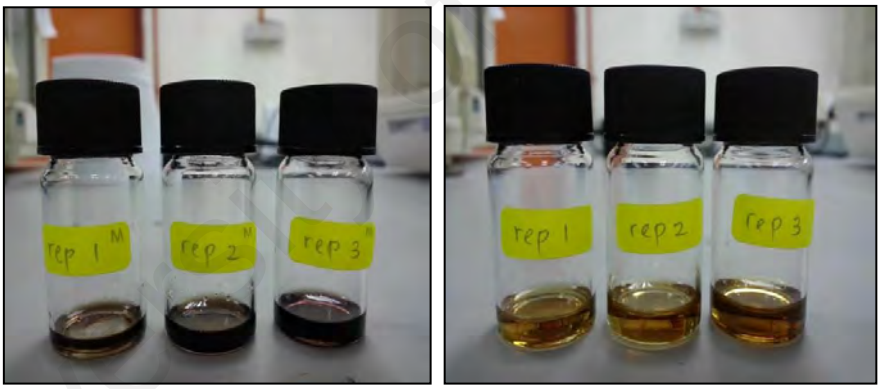
The chemical constituents of essential oils of *C. zedoaria* were subjected to gas chromatography - Flame Ionization Detector (GC-FID) and gas chromatography / mass spectroscopy (GC/MS) analysis for their detail identification of each components in the complex mixture. In this study, the HP-5 capillary column was selected to be used in GC-FID and CBP-5 capillary column for GC/MS. Besides HP-5 column, other equivalent non-polar column such as DB-5, BP-5 and OV-5 are commonly utilised in the analysis of essential oils.

The composition of the essential oils was determined using GC/MS and individual components were identified by comparison with those data in NIST08 library. The Kovat retention indices (KI) of each identified component was calculated based on alkane series C_7 to C_{30} and confirmed by comparison of the retention indices with data in literature.

Table 4.11: The yield of essential oils of *Curcuma zedoaria*

Samples	Yield (mg and %): (n = 3) *			Total of yield mg / %	Average	± SD
	Rep 1	Rep 2	Rep 3			
	Yield	Yield	Yield			
Malaysian *C. zedoaria* oil	142.9 mg, 0.071 %	322.6 mg, 0.16 %	389.9 mg, 0.19 %	855.4 mg, 0.14 %	285.1 mg @ 0.29 g	± 0.13
Indonesian *C. zedoaria* oil	587.1 mg, 0.29 %	845.2 mg, 0.42 %	890.8 mg, 0.45 %	2323.1 mg. 0.39 %	774.4 mg @ 0.77 g	± 0.16

* 200g of dried rhizome / replicate



(A) **(B)**

Figure 4.12: Rhizome essential oils of *Curcuma zedoaria* collected from (A) Malaysia and (B) Indonesia

4.6.1 Malaysian *Curcuma zedoaria* Essential Oil

In this present study, the rhizome oil of Malaysian *Curcuma zedoaria* was collected from Temerloh, Pahang, Malaysia. The yield was about 0.29 gram (0.14%) from 200g of dry plant materials (Table 4.11). The oil imparts pungent and turmeric herbs like odour and the colour was dark yellow (Figure 4.12). Table 4.12 list the chemical constituents identified in the Malaysian *C. zedoaria* essential oils.

A total of twenty-one (21) compounds were identified constituting 95.5% of the *C. zedoaria* rhizome oil from Malaysia (Table 4.12). Essential oil is a complex mixture of compounds commonly consist of monoterpenes and sesquiterpenes. Malaysian *C. zedoaria* oil showed a high content of oxygenated sesquiterpenes (56.1% - consisting of ten compounds), followed by oxygenated monoterpenes (26.5% - 5 compounds), sesquiterpene hydrocarbons (7.6% - 3 compounds), a non-terpene compound (benzyl benzoate, 4.3%) and monoterpene (1.0% - 2 compounds), made up 94.8% of the total components. The main compound was camphor (17.6%) followed by zerumbone (17.2%) and curzerenone (10.2%). Other constituents present in the essential oil with significant amounts were *ar*-turmerone (6.1%), isovelleral (6.6%), β-eudesmol (5.4%) and isoborneol (5.1%). The presence of other compounds with their percentages less than 5.0% were identified as β-pinene, α-selinene and β-caryophyllene oxide. Figure 4.13 displayed the GC-FID chromatogram of Malaysian *C. zedoaria* essential oil.

Table 4.12: Chemical composition of Malaysian *Curcuma zedoaria* essential oil

Compounds (Rt., min.)	KI [a]	KI [b]	Average (%) ± SD	Method of Identification
Monoterpene				
Camphene (8.74)	960	954	1.0 ± 0.07	MS, KI
β-Pinene (10.12)	989	980	t	MS, KI
Oxygenated monoterpene				
Alcohol				
1, 8 – Cineole (13.08)	1044	1031	1.1 ± 0.08	MS, KI
Camphor (18.61)	1155	1146	**17.6 ± 0.19**	MS, KI
Isoborneol (19.17)	1167	1162	5.1 ± 0.03	MS, KI
Borneol (19.57)	1176	1169	2.0 ± 0.01	MS, KI
α -Terpineol (26.90)	1204	1189	t	MS, KI
Sesquiterpene hydrocarbon				
β-Elemene (28.13)	1402	1391	3.9 ± 0.01	MS, KI
α -Curcumene (30.99)	1494	1481	3.7 ± 0.55	MS, KI
α-Selinene (31.20)	1501	1498	t	MS, KI
Oxygenated sesquiterpene				
Ether				
Curzerene (31.50)	1509	1499	1.7 ± 0.17	MS, KI
β-Caryophyllene oxide (34.08)	1599	1583	t	MS, KI
Alcohol				
Spathulenol (35.32)	1645	1608	1.8 ± 0.28	MS, KI
β-Eudesmol (36.03)	1668	1578	5.4 ± 0.03	MS, KI
α-Cuparenol (38.52)	1765	-	4.1 ± 0.75	MS
Aldehyde				
Isovelleral (39.70)	1810	-	6.6 ± 0.52	MS
Ketone				
Curzerenone (34.64)	1620	1606	**10.2 ± 0.06**	MS, KI
ar-Turmerone (36.48)	1686	1651	6.1 ± 0.51	MS, KI
Germacrone (37.18.)	1713	1694	3.0 ± 0.09	MS, KI
Zerumbone (38.20)	1752	1734	**17.2 ± 0.12**	MS, KI
Non-terpene				
Ester				
Benzyl benzoate (38.91)	1780	1760	4.3 ± 0.09	MS, KI
		Total	**94.8%**	

[a] Kovat retention indices relative to *n*-alkanes on the HP-5 capillary column (*n* = 3)
[b] Kovat retention indices referred to in the literature (Adams, 2001)
t Refers to compound present at a composition less than 0.05%
Rt.: Retention time

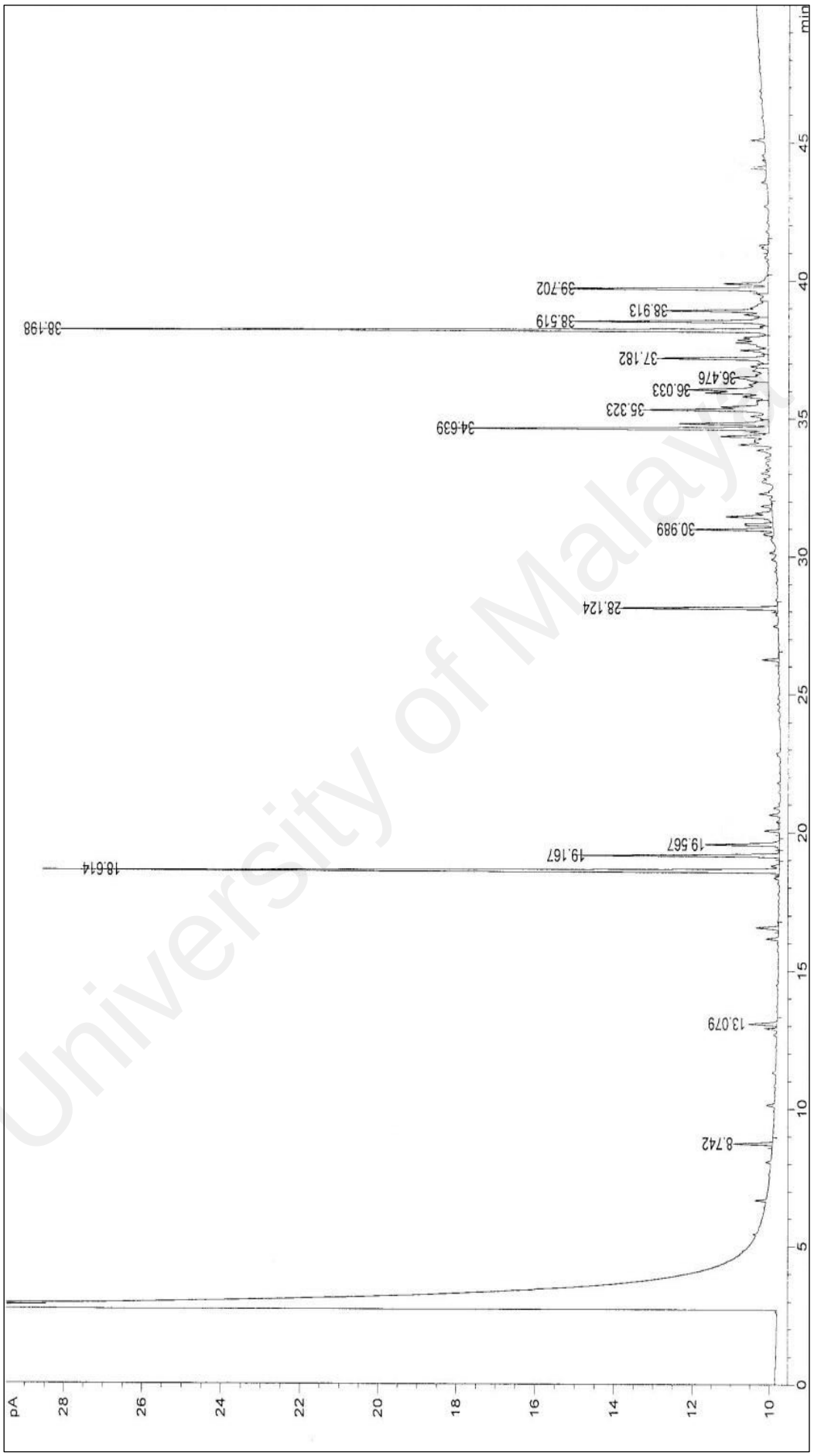


Figure 4.13: Gas chromatogram of Malaysian *Curcuma zedoaria* essential oil

4.6.2 Indonesian *Curcuma zedoaria* Essential Oil

The rhizomes of Indonesian *Curcuma zedoaria* were collected from Tawangmangu, Solo, Indonesia. The yield was recorded as 0.77 gram (0.39%). In comparison with Malaysian *C. zedoaria* oil, the percentage of yield of *C. zedoaria* oil from Indonesia was three times higher. This oil also imparted pungent and turmeric herbs like odour and the colour was golden yellow (Figure 4.12). Table 4.13 listed the chemical constituents identified in the rhizome oil of *C. zedoaria* from Indonesia.

Twenty-one (21) compounds were identified by GC/MS and each compound was confirmed by Kovat indices. This oil consists of mainly oxygenated sesquiterpenes (50.2% - consisting 10 compounds) followed by oxygenated monoterpenes (35.7% - 4 compounds), sesquiterpene hydrocarbons (5.4% - 3 compounds), monoterpenes (3.1% - 2 compounds) and a non-terpene compound. This oil was composed mainly by camphor (19.7%), zerumbone (12.1%), α-cuparenol (8.9%), 1, 8-cineole (8.4%), curzerenone (7.4%), β-eudesmol (5.6%), isoborneol (5.1%) and spathulenol (5.0%). These components made up to 95.1% of the total components detected in the GC-FID chromatogram of the oil. Three compounds present in trace amount (<0.5%) which were identified as β-elemene, curzerene and benzyl benzoate. This oil showed the presence of α-terpineol which was absent in Malaysian *C. zedoaria* oil. Figure 4.14 showed the GC-FID chromatogram of Indonesian *C. zedoaria* oil.

Table 4.13: Chemical composition of Indonesian *Curcuma zedoaria* essential oil

Compounds (Rt., min.)	KI [a]	KI [b]	Average (%) ± SD	Method of Identification
Monoterpene				
Camphene (8.75)	960	954	2.5 ± 0.33	MS, KI
β-Pinene (10.12)	989	980	0.6 ± 0.41	MS, KI
Oxygenated monoterpene				
Alcohol				
1, 8 – Cineole (13.07)	1044	1031	**8.4 ± 1.20**	MS, KI
Camphor (18.93)	1155	1146	**19.7 ± 3.05**	MS, KI
Isoborneol (19.18)	1167	1162	5.1 ± 0.69	MS, KI
Borneol (19.58)	1176	1169	2.5 ±0.49	MS, KI
α -Terpineol (26.90)	1204	1189	1.3 ±0.24	MS, KI
Sesquiterpene hydrocarbon				
β-Elemene (28.13)	1402	1391	t	MS, KI
α -Curcumene (31.00)	1494	1481	3.9 ± 1.10	MS, KI
α-Selinene (31.20)	1501	1498	1.5 ± 0.27	MS, KI
Oxygenated sesquiterpene				
Ether				
Curzerene (31.45)	1509	1499	t	MS, KI
β-Caryophyllene oxide (34.08)	1599	1583	2.3 ± 0.33	MS, KI
Alcohol				
Spathulenol (35.33)	1645	1608	5.0 ± 0.65	MS, KI
β-Eudesmol (35.97)	1668	1578	5.6 ± 1.12	MS, KI
α-Cuparenol (38.53)	1765	-	**8.9 ± 1.53**	MS
Aldehyde				
Isovelleral (39.71)	1810	-	2.5 ± 0.74	MS
Ketone				
Curzerenone (34.65)	1620	1606	7.4 ± 1.25	MS, KI
ar-Turmerone (36.48)	1686	1651	2.0 ± 0.40	MS, KI
Germacrone (37.19)	1713	1694	4.4 ± 0.08	MS, KI
Zerumbone (38.20)	1752	1734	**12.1 ± 1.28**	MS, KI
Non-terpene				
Ester				
Benzyl benzoate (38.91)	1780	1760	t	MS, KI
		Total	**95.7%**	

[a] Kovat retention indices relative to *n*-alkanes on HP-5 capillary column ($n = 3$)
[b] Kovat retention indices referred to in the literature (Adams, 2001)
t Refers to compound present at a composition less than 0.05%
Rt.: Retention time

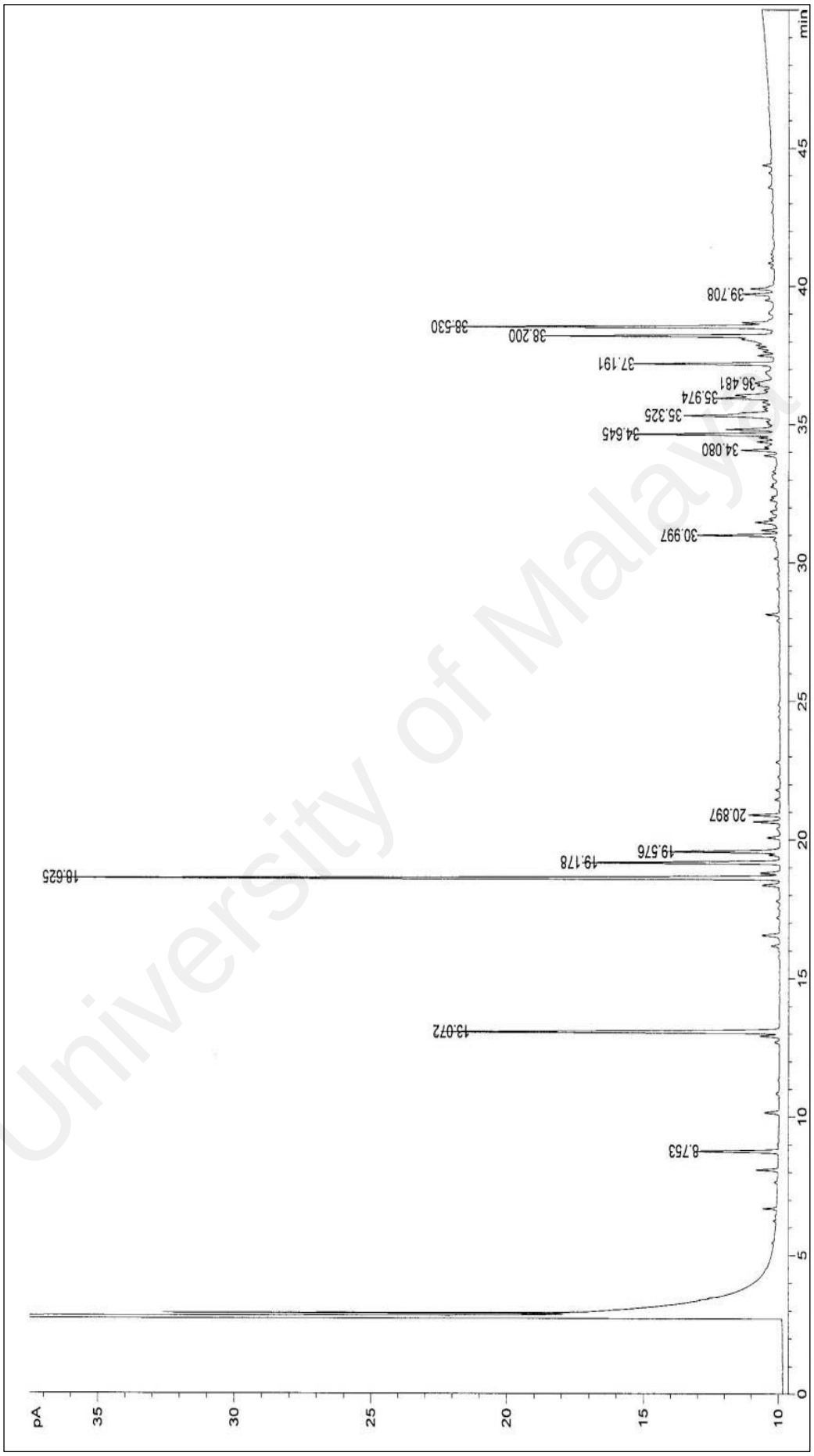


Figure 4.14: Gas chromatogram of Indonesian *Curcuma zedoaria* essential oil

4.6.3 Comparison on Chemical Constituents of Malaysian and Indonesian *Curcuma zedoaria* Essential Oils

The essential oil composition of *Curcuma zedoaria* from Malaysia and Indonesia is listed in Table 4.14, the percentages of each compounds were compared. Figure 4.15 showed the CG-FID chromatogram of both oils and in general, most of the compounds identified in Malaysian *C. zedoaria* oil were also presented in the Indonesian *C. zedoaria* oil except for some variations in their quantity.

The results showed that the major compounds identified in both oils are camphor, zerumbone and curzerenone. Some other compounds such as isoborneol (5.1%), *β*-eudesmol (5.4%), *ar*-turmerone (6.1%), isovelleral (6.6%) also appear in significant amount in Malaysian *C. zedoaria* oil. Apart from the three major components, Indonesian *C. zedoaria* oil contain spathulenol (5.0%), isoborneol (5.1%), *β*-eudesmol (5.6%), 1, 8–cineole (8.4%) and *α*-cuparenol (8.9%) as their other main compounds. Meanwhile, the other components common to both were present in less than 5%. Compounds presented at trace amount in the Malaysian *C. zedoaria* oil including *β*-pinene, *α*-selinene and *β*caryophyllene oxide. Meanwhile, *β*-elemene, curzerene and benzyl benzoate were the compounds presented at trace amount in *C. zedoaria* oil from Indonesia. All compounds identified mainly consist of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes and a non-terpene compound.

Table 4.14: Chemical composition of essential oils of *Curcuma zedoaria* from Malaysia and Indonesia

No	Compounds	GC-FID Rt (min)	KI [a]	KI [b]	Average (%) ± SD	
					Malaysian oils	Indonesian oils
1	Camphene	8.75	960	954	1.0 ± 0.07	2.5 ± 0.33
2	*β*-Pinene	10.12	989	980	t	0.6 ± 0.41
3	1, 8–Cineole	13.07	1044	1031	1.1 ± 0.08	8.4 ± 1.20
4	Camphor	18.63	1155	1146	17.6 ± 0.19	19.7 ± 3.05
5	Isoborneol	19.18	1167	1162	5.1 ± 0.03	5.1 ± 0.69
6	Borneol	19.58	1176	1169	2.0 ± 0.01	2.5 ± 0.49
7	*α*-Terpineol	26.90	1204	1189	t	1.3 ± 0.24
8	*β*-Elemene	28.13	1402	1391	3.9 ± 0.01	t
9	*α*-Curcumene	31.00	1494	1481	3.7 ± 0.55	3.9 ± 1.10
10	*α*-Selinene	31.20	1501	1498	t	1.5 ± 0.27
11	Curzerene	31.45	1509	1499	1.7 ± 0.17	t
12	*β*-Caryophyllene oxide	34.08	1599	1583	t	2.3 ± 0.33
13	Curzerenone	34.65	1620	1606	10.2 ± 0.06	7.4 ± 1.25
14	Spathulenol	35.33	1645	1608	1.8 ± 0.28	5.0 ± 0.65
15	*β*-Eudesmol	35.97	1668	1578	5.4 ± 0.03	5.6 ± 1.12
16	*ar*-Turmerone	36.48	1686	1651	6.1 ± 0.51	2.0 ± 0.40
17	Germacrone	37.19	1713	1694	3.0 ± 0.09	4.4 ± 0.08
18	Zerumbone	38.20	1752	1734	17.2 ± 0.12	12.1 ± 1.28
19	*α*-Cuparenol	38.53	1765	-	4.1 ± 0.75	8.9 ± 1.53
20	Benzyl benzoate	38.91	1780	1760	4.3 ± 0.09	t
21	Isovelleral	39.71	1810	-	6.6 ± 0.52	2.5 ± 0.74
				Monoterpene hydrocarbons	1.0%	3.1%
				Oxygenated monoterpenes	25.8%	37.0%
				Sesquiterpene hydrocarbons	7.6%	5.4%
				Oxygenated sesquiterpenes	56.1%	50.2%
				Non-terpenes	4.3%	t
				Total	**94.8 %**	**95.7 %**

[a] Kovat retention indices relative to *n*-alkanes on HP-5 capillary column (*n* = 3)
[b] Kovat retention indices referred to in the literature (Adams, 2001)
t refers to compound present at a composition less than 0.05%

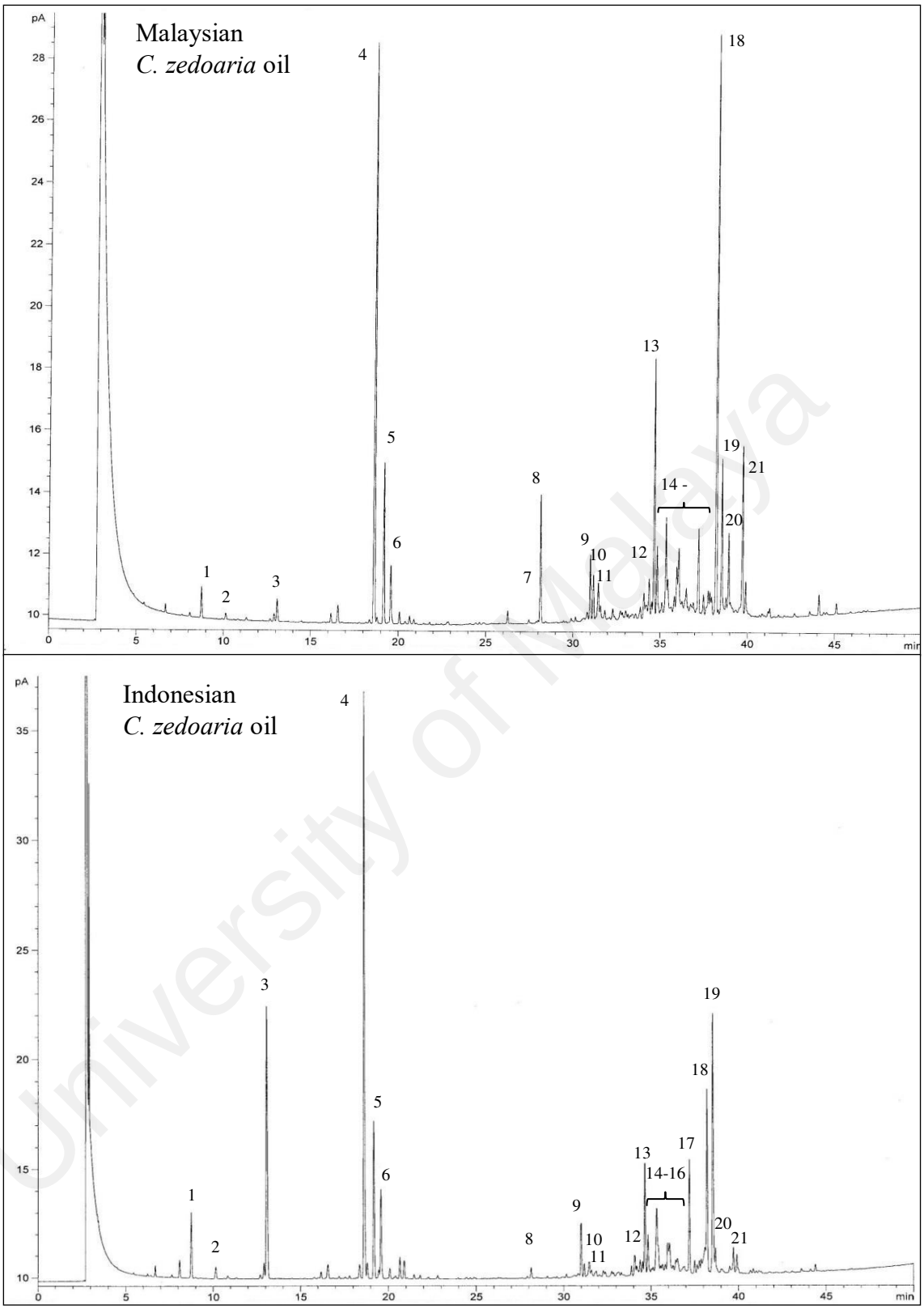


Figure 4.15: Gas chromatograms of Malaysian and Indonesian *Curcuma zedoaria* essential oils

4.6.4 Chemical Group of Constituents in Malaysian and Indonesian *Curcuma zedoaria* Essential Oils

Total yield of chemical compounds in Malaysian and Indonesian *Curcuma zedoaria* oils are 94.8% and 95.7%, respectively. In summary, both essential oils mainly composed of oxygenated sesquiterpenes with 56.1% and 50.2%, respectively, followed by oxygenated monoterpenes (25.8% and 37.0%), sesquiterpene hydrocarbons (7.6% and 5.4%), monoterpene hydrocarbons (1% and 3.1%) and non-terpenes compound (4.3% and 0%), respectively. The major compound, camphor belongs to oxygenated monoterpenes which is included in the group of alcohols. Meanwhile, the other major components, zerumbone and curzerenone belong to ketone from the group of oxygenated sesquiterpenes. Figure 4.16 showed the yield distribution of chemical group between Malaysian and Indonesian *C. zedoaria* essential oils. Malaysian oil showed higher yield of sesquiterpene hydrocarbons (7.6%) and oxygenated sesquiterpene (ether, alcohol, aldehyde and ketone; 56.1%) and a non-terpene compounds (4.3%) as compared to Indonesian *C. zedoaria* essential oil. Vice versa, Indonesian essential oil contains more in monoterpene hydrocarbons (3.1%) and oxygenated monoterpene (ether, alcohol, aldehyde and ketone; 37.0%). The structure of each identified compound and molecular formula is presented in Table 4.15.

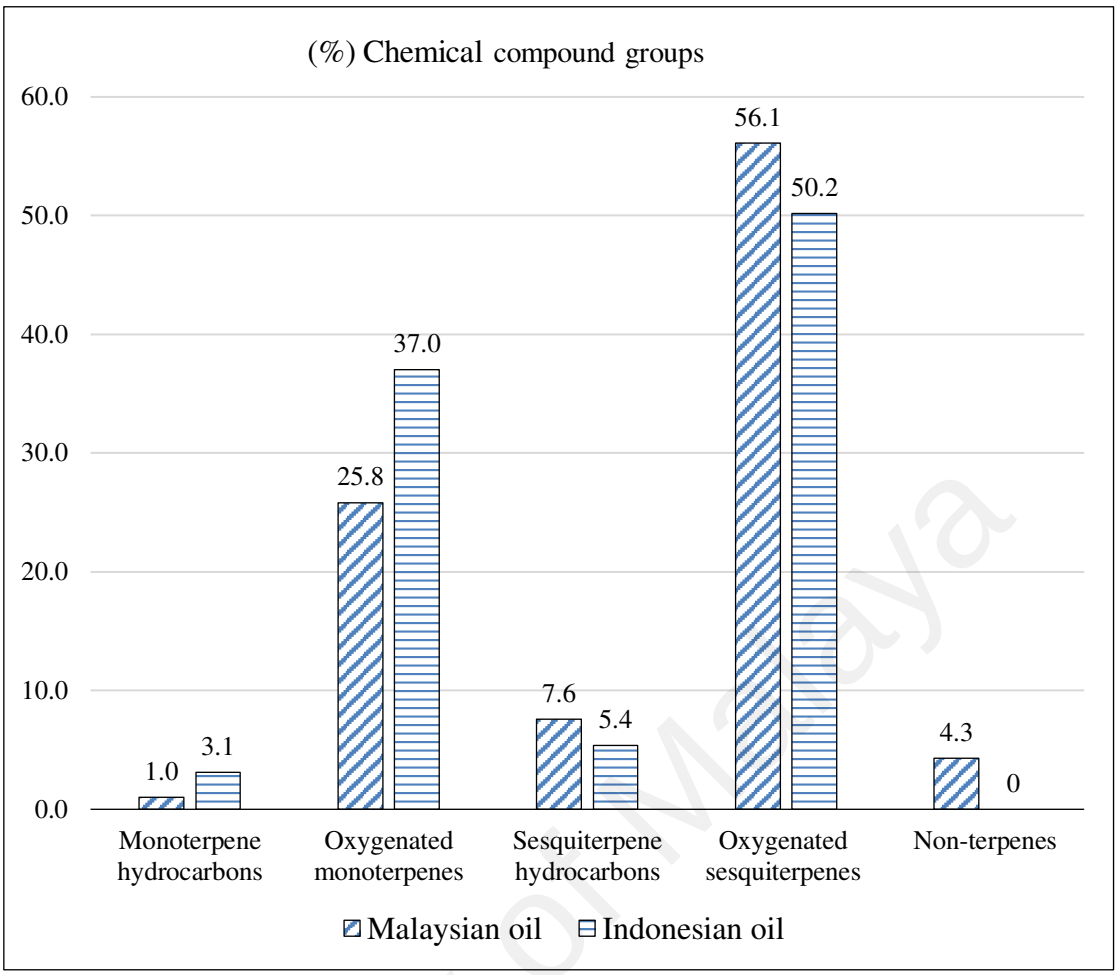
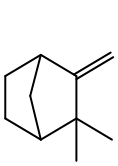


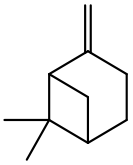
Figure 4.16: Chemical compound groups of Malaysian and Indonesian *Curcuma zedoaria* essential oils

Table 4.15: Chemical structure of constituents in the rhizome essential oils of Malaysian and Indonesian *Curcuma zedoaria*

Monoterpene hydrocarbons

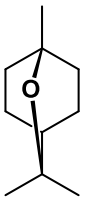


Camphene
$C_{10}H_{16}$

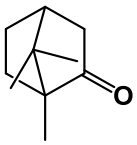


β-pinene
$C_{10}H_{16}$

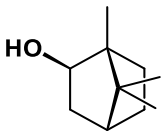
Oxygenated monoterpenes



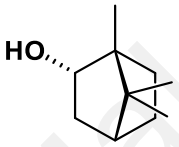
1,8-Cineole
$C_{10}H_{18}O$



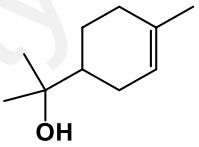
Camphor
$C_{10}H_{16}O$



Isoborneol
$C_{10}H_{18}O$

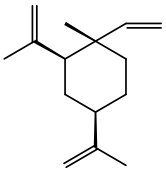


Borneol
$C_{10}H_{18}O$

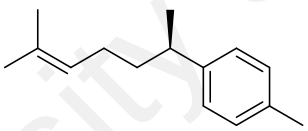


α-Terpineol
$C_{10}H_{18}O$

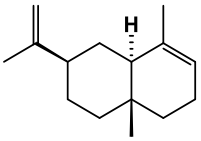
Sesquiterpene hydrocarbons



β-Elemene
$C_{15}H_{24}$

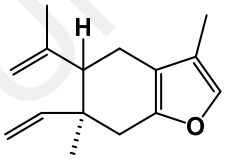


α-Curcumene
$C_{15}H_{22}$

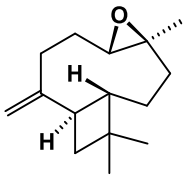


α-Selinene
$C_{15}H_{24}$

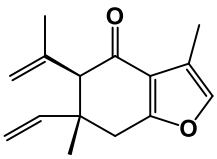
Oxygenated sesquiterpenes



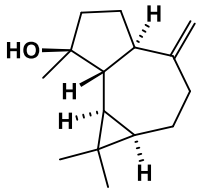
Curzerene
$C_{15}H_{20}O$



β-Caryophyllene oxide
$C_{15}H_{24}O$



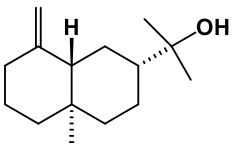
Curzerenone
$C_{15}H_{18}O_2$



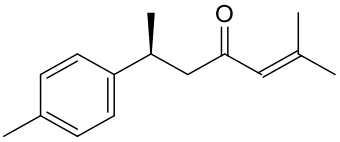
Spathulenol
$C_{15}H_{24}O$

'Table 4.15, continued'

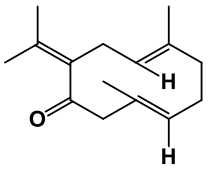
Oxygenated sesquiterpenes



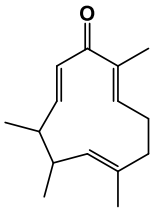
β-Eudesmol
$C_{15}H_{26}O$



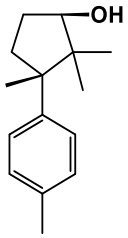
ar-Turmerone
$C_{15}H_{20}O$



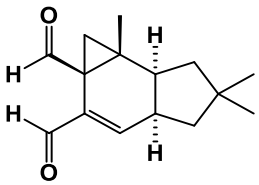
Germacrone
$C_{15}H_{22}O$



Zerumbone
$C_{15}H_{22}O$

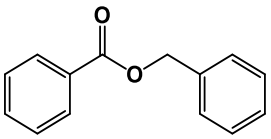


α-Cuparenol
$C_{15}H_{22}O$



Isovelleral
$C_{15}H_{22}O_2$

Non-terpenes



Benzyl benzoate
$C_{14}H_{12}O_2$

4.7 Cytotoxic Activity of the Essential Oils

The cytotoxic activity of the rhizome essential oils of *Curcuma zedoaria* collected from Malaysia and Indonesia was performed using the MTT cell viability assay against cancer cell lines; breast (MCF-7 and MDA-MB-231), lung (A549 and SK-LU-1) and cervical (HeLa S3 and SiHa). Further, live dead viability assay was performed on the most active oil towards selected cell lines. Normal lung cell (MRC-5) was used as the control cell line.

4.7.1 MTT Cell Viability Assay

The results of cytotoxic activity of essential oil from Malaysia and Indonesia are given in Table 4.16. Essential oil or crude extracts is generally considered to have an active cytotoxic activity if the IC_{50} of cancer cells is equal or less than 20 µg/mL but for pure compounds the value should be equal or less than 4 µg/mL (Mahavorasirikul et al., 2010).

The rhizome oil of Malaysian *C. zedoaria* was found to be most active towards cancer cell lines tested as compared to the Indonesian *C. zedoaria* oil. The IC_{50} values of Malaysian *C. zedoaria* oil showed highest cytotoxic activity on HeLa S3 (IC_{50}: 6.4 µg/mL) followed by MCF-7 (8.73 µg/mL), SiHa (9.8 µg/mL), SK-LU-1 (9.9 µg/mL) and A549 (13.9 µg/mL), while MDA-MB-231 cell lines showed moderate cytotoxic activity with IC_{50} value of 22.0 µg/mL.

Indonesian *C. zedoaria* essential oil inhibited SiHa at IC_{50} value of 11.5 µg/mL, followed by A549 (12.1 µg/mL), SK-LU-1 (14.0 µg/mL) and MCF-7 (16.5 µg/mL). Less cytotoxic activity was found on breast cancer cell (MDA-MB-231) and cervical cancer cell (HeLa S3) with IC_{50} value more than 20 µg/mL. From the results, we found that there was cytotoxic effect on MRC-5 (normal cell) after treatment with both Malaysian and Indonesian *C. zedoaria* oils (25.7 µg/mL ± 3.7 and 27.0 µg/mL ± 2.8, respectively).

However, the cytotoxic effect of both oils on MRC-5 was less compared to the cancer cell lines used in this study (Table 4.16).

In this study, the common drug used in chemotherapy treatment, doxorubicin was used as positive control. The results demonstrated that the cytotoxic activity of Malaysian *C. zedoaria* essential oils were higher compared to doxorubicin for MCF-7 with two-fold activity, MDA-MB-231 (1.6 fold), SK-LU-1 (3.6 fold) and HeLa (1.0 fold) cell lines (Table 4.16). In the case of Indonesian *C. zedoaria* oil, higher cytotoxic activity was detected on MCF-7 (1.2 fold), MDA-MB-231 (1.6 fold) and SK-LU-1 (3.7 fold) compared to doxorubicin.

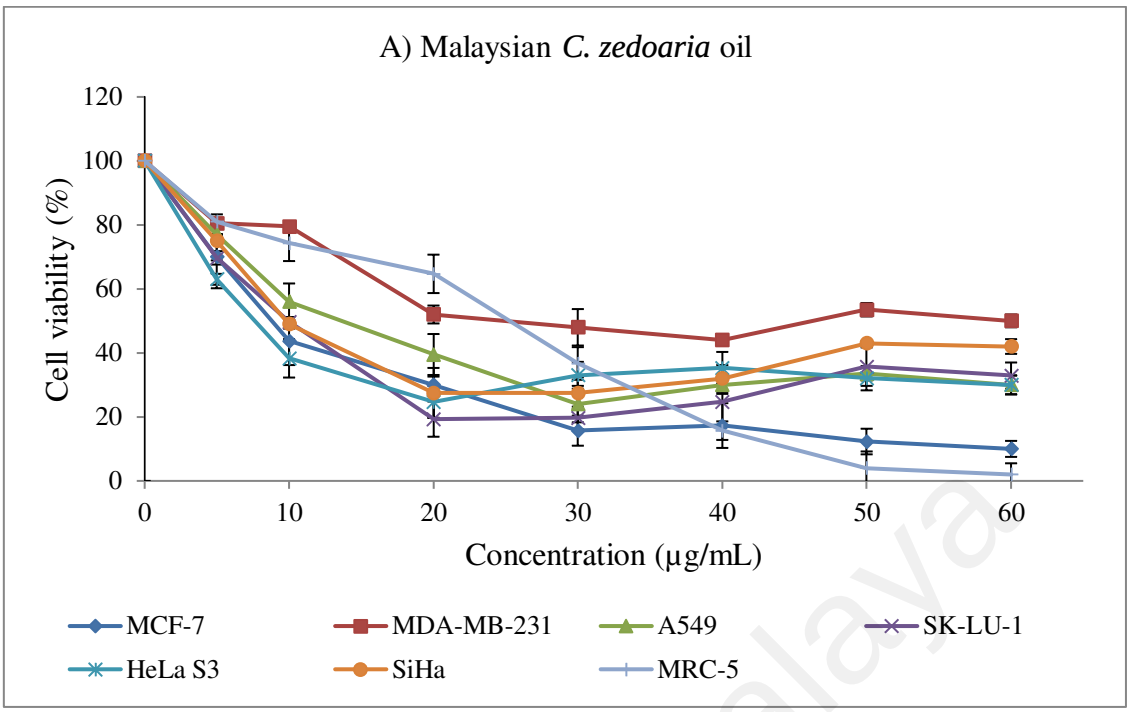
Selectivity index (SI) indicated selectivity of the samples to the cell lines tested. In this case, MRC-5 used as normal cell control for SI values calculation. SI values for Malaysian and Indonesian *C. zedoaria* oils ranged from 1.2 to 4.0. Interestingly, cervical cancer cell, HeLa S3 treated with Malaysian *C. zedoaria* oil revealed high selectivity index toward MRC-5 cells (SI value: 4.0).

According to the American National Centre Institute (Oliveira et al., 2015), both the Malaysian and Indonesian oils constitute promising anti-cancer agents for drug development since their IC_{50} values were recorded to be lower than 20 µg/mL against cancer cell lines tested in the current study (Table 4.16 and Figure 4.17).

Table 4.16: IC_{50} values of Malaysian and Indonesian *Curcuma zedoaria* essential oils on selected cancer cell lines via MTT cell viability assay

Type of cancer / Cell lines		Cell viability assay: IC_{50} (µg/mL) ± SD (n = 3) *					
		Malaysian *C. zedoaria* oil	SI	Indonesian *C. zedoaria* oil	SI	Doxorubicin	SI
Breast	MCF-7	8.7 ± 1.5	2.9	16.5 ± 4.9	1.6	19.6 ± 6.5	> 3.1
	MDA-MB-231	22.0 ± 2.8	1.2	20.4 ± 0.6	1.3	32.9 ± 11.8	> 1.8
Lung	A549	13.9 ± 2.9	1.8	12.1 ± 4.9	2.2	9.3 ± 4.6	> 6.5
	SK-LU-1	9.9 ± 0.3	2.6	14.0 ± 0.8	1.9	51.5 ± 2.1	> 1.2
Cervical	HeLa S3	6.4 ± 0.7	4.0	21.6 ± 2.5	1.3	6.5 ± 0.3	> 9.2
	SiHa	9.8 ± 0.2	2.6	11.5 ± 1.9	2.4	7.8 ± 0.7	> 7.7
Normal cell	MRC-5	25.7 ± 3.7	-	27.0 ± 2.8	-	> 60.0	-

Doxorubicin were use as positive control
SI: Selective index (IC_{50} of normal cells / IC_{50} of cancer cells)
Data presented as mean ± SD after deduction of DMSO solvent induce cytotoxicity of three independent experiments (n = 3)
* After 24 h of treatment



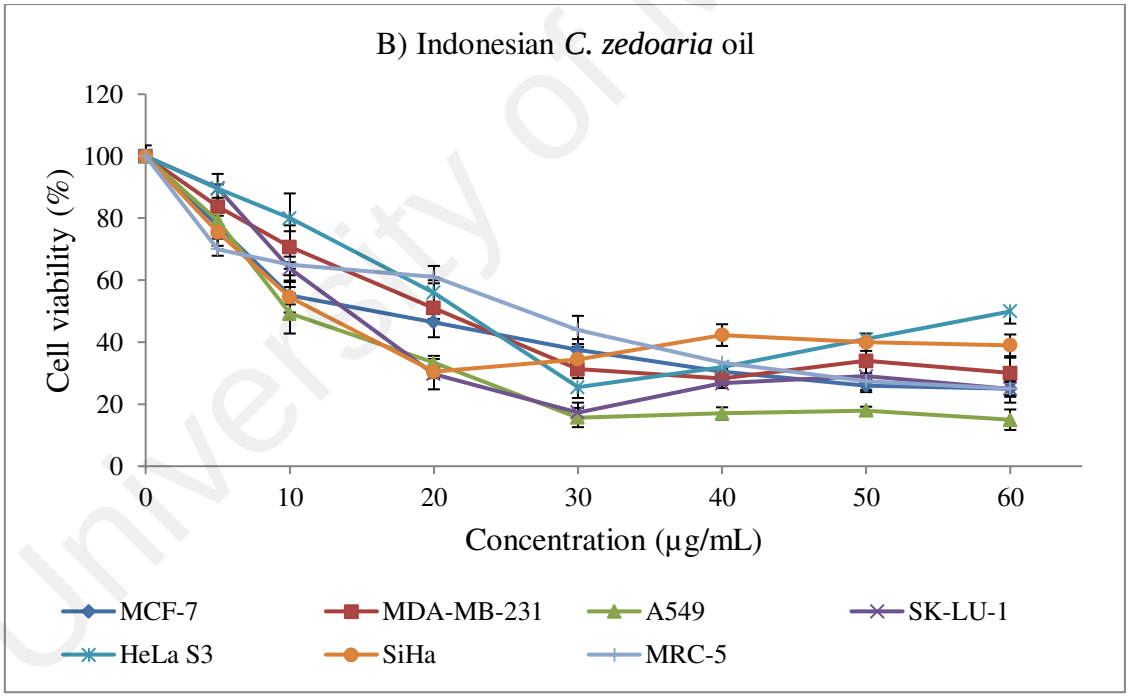
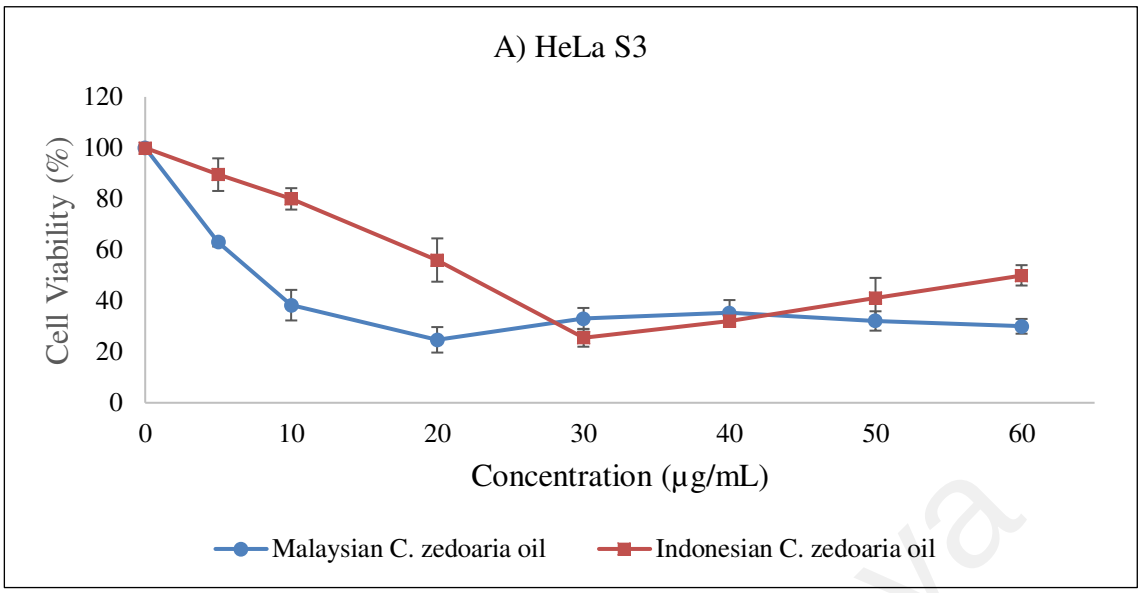


Figure 4.17: Cytotoxic effect of (A) Malaysian and (B) Indonesian *Curcuma zedoaria* essential oil against human cancer cell lines: breast (MCF-7 and MDA-MB-231), lung (A549 and SK-LU-1), cervical (HeLa S3 and SiHa) and normal cell (MRC-5)

4.7.2 Cytotoxic Effect of Malaysian and Indonesian *Curcuma zedoaria* Essential Oils on HeLa S3 and SiHa Cells

Previously, the preliminary MTT cell viability assay on the rhizome essential oils of *Curcuma zedoaria* from Malaysia and Indonesia were conducted on MCF-7 and MDA-MB-231 (breast cancer cell lines), A549 and SK-LU-1 (lung cancer cell lines) and HeLa S3 and SiHa (cervical cancer cell lines) (Table 4.16). Based on the results, cervical cell lines, HeLa S3 and SiHa were selected as the target cells for further downstream investigation.

Incubation of HeLa S3 and SiHa with different concentrations (0-60 µg/mL) of the Malaysian and Indonesian *C. zedoaria* oils for 24 h resulted in concentration dependent cytotoxic effect upon treatment (Figure 4.18). Results suggested that Malaysian *C. zedoaria* oil displayed higher cytotoxic activity than the Indonesian oil. The IC_{50} values of Malaysian *C. zedoaria* oil on HeLa S3 was at 6.4 µg/mL ± 0.7 and 9.8 µg/mL ± 0.2 on SiHa. Meanwhile, Indonesian *C. zedoaria* oil showed the IC_{50} values of 21.6 µg/mL ± 2.5 and 11.5 µg/mL ± 1.9 on HeLa S3 and SiHa cells, respectively. Based on the results, *C. zedoaria* oil from Malaysia was suggested for further investigations in subsequent experiment.



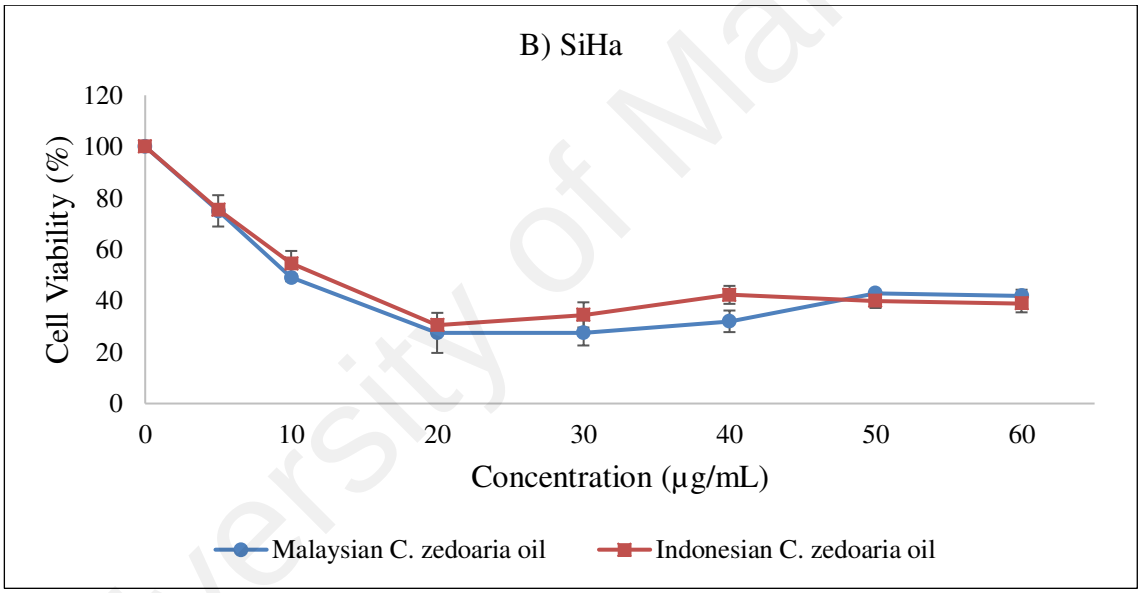


Figure 4.18: Cytotoxic effect of Malaysian and Indonesian *Curcuma zedoaria* oil on (A) HeLa S3 and (B) SiHa at different concentrations for 24 h ($n = 3$)

4.7.3 Live Dead Cell Viability Assay

Live dead viability cytotoxicity assay was performed to further confirm the cell death or cytotoxic effects on cervical cell lines; HeLa S3 and SiHa upon treatment with Malaysian *Curcuma zedoaria* oil and observed under florescent microscope. This assay recognizes two colour fluorescence that simultaneously determines the number of live and dead cells. Viable cells are distinguished by the presence of ubiquitous intracellular esterase activity which was determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. Viable cell will produce an intense uniform green fluorescence in the cells. Detection of dead cells are based on the loss of membrane integrity, ethidium homodimer (EthD-1) dye will enter the cell and bind to the nucleic acid then producing a bright red fluorescence in dead cells. EthD-1 is excluded from live cells due to the intact plasma membranes.

Figure 4.19 indicateed the live and dead cells of cervical cancer cell lines, HeLa S3 and SiHa upon exposure to Malaysian *C. zedoaria* oil. Previously, the MTT assay conducted on these two cells at 6 h with IC_{50} values for HeLa S3 and SiHa are 25.0 µg/mL and 29.5 µg/mL, respectively. The results exhibited a significant cell death of HeLa S3 and SiHa cells after treatment. The percentage of viable cell on HeLa S3 cells was reduced from 98.6% to 63.5%, while on SiHa cells was reduced from 89.1% to 59.6%. DMSO solvent controls indicated minimal viability drop between 5.0% and 2.0% respectively on HeLa S3 and SiHa cells. Statistical analysis indicated significant cell death at $p < 0.01$.

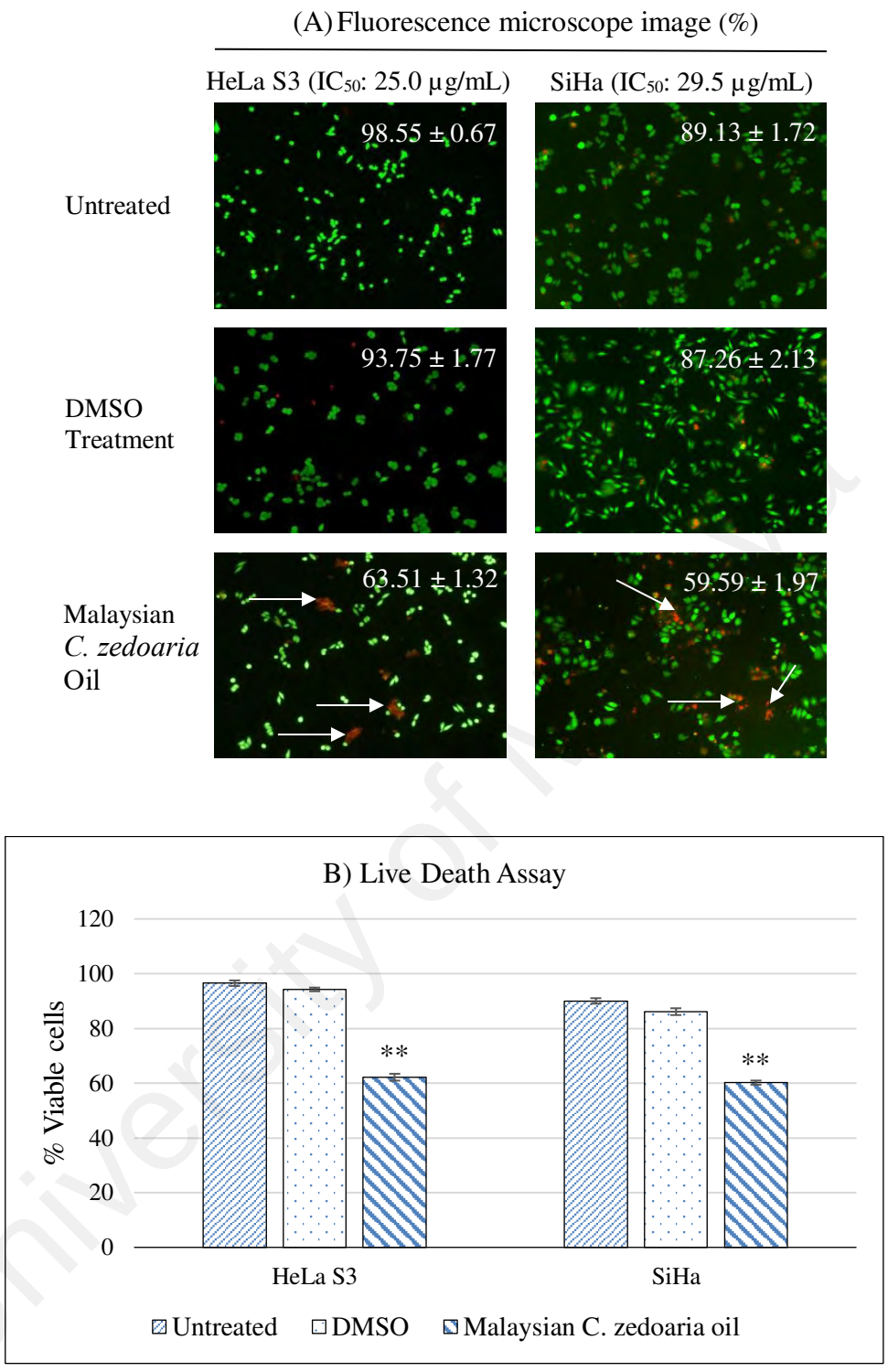


Figure 4.19: Live dead viability cytotoxicity assay upon treatment with Malaysian *Curcuma zedoaria* essential oil for 6 h on HeLa S3 and SiHa (DMSO as control). (A) Fluorescence microscope image of viable cells, (B) percentage of viable cells as calculated under a fluorescence microscope. All data are presented as mean ± SD ($n = 4$), ** $p < 0.01$. Arrows indicates the dead cells.

4.8 Determination of Apoptosis

Determination of apoptosis by Malaysian *Curcuma zedoaria* oil has been evaluated by Annexin-V-FITC/PI assay and Caspase-3/7 assay.

4.8.1 Annexin V-FITC/PI Assay

The early and late stage of apoptotic cells upon treatment with Malaysian *Curcuma zedoaria* oil were determined and quantified using Annexin-V-FITC/PI staining by detection of phosphatidylserine (PS) on the outer leaflet of apoptotic cell membrane. The potential of Malaysian *C. zedoaria* oil to induce apoptosis on HeLa S3 and SiHa was conducted by flow cytometry assay. A double fluorescence staining of Annexin-V-FITC/PI conjugate and propidium iodide (PI) was performed on untreated cancer cell, solvent treatment (DMSO) and Malaysian *C. zedoaria* oil treatment (10, 20, 30, 40 and 50 µg/mL). After 24 h of treatment, the population of dead and viable cells were observed on four quadrants indicated a shift from viable cells (bottom left) to early stage of apoptosis (lower right), late stage of apoptosis (upper right) and followed by necrosis (upper left).

Our finding after using Annexin-V-FITC/PI on HeLa S3 cells, indicated that the number of apoptotic cells that undergo apoptosis (early stage and late stage) increased with a higher concentration of Malaysian *C. zedoaria* oil (Figure 4.20). The percentage on the treatments are 3.9% ± 1.2 (10 µg/mL), 4.3% ± 1.0 (20 µg/mL), 9.4% ± 1.8 (30 µg/mL), 15.3% ± 4.3 (40 µg/mL), 23.9% ± 1.5 (50 µg/mL). Annexin-V-FITC/PI double staining analysis showed that the number of apoptotic cells of HeLa S3 increase significantly (Figure 4.21). At concentration of 20 µg/mL and 30 µg/mL, the results exhibited statistically significant at $p < 0.05$ (*) while at concentration of 40 µg/mL and 50 µg/mL, significant were at $p < 0.01$ (**) as compared to the untreated cells.

On the other hand, another cervical cancer cell line, SiHa was also tested with the same concentrations of Malaysian *C. zedoaria* oil (Figure 4.22). The percentages of apoptotic cells after treatment are 5.1% ± 1.2 (10 µg/mL), 5.4% ± 1.0 (20 µg/mL), 5.4% ± 1.8 (30 µg/mL), 8.4% ± 4.3 (40 µg/mL). Figure 4.23 displays the analysis of the apoptotic cell on SiHa. The percentages of apoptotic cells on SiHa were significantly higher when treated with 40 and 50 µg/mL of Malaysian *C. zedoaria* oil as compared to the DMSO treated cells and untreated cells. The percentage of apoptotic cells (early stage and late stage) on SiHa cells was 9.4% ± 1.6 after treatment at the highest concentration, 50 µg/mL after 24 h of incubation.

HeLa S3

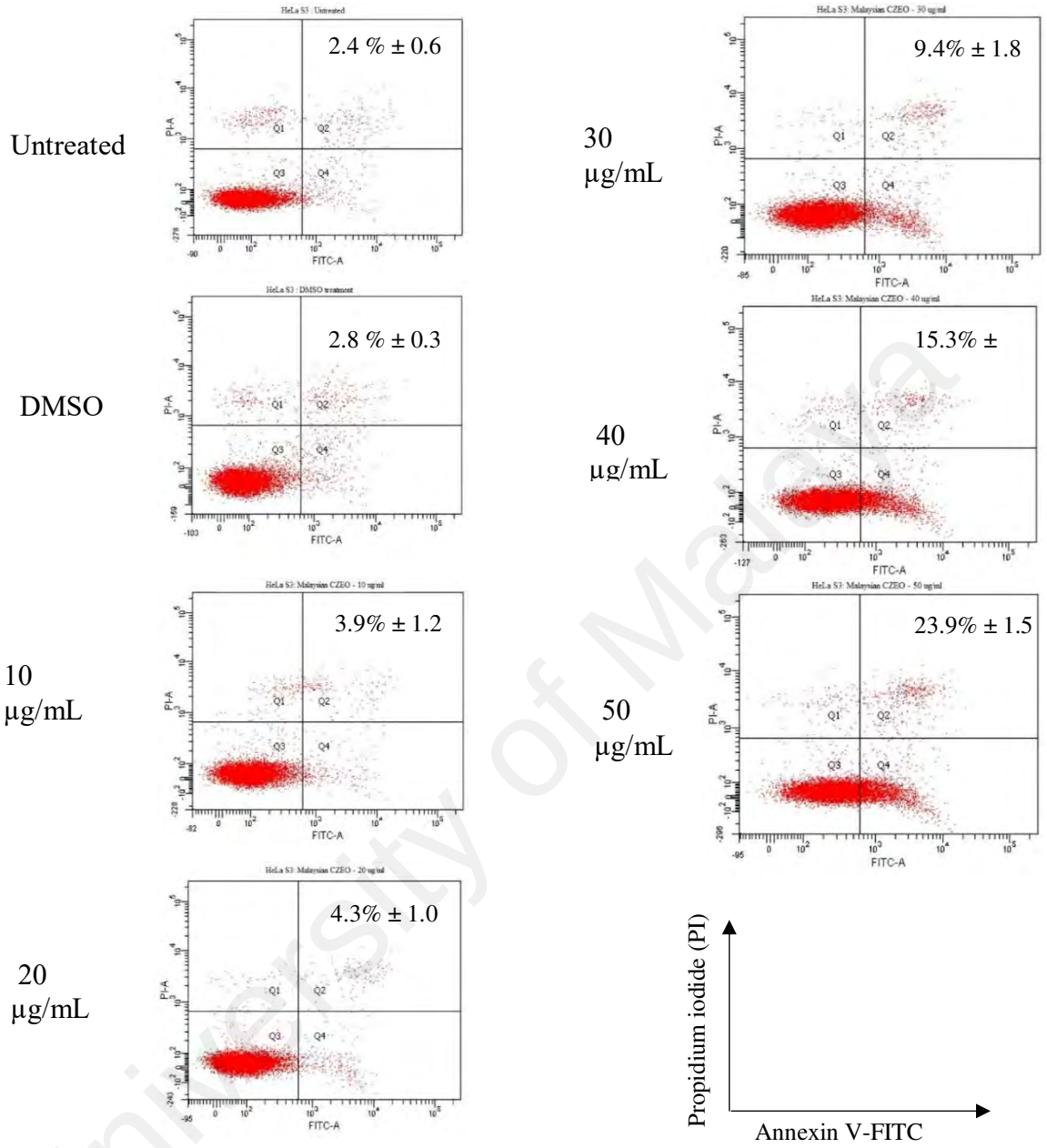


Figure 4.20: Detection of early and late apoptotic cells using Annexin-V-FITC/PI staining on HeLa S3 cells upon treatment with Malaysian *Curcuma zedoaria* oil (10, 20, 30, 40 and 50 µg/mL) for 24 h (*n*=3). Percentage of apoptosis was calculated based on upper right and bottom right of quadrants.

Table 4.17: Apoptotic effects (%) of Malaysian *Curcuma zedoaria* oil obtained from flow cytometer on HeLa S3

Treatment (µg/mL)	% Apoptosis (early + late apoptosis) ± SD)
Untreated	2.4 ± 0.6
DMSO	2.8 ± 0.3
10	3.9 ± 1.2
20	4.3 ±1.0
30	9.4 ± 1.8
40	15.3 ± 4.3
50	23.9 ± 1.5

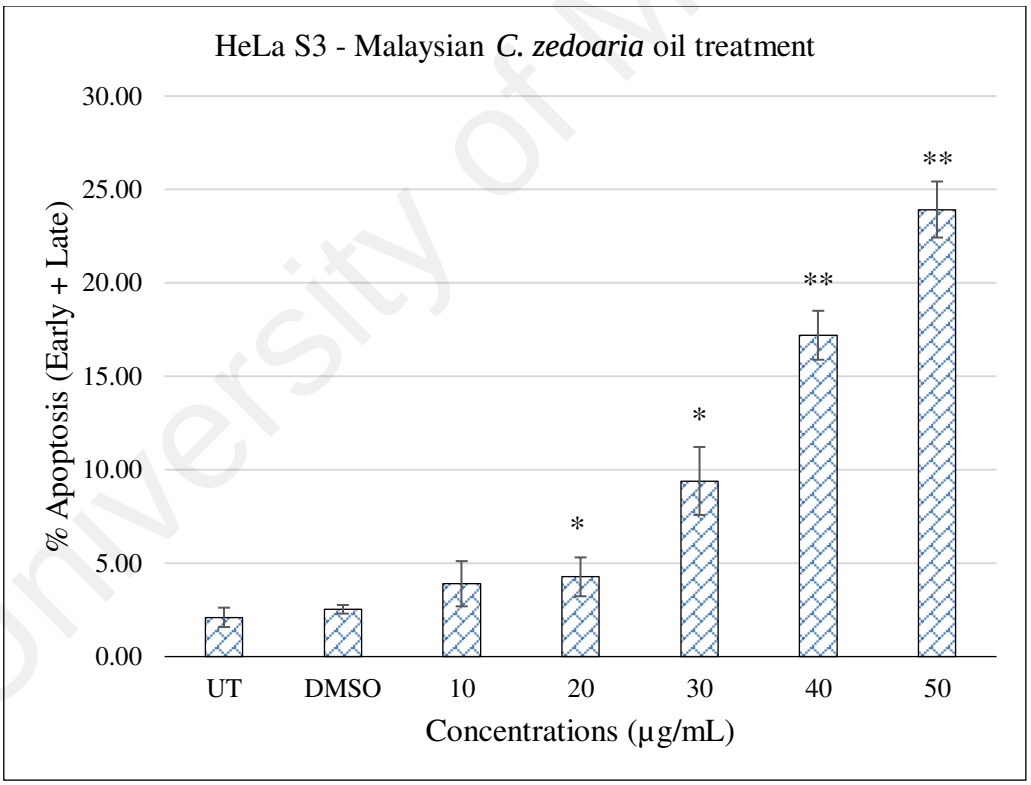


Figure 4.21: Annexin-V-FITC/PI flow cytometry on HeLa S3 upon treatment with Malaysian *Curcuma zedoaria* essential oil for 24 h (*$p < 0.05$, **$p < 0.01$)

SiHa

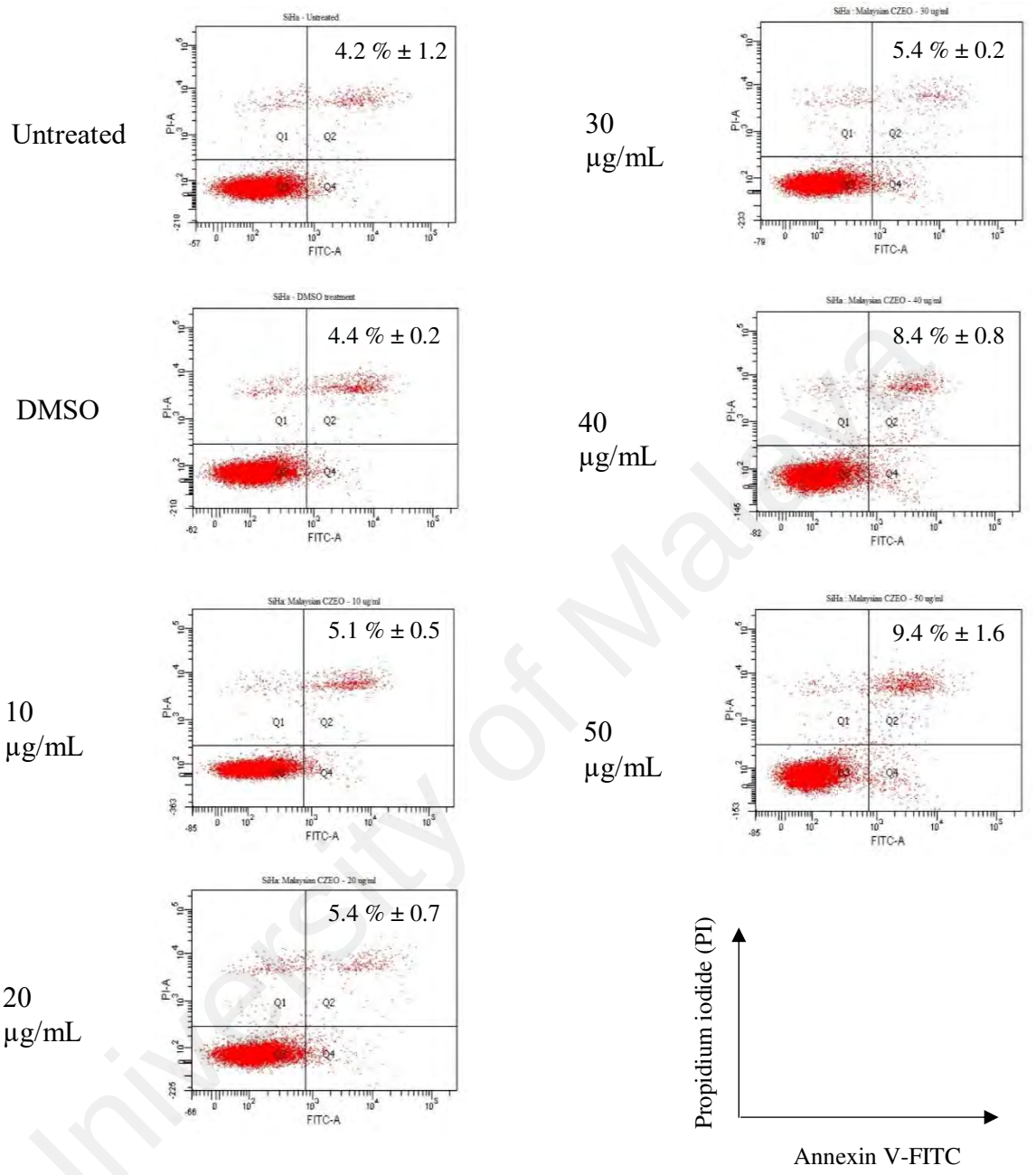


Figure 4.22: Detection of early and late apoptotic cells using Annexin-V-FITC/PI staining on SiHa cells upon treatment with Malaysian *Curcuma zedoaria* oil (10, 20, 30, 40 and 50 µg/mL) for 24 h (*n*=3). Percentage of apoptosis was calculated based on top right and bottom right of quadrants.

Table 4.18: Apoptotic effects (%) of Malaysian *Curcuma zedoaria* oil obtained from flow cytometer on SiHa

Treatment (µg/mL)	% Apoptosis (early + late apoptosis) ± SD
Untreated	4.2 ± 1.2
DMSO	4.4 ± 0.2
10	5.1 ± 0.5
20	5.4 ± 0.7
30	5.4 ± 0.2
40	8.4 ± 0.8
50	9.4 ± 1.6

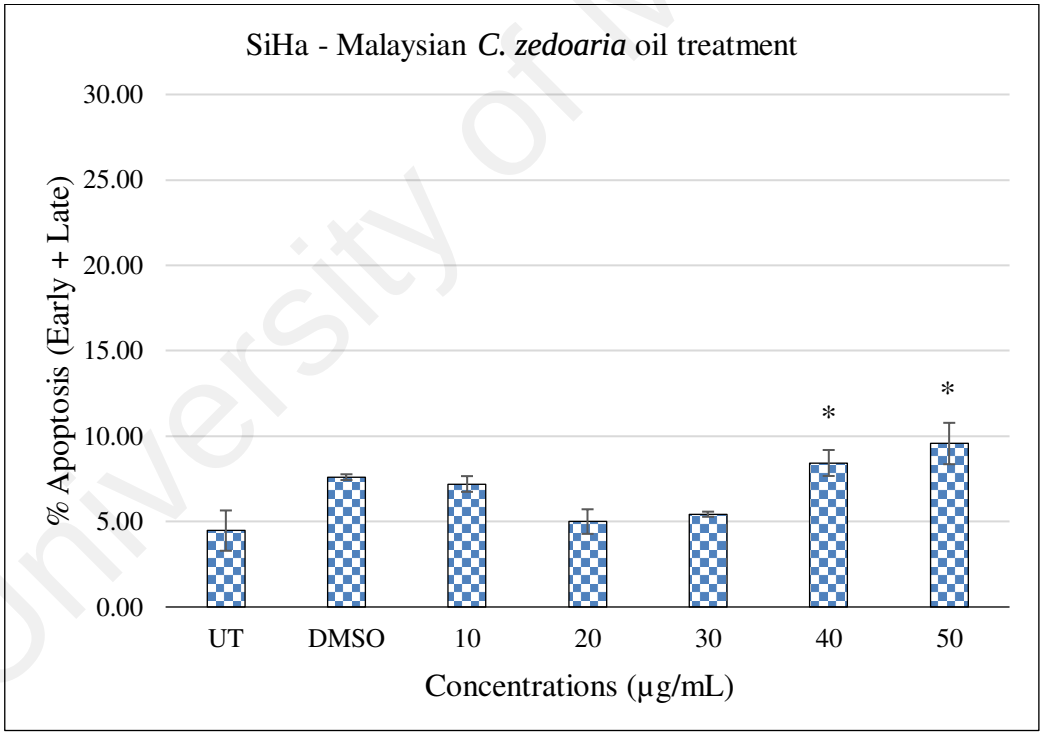


Figure 4.23: Annexin-V-FITC/PI flow cytometry on SiHa upon treatment with Malaysian *Curcuma zedoaria* oil for 24 h (* $p < 0.05$, ** $p < 0.01$)

4.8.2 Caspase-3/7 assay

HeLa S3 and SiHa cell lines were treated with Malaysian *C. zedoaria* oil for 5 h at different concentration of 30, 40, and 50 µg/mL, while DMSO treated cells were used as control. Data is representative of three independent experiments (n = 3). Statistically significant differences between the treatment groups and DMSO control is denoted with (*) for $p \leq 0.05$ and (**) for $p \leq 0.01$.

In HeLa S3, Caspase-3/7 activity assay shows significantly higher caspase-3/7 activity when treated with 40 and 50 µg/mL of Malaysian *C. zedoaria* oil compared to the negative control and untreated cells. Meanwhile in SiHa, no significant difference was detected between all treatment groups when compared to both negative control and untreated cells.

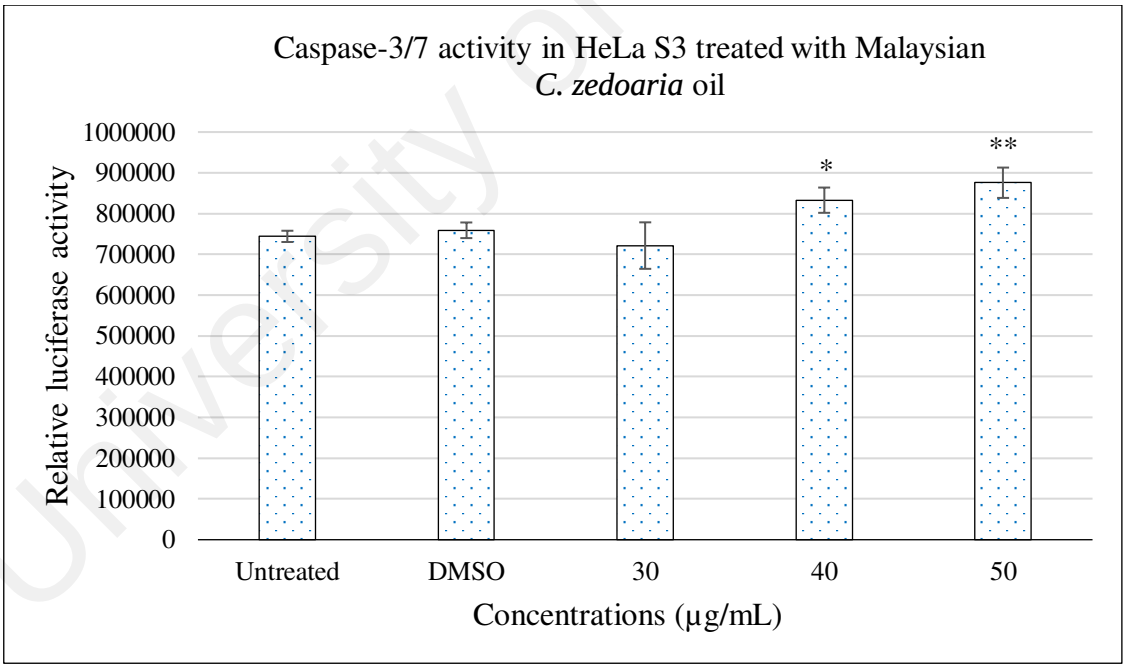


Figure 4.24: Measurement of caspase-3/7 activity in HeLa S3 cell line after being treated with different concentrations of Malaysian *Curcuma zedoaria* oil for 5 h

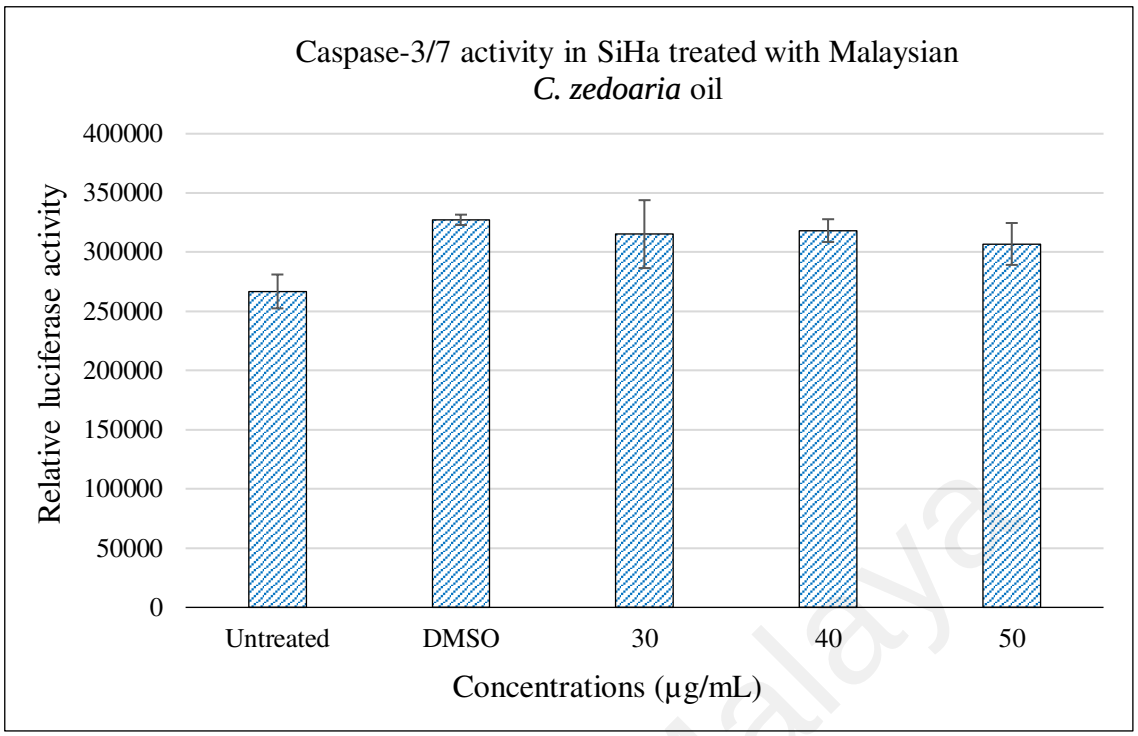


Figure 4.25: Measurement of caspase-3/7 activity in SiHa cell line after being treated with different concentrations of Malaysian *Curcuma zedoaria* oil for 5 h

CHAPTER 5: DISCUSSION

Recently, cancer remains as one of the major and challenging health problems and is one of the main causes of death. The conventional treatments of cancer like surgery and chemotherapy with synthetic drugs are not only expensive, but also evoke severe side effects such as immuno-suppression and organ failures which in many cases cause the death of patients after recovery from cancer. Therefore, combination in the treatment of modern and traditional medicine is an alternative to improve health, to reduce severe side effects and to enhance the efficacy of conventional cancer therapies. Today, many cancer treatments involve the combination of both conventional drugs such as taxol, resveratrol and sulforaphane (Haldar et al., 1996; Kaminski et al., 2012) and the complimentary usage of traditional herbs (Tuna et al., 2011; Yin et al., 2013).

From the dawn of human civilisation, plants have been the source of medication. Until today, medicinal plants are still of great use and an estimated 70% of the world population still use traditional remedies. In South East Asia, particularly Malaysia and Indonesia, the family Zingiberaceae is one of the important plant family used in general health maintenance and the treatment of various maladies such as cancer. Many of plants used in traditional medicines are still utilised especially in the rural areas of developing countries worldwide.

Natural products from plants such as extracts or pure compounds, provide unlimited opportunities for new drugs development (Sasidharan et al., 2011). In the field of cancer research, natural products have been the source of most of the active ingredients for medicine. More than 80% of drug substances were natural products or inspired by natural compound. Screening programs to identify plant-derived agents for the treatment of cancer, led to the identification of pure active phytochemical compounds such as vincristine, vinblastine, paclitaxel, doxotaxel, topotecan, irinotecan, flavopiridol,

acronyciline, bruceantian and thalicarpin which has been successfully employed in cancer treatment (da Rocha et al., 2001). Basically, phytochemicals are defined as naturally occurring, non-nutritive biologically active chemical compounds in plant which act as a natural defence system for host plants and provide colour, aroma and flavour (Liu, 2003).

Curcuma zedoaria Rosc. was selected as plant material used in this present study belongs to the genus of *Curcuma* from Zingiberaceae family. This plant is one of the herbs known to be used in traditional medicine and believed as a potential alternative in the treatment of cancer. The rhizomes are traditionally used and reported for the treatment of menstrual disorders, dyspepsia, vomiting, to relieve flatulence, expectorant and used in the treatment of cancer (Wilson et al., 2005; Lobo et al., 2009). In China, it is also traditionally used for the treatment of cough and fever (Chen et al., 2011). A wide variety of biological activity also have been reported such as anti-oxidant, anti-bacterial and anti-inflammatory (Mau et al., 2003; Rahman et al., 2014).

Phytochemical investigations on *C. zedoaria* by several researchers revealed that the occurrence of many types of sesquiterpenoids; eudesmane, carabrene (Yoshikawa et al., 1998; Matsuda et al., 2001) and germacran (Shiobara et al., 1985). *C. zedoaria* is a rich source of essential oil and was reported to have volatile constituents such as camphene, 1,8-cineole, α-pinene, β-pinene, camphor, curzerenone, curzeone and germacrone. In this present study, a bioassay-guided isolation from the rhizome of *C. zedoaria*, the essential oils analysis, cytotoxic activity and the apoptotic investigation have been performed. The discussion of the results obtained is presented in the following subchapters.

5.1 Characterization of Compounds from *Curcuma zedoaria* from Indonesia using Bioassay-guided Isolation

The objectives of this part were to characterize the compounds from *Curcuma zedoaria* using bioassay-guided isolation and to test their cytotoxic activity on cancer cell lines. Bioactivity-guided fractionation or isolation are categorised as the most efficient method of discovering drugs from natural products resources (Pezzuto, 1997).

In Indonesia, *C. zedoaria* is commonly used as an ingredient in *jamu* (traditional tonic). Therefore, in this study, a bioassay-guided isolation was conducted on *C. zedoaria* collected from Solo, Indonesia according to their *in vitro* cytotoxic activity. Generally, cytotoxic activity can be measured by several methodologies such as 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide test (MTT) (Awang et al., 2010; Hasima et al., 2010; Subramaniam et al., 2016), neutral red uptake test (Repetto et al., 2008; Rahman et al., 2013), trypan blue exclusion test, resazurin test or Hoechst 33342 and propidium iodide test. However, MTT cell viability assay is a common assay for detection for cytotoxic effect of extracts or compounds. Thus, in this study, MTT assay was selected to determine the cytotoxic activity. This assay is based on the conversion of MTT into formazan crystals by living cells which determines mitochondrial activity.

The ethanol extract (EE) and the fractions which were obtained by solvent partitioning method from EE namely hexane soluble fractions (HSF), dichloromethane soluble fractions (DSF) and the residue were evaluated for their cytotoxic effects screening on various cancer cell lines namely lung (A549), liver (HepG2), oral (HSC-4), breast (MCF-7), prostate (PC-3), cervical (SiHa) and lung normal cells (MRC-5). Based on these results, HSF showed significant cytotoxic activity as compared to the other extracts and lung cancer cell, A549 was selected as cell target for further cytotoxic activity. These results suggested that HSF could contain nonpolar compounds which may be responsible for the cytotoxic activities. From previous investigations, hexane extracts

from *Curcuma* spp. demonstrated potent cytotoxic activity against several cancer cell lines (Mohammad et al., 2010; Malek et al., 2011; Rahman et al., 2013; Hamdi et al., 2014; Hong et al., 2016).

Further, the chemical profiling was conducted on the non-polar fraction, HSF by GC-FID, GC/MS and Kovat retention index (KI) analysis to identify the chemical components. Approximately twenty-three (23) significant peaks were observed comprising of curcumenol (20.6%), velleral (13.4%), dehydrocurdione (9.1%), germacrone (7.4%) and curcumenone (7.0%) as major compounds from the total 93.0%. The other compounds such as camphor, sphatulenol, curzerenone were present at 4.9%, 3.2% and 3.0%, respectively. These analyses suggested that most of the compounds in HSF are non-polar compounds from the type of monoterpene and sesquiterpene.

Column chromatography of the HSF led to a set of fractions (fraction H1 to fraction H6) using solvents with different polarity. The process was followed by MTT assay of each fraction against lung cancer cell, A549. The fraction with potent cytotoxic effect was selected for further column chromatography and yielded for the seven sub-fractions labelled as fraction H1.1 to fraction H1.7. Fraction H1.2 showed the highest potency and purification of compounds led to the isolation and characterization of four compounds; 1, 2, 3 and 4 are identified from the group of sesquiterpene. These compounds were identified as spathulenol, β-eudesmol, dehydrocurdione and curcumenone. Compounds 1 and 2 were identified based on the comparison of mass data (EI/MS) and KI values were comparable to the literature (Adams, 2001; Jirovetz et al., 2002; Wang et al., 2003; Hamdi et al., 2015). Compound 3 was successfully isolated and identified as dehydrocurdione, a germacran type of sesquiterpenes, based on the mass data, KI value (references: Hamdi et al., 2015; Rahman et al., 2013) and confirmed by ^{1}H NMR and ^{13}C NMR spectral data. The NMR data were comparable to the literature values (Kuroyanagi et al., 1987; Diastuti et al., 2014; Hamdi et al., 2015). Meanwhile, compound

4 was identified as curcumenone based on their mass spectral data (references: Hamdi et al., 2015; Rahman et al., 2013; Yang et al., 2011). However, curcumenone was unsuccessful to be isolated.

MTT cell viability assay were performed on spathulenol, *β*-eudesmol and dehydrocurdione against two different lung cancer cell lines, A549 and SK-LU-1. Due to insufficient quantity of spathulenol and *β*-eudesmol, both compounds were purchased from Biopurify Phytochemicals Ltd. (Chengdu, Sichuan, China) for the cytotoxic activity test. Curcumenone was not tested for the cytotoxic activity due to insufficient quantity.

Among the compounds, spathulenol was found to be more cytotoxic towards A549 as compared to SK-LU-1 with IC_{50} values of 11.0 µg/mL and 22.3 µg/mL, respectively. Interestingly, the cytotoxic activity of spathulenol against these two cell lines are 2.5 and 1.0-fold higher than the drug control, doxorubicin. The second highest cytotoxic activity was displayed by *β*-eudesmol with IC_{50} values of 12.2 µg/mL and 22.9 µg/mL against A549 and SK-LU-1, respectively. Both compounds, spathulenol and *β*-eudesmol exhibited less toxicity to the normal cell, MRC-5 as compared to the cancer cells. The SI values of spathulenol towards A549 and SK-LU-1 cells are 2.9 and 1.5, respectively. Meanwhile, SI values for *β*-eudesmol were calculated as 2.1 and 1.1 towards A549 and SK-LU-1 cells. The SI values against SK-LU-1 for both compounds were comparable to that of doxorubicin (between 1.1 to 1.5). However, doxorubicin has a higher selective index value towards A549 as compared to spathulenol and *β*-eudesmol (Table 4.10, page 94). Dehydrocurdione showed IC_{50} values of 54.6 µg/mL and 44.3 µg/mL towards A549 and SK-LU-1, respectively indicating a 5.8-fold less toxicity (A549) and 0.8-fold toxicity (SK-LU-1) as compared to doxorubicin. SI values for this compound are >1.1 and >1.4 against A549 and SK-LU-1, respectively.

From the results of MTT assay on compounds suggested that the cytotoxic activity of spathulenol and *β*-eudesmol are greater than the original extract, HSF. Previously,

MTT screening of HSF (Table 4.1, page 70) displayed IC_{50} value of 44.1 µg/mL against A549. However, HSF showed no cytotoxic effect to the normal cells, MRC-5 (IC_{50}: >120 µg/mL) and the selectivity index (SI) was calculated at more than 3.8 towards A549 cells. As mentioned earlier, an SI value greater or equal value of 3.0, the extracts or compounds are considered to be highly selective toward cancer cells (Mahavorasirikul et al., 2010; Ramasamy et al., 2012; Ramasamy et al., 2013). In certain cases such as for the crude extracts and essential oils, the value greater or equal to 2.0 can be considered to indicate a significant selective indices (Oliveira et al., 2015).

Spathulenol is a sesquiterpene alcohol commonly present in essential oil of many plant families. From literature, spathulenol was suggested as one of the immuno-modulatory compounds (Ziaei et al., 2011) and cytotoxic against gastric adenocarcinoma cells (AGS) with IC_{50} value of 23.0 µM (Areche et al., 2009). *β*-eudesmol is a sesquiterpene alcohol with an eudesmane skeleton known to exhibit several biological activities such as cytotoxic properties, hypotensive activity, anti-epileptic agent, insecticidal and anti-microbial agent (Kusuma et al., 2004; Bomfim et al., 2013). *β*-Eudesmol have been reported to exhibit cytotoxic effects against B16-F10, K562 and HepG2 with IC_{50} values ranging from 16.5 µg/mL to 24.6 µg/mL, respectively (Bomfim et al., 2013). Another study reported the cytotoxic effect of *β*-eudesmol on the human leukemia (HL60) cells including the molecular mechanisms involved (Li et al., 2013). The cytotoxic effect on HL60 cells was associated with apoptosis, which was characterised by the presence of DNA fragmentation, cleavage of caspase-3, caspase-9, and poly (ADP-ribose) polymerase, downregulation of *bcl-2* expression, release of cytochrome *c* from mitochondria and decrease in mitochondrial membrane potential (MMP). Hence, from the literature, one may deduce that *β*-eudesmol induced apoptosis in HL60 cells via the mitochondrial apoptotic pathway.

Dehydrocurdione is a sesquiterpenes with germacrane skeleton was first reported in 1972 by (Hikino et al., 1972). In other recent work on *C. zedoaria*, dehydrocurdione exhibited potent cytotoxic effect with IC_{50} values of 33.0 µg/mL, 21.7 µg/mL, 19.1 µg/mL and 22.7 µg/mL against MCF-7, CaSki, PC-3 and HT-29, respectively (Hamdi et al., 2014). In contrary, in this present study, dehydrocurdione showed a weak cytotoxic activity against the two lung cancer cell lines, A549 and SK-LU-1 tested. Consequently, it may be suggested that dehydrocurdione has a wide spectrum of cytotoxic activity towards various cancer cell lines.

Overall, the present study indicated that the compounds identified and isolated (spathulenol, *β*-eudesmol and dehydrocurdione) from the active fraction of rhizome of *C. zedoaria* has a dose-dependent cytotoxic effect against lung cancer cell lines. The order of potency of these compounds towards the lung cancer cell lines, A549 and SK-LU-1 can be suggested to be as follows: spathulenol > *β*-eudesmol > dehydrocurdione.

5.2 Essential Oils Analysis of *Curcuma zedoaria*

In the second part of this study, one of the objectives is to identify the chemical constituents in the rhizome essential oils of *C. zedoaria* collected from Temerloh, Pahang, Malaysia and Tawangmangu, Solo, Indonesia.

In general, essential oils can be defined as a natural, complex mixture of odorous and volatile compounds composed mainly of monoterpenes, sesquiterpenes and in addition to some other non-terpene components. Essential oil can be extracted from different parts of the aromatic plants. Several techniques can be used to extract essential oil including water or steam distillation, solvent extraction, expression under pressure, supercritical fluid and subcritical water extraction (Edris, 2007). However, the term essential oil only can be used if the sample was extracted using water known as hydro distillation or steam distillation. Essential oil is a hydrophobic liquid that tends not to

dissolve in water. Heat and light affected the colour, fragrance and chemical constituents in the oil. Some of the compounds might change to other compounds by chemical reaction due to high degree of temperature and light. Due to this reason, essential oil is commonly kept in -20ºC refrigerator and in amber vial before used for further downstream assays.

In the field of medical and cancer research, plant essential oils are reported to have ability to reduce the risk of cancer (Manosroi et al., 2006; Loizzo et al., 2007; Sharma et al., 2009). Essential oil contains great number of compounds and have no specific cellular targets. In recent years, considerable attention has been focused in the development of drugs vaccines for combating tumour cell growth, metastasis, proliferation in the associated stromal microenvironment (Aravindaram & Yang, 2010). Therefore, research on essential oils and their mechanism of action should be considered as alternative medicine, helping to prevent and subsequently to treat many diseases including cancer.

Essential oils mainly contain monoterpenes and sesquiterpenes. Monoterpenes are a class of terpenes that consist of two isoprene units and have the molecular formula of $C_{10}H_{16}$. They may be linear (acyclic) or contain rings. Biochemical modifications such as oxidation or rearrangement produce the related monoterpenoids. Monoterpenes are found in the essential oils of many plant parts including fruits, vegetables and herbs (Gould, 1997). Studies have shown that monoterpenes exert anti-tumour activities and suggest that many monoterpenes compounds are a good candidate of cancer chemo-preventive agents (Crowell, 1999). In addition, monoterpenes was reported to be effective in treating early and advanced cancer (Gould, 1997). Meanwhile, sesquiterpenes are a class of terpenes that consist of three isoprene unit with the molecular formula $C_{15}H_{24}$. As monoterpenes, sesquiterpenes also can be in the forms of acyclic or containing ring. Resent research has been reported that sesquiterpene lactones have significant anti-cancer potentials (Zhang et al., 2005; Taylor et al., 2008; Rasul et al., 2012; Gach et al., 2015; Shoaib et al., 2017).

In view of this, the dried rhizome of *C. zedoaria* from Temerloh, Pahang and Tawangmangu, Solo, Indonesia was hydrodistilled to extract their essential oils using Clevenger apparatus for 4 h. Both oils have a pungent and pleasant odour like turmeric odour. In other characteristics, Malaysian *C. zedoaria* oil has darker yellow colour while Indonesian *C. zedoaria* oil has golden yellow colour. In comparison of the yield, Indonesian *C. zedoaria* oil (0.39%) was almost three-fold higher yield as compared to Malaysian *C. zedoaria* oil (0.14%).

In the present study, through GC-FID, GC/MS and Kovat retention index (KI) analyses, twenty-one (21) components were successfully identified in Malaysian and Indonesian *C. zedoaria* oil. The results displayed that both essential oils possessed basically the same components, however their chromatographic profiles clearly showed quantitatively compositional differences. Oxygenated sesquiterpenes were the most abundant chemical group in Malaysian and Indonesian *C. zedoaria* oil (56.1% and 50.2%, respectively), followed by oxygenated monoterpenes (25.8% and 37.0%, respectively). Other classifications are sesquiterpene hydrocarbons (7.6% and 5.4%, respectively), monoterpene hydrocarbons (1.0% and 3.1%, respectively) and a non-terpene compound (4.3% in Malaysian *C. zedoaria* oil).

The chemical investigation revealed that both oils characterised by camphor, zerumbone, curzerenone, β-eudesmol and isoborneol as their main constituents. Concerning the major components in essential oil of Malaysian *C. zedoaria*, the results showed consistency with data from the previous study from India and Indonesia (Purkayastha et al., 2006; Retnowati et al., 2014). Camphor, the monoterpene compound commonly isolated from the wood of the *Cinnamomum camphora* known as *kapur* tree presented as the major compound in the essential oil of many aromatic plants. In this present study, camphor was detected abundantly in the oils, 17.6% ± 0.19 in Malaysian *C. zedoaria* oil and 19.7% ± 3.05 in Indonesian *C. zedoaria* oil. Camphor has

camphoraceous, fresh, warm-minty and ethereal odour (Chizzola, 2013). In addition, from literature, the leaf volatile extract of *Ricinus communis* which contained camphor as the major compound showed cytotoxicity to several human tumour cell lines with IC_{50} values ranging between 10 to 40 µg/mL (Darmanin et al., 2009). It may suggest that camphor might play an important role in cytotoxic activity of the oils.

The second major compound is zerumbone and followed by curzerenone. Both compounds are from ketone group (oxygenated sesquiterpenes) which commonly present in plants essential oils including Zingiberaceae family. Zerumbone was reported as a major compound in essential oil from *Zingiber zerumbet* (locally known as *lempoyang*) (Srivastava et al., 2000; Batubara et al., 2013). Meanwhile, curzerenone was reported as a predominant compound of essential oil of *Curcuma aeruginosa* from Thailand (Jarikasem et al., 2003; Theanphong et al., 2015) and from India (Jirovetz et al., 2000).

From the previous report, their main constituents showed consistency with the main components revealed in this study, namely camphor, curzerenone and curzerene. A study of the Indonesian *C. zedoaria* rhizome oil in 2014 revealed a high content of monoterpenoids (73.9%) (Retnowati et al., 2014). They reported the present of the same major compound, camphor (49.1%). In contrary, they did not detect zerumbone, curzerenone, α-cuparenol, spathulenol and β-eudesmol. Another compound, 1,8-cineole, a monoterpene which characterise the Indonesian *C. zedoaria* oil (8.4%) in this present study, was only detected at a concentration of 3.4% in the reports by Retnowati et al., (2014). The marked differences in the composition of the rhizome oil could be attributed to the source, cultivation, vegetative stage and growing season of the plant under investigation (Sivasothy et al., 2011; Sivasothy et al., 2012). Thus, the evidence of these qualitative and quantitative differences reinforces the need for establishing the chemical profile of essential oil prior to a biological assays (Döll-Boscardin et al., 2012).

In India, the rhizome oil was also verified to contain curzerenone (22.3%) as the major component, followed by 1,8-cineole (15.9%) and germacrone (9.0%) (Purkayastha et al., 2006). Similarly, other report from India which presented that the chemical constituents of the rhizome of *C. zedoaria* was dominated by curzerenone (31.6%), germacrone (10.8%) and camphor (10.3%) (Singh et al., 2013). In contrast to this, epicurzerenone and curzerene were found as the first and second highest amounts (24.2% and 10.4%) in the *C. zedoaria* oil reported from Taiwan (Mau et al., 2003). Generally, essential oils have been widely used in aromatherapy products, perfumery products, soap, lotion, massage oil and many others. Essential oils also have long been used by various cultures as a natural remedy to treat various health problems. As mentioned earlier, cancer is a major health problem worldwide with increasing number of cases every year. Due to the reason, nowadays there is increasing of interest to study the effectiveness of essential oil in cancer research by modern pharmacological assays.

5.3 Cytotoxic Properties of *Curcuma zedoaria* Essential Oils

Cytotoxic activity refers to the toxicity level of the compounds or secondary metabolite to the cells by measuring their IC_{50} value which is the concentration of those compounds inhibited the proliferation of cancer cells at 50%. Recent cytotoxic studies on essential oils have shown that essential oils extracted from many plants could elicit cytotoxicity and induce apoptosis in cancer cells (Sharma et al., 2009; Soeur et al., 2011; Manjamalai et al., 2012; Kumar et al., 2015). In the literature, several essential oils revealed the positive activity against cancer cell lines. As example the essential oil of *Cymbopogon flexuosus* (Poaceae family) inhibited various human cancer cell lines with IC_{50} values of the oil ranging from 4.2 µg/mL to 79.0 µg/mL (Sharma et al., 2009); essential oil of rosewood *Aniba rosaeodora* (Lauraceae family) have cytotoxic effect on the human epidermoid carcinoma cell line A431 and HaCaT cells (Soeur et al., 2011);

MTT assay of *Tridax procumbens* (Asteraceae family) essential oil showed cytotoxicity to be high as 70.2% of cancer cell death within 24 h (Manjamalai et al., 2012).

The essential oil of many species from Zingiberaceae family are mainly composed by monoterpenes and sesquiterpenes, which have shown different pharmacological activities such as anti-microbial (Santos et al., 2012; Chen et al., 2016), anti-oxidant (Avanço et al., 2017), anti-inflammatory (Funk et al., 2016) including anti-cancer. As an example, essential oil of *Curcuma purpurascens* (locally known as *temu tis*) with high content of oxygenated sesquiterpenes demonstrated wide spectrum of cytotoxic effects on CaSki, A549, HCT116 and HT29 cell lines (Hong et al., 2014). Other than *Curcuma*, the essential oils from *Etlingera* spp., *E. pyramidosphaera* and *E. brevilabrum* exhibited cytotoxic activity against MCF-7 (LC_{50}: 7.5 ± 0.5 mg/mL) and HL-60 (LC_{50}: 5.0 mg/mL) (Vairappan et al., 2012).

In this present study, both essential oils obtained by hydro distillation, have been investigated for their cytotoxic effect on human cancer cell lines namely breast (MCF-7 and MDA-MB-231), lung (A549 and SK-LU-1), cervical (HeLa S3 and SiHa) and lung normal cell line (MRC-5) by measuring the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye metabolism. The dose dependant manner assay tested at various concentrations of *C. zedoaria* essential oils for 24 h of incubation was conducted. The common drug used in chemotherapy treatment for cancer patients, doxorubicin was used as standard drug in the cytotoxicity assay.

In this study, the most potent *in vitro* cytotoxic activity on various cancer cell lines upon treatment with Malaysian *C. zedoaria* essential oil was exhibited by cervical cancer cell line, HeLa S3 (6.4 µg/mL ± 0.79), whereas, the IC_{50} value for Indonesian *C. zedoaria* essential oil was demonstrated slightly higher (21.6 µg/mL ± 2.55) when tested on HeLa S3. Malaysian *C. zedoaria* essential oil also showed strong cytotoxicity against MCF-7 (8.7 µg/mL), SiHa (9.8 µg/mL) and SK-LU-1 (9.9 µg/mL) cells. It was suggested that the

combination of major and minor constituents in the oil might contribute to the cytotoxic activity synergistically. Interestingly, Malaysian essential oil showed less toxicity when tested against normal cell MRC-5. The selectivity index for HeLa S3 upon treatment with Malaysian *C. zedoaria* essential oil as compared to MRC-5 is 4.0. As compared to doxorubicin, Malaysian essential oil exhibited highest cytotoxic activity against MCF-7, MDA-MB-231, SK-LU-1 and HeLa S3.

Overall, both oils showed promising potential as anti-cancer agents since their IC_{50} values were lower than 30 µg/mL against all cancer cell lines tested in the current study according to the American National Centre Institute (Oliveira et al., 2015). Based on the MTT results, it was concluded that Malaysian *C. zedoaria* oil showed more potent cytotoxic effect and selected for further assay as compared to the Indonesian *C. zedoaria* oil. In addition, cervical cell lines (HeLa S3 and SiHa) was selected out of six cell lines investigated in this present study for further investigation due to the promising results shown in the preliminary cytotoxic assay.

Live dead assay was performed to confirm the cytotoxic effects of Malaysian *C. zedoaria.* From MTT assay of 6 h of treatment, the IC_{50} were observed at 25.0 µg/mL for HeLa S3 cells and 30.0 µg/mL for SiHa cells. The cell death of HeLa S3 and SiHa cell lines was observed after treatment with IC_{50} values for 6 h. Upon treatment with Malaysian *C. zedoaria* oil, the cell viability of HeLa S3 was decreased from 98.6% to 63.5%. Meanwhile, cell viability of SiHa cells decreased approximately as much as 29.5 % as compared to the untreated cells. From here, this result confirmed that Malaysian *C. zedoaria* oil can annihilate cervical cancer cells as early as 6 h upon treatments.

5.4 Apoptotic Properties of Malaysian *Curcuma zedoaria* Essential Oil

Pattern of cell death have been divided into apoptosis, which is one of the programmed cell deaths executed by specific proteases, the caspases and accidental necrosis. The concept of apoptosis represent the major mechanism by which cancer cells are eliminated but other mechanism of cell death such as caspase independent apoptosis and others have also to be considered as cellular response to anti-cancer therapy (Brown & Wilson, 2003). Apoptosis can be characterised by the typical morphological and biochemical hallmarks including cell shrinkage, nuclear DNA fragmentation and membrane blebbing (Hengartner, 2000). Induction of apoptosis in cancer cells has been developed as an indicator for the ability of naturally derived active compounds to treat cancer (Shu et al., 2010). Pharmacological agents that induce apoptosis might be effective against many cancers by inducing death in cancer cells (Hu & Kavanagh, 2003). In this study, considering the potent cytotoxicity MTT results of Malaysian *C. zedoaria* oil, apoptosis assays were conducted to determine if the cytotoxic effect of Malaysian *C. zedoaria* oil is associated with induction of apoptosis in the cervical cancer cell lines, HeLa S3 and SiHa. Subsequently, Annexin-V-FITC/PI double staining flow cytometry assay and Caspase 3/7 assay were performed.

Annexin-V-FITC/PI assay was used to detect the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell (Vermes et al., 1995). By staining the cells with combination of FITC-conjugated annexin-V and propidium iodide (PI), it is possible to detect non-apoptotic cells (lower left quadrant), early apoptotic cells (lower right), late apoptotic (upper right) and necrotic cells (upper left). Percentage of apoptotic cells in HeLa S3 cells upon treatment with various concentrations of Malaysian *C. zedoaria* oil for 24 h showed significant concentration dependent manner. These results confirmed that Malaysian *C. zedoaria* oil induce translocation of

phosphatidylserine leading to apoptosis indicating that extensive damage of cell membrane has occurred in HeLa S3 cells with up to 23.9% apoptotic cells at 50 µg/mL. However, SiHa cells only showed a percentage of apoptotic cells at 9.4 % ± 1.6 (early + late apoptosis population) at the maximum concentration used in this assay (50 µg/mL).

Caspase-3 and caspase-7 are executioner caspases that are involved in the activation of caspase-dependent apoptosis. They can be activated by initiator caspases from either the intrinsic or extrinsic apoptotic pathways, eventually resulting in controlled cellular degradation during apoptosis. Measurement of caspase-3/7 activity using Caspase-Glo® 3/7 Assay kit uses caspase substrate which results in the release of luminescent signal proportional to activated caspase activity. From the results, it was observable that treatment with 40 and 50 µg/mL of Malaysian *C. zedoaria* oil causes increased caspase activity, which translates to higher rate of apoptosis in HeLa S3. This matches the findings from the flow cytometry assay (Figure 4.20), which analyses cell death events following the activation of caspases, such as the translocation of phosphatidylserine to the external surface of the plasma membrane and degradation of cell membranes. Meanwhile, similar results were not achieved in SiHa as are evident from both caspase-3/7 activity assay and flow cytometry assay, suggesting that the Malaysian *C. zedoaria* oil is not as effective in inducing apoptosis when used on this cell line.

CHAPTER 6: CONCLUSION

Herbal consumption among Asian people mainly contribute to general health and sometimes as an alternative to modern medicine to treat diseases including cancer. Zingiberaceae species particularly the genus *Curcuma* plays an important role in Malay traditional medicine which are inherited from our ancestors. In Malaysia and Indonesia, *C. zedoaria* is commonly used as one of the ingredients in *jamu* (traditional tonic). Consumption of *jamu* helps in the maintenance of general health, refreshes the body and as an alternative medicine to treat various diseases including cancer.

Phytochemistry and pharmacological studies reported in Chapter 2 (Literature Review) support the therapeutic values of *Curcuma* spp. In this present study, *Curcuma zedoaria* (white turmeric) locally known as *temu putih*, was selected to be investigated for its cytotoxic and apoptotic potential against cancer cell lines. Overall, the study was divided into two parts. Part A is the characterization of compounds from *C. zedoaria* from Indonesia using bioassay-guided isolation and Part B involved the essential oils analysis of *C. zedoaria* from Malaysian and Indonesian and characterization of their cytotoxic and apoptosis abilities.

In Part A, the most active fraction; hexane soluble fraction (HSF) was subjected to GC-FID and GC/MS analysis for screening of the phytochemical compounds. It was found that HSF was dominated by terpenoid compounds, that may be responsible for the cytotoxic activity. Bioassay-guided isolation led to the isolation and characterisation of four compounds; spathulenol, *β*-eudesmol, dehydrocurdione and curcumenone. This is the first report on the cytotoxic activity against the two lung cancer cell lines; A549 and SK-LU-1 for all three compounds; spathulenol, *β*-eudesmol and dehydrocurdione with spathulenol being the most potent ($IC_{50:}$ 11.0 μg/mL for A549 and 22.3 μg/mL for SK-LU-1, respectively). *β*-Eudesmol showed similar IC_{50} value of 12.2 μg/mL and 22.9

μg/mL, respectively while dehydrocurdione showed moderate activity at IC_{50} value of 54.6 μg/mL and 44.3 μg/mL, respectively. In another study, dehydrocurdione was reported to possess cytotoxic activity against breast (MCF-7), cervical (CaSki), prostate (PC-3) and colon (HT-29) cancer cell lines (Hamdi et al., 2014).

In Part B, the analysis of *C. zedoaria* rhizome oils from Malaysia and Indonesia were carried out and identification of the chemical constituents revealed the presence of twenty-one (21) components. Our findings showed that the essential oils were mostly dominated by oxygenated sesquiterpenes and displayed the same major components; camphor, curzerenone and curzerene. However, there are previous studies on the chemical constituents of *C. zedoaria* rhizome oil from Malaysia and India that reported 1, 8- cineole as the major compound with composition more than 15.0% (Abdullah et al., 2002; Singh et al., 2002; Purkayastha et al., 2002). In this present study, both the Malaysian and Indonesian oils contain 1, 8- cineole as a minor component with only 1.1% and 8.4%, respectively. This study suggested that geographical locations may play an important role in the differences of compound compositions in the oils.

Malaysian *C. zedoaria* oil also exhibited potent cytotoxic activity against two types of cervical cancer cell lines, HeLa S3 with IC_{50} values of 6.4 μg/mL and 9.8 μg/mL for SiHa, while the IC_{50} values for Indonesian *C. zedoaria* oil are 21.6 μg/mL and 11.5 μg/mL, respectively. Additionally, another study of *Curcuma* spp., *C. purpurascens* has also shown that essential oils containing camphor and curzerene possess significant cytotoxic activity (Hong et al., 2014). This present study was further with apoptosis assays such as Annexin-V-FITC/PI and Caspase-3/7. The results showed significant apoptosis pathway was induced on HeLa S3 cells upon treatment with the Malaysian *C. zedoaria* oil.

Even though there are previous studies of *C. zedoaria* worldwide, the reports on phytochemical and biological activities are still needed due to the promising potential of

this species as herbal and drug medicine. Future work of *C. zedoaria* in cancer research could involve multiple analysis of killing mechanisms on the active pure compounds, extracts and essential oils such as caspase independent pathway study and autophagy. In addition, phytochemical studies of different sample parts such as the aerial part of *C. zedoaria*, instead of the rhizome, could be considered.

In conclusion, the present findings support the traditional uses of the rhizome of *C. zedoaria* as a potential preventive and therapeutic agent for cancer which require extensive *in vitro* and *in vivo* experiments to fully understand its clinical anti-cancer benefits.

REFERENCES

Abas, F., Lajis, N.H., Shaari, K., Israf, D.A., Stanslas, J., Yusuf, U.K., and Raof, S.M. (2005). A labdane diterpene glucoside from the rhizomes of *Curcuma mangga*. *Journal of Natural Products, 68*(7), 1090-1093.

Abdullah, F., Malek, S.N.A., Ali, N.A., and Ibrahim, H. (2002). *The essential oil of Curcuma zedoaria*. Paper presented at the Natural Products R & D, Transforming Rainforest Industries: Proceedings of the 18th Seminar of the Malaysian Natural Products Society, Sabah, Malaysia.

Adams, R.P. (2001). *Identification of Essential Oil Components by Gas Chromatography / Quadrupole Mass Spectroscopy* (3rd ed.) (pp. 10-40). USA: Allured Publishing Corporation.

Al-Reza, S.M., Rahman, A., Sattar, M.A., Rahman, M.O., and Fida, H.M. (2010). Essential oil composition and anti-oxidant activities of *Curcuma aromatica* Salisb. *Food and Chemical Toxicology, 48*(6), 1757-1760.

Alan Sheeja, D.B., and Nair, M.S. (2012). Phytochemical constituents of *Curcuma amada*. *Biochemical Systematics and Ecology, 44*, 264-266.

Angel, G.R., Menon, N., Vimala, B., and Nambisan, B. (2014). Essential oil composition of eight starchy *Curcuma* species. *Industrial Crops and Products, 60*, 233-238.

Apisariyakul, A., Vanittanakom, N., and Buddhasukh, D. (1995). Anti-fungal activity of turmeric oil extracted from *Curcuma longa* (Zingiberaceae). *Journal of Ethnopharmacology, 49*(3), 163-169.

Aravindaram, K., and Yang, N.S. (2010). Anti-inflammatory plant natural products for cancer therapy. *Planta Medica, 76*(11), 1103-1117.

Areche, C., Schmeda-Hirschmann, G., Theoduloz, C., and Rodríguez, J.A. (2009). Gastroprotective effect and cytotoxicity of abietane diterpenes from the Chilean Lamiaceae *Sphacele chamaedryoides* (Balbis) Briq. *Journal of Pharmacy and Pharmacology, 61*(12), 1689-1697.

Avanço, G.B., Ferreira, F.D., Bomfim, N.S., Santos, P.A. d. S. R. d., Peralta, R.M., Brugnari, T., Mallmann, C.A., Abreu Filho, B.A. d., Mikcha, J.M.G., and Machinski Jr, M. (2017). *Curcuma longa* L. essential oil composition, anti-oxidant effect and effect on *Fusarium verticillioides* and fumonisin production. *Food Control, 73,* Part B, 806-813.

Awang, K., Azmi, M.N., Aun, L.I., Aziz, A.N., Ibrahim, H., and Nagoor, N.H. (2010). The apoptotic effect of 1's-1'-acetoxychavicol acetate from *Alpinia conchigera* on human cancer cells. *Molecules, 15*(11), 8048-8059.

Bamba, Y., Yun, Y.S., Kunugi, A., and Inoue, H. (2011). Compounds isolated from *Curcuma aromatica* Salisb. inhibit human P450 enzymes. *Journal of Natural Medicines, 65*(3-4), 583-587.

Batubara, I., Suparto, I.H., Sadiah, S., Matsuoka, R., and Mitsunaga, T. (2013). Effect of *Zingiber zerumbet* essential oils and zerumbone inhalation on body weight of Sprague Dawley rat. *Pakistan Journal of Biological Sciences, 16*(19), 1028-1033.

Bauer, K., Dorothea G., and Horst S. (2001). *Common Fragrance and Flavor Materials* (4th ed.) (pp. 7-165). Germany: Wiley-VCH.

Bayala, B., Bassole, I.H.N., Scifo, R., Gnoula, C., Morel, L., Lobaccaro, J.M.A., and Simpore, J. (2014). Anti-cancer activity of essential oils and their chemical components - a review. *American Journal of Cancer Research, 4*(6), 591-607.

Bhardwaj, R.S., Bhardwaj, K.S., Ranjeet, D. and Ganesh, N. (2011). *Curcuma longa* leaves exhibits a potential anti-oxidant, anti-bacterial and immuno-modulating properties. *International Journal of Phytomedicine, 3*(2), 270-278.

Bold, R.J., Termuhlen, P.M., and McConkey, D.J. (1997). Apoptosis, cancer and cancer therapy. *Surgical Oncology, 6*(3), 133-142.

Bomfim, D.S., Ferraz, R.P., Carvalho, N.C., Soares, M.B., Pinheiro, M.L., Costa, E.V., and Bezerra, D.P. (2013). Eudesmol isomers induce caspase-mediated apoptosis in human hepatocellular carcinoma HepG2 cells. *Basic & Clinical Pharmacology & Toxicology, 113*(5), 300-306.

Boujrad, H., Gubkina, O., Robert, N., Krantic, S., and Susin, S.A. (2007). AIF-mediated programmed necrosis: a highly regulated way to die. *Cell Cycle, 6*(21), 2612-2619.

Bowles, E.J. (2004). *The Chemistry of Aromatherapeutic Oils* (3rd ed.) (pp. 6-179). Australia: Allen & Unwin Academic.

Brown, J.M., and Wilson, G. (2003). Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us?. *Cancer Biology & Therapy, 2*(5), 477-490.

Burkill, I.H. (2002). *A Dictionary of the Economic Products of the Malay Peninsula* (4th ed.) (pp. 871-999). Malaysia: Ministry of Agriculture Malaysia.

Carvalho, F., Vassão, R., Nicoletti, M., and Maria, D. (2010). Effect of *Curcuma zedoaria* crude extract against tumor progression and immuno-modulation. *Journal of Venomous Animals and Toxins Including Tropical Diseases, 16*, 324-341.

Chaiwongsa, R., Ongchai, S., Boonsing, P., Kongtawelert, P., Panthong, A., and Reutrakul, V. (2013). Active compound of *Zingiber Cassumunar* Roxb. down-regulates the expression of genes involved in joint erosion in a human synovial fibroblast cell line. *African Journal of Traditional, Complementary and Alternative Medicines, 10*(1), 40-48.

Chen, C.C., Chen, Y., Hsi, Y.T., Chang, C.S., Huang, L.F., Ho, C.T., Way, T.D., and Kao, J.Y. (2013). Chemical constituents and anti-cancer activity of *Curcuma zedoaria* Roscoe essential oil against non-small cell lung carcinoma cells *in vitro* and *in vivo*. *Journal of Agriculture and Food Chemistry, 61*(47), 11418-11427.

Chen, W., Lu, Y., Gao, M., Wu, J., Wang, A., and Shi, R. (2011). Anti-angiogenesis effect of essential oil from *Curcuma zedoaria in vitro* and *in vivo*. *Journal of Ethnopharmacology, 133*(1), 220-226.

Chen, Z., He, B., Zhou, J., He, D., Deng, J., and Zeng, R. (2016). Chemical compositions and anti-bacterial activities of essential oils extracted from *Alpinia guilinensis* against selected foodborne pathogens. *Industrial Crops and Products, 83*, 607-613.

Chinnaiyan, A.M. (1999). The apoptosome: heart and soul of the cell death machine. *Neoplasia, 1*(1), 5-15.

Chizzola, R. (2013). Regular monoterpenes and sesquiterpenes (Essential Oils). In Ramawat, G.K. & Mérillon, J.M. (Eds.), *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes* (pp. 2973-3008). Berlin, Heidelberg: Springer Berlin Heidelberg.

Cho, W.Y., and Kim, S.J. (2012). Anti-oxidative actions of *Curcuma zedoaria* extract with inhibition of inducible nitric oxide synthase (iNOS) induction and lipid peroxidation. *Journal of Medicinal Plants Research, 6*(22), 3837-3844.

Choudhury, S.N., Rabha, L.C., Kanjilal, P.B., Ghosh, A.C., and Leclercq, P.A. (1996). Essential oil of *Curcuma amada* Roxb. from Northeastern India. *Journal of Essential Oil Research, 8*(1), 79-80.

Crowell, P.L. (1999). Prevention and therapy of cancer by dietary monoterpenes. *The Journal of Nutrition, 129*(3), 775.

da Rocha, A.B., Lopes, R.M., and Schwartsmann, G. (2001). Natural products in anti-cancer therapy. *Current Opinion in Pharmacology, 1*(4), 364-369.

Darmanin, S., Wismayer, P.S., Camilleri Podesta, M.T., Micallef, M.J., and Buhagiar, J.A. (2009). An extract from *Ricinus communis* L. leaves possesses cytotoxic properties and induces apoptosis in SK-MEL-28 human melanoma cells. *Natural Product Research, 23*(6), 561-571.

Das, K., and Rahman, M.A. (2012). Analgesic and anti-microbial activities of *Curcuma zedoaria*. *Chemical Analysis, 9*, 20.

Degterev, A., Boyce, M., and Yuan, J. (2003). A decade of caspases. *Oncogene, 22*(53), 8543-8567.

Diastuti, H., Syah, Y.M., Juliawaty, L.D., and Singgih, M. (2014). Antibacterial activity of germacrane type sesquiterpenes from *Curcuma heyneana* rhizomes. *Indonesian Journal of Chemistry, 14*(1), 32-36.

Döll-Boscardin, P.M., Sartoratto, A., Sales Maia, B.H.L.d.N., Padilha de Paula, J., Nakashima, T., Farago, P.V., and Kanunfre, C.C. (2012). *In vitro* cytotoxic potential of essential oils of *Eucalyptus benthamii* and its related terpenes on tumor cell lines. *Evidence-Based Complementary and Alternative Medicine, 2012*, 8.

Edris, A.E. (2007). Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. *Phytotherapy Research, 21*(4), 308-323.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology, 35*(4), 495-516.

Eng-Chong, T., Yean-Kee, L., Chin-Fei, C., Choon-Han, H., Sher-Ming, W., Li-Ping, C. T., Gen-Teck, F., Khalid, N., Abd Rahman, N., Karsani, S.A., Othman, S., Othman, R., and Yusof, R. (2012). *Boesenbergia rotunda*: from ethnomedicine to drug discovery. *Evidence-Based Complementary and Alternative Medicine, 2012*, 25.

Eun, S., Choi, I., and Shim, S.H. (2010). A new sesquiterpenoid from the rhizome of *Curcuma zedoaria*. *Bulletin of the Korean Chemical Society, 31*(5), 1387-1388.

Ferreira, F.D., Mossini, S.A.G., Ferreira, F.M.D., Arroteia, C.C., da Costa, C.L., Nakamura, C.V., and Machinski Junior, M. (2013). The inhibitory effects of *Curcuma longa* L. essential oil and curcumin on *Aspergillus flavus* Link growth and morphology. *The Scientific World Journal, 2013*, 6.

Firman, K., Kinoshita, T., Itai, A., and Sankawa, U. (1988). Terpenoids from *Curcuma heyneana*. *Phytochemistry, 27*(12), 3887-3891.

Fulda, S. (2009). Caspase-8 in cancer biology and therapy. *Cancer Letters, 281*(2), 128-133.

Fulda, S., and Debatin, K.M. (2004). Targeting apoptosis pathways in cancer therapy. *Current Cancer Drug Targets, 4*(7), 569-576.

Fulda, S., and Debatin, K.M. (2006). Extrinsic versus intrinsic apoptosis pathways in anti-cancer chemotherapy. *Oncogene, 25*(34), 4798-4811.

Funk, J.L., Frye, J.B., Oyarzo, J.N., Chen, J., Zhang, H., and Timmermann, B.N. (2016). Anti-inflammatory effects of the essential oils of ginger *(Zingiber officinale* Roscoe) in experimental rheumatoid arthritis. *Pharma Nutrition, 4*(3), 123-131.

Gach, K., Długosz, A., and Janecka, A. (2015). The role of oxidative stress in anti-cancer activity of sesquiterpene lactones. *Naunyn-Schmiedeberg's Archives of Pharmacology, 388*(5), 477-486.

Gao, X.F., Li, Q.L., Li, H.L., Zhang, H.Y., Su, J.Y., Wang, B., Liu, P., and Zhang, A.Q. (2014). Extracts from *Curcuma zedoaria* inhibit proliferation of human breast cancer cell MDA-MB-231 *in vitro*. *Evidence-Based Complementary and Alternative Medicine, 2014,* 730678.

Garg, S.N., Naquvi, A.A., Bansal, R.P., Bahl, J.R., and Kumar, S. (2005). Chemical composition of the essential oil from the leaves of *Curcuma zedoaria* Rosc. of Indian origin. *Journal of Essential Oil Research, 17*(1), 29-31.

Golstein, P., and Kroemer, G. (2007). Cell death by necrosis: towards a molecular definition. *Trends in Biochemical Sciences, 32*(1), 37-43.

Gosslau, A., and Chen, K.Y. (2004). Nutraceuticals, apoptosis and disease prevention. *Nutrition, 20*(1), 95-102.

Gould, M.N. (1997). Cancer chemoprevention and therapy by monoterpenes. *Environmental Health Perspectives, 105*(Suppl 4), 977.

Green, D.R., and Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science, 305*(5684), 626-629.

Gupta, A., Mahajan, S., and Sharma, R. (2015). Evaluation of anti-microbial activity of *Curcuma longa* rhizome extract against *Staphylococcus aureus*. *Biotechnology Reports, 6*, 51-55.

Haldar, S., Chintapalli, J., and Croce, C. M. (1996). Taxol induces *bcl-2* phosphorylation and death of prostate cancer cells. *Cancer Research, 56*(6), 1253.

Hamdi, O.A.A, Rahman, S.N.S.A, Awang, K., Wahab, N.A, Looi, C.Y., Thomas, N.F., and Malek, S.N.A (2014). Cytotoxic constituents from the rhizomes of *Curcuma zedoaria*. *The Scientific World Journal*, 321943.

Hamdi, O.A.A., Ye, L.J., and Alfarizal, M.N. (2015). Neuroprotective and antioxidant constituents from *Curcuma zedoaria* rhizomes. *Records of Natural Products, 9*(3), 349-355.

Hartati, R., Suganda, A.G., and Fidrianny, I. (2014). Botanical, phytochemical and pharmacological properties of *Hedychium* (Zingiberaceae) – a review. *Procedia Chemistry, 13*, 150-163.

Hasima, N., Aun, L.I.L., Azmi, M.N., Aziz, A.N., Thirthagiri, E., Ibrahim, H., and Awang, K. (2010). 1′S-1′-Acetoxyeugenol acetate: a new chemotherapeutic natural compound against MCF-7 human breast cancer cells. *Phytomedicine, 17*(12), 935-939.

Hengartner, M.O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776.

Hikino, H., Konno, C., and Takemoto, T. (1972). Structure of dehydrocurdione, a sesquiterpenoid of *Curcuma zedoaria*. *Chemical & Pharmaceutical Bulletin, 20*(5), 987-989.

Hill, M.M., Adrain, C., Duriez, P.J., Creagh, E.M., and Martin, S.J. (2004). Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. *The EMBO Journal, 23*(10), 2134-2145.

Hisashi, K., Tetsuya, Y., and Akihiro, T. (1998). Essential oil constituents from Japanese and Indian *Curcuma aromatica* rhizomes. *Planta Medica, 64*(4), 380-381.

Holttum, R.E. (1950). *The Zingiberaceae of the Malay Peninsula*. Singapore: The Gardens' Bulletin.

Hong, C.H., Kim, Y., and Lee, S.K. (2001). Sesquiterpenoids from the rhizome of *Curcuma zedoaria*. *Archives of Pharmacal Research, 24*(5), 424-426.

Hong, S.L., Lee, G.S., Rahman, S.N.S.A., Hamdi, O.A.A., Awang, K., Nugroho, N.A, and Malek, S.N.A (2014). Essential oil content of the rhizome of *Curcuma purpurascens* Bl. (temu tis) and its antiproliferative effect on selected human carcinoma cell lines. *The Scientific World Journal, 2014*, 7.

Hong, G.W., Hong, S.L., Lee, G.S., Yaacob, H., and Malek, S.N.A. (2016). Non-aqueous extracts of *Curcuma mangga* rhizomes induced cell death in human colorectal adenocarcinoma cell line (HT29) via induction of apoptosis and cell cycle arrest at G0/G1 phase. *Asian Pacific Journal of Tropical Medicine, 9*(1), 8-18.

Hossain, C.F., Al-Amin, M., Sayem, A.S.M., Siragee, I.H., Tunan, A.M., Hassan, F., Kabir, M.M., and Sultana, G.N.N. (2015). Antinociceptive principle from *Curcuma aeruginosa*. *BMC Complementary and Alternative Medicine, 15*(1), 1-7.

Hu, W., and Kavanagh, J.J. (2003). Anti-cancer therapy targeting the apoptotic pathway. *The Lancet Oncology, 4*(12), 721-729.

Huang, W., Zhang, P., Jin, Y.C., Shi, Q., Cheng, Y.Y., Qu, H.B., and Ma, Z.J. (2008). Cytotoxic diterpenes from the root tuber of *Curcuma wenyujin*. *Helvetica Chimica Acta, 91*(5), 944-950.

Hussain, A.I., Anwar, F., Chatha, S.A.S., Jabbar, A., Mahboob, S., and Nigam, P.S. (2010). *Rosmarinus officinalis* essential oil: anti-proliferative, anti-oxidant and anti-bacterial activities. *Brazilian Journal of Microbiology, 41*(4), 1070-1078.

Ibrahim, H., Syamsir, D.R., Aziz, A.N., Awang, K., Azah, M.A.N., Mastura, M., and Rasadah, M.A. (2009). Essential oils of *Elettariopsis curtisii* (Zingiberaceae) and their anti-microbial activities. *Journal of Essential Oil Research, 21*(5), 464-466.

Jaju, S., Indurwade, N., Sakarkar, D., Fuloria, N., and Al, M. (2009). Isolation of galangogalloside from rhizomes of *Alpinia galanga*. *International Journal of Green Pharmacy (IJGP), 3*(2).

Jamal, A., Javed, K., Aslam, M., and Jafri, M.A. (2006). Gastroprotective effect of cardamom, *Elettaria cardamomum* Maton. fruits in rats. *Journal of Ethnopharmacology, 103*(2), 149-153.

Jambunathan, S., Bangarusamy, D., Padma, P.R., and Sundaravadivelu, S. (2014). Cytotoxic activity of the methanolic extract of leaves and rhizomes of *Curcuma amada* Roxb against breast cancer cell lines. *Asian Pacific Journal of Tropical Medicine, 7*, S405-S409.

Jarikasem, S., Thubthimthed, S., Chawananoraseth, K., and Suntorntanasat, T. (2003). *Essential oils from three Curcuma species collected in Thailand.* Paper presented at the III WOCMAP Congress on Medicinal and Aromatic Plants (Volume 1): Bioprospecting and Ethnopharmacology, 675.

Jantan, I., Saputri, F.C., Qaisar, M.N., and Buang, F. (2012). Correlation between chemical composition of *Curcuma domestica* and *Curcuma xanthorrhiz*a and their antioxidant effect on human low-density lipoprotein oxidation. *Evidence-Based Complementary and Alternative Medicine, 2012*, 10.

Jantan, I., Ahmad, A.S., Ali, N.A.M., Ahmad, A.R., and Ibrahim, H. (1999). Chemical composition of the rhizome oils of four *Curcuma* species from Malaysia. *Journal of Essential Oil Research, 11*(6), 719-723.

Jantan, I., Yassin, M.S.M., Chin, C.B., Chen, L.L., and Sim, N.L. (2003). Antifungal activity of the essential oils of nine Zingiberaceae species. *Pharmaceutical Biology, 41*(5), 392-397.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., and Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians, 61*(2), 69-90.

Jirovetz, L., Buchbauer, G., Puschmann, C., Shafi, M.P., and Geetha Nambiar, M. (2000). Essential oil analysis of *Curcuma aeruginosa* Roxb. leaves from South India. *Journal of Essential Oil Research, 12*(1), 47-49.

Jirovetz, L., Buchbauer, G., Ngassoum, M.B., and Geissler, M. (2002). Aroma compound analysis of *Piper nigrum* and *Piper guineense* essential oils from Cameroon using solid-phase microextraction–gas chromatography, solid-phase microextraction–gas chromatography–mass spectrometry and olfactometry. *Journal of Chromatography A, 976*(1–2), 265-275.

Kaminski, B. M., Steinhilber, D., Stein, J. M., and Ulrich, S. (2012). Phytochemicals resveratrol and sulforaphane as potential agents for enhancing the anti-tumor

activities of conventional cancer therapies. *Current Pharmaceutical Biotechnology, 13*(1), 137-146.

Kaufmann, S.H., and Hengartner, M.O. (2001). Programmed cell death: alive and well in the new millennium. *Trends in Cell Biology, 11*(12), 526-534.

Kaushik, M., and Jalalpure, S.S. (2011). Anti-inflammatory efficacy of *Curcuma zedoaria* Rosc root extracts. *Asian Journal of Pharmaceutical and Clinical Research, 4*(3), 90-92.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer, 26*(4), 239-257.

Kress, W.J., Prince, L.M., and Williams, K.J. (2002). The phylogeny and a new classification of the gingers (Zingiberaceae): evidence from molecular data. *American Journal of Botany, 89*(10), 1682-1696.

Kumar, D., Sukapaka, M., Babu, G.D., and Padwad, Y. (2015). Chemical composition and *in vitro* cytotoxicity of essential oils from leaves and flowers of *Callistemon citrinus* from Western Himalayas. *PLoS One, 10*(8), 0133823.

Kuroyanagi, M., Ueno, A., Ujiie, K., and Sato, S. (1987). Structures of sesquiterpenes from *Curcuma aromatica* Salisb. *Chemical and Pharmaceutical Bulletin, 35*(1), 53-59.

Kusuma, I. W., Ogawa, T., Itoh, K., and Tachibana, S. (2004). Isolation and identification of an antifungal sesquiterpene alcohol from Amboyna wood. *Pakistan Journal of Biological Sciences, 7*(10), 1735-1740.

Lai, E.Y.C., Chyau, C.C., Mau, J.L., Chen, C.C., Lai, Y.J., Shih, C.F., and Lin, L.L. (2004). Anti-microbial activity and cytotoxicity of the essential oil of *Curcuma zedoaria*. *The American Journal of Chinese Medicine, 32*(02), 281-290.

Lakshmi, S., Padmaja, G., and Remani, P. (2011). Anti-tumour effects of isocurcumenol isolated from *Curcuma zedoaria* rhizomes on human and murine cancer cells. *International Journal of Medicinal Chemistry, 2011, Article ID 253962.*

Larsen, K., Friis, I., and Balslev, H. (2005). Distribution patterns and diversity centres of Zingiberaceae in SE Asia. *Biologiske Skrifter, 55*, 219-228.

Larsen, K., Ibrahim, H., Khaw, S., and Saw, L. (1999). *Gingers of Peninsular Malaysia and Singapore* (pp. 10-52). Borneo Malaysia: Natural History Publications.

Leland, J.C., Ara K., Peter B.K., Sara W., James, A.D., and Harry, L.B. (2006). *Natural products from plants* (2nd ed.) (pp. 15-36). Florida, USA: Taylor and Francis Group.

Levenson, A.S., and Jordan, V.C. (1997). MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Research, 57*(15), 3071-3078.

Li, Y., Li, T., Miao, C., Li, J., Xiao, W., and Ma, E. (2013). Beta-eudesmol induces JNK-dependent apoptosis through the mitochondrial pathway in HL60 cells. *Phytotheraphy Research, 27*(3), 338-343.

Lim, G.C.C. (2002). Overview of cancer in Malaysia. *Japanese Journal of Clinical Oncology, 32*(suppl 1), S37-S42.

Lin, S.C., Teng, C.W., Lin, C.C., Lin, Y.H., and Supriyatna, S. (1996). Protective and therapeutic effect of the Indonesian medicinal herb *Curcuma xanthorrhiza* on *β*-D-galactosamine-induced liver damage. *Phytotherapy Research, 10*(2), 131-135.

Liu, R.H. (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American Journal of Clinical Nutrition, 78*(3 Suppl), 517-520.

Lobo, R., Prabhu, K.S., Shirwaikar, A., and Shirwaikar, A. (2009). *Curcuma zedoaria* Rosc. (white turmeric): a review of its chemical, pharmacological and ethnomedicinal properties. *Journal of Pharmacy and Pharmacology, 61*(1), 13-21.

Locksley, R.M., Killeen, N., and Lenardo, M.J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell, 104*(4), 487-501.

Loizzo, M.R., Tundis, R., Menichini, F., Saab, A.M., Statti, G.A., and Menichini, F. (2007). Cytotoxic activity of essential oils from Labiatae and Lauraceae families against *in vitro* human tumor models. *Anti-cancer research, 27*(5A), 3293-3299.

López-Terrada, D., Cheung, S.W., Finegold, M.J., and Knowles, B.B. (2009). Hep-G2 is a hepatoblastoma-derived cell line. *Human pathology, 40*(10), 1512-1515.

Lorenzo, H.K., and Susin, S.A. (2007). Therapeutic potential of AIF-mediated caspase-independent programmed cell death. *Drug Resistance Updates, 10*(6), 235-255.

Los, M., Mozoluk, M., Ferrari, D., Stepczynska, A., Stroh, C., Renz, A., Herceg, Z., Wang, Z.Q., and Schulze-Osthoff, K. (2002). Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. *Molecular Biology of the Cell, 13*(3), 978-988.

Lou, Y., Zhao, F., Wu, Z., Peng, K.F., Wei, X.C., Chen, L.X., and Qiu, F. (2009). Germacrane-type sesquiterpenes from *Curcuma wenyujin*. *Helvetica Chimica Acta, 92*(8), 1665-1672.

Ma, J., Wang, Y., Liu, Y., Gao, S., Ding, L., Zhao, F., Chen, L., and Qiu, F. (2015). Cadinane sesquiterpenes from *Curcuma phaeocaulis* with their inhibitory activities on nitric oxide production in RAW 264.7 cells. *Fitoterapia, 103*, 90-96.

Ma, Z.J., Meng, Z.K., and Zhang, P. (2009). Chemical constituents from the radix of *Curcuma wenyujin*. *Fitoterapia, 80*(6), 374-376.

Madhu, S.K., Shaukath, A.K., and Vijayan, V.A. (2010). Efficacy of bioactive compounds from *Curcuma aromatica* against mosquito larvae. *Acta Tropica, 113*(1), 7-11.

Mahavorasirikul, W., Viyanant, V., Chaijaroenkul, W., Itharat, A., and Na-Bangchang, K. (2010). Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells *in vitro*. *BMC Complementary and Alternative Medicine, 10*(1), 1-8.

Majno, G., and Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *The American Journal of Pathology, 146*(1), 3-15.

Makabe, H., Maru, N., Kuwabara, A., Kamo, T., and Hirota, M. (2006). Anti-inflammatory sesquiterpenes from *Curcuma zedoaria*. *Natural Product Research, 20*(7), 680-685.

Makin, G., and Dive, C. (2001). Apoptosis and cancer chemotherapy. *Trends in Cell Biology, 11*(11), S22-26.

Malek, S.N., Seng, C.K., Zakaria, Z., Ali, N.A., Ibrahim, H., and Jalil, M.N. (2006). The essential oil of *Curcuma inodora* aff. Blatter from Malaysia. *Journal of Essential Oil Research, 18*(3), 281-283.

Malek, S.N.A., Lee, G.S., Hong, S.L., Yaacob, H., Wahab, N.A., Faizal Weber, J.F., and Shah, S.A.A. (2011). Phytochemical and cytotoxic investigations of *Curcuma mangga* rhizomes. *Molecules, 16*(6), 4539.

Manjamalai, A., Kumar, M.J., and Grace, V.M. (2012). Essential oil of *Tridax procumbens* L induces apoptosis and suppresses angiogenesis and lung metastasis of the B16F-10 cell line in C57BL/6 mice. *Asian Pacific Journal Cancer Prevention, 13*(11), 5887-5895.

Manosroi, J., Dhumtanom, P., and Manosroi, A. (2006). Anti-proliferative activity of essential oil extracted from Thai medicinal plants on KB and P388 cell lines. *Cancer Letters, 235*(1), 114-120.

Matsuda, H., Morikawa, T., Ninomiya, K., and Yoshikawa, M. (2001). Hepatoprotective constituents from Zedoariae Rhizoma: absolute stereostructures of three new carabrane-type sesquiterpenes, curcumenolactones A, B, and C. *Bioorganic & Medicinal Chemistry, 9*(4), 909-916.

Mau, J.L., Lai, E.Y.C., Wang, N.P., Chen, C.C., Chang, C.H., and Chyau, C.C. (2003). Composition and anti-oxidant activity of the essential oil from *Curcuma zedoaria*. *Food Chemistry, 82*(4), 583-591.

Melino, G., Knight, R.A., and Nicotera, P. (2005). How many ways to die? How many different models of cell death?. *Cell Death Differ, 12*(2), 1457-1462.

Mohammad, P., Nosratollah, Z., Mohammad, R., Abbas, A., and Javad, R. (2010). The inhibitory effect of *Curcuma longa* extract on telomerase activity in A549 lung cancer cell line. *African Journal of Biotechnology, 9*(6), 912-919.

Moretti, L., Yang, E.S., Kim, K.W., and Lu, B. (2007). Autophagy signaling in cancer and its potential as novel target to improve anti-cancer therapy. *Drug Resistance Updates, 10*(4-5), 135-143.

Mustafa, A., Ali, M., and Khan, N.Z. (2005). Volatile oil constituents of the fresh rhizomes of *Curcuma amada* Roxb. *Journal of Essential Oil Research, 17*(5), 490-491.

National Cancer Registry, Ministry of Health (Malaysia). *Malaysia National Cancer Registry Report 2007. Kuala Lumpur,* Malaysia: National Cancer Registry, Ministry of Health (Malaysia), 2011.

Oguntimein, B.O., Weyerstahl, P., and Marschall, W.H. (1990). Essential oil of *Curcuma longa* L. leaves. *Flavour and Fragrance Journal, 5*(2), 89-90.

Ohshiro, M., Kuroyanagi, M., and Ueno, A. (1990). Structures of sesquiterpenes from *Curcuma longa*. *Phytochemistry, 29*(7), 2201-2205.

Okada, H., and Mak, T.W. (2004). Pathways of apoptotic and non-apoptotic death in tumour cells. *Nature Reviews Cancer, 4*(8), 592-603.

Oliveira, P.F.D., Alves, J.M., Damasceno, J.L., Oliveira, R.A.M., Dias, H.J., Crotti, A.E. M., and Tavares, D.C. (2015). Cytotoxicity screening of essential oils in cancer cell lines. *Revista Brasileira de Farmacognosia, 25*(2), 183-188.

Ong, H.C., and Nordiana, M. (1999). Malay ethno-medico botany in Machang, Kelantan, Malaysia. *Fitoterapia, 70*(5), 502-513.

Oonmetta-aree, J., Suzuki, T., Gasaluck, P., and Eumkeb, G. (2006). Anti-microbial properties and action of galangal (*Alpinia galanga* Linn.) on *Staphylococcus aureus*. *LWT - Food Science and Technology, 39*(10), 1214-1220.

Padalia, R.C., Verma, R. S., Sundaresan, V., Chauhan, A., Chanotiya, C. S., and Yadav, A. (2013). Volatile terpenoid compositions of leaf and rhizome of *Curcuma amada* Roxb. from Northern India. *Journal of Essential Oil Research, 25*(1), 17-22.

Pandey, A.K., and Chowdhury, A.R. (2003). Volatile constituents of the rhizome oil of *Curcuma caesia* Roxb. from central India. *Flavour and Fragrance Journal, 18*(5), 463-465.

Pant, N., Jain, D., Bhakuni, R., Prajapati, V., Tripathi, A., and Kumar, S. (2001). Zederone: a sesquiterpenic keto-dioxide from *Curcuma aromatica*. *Indian Journal of Chemistry Section B, 40*(1), 87-87.

Pant, N., Misra, H., and Jain, D.C. (2013). Phytochemical investigation of ethyl acetate extract from *Curcuma aromatica* Salisb. rhizomes. *Arabian Journal of Chemistry, 6*(3), 279-283.

Paramapojn, S., and Gritsanapan, W. (2009). Free radical scavenging activity determination and quantitative analysis of curcuminoids in *Curcuma zedoaria* rhizome extracts by HPLC method. *Current Science, 97*(7), 1069-1073.

Park, G.G., Eun, S., and Shim, S.H. (2012). Chemical constituents from *Curcuma zedoaria*. *Biochemical Systematics and Ecology, 40*, 65-68.

Pemberton, R.W. (2006). A tropical garden flora. Plants cultivated in the Hawaiian Islands and other tropical places. *Economic Botany, 60*(2), 193-194.

Perry, L.M., and Metzger, J. (1980). *Medicinal plants of east and southeast Asia: attributed properties and uses* (pp. 60-420). Cambridge, UK: MIT Press.

Pezzuto, J.M. (1997). Plant-derived anti-cancer agents. *Biochemical Pharmacology, 53*(2), 121-133.

Policegoudra, R., Divakar, S., and Aradhya, S. (2007). Identification of difurocumenonol, a new anti-microbial compound from mango ginger (*Curcuma amada* Roxb.) rhizome. *Journal of Applied Microbiology, 102*(6), 1594-1602.

Policegoudra, R.S., Aradhya, S.M., and Singh, L. (2011). Mango ginger (*Curcuma amada* Roxb.)-a promising spice for phytochemicals and biological activities. *Journal of Biosciences, 36*(4), 739-748.

Purkayastha, J., Nath, S.C., and Klinkby, N. (2006). Essential oil of the rhizome of *Curcuma zedoaria* (Christm.) Rose. native to Northeast India. *Journal of Essential Oil Research, 18*(2), 154-155.

Qu, Y., Xu, F., Nakamura, S., Matsuda, H., Pongpiriyadacha, Y., Wu, L., and Yoshikawa, M. (2009). Sesquiterpenes from *Curcuma comosa*. *Journal of Natural Medicines, 63*(1), 102-104.

Rahman, S.N.A.A., Wahab, N.A., and Malek, S.N.A. (2013). *In vitro* morphological assessment of apoptosis induced by antiproliferative constituents from the rhizomes of *Curcuma zedoaria*. *Evidence-Based Complementary and Alternative Medicine, 2013*, 14.

Rahman, A., Afroz, M., Islam, R., Islam, K. D., Hossain, M.A., and Na, M., (2014). *In vitro* anti-oxidant potential of the essential oil and leaf extracts of *Curcuma zedoaria* Rosc. *Journal of Applied Pharmaceutical Science, 4*(2), 107-111.

Ramasamy, S., Abdul Wahab, N., Zainal Abidin, N., and Manickam, S. (2013). Effect of extracts from *Phyllanthus watsonii* Airy Shaw on cell apoptosis in cultured human breast cancer MCF-7 cells. *Experimental and Toxicologic Pathology, 65*(3), 341-349.

Ramasamy, S., Wahab, N.A., Abidin, N.Z., Manickam, S., and Zakaria, Z. (2012). Growth inhibition of human gynecologic and colon cancer cells by *Phyllanthus watsonii* through apoptosis induction. *PLoS One, 7*(4), e34793.

Rasul, A., Parveen, S., and Ma, T. (2012). Costunolide: a novel anti-cancer sesquiterpene lactone. *Bangladesh Journal of Pharmacology*, *7*(1), 6-13.

Rameshkumar, K., Alan Sheeja, D., Nair, M. S., and George, V. (2015). *Curcuma ecalcarata*–new natural source of pinocembrin and piperitenone. *Natural Product Research, 29*(13), 1276-1279.

Ravindran, P.N., Babu, K.N., and Sivaraman, K. (2007). *Turmeric: the genus Curcuma* (pp. 54-200). New York, USA: CRC Press.

Repetto, G., del Peso, A., and Zurita, J.L. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols, 3*(7), 1125-1131.

Retnowati, R., Rahman, M. F., and Yulia, D. (2014). Chemical constituents of the essential oils of white turmeric (*Curcuma zedoaria* (Christm.) Roscoe) from Indonesia and its toxicity toward *Artemia salina* leach. *Journal of Essential Oil Bearing Plants, 17*(3), 393-396.

Riswan, S., and Setyowati, F.M. (1996). *Ethnobotanical study on Zingiberaceae in Indonesia.* Paper presented at the Proc 2nd Symp Fam Zingiberaceae South China Inst Bot.

Roosita, K., Kusharto, C.M., Sekiyama, M., Fachrurozi, Y., and Ohtsuka, R. (2008). Medicinal plants used by the villagers of a Sundanese community in West Java, Indonesia. *Journal of Ethnopharmacology, 115*(1), 72-81.

Saelens, X., Festjens, N., Vande W.L., Van G.M., Van L.G., and Vandenabeele, P. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene, 23*(16), 2861-2874.

Santos, G.K.N., Dutra, K.A., Barros, R.A., da Câmara, C.A.G., Lira, D.D., Gusmão, N.B., and Navarro, D.M.A.F. (2012). Essential oils from *Alpinia purpurata* (Zingiberaceae): chemical composition, oviposition deterrence, larvicidal and antibacterial activity. *Industrial Crops and Products, 40*, 254-260.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M., and Yoga L.L. (2011). Extraction, isolation and characterization of bioactive compounds from plants'

extracts. *African Journal of Traditional, Complementary and Alternative Medicines, 8*(1), 1-10.

Sharma, R.K., Misra, B.P., Sarma, T.C., Bordoloi, A.K., Pathak, M.G., and Leclercq, P.A. (1997). Essential oils of *Curcuma longa* L. from Bhutan. *Journal of Essential Oil Research, 9*(5), 589-592.

Sharma, P.R., Mondhe, D.M., Muthiah, S., Pal, H.C., Shahi, A.K., Saxena, A.K., and Qazi, G.N. (2009). Anti-cancer activity of an essential oil from *Cymbopogon flexuosus*. *Chemico Biological Interactions, 179*(2-3), 160-168.

Shin, Y., and Lee, Y. (2013). Cytotoxic activity from *Curcuma zedoaria* through mitochondrial activation on ovarian cancer cells. *Toxicological research, 29*(4), 257.

Shinobu-Mesquita, C.S., Bertoni, T.A., Guilhermetti, E., and Svidzinski, T.I. (2011). Anti-fungal activity of the extract of *Curcuma zedoaria* (Christm.) Roscoe, Zingiberaceae, against yeasts of the genus *Candida* isolated from the oral cavity of patients infected with the human immunodeficiency virus. *Revista Brasileira de Farmacognosia, 21*(1), 128-132.

Shiobara, Y., Asakawa, Y., Kodama, M., Yasuda, K., and Takemoto, T. (1985). Curcumenone, curcumanolide A and curcumanolide B, three sesquiterpenoids from *Curcuma zedoaria*. *Phytochemistry, 24*(11), 2629-2633.

Shiobara, Y., Asakawa, Y., Kodama, M., and Takemoto, T. (1986). Zedoarol, 13-hydroxygermacrone and curzeone, three sesquiterpenoids from *Curcuma zedoaria*. *Phytochemistry, 25*(6), 1351-1353.

Shoaib, M., Shah, I., Ali, N., Adhikari, A., Tahir, M.N., Shah, S.W.A., Ishtiaq, S., Khan, J., Khan, S., and Umer, M.N. (2017). Sesquiterpene lactone a promising antioxidant, anti-cancer and moderate antinociceptive agent from *Artemisia macrocephala* Jacquem. *BMC Complementary and Alternative Medicine, 17*(1), 27.

Shu, L., Cheung, K.L., Khor, T.O., Chen, C., and Kong, A.N. (2010). Phytochemicals: cancer chemoprevention and suppression of tumor onset and metastasis. *Cancer Metastasis Review, 29*(3), 483-502.

Singh, P., Singh, S., Kapoor, I.P.S., Singh, G., Isidorov, V., and Szczepaniak, L. (2013). Chemical composition and anti-oxidant activities of essential oil and oleoresins from *Curcuma zedoaria* rhizomes, part-74. *Food Bioscience, 3*, 42-48.

Singh, G., Singh, O.P., and Maurya, S. (2002). Chemical and biocidal investigations on essential oils of some Indian *Curcuma* species. *Progress in Crystal Growth and Characterization of Materials, 45*(1), 75-81.

Sirat, H.M., Jamil, S., and Hussain, J. (1998). Essential oil of *Curcuma aeruginosa* Roxb. from Malaysia. *Journal of Essential Oil Research, 10*(4), 453-458.

Sirat, H.M., Jamil, S., and Rahman, A.A. (1998). Sesquiterpenes from *Curcuma aeruginosa*. *Planta Medica, 64*(6), 584-585.

Sirat, H.M., and Nordin, A.B. (1995). Chemical composition of the rhizome oil of *Alpinia conchigera* Griff from Malaysia. *Journal of Essential Oil Research, 7*(2), 195-197.

Sirirugsa, P., Larsen, K., and Maknoi, C. (2007). The genus *Curcuma* L.(Zingiberaceae): distribution and classification with reference to species diversity in Thailand. *Garden Bulletin Singapore, 59*(1-2), 203-220.

Sivasothy, Y., Chong, W.K., Hamid, A., Eldeen, I. M., Sulaiman, S.F., and Awang, K. (2011). Essential oils of *Zingiber officinale* var. *rubrum* Theilade and their anti-bacterial activities. *Food Chemistry, 124*(2), 514-517.

Sivasothy, Y., Awang, K., Ibrahim, H., Thong, K.L., Fitrah, N., Koh, X.P., and Tan, L.K. (2012). Chemical composition and anti-bacterial activities of essential oils from *Zingiber spectabile* Griff. *Journal of Essential Oil Research, 24*(3), 305-313.

Soeur, J., Marrot, L., Perez, P., Iraqui, I., Kienda, G., Dardalhon, M., Meunier, J.R., Averbeck, D., and Huang, M. E. (2011). Selective cytotoxicity of *Aniba rosaeodora* essential oil towards epidermoid cancer cells through induction of apoptosis. *Mutation Research, 718*(1-2), 24-32.

Sothilingam, S., Sundram, M., Malek, R., and Sahabuddin, R.M. (2010). Prostate cancer screening perspective, Malaysia. *Urologic Oncology, 28*(6), 670-672.

Soule, H.D., Vazguez, J., Long, A., Albert, S., and Brennan, M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute, 51*(5), 1409-1416.

Srivastava, A.K., Srivastava, S.K., and Shah, N.C. (2000). Essential oil composition of *Zingiber zerumbet* (L.) Sm. from India. *Journal of Essential Oil Research, 12*(5), 595-597.

Strasser, A., O'Connor, L., and Dixit, V.M. (2000). Apoptosis signaling. *Annual Review of Biochemistry, 69*, 217-245.

Subramaniam, M., In, L.L.A., Kumar, A., Ahmed, N., and Nagoor, N.H. (2016). Cytotoxic and apoptotic effects of heat killed *Mycobacterium indicus pranii* (MIP) on various human cancer cell lines. *Scientific Reports, 6*, 19833.

Sukari, A.H., Lajis, N.H., Muse, R., and Riyanto, S. (2007). Chemical constituents and bioactivity of *Curcuma aeruginosa* Roxb. *Natural Product Sciences, 13*(3), 175-179.

Sumathi, S., Iswariya, G., Sivaprabha, B., Dharani, B., Radha, P., and Padma, P. (2013). Comparative study of radical scavenging activity and phytochemical analysis of fresh and dry rhizomes of *Curcuma zedoaria*. *International Journal of Pharmaceutical Sciences and Research, 4*(3), 1069.

Suparji, N.S., Chan, G., Sapili, H., Arshad, N.M., In, L.L.A., Awang, K., and Nagoor, N.H. (2016). Geranylated 4-phenylcoumarins exhibit anti-cancer effects against human prostate cancer cells through caspase-independent mechanism. *PLoS One, 11*(3).

Takano, I., Yasuda, I., Takeya, K., and Itokawa, H. (1995). Guaiane sesquiterpene lactones from *Curcuma aeruginosa*. *Phytochemistry, 40*(4), 1197-1200.

Tan, C.P., Lu, Y.Y., Ji, L.N., and Mao, Z.W. (2014). Metallomics insights into the programmed cell death induced by metal-based anti-cancer compounds. *Metallomics, 6*(5), 978-995.

Taylor, P.G., Dupuy Loo, O.A., Bonilla, J.A., and Murillo, R. (2008). Anti-cancer activities of two sesquiterpene lactones, millerenolide and thieleanin isolated from *Viguiera sylvatica* and *Decachaeta thieleana*. *Fitoterapia, 79*(6), 428-432.

Theanphong, O., Mingvanish, W., and Kirdmanee, C. (2015). Chemical constituents and biological activities of essential oil from *Curcuma aeruginosa* Roxb. rhizome. *Bulletin of Health, Science and Technology, 13*(1).

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistics, 2012. *CA: A Cancer Journal for Clinicians, 65*(2), 87-108.

Tuna, S., Dizdar, O., and Calis, M. (2011). The prevalence of usage of herbal medicines among cancer patients. *Journal of the Balkan Union of Oncology, 83*, 32.

Ullah, H.A., Zaman, S., Juhara, F., Akter, L., Tareq, S.M., Masum, E.H., and Bhattacharjee, R. (2014). Evaluation of anti-nociceptive, *in-vivo & in-vitro* anti-inflammatory activity of ethanolic extract of *Curcuma zedoaria* rhizome. *BMC Complementary and Alternative Medicine, 14*(1), 1.

Vairappan, C.S., Nagappan, T., and Palaniveloo, K. (2012). Essential oil composition, cytotoxic and anti-bacterial activities of five *Etlingera* species from Borneo. *Natural Product Communications, 7*(2), 239-242.

Verma, S.K., Jain, V., and Katewa, S.S. (2009). Blood pressure lowering, fibrinolysis enhancing and anti-oxidant activities of cardamom (*Elettaria cardamomum*). *Indian Journal of Biochemistry and Biophysics, 46*(6), 503-506.

Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of Immunological Methods, 184*(1), 39-51.

Villaflores, O.B., Macabeo, A.P.G., Gehle, D., Krohn, K., Franzblau, S.G., and Aguinaldo, A.M. (2010). Phytoconstituents from *Alpinia purpurata* and their *in vitro* inhibitory activity against *Mycobacterium tuberculosis*. *Pharmacognosy Magazine, 6*(24), 339-344.

Vitasari, R.A., Wibowo, F.R., Marliyana, S.D., and Wartono, M.W. (2016). *Isolation and identification of curcumin and bisacurone from rhizome extract of temu glenyeh (Curcuma soloensis. Val).* Paper presented at the IOP Conference Series: Materials Science and Engineering.

Wang, Y., Yuan, C.S., Han, Y.F., and Jia, Z.J. (2003). Sesquiterpenes from roots of *Lingularia veitchiana*. *Die Pharmazie- An International Journal of Pharmaceutical Sciences, 58*(5), 349-352.

Waterman, P. G. (1993). The chemistry of volatile oils. *In: Volatile Oil Crops: Their Biology, Biochemistry and Production* (pp. 5-60). New York, USA: Wiley.

Wei, A., and Shibamoto, T. (2010). Anti-oxidant/lipoxygenase inhibitory activities and chemical compositions of selected essential oils. *Journal of Agricultural and Food Chemistry, 58*(12), 7218-7225.

Wilson, B., Abraham, G., Manju, V.S., Mathew, M., Vimala, B., Sundaresan, S., and Nambisan, B. (2005). Anti-microbial activity of *Curcuma zedoaria* and *Curcuma malabarica* tubers. *Journal of Ethnopharmacology, 99*(1), 147-151.

Wong, K.C., Chong, T.C., and Chee, S.G. (1999). Essential oil of *Curcuma mangga* Val. and van Zijp rhizomes. *Journal of Essential Oil Research, 11*(3), 349-351.

Xiao, Y., Yang, F.Q., Li, S.P., Hu, G., Lee, S.M.Y., and Wang, Y.T. (2008). Essential oil of *Curcuma wenyujin* induces apoptosis in human hepatoma cells. *World Journal of Gastroenterology, 14*(27), 4309-4318.

Xu, Z., and Chang, L. (2017). *Zingiberaceae. In: Identification and Control of Common Weeds, Volume 3* (pp. 909-911). Singapore: Springer.

Ya-Nan, W., Jia-Liang, W., Dan-Wei, M., Jiao, L., and Du-Yu, Z. (2015). Anti-cancer effects of *Chenopodium ambrosiodes* L. essential oil on human breast cancer MCF-7 cells *in vitro*. *Tropical Journal of Pharmaceutical Research, 14*(10), 1813-1820.

Yang, F., Wang, H., Chen, H., Chen, J., and Xia, Z. (2011). Fractionation of volatile constituents from *Curcuma* rhizome by preparative gas chromatography. *Journal of Automated Methods and Management in Chemistry, 2011* (Article ID 942467).

Yin, S.-Y., Wei, W.-C., Jian, F.-Y., and Yang, N.-S. (2013). Therapeutic applications of herbal medicines for cancer patients. *Evidence-Based Complementary and Alternative Medicine, 2013* (Article ID 302426), 1-15.

Yob, N. J., Jofrry, S.M., Affandi, M.M.R.M.M., Teh, L.K., Salleh, M.Z., and Zakaria, Z.A. (2011). *Zingiber zerumbet* (L.) Smith: a review of its ethnomedicinal, chemical, and pharmacological uses. *Evidence-Based Complementary and Alternative Medicine, 2011*, 12.

Yonzon, M., Lee, D.J., Yokochi, T., Kawano, Y., and Nakahara, T. (2005). Anti-microbial activities of essential oils of Nepal. *Journal of Essential Oil Research, 17*(1), 107-111.

Yoshikawa, M., Murakami, T., Morikawa, T., and Matsuda, H. (1998). Absolute stereostructures of carabrane-type sesquiterpenes, curcumenone, 4s-dihydrocurcumenone and curcarabranols A and B: vasorelaxant activity of zedoary sesquiterpenes. *Chemical & Pharmaceutical Bulletin, 46*(7), 1186-1188.

Yostawonkul, J., Surassamo, S., Boonrungsiman, S., Namdee, K., Boonthum, C., Pagseesing, S., Chatdarong, K., and Yata, T. (2017). Nanocarrier-mediated delivery of α-mangostin for non-surgical castration of male animals. *The Thai Journal of Veterinary Medicine, 47*, 315-316.

Zaridah, S. (2014). A review of cervical cancer research in Malaysia. *Medical Journal of Malaysia, 69* Suppl. A, 33-41.

Zhang, S., Won, Y. K., Ong, C. N., and Shen, H. M. (2005). Anti-cancer potential of sesquiterpene lactones: bioactivity and molecular mechanisms. *Current Medicinal Chemistry Anti-cancer Agents, 5*(3), 239-249.

Zhang, P., Huang, W., Song, Z., Zhang, M., Cheng, L., Cheng, Y., Qu, H., and Ma, Z. (2008). Cytotoxic diterpenes from the radix of *Curcuma wenyujin*. *Phytochemistry Letters, 1*(2), 103-106.

Ziaei, A., Ramezani, M., Wright, L., Paetz, C., Schneider, B., and Amirghofran, Z. (2011). Identification of spathulenol in *Salvia mirzayanii* and the immuno-modulatory effects. *Phytotherapy Research, 25*(4), 557-562.

LIST OF PUBLICATION AND PAPER PRESENTED

Article published in ISI-cited journal:

Devi Rosmy Syamsir, Yasodha Sivasothy, Hazrina Hazni, Sri Nurestri Abdul Malek, Noor Hasima Nagoor, Halijah Ibrahim and Khalijah Awang (2017). Chemical constituents and evaluation of cytotoxic activities of *Curcuma zedoaria* (Christm.) Roscoe oils from Malaysia and Indonesia. *Journal of Essential Oil Bearing Plants, 20* (4), 972-982.

Paper presented:

Devi Rosmy Syamsir, Halijah Ibrahim, Noor Hasima Nagoor and Khalijah Awang. Bioassay-guided isolation of *Curcuma zedoaria,* the cytotoxic and apoptotic investigation of the bioactive pure compounds. The 3rd International Seminar on Chemistry 2014, Bandung, Indonesia, 20-21 November 2014.