CHAPTER 1: INTRODUCTION

1.1 Introduction

Malaysia, is one of the 12 megadiversity countries of the world and embraces about 60% of the world's known species (Mohamed, 2005). About 10% out of 15000 species of flowering plants are classified as medicinal plants (Ibrahim *et al*., 2001a; Ibrahim *et al*., 2001b). A Dictionary of the Economic Products of the Malay Peninsular written by Burkill in 1953 gave a comprehensive knowledge about Malaysian forest diversity and the medicinal plants distribution in Malaysia and it is a valuable reference for scientists doing research in the field of medicinal plants (Burkill, 1966).

In recent years, medicinal plants play an important and vital role in traditional medicine to treat a wide spectrum of diseases and infections. Additionally, they are also widely consumed as home remedies by rural people and aborigines. Many studies have shown that medicinal plants are consumed worldwide for the treatment of several diseases such as diabetes, high blood pressure, bronchitis, jaundice, diarrhea, gastric disorder, emesis, hepatitis B, skin infection and a medication for post-partum (Bhuiyan *et al*., 2010; Ebrahim *et al*., 2012; Lee *et al*., 2010; Ong & Nordiana, 1999; Subramamian *et al*., 2012; Rana *et al*., 2010).

The global market of herbal products throughout the world has increased over the years. In 2003, it is reported that the market is worth about an estimated US$60 billion per year with growth rate of 7 percent and is expected to reach 5 trillion by 2050 (WHO, 2003). The annual expenditures on traditional medicine rose up to US$7.4 billion in 2009 from US$4.4 billion in 2004. In 2013, it is reported that the output of Chinese material medica

has increased as much as 20% which accounted for about US$83.1 billion in 2012. While, United States spent about US$14.8 billion in 2008 for the natural products expenditures (WHO, 2013). Most herbal medicines are affordable, easily accessible and have low side effects compared to the synthetic drugs and as well as the cost is cheaper than the conventional drugs (Ajasa *et al*., 2004; Al-Adhroey *et al*., 2010).

Jamal *et al.* (2011) reported the investigation on medicinal plants used for postnatal care in Malay traditional medicine in the Peninsular Malaysia. For example, the rhizomes of *Alpinia conchigera* were mixed with hot water and given to the women during confinement to improve blood circulation, to make the body feel warm, to encourage contraction of the uterus, to expel wind, to prevent fit and as a laxative (Jamal *et al*., 2011). Therefore, medicinal plants are important raw materials in pharmaceutical industries especially for the production of phytopharmaceuticals (Ajasa *et al*., 2004). The active compounds of the plants are metabolic products of plant cells and a number of trace elements play an important role in the metabolism (Rajurkar & Damame, 1997).

In the terms of medicinal properties, each plant consists of active compounds which can interact with the human cell body to cure some diseases. The active compounds work either binding itself or bind with other material (such as trace metal or other compound) to enhance the activity of the compound for biological activity. Biological activity like antifungal, antimicrobial, anti-inflammatory and anti-oxidant of the plants extract is the preliminary study to determine the medicinal properties of the medicinal plant studied.

There have been many reports on medicinal treatment of Zingiberaceae family. For example, in Malaysia, gingers of the Zingiberaceae family, such as the leaves of *Alpinia galanga* are consumed to treat diarrhoea, stems of *Costus speciosus* is believed to relieve

skin rashes, juice obtained from the rhizome of *Curcuma xanthoriza* is applied on the face to cure pimples and the rhizomes of *Zingiber zerumbet* were used to treat stomach ache (Khalid *et al.*, 2011; Ong & Nordiana, 1999). The plants belonging to Zingiberaceae family such as *Alpinia* species, *Costus* species, *Etlingera* species and *Zingiber* species have also reported to have an antioxidant, antimicrobial and anti-inflammatory properties (Aziz *et al*., 2013; Chan *et al*., 2008; Chan *et al*., 2011; Habsah *et al*., 2000; Yu *et al*., 2009). Some investigations of the plants used to treat malaria in Peninsular Malaysia have been done by Al-Adhroey *et al*. (2010). The investigation showed fifteen plants included *Languas galanga* are used orally and four plants included *Curcuma domestica* are used externally to treat the malaria disease. Anti-tuberculosis potential of some selected medicinal plants in Malaysia have been studied by Mohamad *et al.* (2011). However, they reported that there is no inhibition of the mycobacterial growth at the highest test concentration of 1600 µg/ml for *Alpinia galanga* and *Zingiber officinale* (Mohamad *et al.*, 2011). On the other hand, the methylene chloride extract of *Zingiber officinale* (rhizome) exhibited high anti-tuberculosis activity which is 85% inhibition at 100 µg/ml of the test concentration using BACTEC system assay as reported by Hiserodt *et al.* (1998).

According to Lee and Houghton (2005), *Alpinia galanga* from Malaysia showed weaker cytotoxicity profile as compared to those from Thailand on human non-small cell lung cancer (NSCLC) COR L23 and human breast adenocarcinoma MCF7 cell lines. The Thai sample of *Alpinia galanga* also presented much more 1'-acetoxychavicol acetate than the Malaysian sample. This phenylpropanoids, in particular, are known to exhibit various activities such as anticancer, antifungal, antimicrobial, heaptoprotective and antioxidant. These compounds are also contribute in the preparation of neurotropic, adaptogeni and immunostimulating (Kurkin, 2013).

Besides the phytochemical screening and chemical studies, the macronutrients and metal accumulation in medicinal plants are also necessary to be studied. The macronutrients and metal content in medicinal plants will ascertain the plants are safe and healthy for consumption as remedies. The nutrients in the plants are important for the plants growth and to propagate so that human can get the benefit too. The content or the concentration of the metal accumulation is also important to be studied to maintain the value of the medicinal plants. The admitted maximum limit of the metal content in foodstuff and vegetable were established by the Food and Nutrition Board, Commission of European Community, World Health Organization (WHO) and Ministry of Health of Malaysia (MOH).

Various publications have documented the studies of macronutrients and metal analysis in the medicinal plants. Djama *et al.* (2012) reported that the heavy metal analysis of some anti-diabetic medicinal plants in Côte d'Ivoire namely *Boerhavia diffusa* L., *Catharanthus roseus* (L.) *Sw*., *Phyllanthus amarus schumach* and *Solenostenum monostacyus*. They reported the element such as manganese (Mn), chromium (Cr), vanadium (V) and zinc (Zn) detected in these medicinal plants are implicated in the regulation of insulin and control of the blood sugar levels in human body. Hence, these medicinal plants are suitable for management of diabetes (Djama *et al*., 2012).

Ten elements which are zinc (Zn), copper (Cu), iron (Fe), manganese (Mn), sodium (Na), lead (Pb), potassium (K), magnesium (Mg), calcium (Ca) and phosphorus (P) were determined against ten different herbal plants in Nigeria. The results showed that the herbal plants contain large amount of nutrients and are rich in Fe, Mg, Ca, Na, K and P. These plants from different collecting sites are related to the different concentration of the elements (Ajasa *et al*., 2004). The analysis of mineral and heavy metal in some medicinal

plants in local market in Salem, Tamil Nadu, India were determined by Subramanian *et al*. (2012). The results indicated that the plants are rich in Fe, Na, Mg and Mn. It is also notable that Pb, Cd, Cu and Zn in these 15 medicinal plants were within the prescribed medicine.

Rai *et al.* (2001) has analysed the heavy metals accumulation in 34 samples belonging to 9 different plant species. The reports acquired the presents of higher concentration in heavy metals are related to the place where the plant was collected. For the sample situated in Lucknow, it showed that the highest concentration in Pb, Cd, Cu and Zn in *Alpinia galanga*. Lucknow is one of the cities with the higher vehicle pollution. Therefore, the results with the higher concentration of those metals are suspected (Rai *et al*., 2005; Rai *et al*., 2001).

Since there were no reported on the study of metal analysis of *Alpinia conchigera* Griff., and based on the research finding of the Zingiberaceae that mentioned above, thus this work will include metal analysis study on the medicinal plants of *Alpinia conchigera* Griff.

The objectives of this study are listed below:

1. To extract the leaves and rhizomes of *Alpinia conchigera* using supercritical fluid extraction technique.

2. To analyse the volatile constituents of the leaves and rhizomes of *Alpinia conchigera* crude extracts using gas chromatography flame ionization detector (GC-FID) and gas chromatography mass spectrometry (GC-MS).

3. To determine the macronutrients and trace metal accumulation in *Alpinia conchigera* by microwave digestion method using Flame Atomic Absorption Spectroscopy.

4. To investigate the antifungal activity of the crude extracts of *Alpinia conchigera* against *Microsporum canis*, *Trichophyton mentagrophytes* and *Tricophyton rubrum* using Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) assay.

1.2 Zingiberaceae : Distribution and Habitat

The Zingiberaceae also known as the Ginger family is one of the largest families in the order of Zingiberales, which are distributed about 50 genera and 1500 species throughout tropical Africa, Asia and Americas with its greatest diversity in Southeast Asia. In Peninsular Malaysia the Zingiberaceae are a component of the herbaceous ground flora of the rainforest. It is estimated that there are 150 species of ginger belonging to 23 genera found in Peninsular Malaysia (Holttum, 1950).

Zingiberaceae species grow naturally on the ground flora of the primitive forest of Peninsular Malaysia. The plant which can be found anywhere in the forest usually known as scattered plants, seldom as a thicket. Most of the family is terrestrial, perennial and aromatic herbs growing in lowland and midmountain forest and only a few of the family can be seen on high mountain ridges. The genus *Alpinia* of the tribe *Alpineae* has been recorded to show one of the highest diversity of Zingiberaceae family.

1.3 Zingiberaceae : General Appearance and Morphology

Zingiberaceae are recognized as herbs perennial, terrestrial, rarely epiphytic, aromatic with fleshy, tuberous or non-tuberous rhizomes and often with tuber-bearing roots. Stems are usually short and replaced by pseudostems formed by leaf sheaths. The leaves are distichous, simple, those towards base of plant usually bladeless and reduced to sheaths. Leaf blade suborbicular or lanceolate to narrowly strap-shaped, rolled longitudinally in bud, glabrous or hairy, midvein prominent, lateral veins usually numerous, pinnate, parallel and margin entire. Most members of the family are easily recognized by the characteristic aromatic leaves and fleshy rhizome when both of them are crushed and also by elliptic-oblong leaves arranged in two ranks on the leaf-shoot. The characteristic of the Zingiberaceae were identified by Kress *et al.* (2002) based on vegetative and floral characteristics. The summary of the characteristic of the subfamilies and tribes of the Zingiberaceae is presented in Table 1.1 (Kress, Prince, & Williams, 2002).

Zingiberaceae provides a potential material for ornamental and it has potential resources of a variety uses ranging from medicine to food (Ibrahim, 1999). Some of the species in this family are believed to be useful in the treatment of several type of cancer. The rhizomes are also externally applied for treatment of rheumatism, wounds, sores and ringworm.

Table 1.1: Characteristics of the subfamilies and tribes of the new classification of the Zingiberaceae (Kress *et al*., 2002).

Character	Subfamilies and tribes					
	Siphonochiloideae: Siphonochileae	Tamijoideae: Tamijieae	Alpinioideae: Riedelieae	Alpinioideae: Alpinieae	Zingiberoideae: Zingibereae	Zingiberoideae: Globbeae
Seasonality	Dormancy period	Evergreen	Evergreen	Evergreen	Dormancy period	Dormancy period
Rhizomes	Fleshy	Fibrous	Fibrous	Fibrous	Fleshy	Fleshy
Plane of distichy of leaves	Perpendicular to rhizome	Perpendicular to rhizome	Perpendicular to rhizome	Perpendicular to rhizome	Parallel to rhizome	Parallel to rhizome
Extrafloral nectaries	Absent	Absent	Present on leaf blades	Absent	Absent	Absent
Lateral staminodes	Petaloid, fused to labellum	Petaloid, fused to labellum	Small or absent, never petaloid	Small or absent, never petaloid	Petaloid, free from or fused to labellum	Petaloid, free from labellum and sometimes connate to filament
Labellum	Not connate to filament	Not connate to filament	Not connate to filament	Not connate to filament	Not connate to filament	Connate to filament in slender tube
Filament	Short	Short	Medium	Medium, sometimes arching	Short to long	Short to long, sometimes arching

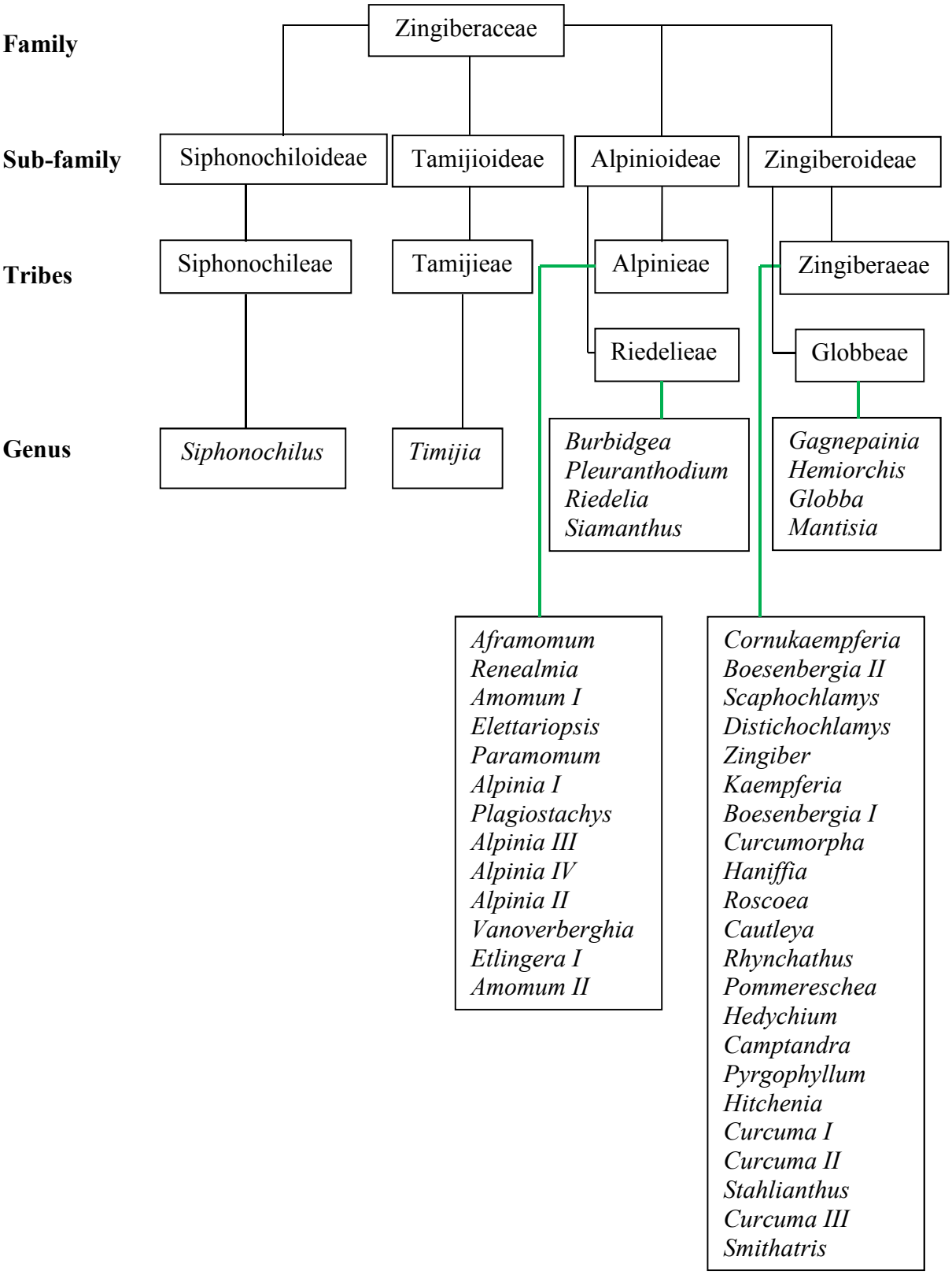
Table 1.1, continued

Character	Subfamilies and tribes					
	Siphonochiloideae: Siphonochileae	Tamijoideae: Tamijieae	Alpinioideae: Riedelieae	Alpinioideae: Alpinieae	Zingiberoideae: Zingibereae	Zingiberoideae: Globbeae
Ovary	3-locular (sometimes incompletely so)	1-locular	1- or 3-locular	3-locular	3-locular (sometimes incompletely so)	1-locular
Anther crest	Petaloid	Petaloid	Petaloid	Petaloid	Absent, petaloid, or well-developed and wrapped around style	Spurred or absent
Placentation	Axial	Parietal	Axial or parietal	Axial or free central	Axial, basal, or free columnar	Parietal
Capsule	Fleshy	Unknown	Silique-like, opening by longitudinal slits	Indehiscent or fleshy	Fleshy and dehiscent	Globbose and dehiscent

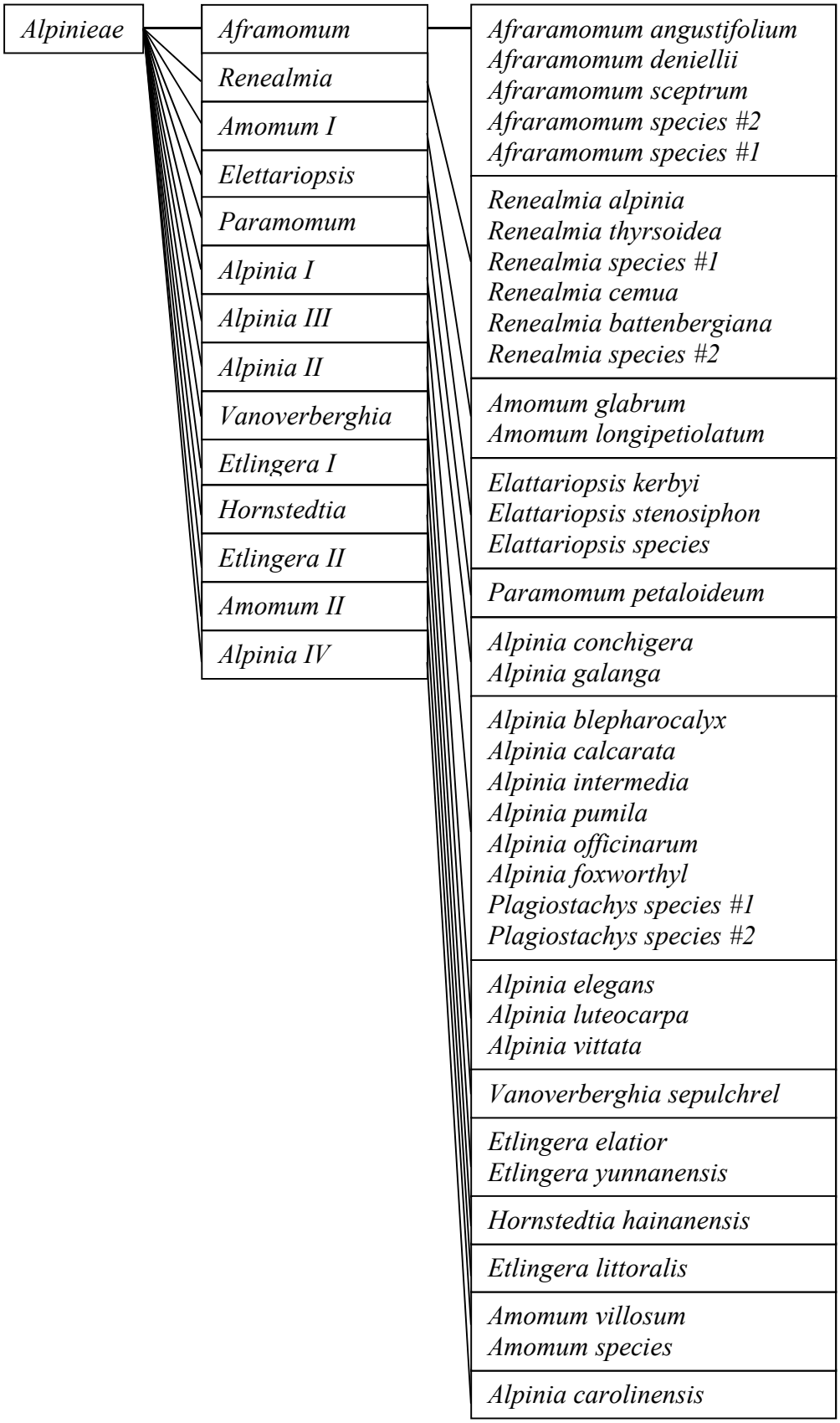
1.4 Zingiberaceae : Classifications of tribes

Classifications of the Zingiberaceae family were proposed in 1889 by Petersen and were refined by other researchers. Four tribes were recognized which are Globbeae, Hedychieae, Alpinieae and Zingibereae. The morphology features like number of locules and placentation in the ovary, development of staminodia, modifications of the fertile anther and rhizome-shoot-leaf orientation have been used to distinguish the four tribes.

The new classification of the Zingiberaceae were defined by Kress *et al.* (2002) with the new phylogenetic analyses based on DNA sequences of the nuclear internal transcribed spacer (ITS) and plastid *matK* regions, suggesting at least some of these morphological traits are homoplasious and three of the tribes are paraphyletic. Scheme 1.1 illustrates the classification of Zingiberaceae and Scheme 1.2 manifests the order of *Alpinia* according to Kress *et al.* (2002).



Scheme 1.1 Classification of Zingiberaceae (Kress *et al.*, 2002).



Scheme 1.2 : Polyphyletic species group in the tribe *Alpinieae* (Kress *et al*., 2002).

1.5 The Genus :*Alpinia*

Alpinia is a large, widespread and taxonomically complex genus in Zingiberaceae with 230 species throughout tropical and subtropical Asia (Kress *et al*., 2005). Table 1.2 listed the species of *Alpinia* and their distributions (Bhuiyan *et al*., 2010; Burkill, 1966; Holttum, 1950; Indrayan *et al*., 2010; Kanjilal *et al*., 2010; Materials, 2003; Rana *et al*., 2010).

Alpinia conchigera Griff. were found in Bangladesh, Cambodia, India, Indonesia, Malaysia, Myanmar, Thailand and Vietnam with height about 600-1100 m. It is also known as *lengkuas ranting*, *lengkuas kecil*, *lengkuas padang*, *lengkuas getting* or *chengkenam* in Malay communities (Burkill, 1966; Jansesen *et al*., 1985; Kress *et al*., 2005).

Table 1.2: List of selected species of *Alpinia* and their distributions.

Species of *Alpinia*	Distributions
Alpinia cannaefolia Ridl.	Not common in Peninsular Malaysia, but found from Penang to Negeri Sembilan.
Alpinia chinensis Rosc.	Cultivated in Malacca.
Alpinia conchigera Griff.	Found in eastern Bengal and southwards to the Peninsular Malaysia and Sumatera.
Alpinia galangal Swartz.	Found from the foot of the eastern Himalaya, southwest of India between the Ghats and the sea, southeast Asia, Java, Laos, Thailand, Malaysia, Borneo, Philippine.
Alpinia javanica Blume.	Plentiful in Peninsular Malaysia, Sumatera and Java.
Alpinia malaccensis Rosc.	Cultivated in Bangladesh, Bhutan, India, Java, West Malaysia, Myanmar, Thailand.
Alpinia melabocarpa Ridl.	East coast and West Coast of the Peninsular Malaysia.
Alpinia mutica Roxb.	Penang and Singapore.
Alpinia nigra Burtt.	Found in China, Bhutan, India, Sri Lanka and Thailand.
Alpinia officinarum Hance	Cultivated in Vietnam and Southern China.
Alpinia rafflesiana Wall.	Peninsular Malaysia.
Alpinia scabra Benth.	Mountains of the Peninsular Malaysia and Java.
Alpinia speciosa Rosc.	A native of north-eastern India, China and Japan.

1.6 *Alpinia conchigera* Griff.

1.6.1 Plant Material

The rhizomes and leaves of *Alpinia conchigera* Griff. were collected from Jeli, Kelantan, Malaysia. It was deposited in Herbarium University of Malaya, Kuala Lumpur, Malaysia with herbarium series number KL 5049. This sample was recognized by Professor Dr. Halijah Ibrahim of the Institute of Biological Sciences, University of Malaya.

1.6.2 Plant description

Alpinia conchigera Griff. is a perennial herb known for its medicinal properties. An example of this would be for the treatment for skin disease, where a paste of rhizome mixed with kerosene is rubbed to the skin affected by fungal infection (Pongboonrod, S. & Thai, M. T. M., 1976; Wannissorn *et al*., 2005; Wuththitum, 1994). The paste of rhizome also can be applied to skin to treat bone aches and pains (Ong & Nordiana, 1999). The young shoots of *Alpinia conchigera* are prepared as food condiment in Peninsular Malaysia and in Thai food dishes (Athamaprasangsa *et al.*, 1994; Ibrahim *et al.*, 2009).

The pseudostems of *Alpinia conchigera* can grow to the height of 1.2 to 2 meter and has a ligule with the diameter of approximately 5 mm. Its leaf shape can be characterized as lanceolate with the measurements of around 20-30 cm in length by 1-10 cm wide. It is tomentose or glabrous, except pubescent along the midvein abaxially and at the margin. The lateral veins are very noticeable and are dense when it dried. The shape is obtuse at the base and acute at the apex. The panicles are about 20 to 30 cm. Generally they only have 1 or 2 branches and have many secondary branches in 1.5 cm. The bracts are around 5 mm and the bracteoles in funnel form are more or less 3 to 4 mm with apex obliquely truncate.

About 3 to 5 mm size of pedicel. The pale green calyx is found in a cupular form and is 3 to 4 mm approximately with 3-cleft apex (Wu Delin, 2000).

Alpinia conchigera has a white or pale blue-green corolla by abaxially pubescent. The tube equaling calyx with lobes measuring 5 to 7 mm and has a central one rounded at its apex. The lateral staminodes are red and its quadrate size is approximately 1.5 mm. The pinkish or pale yellow of the labellum with the red streaks in the obovate at 5 mm (Wu Delin, 2000). It is concave at the base bearing with a purple callosity covering the corolla throat. The filament of *Alpinia conchigera is* pale yellowish to pinkish in colour and slender which are about 5 mm and 2 mm for the anther. An ovary pyriform is glabrous in shape. In a fresh environment, the capsule is globose and becomes oblong when it dried, with a diameter of around 8 to 10 mm. Three to five seeds were produced and gave a strong aromatic odour (Wu Delin, 2000). The illustration of the *Alpinia conchigera*, leaves and rhizomes are shown in Figures 1.1, 1.2 and 1.3 respectively.

Figure 1.1 : The plant of *Alpinia conchigera*.

Figure 1.2 : The rhizomes and leaves of *Alpinia conchigera*.

Figure 1.3 : The flowers *of Alpinia conchigera*.

1.7 Medicinal Properties of *Alpinia* Species.

Many *Alpinia* species have been known to possess medicinal properties. Several of them become a major ingredient in traditionally prepared tonics locally called `Jamu'. Many researchers reported on the medicinal value of *Alpinia conchigera* related to skin diseases (Aziz *et al*., 2013; Ibrahim *et al*., 2009; Ong & Nordiana, 1999; Wannissorn *et al*., 2005) and it is also used as food condiment (Ibrahim *et al.*, 2009). *Alpinia galangal* is famous as one of the ingredient in food preparation. In India, *Alpinia calcarata* was practiced in the traditional remedies to treat diabetes, rheumatism, fever and stomache ache (Hema & Nair, 2009).

Alpinia species are in medium sized to large forest plants with some species reaching a height of over three meter. *Alpinia* is the only genus in Alpinieae that has a terminal inflourescence on the leafy shoots. The flowers are yellowish-green to creamy coloured or red, usually conspicuous. The staminodes are reduced to large teeth with several mm long at the base of the lip. The lip is more or less saccate and not divided, if pale coloured often with yellow blotches or red lines. The capsules are smooth, spherical or ellipsoid (Larsen *et al*., 1999). *Alpinia* species are rich in 1,8-cineol, linalool, β-caryophyllene (E) methyl cinnamate, β-bisabolene, eucalyptol, α-pinene, β-elemene and α-phellandrene (Ibrahim *et al*., 2009; Sirat & Nordin, 1995; Suthisut *et al*., 2011; Xie *et al*., 2013). The medicinal value of *Alpinia* from the previous study are summarized in Table 1.3.

Table 1.3: List of some *Alpinia* species used in traditional medicines for several ailments.

***Alpinia* species that can be applied for treatments**	**Ailments**
Alpinia galanga	Stomach ache, diarrhoea, dysentery, medications for post-partum, heart disease, bronchitis, tuberculosis, diarrhoea, ringworm, vomiting, skin disease, sinus and fever (Burkill, 1966; Perry & Metzger, 1980; Ong & Nordiana, 1999; Sinha, 2001; Rana *et al*., 2010).
Alpinia calcarata	Diabetes, rheumatism, fever and stomach ache (Hema & Nair, 2009; Materials, 2003).
Alpinia conchigera	Bronchitis, jaundice, headache, vertigo, metritis, arthritis, rheumatism, medications for post-partum, bond ache and pains, skin fungal infection, condiment, to relieve gastrointestinal disorders (Athmaprasangsa *et al*., 1994; Bhuiyan *et al*., 2010; Burkill, 1966; Holttum, 1950; Ibrahim *et al*., 2009; Ong & Nordiana, 1999, Wannissorn, 2005).
Alpinia japonica	Stomach ache, diarrhoea, dysentery (Burkill, 1966).
Alpinia katsumadai	Stomach ache, diarrhoea, dysentery (Burkill, 1966).
Alpinia mutica	Stomach ache, diarrhoea, dysentery (Burkill, 1966).
Alpinia officinarum	Stomach ache diarrhoea dysentery, medications for post-partum, treating colds, invigorating the circulatory system and reducing swelling (Burkill, 1966; The State Pharmacopoeia Commission of the People's Republic of China, 2005; Luo *et al*., 2010; Sinha, 2001; Rana *et al*., 2010).
Alpinia chinensis	Asthma and analgesic.
Alpinia malaccensis	Rheumatism, arthritis, to cure wounds, used for bathing feverish person, relieves sores and emetic proposes (Bhuiyan *et al*., 2010 & Kress *et al*., 2005).
Alpinia melanocarpa	Medications for post-partum (Burkill, 1966).
Alpinia cannaefolia	Fever (Burkill, 1966).
Alpinia globosa	Cardiac stimulant.
Alpinia oxyphylla	Treating Parkinson's disease (Li *et al*., 2013).
Alpinia zerumbet	Treating arterial hypertension, rheumatism and catarrhal infection (Elzaawely *et al*., 2007; Indrayan *et al*., 2010; Victorio *et al*., 2009).

CHAPTER 2: CHEMICAL CONSTITUENTS OF THE SFE EXTRACT

2.1 Introduction

In recent years, a supercritical fluid extraction (SFE) has been used in many applications especially in food, pharmaceuticals and cosmetic industries (Araus *et al*., 2009 & Pourmortazavi *et al*., 2005) as an alternative to conventional solvent extraction. The SFE processes are performed in the range of temperature in which thermo labile compounds have no thermal stress (Da Porto *et al*., 2009). Carbon dioxide, CO_2 act as an extraction solvent as it showed strong lyophilic selectivity; therefore extracts are devoid of unwanted compounds (organic and inorganic salts, sugars, amino acids, tannins etc.). The condition such as co-solvent, temperature and pressure were set to get the optimum yield or to achieve the compound of interest. SFE in plant essential and volatile oil analysis by Pourmortazavi and Hajimirsadeghi (2007) were revealed that there are many variables which influence the yield of the extract such as the solubility and mass transfer rate of plants oils. The matrix effect such as the particle size, shape, surface area, porosity, moisture, level of extractable solutes and the nature of the matrix will also affect the results.

Carbon dioxide can be liquefied to a state called supercritical fluid when the pressure and temperature are kept at certain conditions. The characteristic of a supercritical fluid resemble both a gas and liquid. The gas-like characteristics help the fluid diffuse to the matrix and access to the phytochemicals, while the liquid-like characteristics provide good solvation power. Therefore, liquid CO_2 can be a good solvent for SFE techniques. In addition, due to the low critical pressure (1100 psi) and temperature (31°C) of CO_2, it allows working at mild conditions and its gaseous standard state provides a solvent-free

product (Da Porto *et al*., 2009). The CO_2 pressure-temperature phase diagram is shown in Figure 2.1.

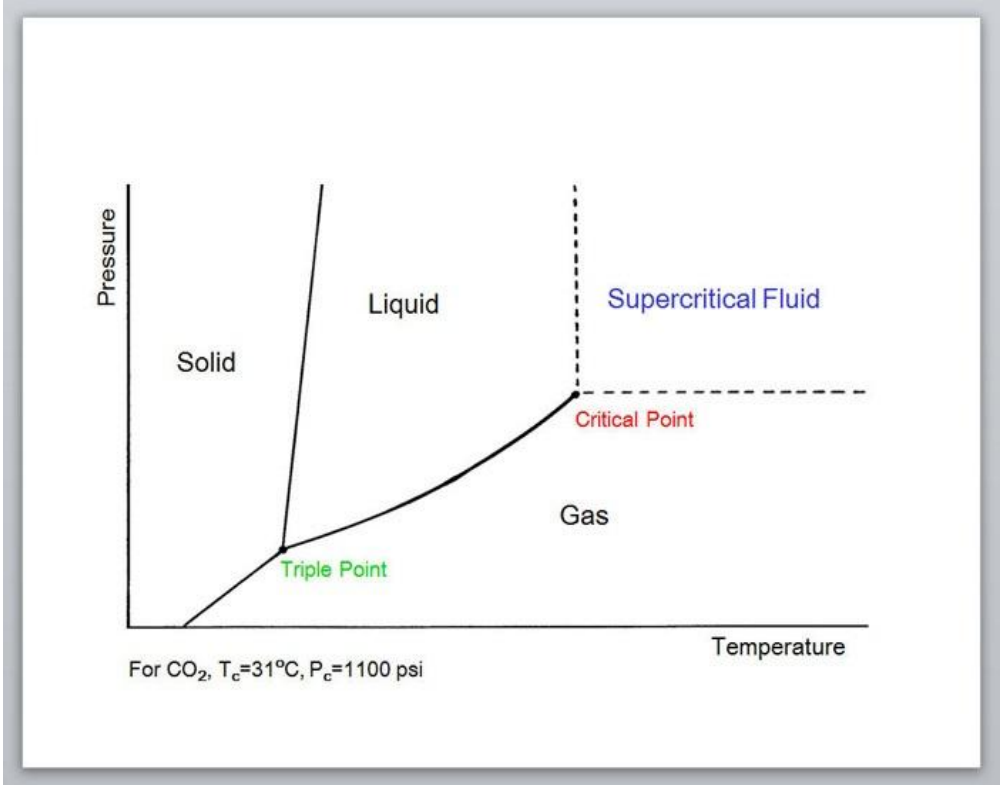
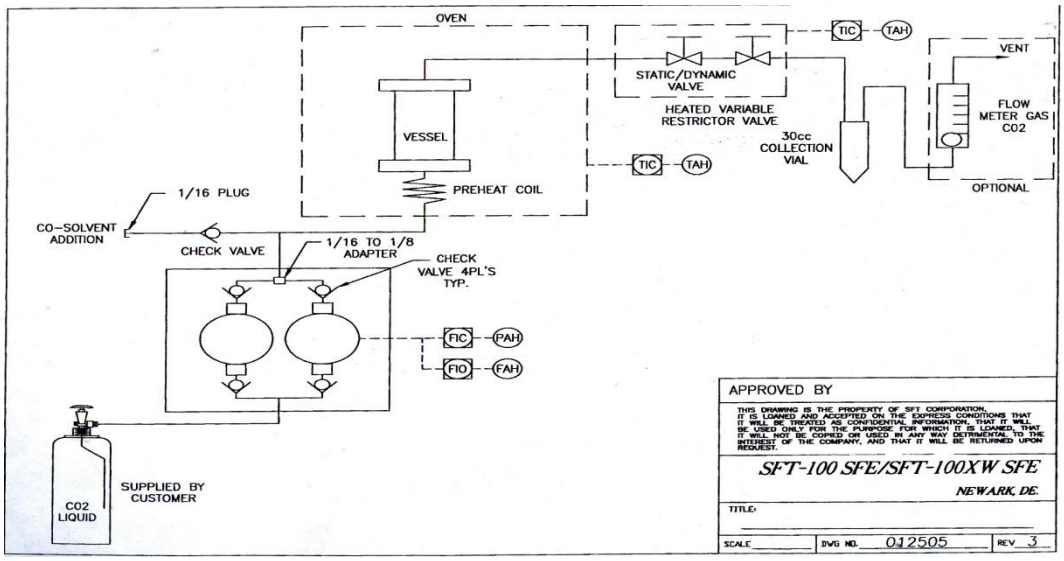


Figure 2.1 : The pressure-temperature phase diagram of carbon dioxide

The advantages of using the supercritical fluid extraction are not only higher extraction rate but it is non-toxic, non-flammable, non-explosive and easy to remove from the extracted materials, so it is an environmental friendly extraction (Liu *et al.*, 2009). It is also the fastest extraction.

The efficiency of CO_2-SFE can be optimized by changing the density of CO_2, modifier as a co-solvent (ethanol, methanol, etc.) modifier percentage, temperature, pressure and other parameters. The schematic diagram (Scheme 2.1) and the picture of SFE apparatus was showed in Figure 2.2, 2.3 and 2.4 (Appendix).



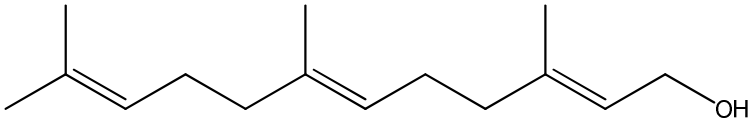
Scheme 2.1 : The schematic diagram of SFE apparatus

Chemical constituents in the SFE extracts can be detected by gas chromatography . Gas chromatography mass spectrometry is one of the major techniques to analyse and identify the volatile constituents in this study. Different parameters are applied to determine the entire compounds in the plants extract.

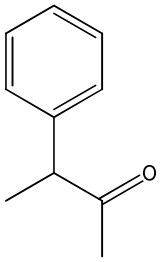
There are several reports regarding the extraction of chemical constituents of *Alpinia* species using SFE (Jin *et al*., 2009 & Luo *et al*., 2010) but to date there is still no report on the SFE extraction of chemical constituents of *Alpinia conchigera*. Jin Hong *et al.* (2009) reported the studies on the fingerprint of volatile oils from *Semen Alpiniae katsumadai* by GC-MS. In their study, five major constituents were identified which were farnesol (**I**) (22.53%), 3-phenyl-2-butanone (**II**) (6.72%), eucalyptol (**III**) (7.48%), 3-carene (**IV**) (19.22%) and β-pinene (**V**) (5.61%). The structures of the chemical constituents of *Semen Alpiniae katsumadai* were shown in Figure 2.5. They also reported that the SFE method was beneficial for the quality control of *Semen Alpiniae katsumadai*.

Luo *et al.* (2010) studied the supercritical extracted diarylheptanoids separation and identification of *Alpinia officinarum* by UPLC-MS-MS. The results revealed the presence

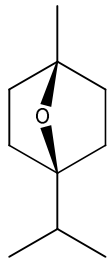
of 23 diarylheptanoids. Among them are, officinarumane C (**VI**), 1,7-bisphenyl-4-en-3-heptanone (**VII**), 1,7-bisphenylhepta-3,5-dione (**VIII**), 5-hydroxy-1,7-bisphenyl-3-heptanone (**IX**) and 3,5-dishydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-phenylheptane (**X**). The structures of the chemical constituents of *Alpinia officinarum* were shown in Figure 2.6. To the best of author's knowledge, there is no report on the chemical constituents of *Alpinia conchigera* extract using SFE technique.



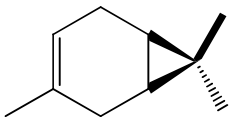
farnesol (**I**)



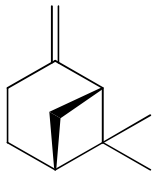
3-phenyl-2-butanone (**II**)



eucalyptol (**III**)

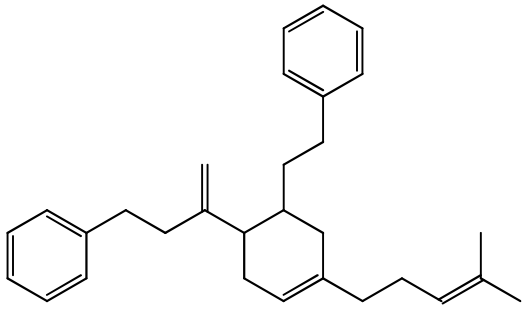


3-carene (**IV**)

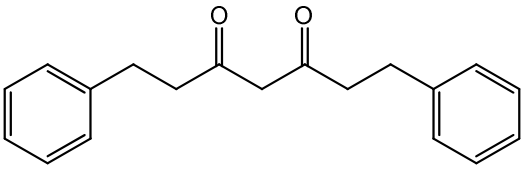


β-pinene (**V**)

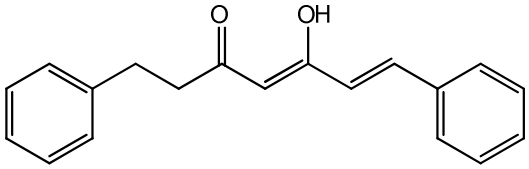
Figure 2.5: The structures of the chemical constituents of *Semen Alpiniae katsumadai*.



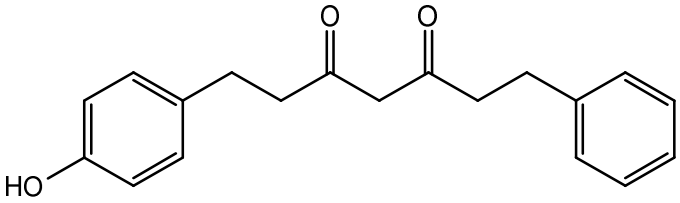
Officinarumane C (**VI**)



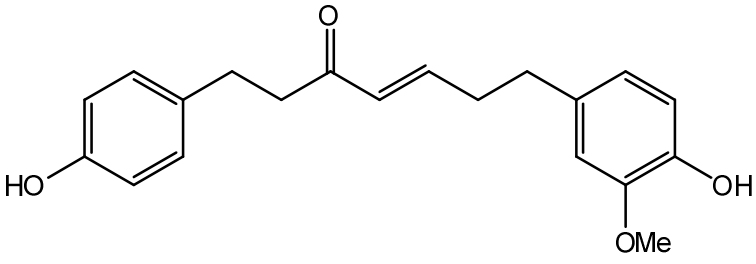
1,7-bisphenyl-4-en-3-heptanone (**VII**)



1,7-bisphenylhepta-3,5-dione (**VIII**)



5-hydroxy-1,7-bisphenyl-3-heptanone (**IX**)



3,5-dishydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-phenylheptane (**X**)

Figure 2.6: The structures of the chemical constituents of *Alpinia officinarum*.

2.2 Methodology

2.2.1 Extraction of leaf and rhizome of *Alpinia conchigera* using SFE technique

In this study, about 100-160 g of the dried rhizomes of *Alpinia conchigera* were placed in 100 ml extraction vessel of supercritical fluid extraction. The supercritical fluid extractor was set up to a temperature of 40°C and pressure of 1500 psi/ 5000 psi. In this research, two conditions of pressure were applied as shown in Table 2.1. This condition was counted according to the low critical temperature and pressure of CO_2 (as described in section 2.1) to get the optimum yield of extract. The extractor was left for one hour for static extraction which meant no liquid CO_2 flowed at that time. Subsequently, the sample materials were subjected to dynamic extraction by flowing the liquid CO_2 into the vessel for 15 minutes. During the extraction, up to ten crude extracts were collected in collection vials, as well as three times sample replicates. These procedures were repeated as well as for the dried leaves of *Alpinia conchigera*. All the crudes were then subjected to gas chromatography analysis and biological activity testing.

The percentage of yield of extraction was calculated using the equation:

$$\text{Yield of extracts} = \frac{m_{\text{extract}}}{m_{\text{raw material}}} \times 100\%$$

where : m_{extract} is mass of plant extract

$m_{\text{raw material}}$ is mass of dried plants fed into the extractor

Table 2.1 : The conditions of supercritical fluid extraction of *Alpinia conchigera* leaves and rhizomes.

Plants material		Temperature (°C)	Pressure (psi)
Leaves of *Alpinia conchigera*	A1	40	1500
	A2	40	5000
Rhizomes of *Alpinia conchigera*	B1	40	1500
	B2	40	5000

2.2.2 Analysis of crude extracts using GC-FID and GC-MS.

GC analysis of the crude extracts was carried out on a Agilent 7683B series injector flame ionization detector (FID) equipped with Agilent 7890A GC system. The HP-5 fused silica capillary column with a 30 mm X 0.320 mm internal diameter and 0.25 µm film thicknesses were fitted to the GC. The oven temperature was set from 70°C and held for 10 minutes and the temperature was increased to 245°C at a rate of 3°C per minute and held for 20 minutes at the end temperature. The nitrogen gas acted as a carrier gas with a flow rate 1ml/min and 1 µl injection sample at 20:1 split ratio. The picture of the Gas Chromatography Flame Ionization Detector (GC-FID) is shown in Figure 2.7 (Appendix).

GC-MS analysis was performed by Shimadzu GC-2010 directly coupled with GCMS-QP 2010 Plus and a Rtx- 5MS capillary column with 30 mm X 0.320 mm internal diameter and 0.25 m film thickness, using helium as a carrier gas. The oven temperature was set from 70°C and held for 10 minutes, then the temperature was increased to 245°C at a rate of 3°C per minute and then held for 20 minutes at the end temperature. The picture of the Gas Chromatography Mass Spectrometry (GC-MS) is shown in Figure 2.8 (Appendix).

The relative percentage of each constituent was calculated by comparing its GC peak area to the total areas that were summed from all detected peaks. The volatiles constituents of the crude extracts were identified by comparing their retention indices relative to *n*-hydrocarbon series (purchased from Sigma-Aldrich consists of 7 carbons to 40 carbons atom) and matching their mass spectra those with from National Institute of Standards and Technology MS library (NIST08) and references. Quantitative determination of the compound as obtained based on peak area measurements. The formula of retention indices are derived as equation:

$$\frac{100\ (t - t_n)}{(t_{n+1} - t_n)} + 100(n)$$

t : retention time of unknown by GC-FID

t_n : retention time of elute before unknown

t_{n+1} : retention time of elute after unknown

2.3 Results and discussion

The percentage of yield of the SFE extract for the leaves and rhizomes of *Alpinia conchigera* are shown in Table 2.2. There are two conditions in which the experiments were run at constant temperature (40°C) and different pressure (1500 psi and 5000 psi).

The yield of the crude extract was increased when the pressure increases based on a dry weight basis (w/w). Different colour of the extracts obtained were according to the part of the plants; the leaves of the *Alpinia conchigera* presented a yellowish green extract and the rhizomes of *Alpinia conchigera* yielded a golden yellow extract. The green colour of the leaves extract might be due to the presence of chlorophyll. Both extracts were in oily form.

Table 2.2: The percentage of yield for *Alpinia conchigera* leaves and rhizomes by SFE extraction.

Plant samples	Sample dry weight (g)	Yield (g)	% yield of crude extracts/g	Descriptions
A1 (leaves)	18.12	0.11	0.60	Oily yellowish green colour
A2 (leaves)	18.81	0.14	0.76	Oily yellowish green colour
B1 (rhizomes)	25.54	0.27	1.06	Oily golden yellow colour
B2 (rhizomes)	25.22	0.29	1.15	Oily golden yellow colour

Where: A1: leaves of *Alpinia conchigera* at temperature 40°C and 1500psi

A2: leaves of *Alpinia conchigera* at temperature 40°C and 5000psi

B1: rhizomes of *Alpinia conchigera* at temperature 40°C and 1500psi

B2: rhizomes of *Alpinia conchigera* at temperature 40°C and 5000psi

The chemical constituents of the leaves and rhizomes of *Alpinia conchigera* were separated using HP-5 capillary column for GC and Rtx-5MS capillary column for GC-MS. The chemical constituents of the leaves and rhizomes of *Alpinia conchigera* in different parameter of SFE are summarized in Table 2.3 – 2.6. Twenty-six volatile compounds and fourty-six volatile compounds were detected in A1 (95%) and A2 (90%), respectively. From the rhizomes, twenty-three volatile compounds were identified in B1 and twenty volatile compounds were identified in B2 accounting for 95% and 98%, respectively. The chromatograms of four samples were shown in Figure 2.9 – 2.12.

Among them, the main constituents were acetoxychavicol acetate (29.63%), *n*-hexadecanoic acid (14.45%), *n*-butylbenzenesulfonamide (5.11%) and *n*-octadecanoic acid (1.66%) were detected in A1. Major compounds in A2 consist of acetoxychavicol acetate (38.32%), *n*-hexadecanoic acid (14.91%), (E)-9-octadecenoic acid (12.77%), *n*-

octadecanoic acid (1.92%) and hexahydrofarnesyl acetone (1.27%). Acetoxychavicol acetate (76.12%) was identified as the major compound in B1 while *β*-bisabolene (5.69%), *trans*-*p*-coumaryl diacetate (1.74%), *n*-hexadecanoic acid (1.61%) and phytol (1.46%) were identified as minor constituents. B2 also revealed the major to be acetoxychavicol acetate (80.28%) while *β*-bisabolene (4.11), *trans*-*p*-coumaryl diacetate (1.85%), *n*-hexadecanoic acid (1.79%) and methyl(acetyloxy)[4-(acetyloxy)phenyl]acetate (1.35%).

Table 2.3 : List of volatile constituents of leaves crude of *Alpinia conchigera* (A1).

Compounds A1	Retention index	Retention time (*t*)	Relatives area (%)	Method of identification
Cumene	**788**	6.139	0.07	MS,RIL
α-terpineol	**1196**	14.488	0.05	MS,RIL
Chavicol	**1257**	17.630	0.05	MS,RIL
Trans-2-decenal	**1262**	17.886	0.04	MS,RIL
Eugenol	**1371**	23.290	0.21	MS,RIL
Caryophyllene	**1413**	25.240	0.20	MS,RIL
Methyl-2,6-dimethylbenzoate	**1426**	25.761	0.06	MS
n-pentadecane	**1498**	28.841	0.17	MS,RIL
β-bisabolene	**1504**	29.083	0.35	MS,RIL
Caryophyllene oxide	**1571**	31.686	0.25	MS,RIL
2-methoxy-3-(2-propenyl)-phenol	**1597**	32.714	0.08	MS,RIL
Acetoxychavicol acetate	**1659**	35.003	29.63	MS,RIL
β-Eusdesmol	**1685**	35.945	0.33	MS
n-heptadecane	**1700**	36.494	0.23	MS,RIL
N-butylbenzenesulfonamide	**1785**	39.417	5.11	MS
Acetoxyeugenol acetate	**1804**	40.075	0.48	MS,RIL
Trans-*p*-coumaryl diacetate	**1845**	41.413	0.97	MS,RIL

n-pentadecanoic acid	**1866**	42.084	0.43	MS,RIL
Cis-9-Octadecenal	**1919**	43.794	0.25	MS
n-hexadecanoic acid	**1991**	46.047	14.45	MS,RIL
n-heptadecanoic acid	**2071**	48.460	0.20	MS,RIL
Phytol	**2113**	49.692	0.68	MS,RIL
9-octadecenoic acid	**2165**	51.174	17.14	MS
n-octadecanoic acid	**2182**	51.667	1.66	MS,RIL
Hexahydrofarnesyl acetone	**2223**	52.800	0.90	MS,RIL
3,7,11,15-tetramethyl-2-hexadecen-1-ol	**2546**	61.221	1.62	MS
Total percentage identified			**94.96**	

MS, identified by mass spectrometry; RIL, identified by retention index and compared with those reported in the literature (Adams, 2001; Arn, 2004; Aziz, 2007; Ibrahim *et al*., 2009; Luo *et al*., 2010; Syamsir, 2009).

Table 2.4: List of volatile constituents of leaves crude of *Alpinia conchigera* (A2).

Compounds A2	Retention index	Retention time (t)	Relatives area (%)	Method of identification
Chavicol	**1257**	17.625	0.67	MS,RIL
Trans-2-decenal	**1262**	17.884	0.17	MS,RIL
Bornyl acetate	**1292**	19.543	0.08	MS,RIL
(E,E)-2,4-decadienal	**1314**	20.621	0.05	MS, RIL
p-hydroxybenzaldehyde	**1349**	22.262	0.15	MS,RIL
Eugenol	**1370**	23.256	0.23	MS,RIL
4-hydroxy-o-anisaldehyde	**1392**	24.286	0.13	MS, RIL
Caryophyllene	**1413**	25.214	0.49	MS, RIL
Methyl-2,6-dimethylbenzoate	**1425**	25.742	0.06	MS
Farnesane	**1463**	27.350	0.10	MS, RIL
α-irone	**1487**	28.367	0.14	MS,RIL
n-pentadecane	**1500**	28.907	0.78	MS, RIL
β-bisabolene	**1515**	29.507	0.20	MS, RIL
Eugenol acetate	**1527**	29.977	0.07	MS, RIL
Dihydroactinidiolide	**1548**	30.792	0.07	MS
Fumaric acid, ethyl-2-methylallyl ester	**1570**	31.669	0.25	MS
Caryophyllene oxide	**1575**	31.835	0.17	MS,RIL
Methyl-2,6-dimethylbenzoate	**1591**	32.478	0.22	MS
Cis-lanceol	**1605**	32.994	0.30	MS, RIL
E-farnesene epoxide	**1617**	33.433	0.08	MS
Spathulenol	**1633**	34.044	0.04	MS, RIL
Acetoxychavicol acetate	**1655**	34.824	38.32	MS,RIL
Methyl(acetyloxy)[4-(acetyloxy)phenyl]acetate	**1659**	34.969	0.40	MS
Isocaryophyllene dioxide	**1674**	35.548	0.39	MS, RIL
β-Eusdesmol	**1684**	35.883	0.42	MS, RIL
n-heptadecane	**1700**	36.486	0.40	MS, RIL

Oplopanone	**1743**	37.977	0.14	MS, RIL
(E,Z)-Farnesol	**1754**	38.357	0.27	MS, RIL
Curcumenol.	**1766**	38.758	0.37	MS, RIL
n-tetradecanoic acid	**1781**	39.283	0.86	MS, RIL
Acetoxyeugenol acetate	**1803**	40.015	0.39	MS, RIL
Nerolidol	**1809**	40.242	0.37	MS, RIL
Trans-*p*-coumaryl diacetate	**1844**	41.384	1.27	MS, RIL
n-pentadecanoic acid	**1864**	42.041	0.51	MS, RIL
Cis-9-Octadecenal	**1919**	43.798	0.27	MS
Methyl palmitate	**1927**	44.052	0.22	MS
Isophytol	**1946**	44.656	0.12	MS, RIL
n-hexadecanoic acid	**1979**	45.681	14.91	MS, RIL
n-heptadecanoic acid	**2039**	47.504	0.05	MS, RIL
Linoleic acid	**2066**	48.307	0.30	MS, RIL
Methyl linoleate	**2083**	48.797	0.11	MS
Oxalic acid, hexadecyl isohexyl ester	**2100**	49.309	0.26	MS
Phytol	**2112**	49.647	0.50	MS, RIL
(E)-9-octadecenoic acid	**2150**	50.735	12.77	MS
n-octadecanoic acid	**2172**	51.364	1.92	MS, RIL
3,7,11,15-tetramethyl-2-hexadecen-1-ol	**2221**	52.755	0.38	MS
4,8,12,16-tetramethylheptadecan-4-olide	**2347**	56.155	0.49	MS
Total percentage identified			90.22	

MS, identified by mass spectrometry; RIL, identified by retention index and compared with those reported in the literature (Adams, 2001; Arn, 2004; Aziz, 2007; Ibrahim *et al*., 2009; Luo *et al*., 2010; Syamsir, 2009).

Table 2.5 : List of volatile constituents of rhizomes crude of *Alpinia conchigera* (B1).

Compounds B1	Retention index	Retention time (*t*)	Relative area (%)	Method of identification
Chavicol	**1254**	25.836	0.83	MS, RIL
Chavicol acetate	**1345**	30.145	0.67	MS, RIL
Eugenol	**1359**	30.774	0.23	MS, RIL
(Z)-cyclodecane	**1380**	31.722	0.21	MS, RIL
Caryophyllene	**1397**	32.470	0.10	MS, RIL
Zingiberene	**1456**	34.907	0.18	MS, RIL
n-pentadecane	**1491**	36.338	0.07	MS, RIL
β-bisabolene	**1509**	37.032	5.69	MS, RIL
(E)-β-farnesene	**1524**	37.624	0.47	MS, RIL
1-hydroxychavicol acetate	**1532**	37.937	0.14	MS, RIL
4-chromanol	**1550**	38.600	1.27	MS, RIL
Caryophyllene oxide	**1563**	39.141	0.08	MS, RIL
2-methoxy-3-(2-propenyl)-phenol	**1585**	39.971	0.14	MS, RIL
Acetoxychavicol acetate	**1650**	42.409	76.12	MS, RIL
Methyl(acetyloxy)[4-(acetyloxy)phenyl]acetate	**1655**	42.594	1.04	MS, RIL
Isocaryophyllene	**1674**	43.298	0.33	MS, RIL
n-heptadecane	**1685**	43.693	0.43	MS, RIL
Phenylglyoxylnitrile	**1768**	46.472	0.43	MS
Acetoxyeugenol acetate	**1801**	47.584	0.39	MS, RIL
Trans-*p*-coumaryl diacetate	**1847**	49.104	1.74	MS, RIL
n-hexadecanoic acid	**1961**	52.720	1.61	MS, RIL
Phytol	**2069**	58.015	1.46	MS, RIL
n-octadecanal	**2081**	58.694	0.3	MS, RIL
Total percentage identified			93.93	

MS, identified by mass spectrometry; RIL, identified by retention index and compared with those reported in the literature (Adams, 2001; Arn, 2004; Aziz, 2007; Ibrahim *et al*., 2009; Luo *et al*., 2010; Syamsir, 2009).

Table 2.6 : List of volatile constituents of rhizomes crude of *Alpinia conchigera* (B2).

Compounds B2	Retention index	Retention time (*t*)	Relative area (%)	Method of identification
Chavicol	**1254**	25.857	0.89	MS, RIL
Chavicol acetate	**1345**	30.163	0.49	MS, RIL
Eugenol	**1359**	30.791	0.41	MS, RIL
(Z)-cyclodecane	**1380**	31.744	0.33	MS, RIL
n-pentadecane	**1498**	36.619	0.33	MS, RIL
β-bisabolene	**1509**	37.040	4.11	MS, RIL
(Z)-β-farnesene	**1524**	37.635	0.33	MS, RIL
Eugenol acetate	**1527**	37.759	0.16	MS, RIL
Hydroxychavicol acetate	**1549**	38.607	1.11	MS, RIL
Acetoxychavicol acetate	**1648**	42.378	80.28	MS, RIL
Methyl(acetyloxy)[4-(acetyloxy)phenyl]acetate	**1654**	42.591	1.35	MS, RIL
Isocaryophyllene	**1673**	43.303	0.28	MS, RIL
n-heptadecane	**1683**	43.700	0.46	MS, RIL
Iodononane	**1709**	44.608	0.22	MS, RIL
Acetamide,N-(3-amina-2,4,6-trimethylphenyl)	**1767**	46.480	0.46	MS, RIL
Acetoxyeugenol acetate	**1801**	47.592	0.41	MS, RIL
Trans-p-coumaryl diacetate	**1847**	49.109	1.85	MS, RIL
n-hexadecanoic acid	**1960**	52.718	1.79	MS, RIL
Phytol	**2070**	58.019	1.26	MS, RIL
n-octadecanal	**2081**	58.701	0.14	MS, RIL
Total percentage identified			96.66	

MS, identified by mass spectrometry; RIL, identified by retention index and compared with those reported in the literature (Adams, 2001; Arn, 2004; Aziz, 2007; Ibrahim *et al*., 2009; Luo *et al*., 2010; Syamsir, 2009).

The results indicated that acetoxychavicol acetate (**a**) was detected in all extracts. The percentage of the acetoxychavicol acetate in rhizomes (about 76% in B1 and about 80% in B2) was two to three times higher than in the leaves (about 30% in A1 and about 41% in A2). The mass spectrum of acetoxychavicol acetate indicated the molecular ion peak at *m/z* 243 as well as fragment ion peaks at *m/z* 192, 150 and 132. Figure 2.13 shows the mass spectrum of acetoxychavicol acetate and Scheme 2.2 shows the possibility of mass fragmentation of acetoxychavicol acetate.

There are reported various medicinal properties of acetoxychavicol acetate including anti allergic (Matsuda *et al*., 2005), anti-human immunodeficiency virus (HIV) (Ye & Li, 2006), anticancer (Awang *et al*., 2010; In *et al*., 2012), antifungal and antimicrobial activity (Aziz *et al*., 2013). More recently, Kato *et al.* (2014) reported the synergestic effect of 1'-acetoxychavicol acetate and sodium butyrate on the death of human hepatocellular carcinoma cells which can provide the development of novel combination therapies against hepatocellular carcinoma. Therefore, the major constituents, acetoxychavicol acetate, could be responsible for various medicinal properties of *Alpinia conchigera*.

Previous study of volatile constituents the *Alpinia conchigera* using hydro distillation technique reported that *β*-bisabolene (**b**) as the major compound (15.3% of composition) (Aziz *et al*., 2013; Ibrahim *et al*., 2009; Sirat & Nordin, 1995). But in this study, *β*-bisabolene was present in a much lower amount; 0.35% in A1, 0.20% in A2, 5.69% in B1 and 4.11% in B2. The difference of amount of *β*-bisabolene is due to the different of extraction technique of *Alpinia conchigera*; SFE and hydrodistillation technique. Since SFE are involved of pressure (1500 psi and 5000 psi) and temperature (40°C) while the hydrodistillation are involving the atmospheric pressure (14.696 psi) and

the temperature which can reach up to 100°C, it might be the high pressure (5000 psi) will caused the evaporation of the compound hence reduce the amount of *β*-bisabolene.. *β*-bisabolene present a molecular ion at *m/z* 204 and fragment ions at *m/z* 161, 119, 109, 93 and 69 as presented in Figure 2.14.

From the GC analysis, acetoxyeugenol acetate (**c**) were detected in all extracts; A1 (0.48%), A2 (0.39%), B1 (0.39%), B2 (0.41%). The molecular ion was detected at m/z 264 while fragment ions appeared at m/z 222, 180, 162, 141, 131, 119, 103, 91, 77 and 55 in mass spectra. The mass spectrum of acetoxyeugenol acetate is shown in Figure 2.15 and the possibility of mass fragmentation of acetoxyeugenol acetate is shown in Scheme 2.3.

Trans-p-coumaryl diacetate (**d**) was also detected in all extracts. A slightly lower composition was identified in A1 (0.97%) compared to A2 (1.27%), B1 (1.74%) and B2 (1.85%). The mass spectrum of *trans-p*-coumaryl diacetate was presented in Figure 2.16 which indicated a molecular ion peak at m/z 234 and fragment ions peak at m/z 192, 149, 133, 121, 107, 94, 77, 65 and 51. Scheme 2.4 shows the possibility of mass fragmentation of *trans-p*-coumaryl diacetate.

On the other hand for the SFE extract obtained at high pressure (5000 psi), eugenol acetate (**e**) was detected at 29.977 min in leaves extract (A2) and at 37.759 min in rhizomes extracts (B2). The mass spectrum of eugenol acetate is shown in Figure 2.17. It showed the molecular ions at m/z 206 and fragment ions at m/z 164, 149, 131, 121, 103, 91, 77, 65, 55 and 50.

Palmitic acid also known as *n*-hexadecanoic acid was identified in leaves extract of *Alpinia conchigera* which contribute about 14-15% of composition. There are variety of biogical activity of *n*-hexadecanoic acid included antioxidant (Omotoso *et al*., 2014), anti-

androgenic (Omotoso *et al*., 2014) and anti-inflammatory (Vasudevan *et al.*, 2012). Vasudevan et al. (2012) was reported the inhibition of phospholipase A_2 is one of the ways to control the inflammation which is the phospholipase A_2 will hydrolyse the ester bond of membrane phospholipids and release the fatty acids to initiate the inflammation. Hence, the finding validates the use of *Alpinia conchigera* for the external treatment of rheumatic and arthritis (Holttum, 1950).

(E)-9-octadecanoic acid was found about 12-17% of composition in leaves extract of *Alpinia conchigera*. This type of fatty acid together with the fennel oil and (+)-fenchone was invented for the insect repellent (Ahn, *et al*. 2002).

The common compounds in all four extracts were chavicol, eugenol, *n*-pentadecane, *β*-bisabolene, acetoxychavicol acetate, *n*-heptadecane, *n*-hexadecanoic acid, acetoxyeugenol acetate and phytol.

Chavicol, *trans*-2-decenal, eugenol, methyl-2,6-dimethylbenzoate, *n*-pentadecane, *β*-bisabolene, caryophyllene oxide, acetoxychavicol acetate, *n*-heptadecane, *n*-hexadecanoic acid, acetoxyeugenol acetate, *n*-pentadecanoic acid, *n*-hexadecanoic acid, *n*-heptadecanoic acid, *n*-octadecanoic acid, 3,7,11,15-tetramethyl-2-hexadecen-1-ol and phytol were identified in leaves extract of *Alpinia conchigera* (A1 and A2).

Additionally, some of the compounds were identified in rhizomes extract of *Alpinia conchigera* (B1 and B2) which are chavicol, chavicol acetate, eugenol, (z)-cyclodecane, *n*-pentadecane, *β*-bisabolene, hydroxychavicol acetate, acetoxychavicol acetate, *n*-heptadecane, *n*-hexadecanoic acid, *n*-octadecanal, *trans*-*p*-coumaryl diacetate, acetoxyeugenol acetate and phytol.

Even though the samples were collected at the same time and location but the differences in the composition of compound could be due to the different technique utilized; SFE and hydrodistillation. In SFE, the pressure is 5000 psi with operating temperature of 40°C whereas hydrodistillation technique applied normal pressure at about 14.7 psi and the operating temperature can goes up to 100°C. From our data analyses, the SFE extract are capable to identify about 70 compounds with acetoxychavicol acetate as the major constituents (80% yield). However in hydrodistillation, there are only 40 compounds were identified and no acetoxychavicol acetate was detected here. This is because the hydrodistillation technique only extracted low boiling point compounds (not more than 100°C) whereas with SFE, most non polar compounds were extracted with the boiling point is more than 100°C. The boiling point for acetoxychavicol acetate is 325.4°C. Therefore, the SFE extracts method extracts a wider range of non-polar compounds which include those with higher boiling point.

Some of major peak are shown at R_t ~84 min in the chromatogram A1 and A2. These peaks are not identified because of the standard hydrocarbon of alkane series can be trigger until R_t ~74 min. Therefore, compounds with R_t ~74 min and above cannot be identified. However, these compounds could be identified if they can be isolated and collected, for example by using preparative gas chromatography and identified with NMR.

.

CHAPTER 3: MACRONUTRIENTS AND TRACE METAL

3.1 Introduction

Macronutrients such as calcium and magnesium whereas trace metals such as iron, copper, zinc and manganese are essential metals and nutrients which play an important role in biological systems. Trace metals, if consumed more than the recommended levels can cause morphological abnormalities, stunted growth and increase mortality and mutagenic effects in human (Onianwa *et al*., 2001). Cu is an essential metal to human body as constituent of some metalloenzymes and it is required in haemoglobin synthesis and in the catalysis of metabolic oxidation (Onianwa *et al*., 2001) The permissible limit for Cu and Zn in agricultural products should be within 4 to 15 ppm and 15 to 200 ppm, respectively (Ajasa *et al*., 2004 & Allaway, 1968).

Zn is an essential metal for the normal functioning of various enzyme systems. Therefore, Zn deficiency might lead to loss of appetite, growth retardation particularly in children, weakness and even stagnation of sexual growth (Saracoglu *et al*., 2009 & Subramanian *et al*., 2012). According to FAO/WHO (2002), the maximum tolerable daily intake of zinc for an adult man is 45 mg/day and for children is 23-28 mg/day while for copper, the daily dietary limit is 0.5 mg/kg of body weight (WHO, 1982; National Coordinating Committee on Food and Nutrition, 2005; Subramanian, 2012).

The Recommended Dietary Allowance (RDA) compiled by Food and Nutrition Board of United States government advocated an average intake of 800, 350, 10, 15 mg per person/day for Ca, Mg, Fe, Zn, respectively. But the RDA for Cu and Mn are between 1.5

to 3 mg and 2 to 5 mg per person/day respectively. This daily nutrient intake is likely to pose no risk of adverse effects (Food and Nutrition Board of United States, 1989).

Despite numerous studies on chemical contents and biological activities, there is yet any report on their macronutrient and trace metal content of *Alpinia conchigera*. Therefore, there is a need to study the macronutrients and trace elements of these plants in Malaysia.

3.2 Flame Atomic Absorption Spectroscopy

Flame Atomic Absorption (FAAS) involved aspiration and combustion process. The samples introduced as a liquid by means the sample is digested or extracted so that it will be aspirated and mixed as fine aerosol with combustible gas such as air-acetylene, acetylene or nitrous oxide. The mixture is ignited to a temperature between 2200°C to 3300°C according to the fuel gas used. During the combustion process, atoms of the element of interest in the samples are reduced to the atomic state. A light beam from a hollow cathode lamp of the element is passed through the flame into a monochromator and detector. The free atom of the element will absorb light at characteristic wavelength. Therefore, the concentration of the element in the sample is measured by the reduction of the light energy at the analytical wavelength.

In spectroscopic analysis, no particular precaution was taken since the measured concentrations satisfied the principal criteria which were; sensitivity, detection limits and working range. The working range is dependent on the metal. The working range examines the sample with different analyte concentrations to determine the concentration range for acceptable calibration linearity so that the measurement uncertainty can be achieved. The detection limit is defined as the low concentration of element that can be detected by the

instrument. The detection limits and working range for the metal tested are listed in Table 3.1.

Table 3.1 : The detection limit and working range of Ca, Mg, Mn, Fe, Zn, Cu and Pb for Atomic Absorption Spectrometry (AAS) with air-acetylene flame gases.

Element	**Wavelength (nm)**	**Slit width (nm)**	**Sensitivity check (mg/L)**	**Working range (µg/L)**
Calcium (Ca)	422.7	0.7	4	3-5
Magnesium (Mg)	285.2	0.7	0.3	0.18-0.25
Manganese (Mn)	279.5	0.2	2.5	0.6-1.0
Iron (Fe)	248.3	0.2	5	2-3
Zinc (Zn)	213.9	0.7	1	0.3-0.75
Copper (Cu)	324.8	0.7	4	1.3-1.6
Lead (Pb)	283.3	0.7	20	8-10

Measured concentrations (mg/L) from Flame Atomic Absorption Spectroscopy (FAAS) were converted to ppm (parts per million) in the form of µg/g to obtain the amount of trace elements in µg present in 1 g of plant samples. Conversion was made by using the equation below:

$$\frac{\text{concentration (mg/L)} \times 10^{-3} \times 0.025\ \text{L (volume of sample solution)} \times \text{dilution factor (ml/ml)}}{\text{0.2 g (weight of samples)}}$$

Macronutrients and trace metal study consisted of two digestion methods. The method validation for these two methods has been done to acquire the most appropriate method for the macronutrients and trace metal analysis.

3.3 Method of digestion

3.3.1 Dry Ashing Digestion Method

Dry ashing method was employed for the digestion described below. About 2-4 g of powdered plant samples were weighed into a quartz crucible. The samples were dried and charred slowly on a hot plate. Complete ashing was achieved once the samples stop smoking. 5 ml of deionised water was added and the charred material was broken up using a glass rod. The samples were evaporated to dryness using a hot plate. Popping and spattering of the samples was avoided during charring. The sample was further heated in a muffle furnace for 24 hours at 450ºC. 1 – 2 ml of deionized water was added and evaporated to dryness followed by heating in the muffle furnace for 2 – 3 hours at 450ºC to remove carbon residues. The ash was then dissolved in 10 ml of suprapur nitric acid (1+9) and the mixture was warmed gently on hot plate for about 15 minutes. The sample was filtered with Whatmann No. 1 (110 mm pores size) filter paper and transferred into a 25 ml volumetric flask and diluted to the volume with deionized water. Finally, the solution was transferred into plastic bottle and kept in refrigerator prior to analysis.

An amount of 0.2 g of sample was placed on porcelain crucible and charred on a hot plate. Then, 5 ml of distilled deionized water was added into the sample and heated again until dry. After that, the sample was transferred into a muffle furnace for 24 hours at 450°C temperature to become ash. The sample was added with 10 ml of 65% suprapure nitric acid and heated for 15 minutes. After the sample was cooled to room temperature, it was transferred into a 25 ml volumetric flask. The sample was diluted with distilled deionized water to the mark and reserved for the analysis using FAAS as shown in Figure 3.1 (Appendix).

3.3.2 Microwave Digestion Method

0.2 g of sample was placed in a microwave vessel. The sample was added with 8 ml of 65% suprapure nitric acid and followed by 2 ml of 30% suprapure hydrogen peroxide supplied by Merck. All the mixtures were then left for an hour to let the acid and sample mix. After that, the sample were placed in the microwave oven (Microwave Accelerated Reaction System, MARS 5 by CEM Corporation, North Carolina USA) and set at the parameters shown in Table 3.2. The sample was cooled down to room temperature and let to stand overnight as in Figure 3.2 (Appendix). Later, the sample was filtered with Whatmann No. 1 (110 mm pore size), transferred into 50 ml of volumetric flask and distilled deionized water was added to the calibration mark. The sample was transferred in a bottle and stored in refrigerator before analysis using Flame Atomic Absorption Spectrometry (FAAS).

Table 3.2 : The parameter of microwave oven

Stage	Max Power	% power	Ramp (min)	T (°C)	Hold (min)
1	400 W	100	15	200	5
2	400 W	100	1	210	5
3	400 W	100	1	220	5

3.4 The standard calibration

All reagents were suprapur reagent grade purchased from Merck, Darmstadt, Germany. All the certified stock standard solution (1000 mg/L, each) for Ca, Mg, Fe, Zn, Mn and Cu were supplied from Merck, Darmstadt, Germany. All the standard solutions were diluted in deionized water with resistivity of 18.2 MΩ cm^{-1} by ELGA Purelab Classic UV MK2 system (provided by ELGA LabWater, United Kingdom) to give calibration solutions within 0.10 ppm until 11 ppm. Standard solutions were prepared for each element according to their working range. In addition, the standard solutions were prepared freshly to avoid any errors to the results. All plastics and glassware were cleaned by soaking in dilute nitric acid (1:9) and were rinsed with deionized water prior to use. Air-acetylene (99.99% of purity) was purchased from Linde Malaysia Sdn Bhd, Malaysia.

3.5 Analytical procedure

CRM 1547 (peach leaves) was digested using the method described in section 3.2.1 and 3.2.2. All measurements were run in triplicate and all elements were determined by FAAS model (Perkin Elmer AAnalyst 400 equipped with WinLab 32 Version 6.5) with PerkinElmer single-element hallow cathode lamp (HCL) for Ca, Zn, Cu, Mg, Mn and Fe. The method validation was applied to find the best method for this study. Therefore, the standard deviation (S.D) and coefficient variation (CV) were counted for get the precise data. The best method was chosen and applied for extraction of both rhizomes and leaves of *Alpinia conchigera*. The t-test and analysis of variance (ANOVA) are the statistical inference to analyse the significant different within two variables and sample in this study.

3.6 Results and discussion

The results obtained using dry ashing digestion method and microwave digestion method is depicted in Tables 3.3. The accurate results within the range of 80% to 120% recoveries were obtained for all target elements except iron, manganese and lead by the dry ashing digestion method using CRM 1547 (peach leaves).

The results acquired using microwave digestion with a mixture of nitric acid and hydrogen peroxide (aqua regia) were in very good agreement with the certified values except for lead. Microwave digestion is also known as close system digestion. This method is recommended due to the fact that the acids used are easily evaporated when heated at high temperatures. Microwave digestion is an adequate method for this study because it reduces the time of experiment. Compared to dry ashing digestion, microwave digestion with nitric acid and hydrogen peroxide digestion increased the elemental recovery for all elements except for lead. Lead is one of the highly volatile elements found in plants. Therefore the microwave digestion method was applied for both rhizome and leaves of *Alpinia conchigera*.

During the experiment, 6 samples and 1 blank were run in triplicates to get accurate results. Table 3.4 and Figure 3.3 showed the results of the metal analysis of *Alpinia conchigera* leaves and rhizomes via the microwave digestion method.The coefficient of variation (CV) of microwave digestion for intraday and interday was below than 5%, indicating the precision of the data collected for microwave digestion method compared to dry ashing method.

Table 3.4 : Metal analysis of leaves and rhizomes of *Alpinia conchigera*.

Alpinia conchigera	**Ca**	**Zn**	**Mg**	**Cu**	**Mn**	**Fe**
Leaves	3929.32± 2.81	55.52± 2.81	2558.56± 1.61	14.37± 1.40	2334.01± 1.78	547.90± 3.70
Rhizomes	295.27± 1.30	51.02± 2.04	4036.53± 2.90	18.10± 1.38	2201.44± 1.73	25.64± 1.39

*(µg element /g dry weight ± S.D.)

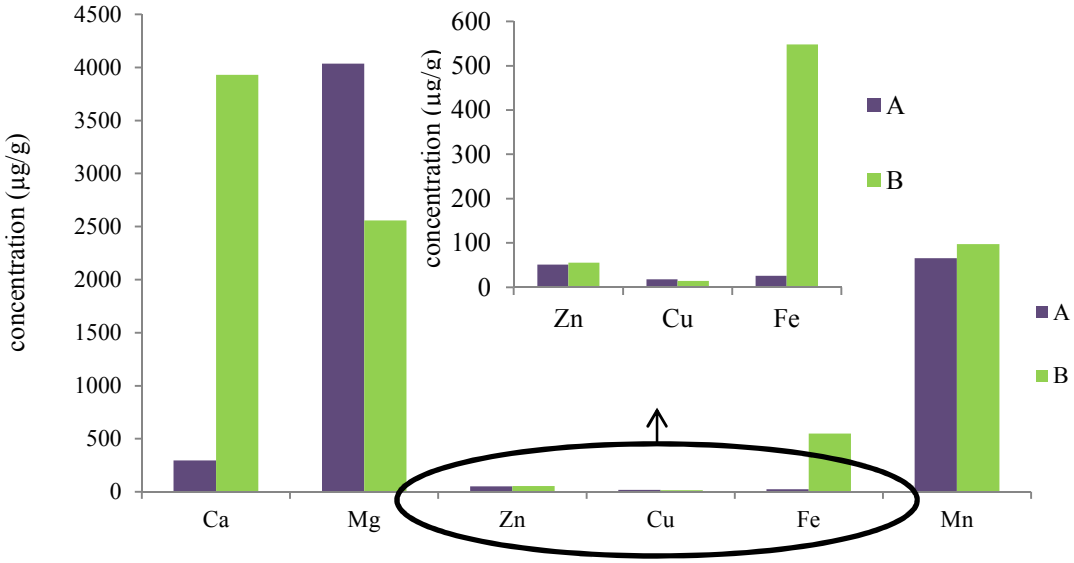


Figure 3.3 : The metal concentration in leaves (A) and rhizomes (B) of *Alpinia conchigera*.

The highest macronutrients in leaves of *Alpinia conchigera* is Ca (3929.32 µg/g) while Mg is dominant in rhizomes of *Alpinia conchigera* (4036.53 µg/g). The trace metal concentration in leaves of *Alpinia conchigera* showed increasing order of Cu (14.37 µg/g), Zn (55.52 µg/g), Fe (547.90 µg/g), Mn (2334.01 µg/g), Mg (2558.56 µg/g) and Ca (3929.32 µg/g). On the other hand, rhizomes of *Alpinia conchigera* showed decreasing order of Mg (4036.53 µg/g), Mn (2201.44 µg/g), Ca (295.27 µg/g), Zn (51.02 µg/g), Fe (25.64 µ/g) and Cu (18.10 µg/g). The abundance of manganese in leaves (2334 µg/g) of *Alpinia conchigera* are expected, and it is less in roots and stems which is as reported by

Ražić, S. (2005). The presence of high quantities of Mn in *Alpinia conchigera* was correlated with therapeutic properties against diabetic to stimulate insulin action and cardiovascular diseases (Ajasa *et al*., 2004).

Both parts of *Alpinia conchigera* showed the lowest concentration of Cu. Cu is an essential metal to human body as constituent of some metalloenzymes and it is required in haemoglobin synthesis and in the catalysis of metabolic oxidation (Onianwa *et al*., 2001). The permissible limit for Zn and Cu in agricultural product should be within 4 to 15 ppm for Cu and 15 to 200 ppm for Zn (Ajasa *et al*., 2004 & Allaway, 1968). The high concentration of Ca was obtained from leaves of *Alpinia conchigera* which is potential to cure hypocalcaemia in malaria's patient. Singh *et al.* (2012) have reported that high Ca content is vital to prevent the malaria's victims from hypocalcaemia which can cause prolonged Q-Tc interval that could be a risk factor for quinine cardiotoxicity and sudden death. Therefore, these findings showed that a high Ca content could be beneficial in the treatment of malaria patients.

Fe is the most abundant in leaves (547.90 μg/g) compared to rhizome (25.64 μg/g). It plays an important role in cellular processes such as the synthesis of DNA, RNA and proteins (National Coordinating Committee on Food and Nutrition, 2005). Fe deficiencies can reduce the productivity where there is a reduction in the tissue oxidative capacity as well as result in a decrease in hemoglobin concentration and cause anemia. In Malaysia, surveys have been conducted by the Ministry of Health Malaysia, in which 18.3% and 20.8% of boys and girls below 5 years old respectively were anemic (Ministry of Health, 2000). There were also reported figures of 25% of females aged between 18 to 60 years old suffering from anemia and 23% in elderly of both sexes (Ministry of Health, 2000). However it must be pointed out that an excess in iron may cause in multi-step processes of

carcinogenesis, pathogenesis of atherosclerosis, or neurodegerative disorders like Parkinson's or Alzheimer's diseases (Connor *et al*., 1992).

The limit of detection (LOD) and the limit of quantitation (LOQ) of the data were calculated and the mean of blank solution were determined for 6 replicate blank solution (n=6). As shown in Table 3.5, the LOD is in the range 0.002 µg/L to 1.543 µg/L and the LOQ is in the range of 0.007 µg/L to 5.143 µg/L for all the elements studied. The correlation coefficient (R^2) of the standard solution for the entire elements tested are >0.9900.

Table 3.5 : Limit of detection (LOD) and limit of quantitation (LOQ) of Ca, Mg, Cu, Fe, Mn and Zn with n = 6; (µg/L).

Element	LOD	LOQ	Correlation coefficient (R^2)
Ca	1.543	5.143	0.9942
Mg	1.208	4.028	0.9995
Cu	0.002	0.007	0.9966
Fe	0.003	0.110	0.9989
Mn	0.111	0.372	0.9997
Zn	0.137	0.457	0.9990

The t-test for leaves and rhizomes of *Alpinia conchigera* showed a significant difference for all macronutrients and trace metal except for Cu and Mg. It is because the t calculated value is higher than t critical (2.021) (t-calculated < t-critical) with $P<0.05$ and sample number, N: 21. Therefore, the mineral composition between two parts of *Alpinia conchigera* is significantly different due to the different of necessity of each macronutrients and trace metal for the different part of plants; leaves and rhizomes. As for leaves, Cu is

involved in production of new growing point and root tips which provides the elasticity and expansion of cell walls to keep growing points from becoming rigid and brittle. The deficiency of Cu will caused stunted growth of the leaves and the leaves become distorted. On the other hand, Mg is essential in photosynthesis process and for the metabolism of carbohydrates. It also regulates uptake of the other macronutrients as it supports as carrier of phosphate compounds throughout the plant and to facilitate the translocation of carbohydrates. The entire plants are turned to yellow with green intervene areas if the plants are lack of Mg

Hence, the macronutrients and trace elements were obtained in this study are in the increasing order of Cu < Zn < Fe < Mn < Mg < Ca for leaves and Cu < Fe < Zn < Ca < Mn < Mg for rhizomes of *Alpinia conchigera*. The significant presence of all the important macronutrients and trace metals which are essential for human health and well-being to a certain extent substantiate their use medicinally in traditional practices.

CHAPTER 4: BIOLOGICAL ACTIVITY

4.1 Introduction

Biological activity such as cytotoxic activity, antifungal, antioxidant, antibacterial and antimicrobial activity can be described as the beneficial or adverse effects of a chemical constituent on living matter such as human and plants. The activity is exerted by the substance's active compound and it can be modified by other constituents. A compound which is an active constituent or a modified compound is considered bioactive if it has an interaction with or effect on any cell tissue in the human body. Natural products of higher plants may give a new source of bioactive agents with possibly novel mechanisms of actions. Much work has been done on ethnomedicinal plants throughout the world (Aziz *et al*., 2013; Oluwafemi & Debiri, 2008; Catapan *et al*., 2001; Elzaawely *et al*., 2007; Ibrahim *et al*., 2009; Indrayan *et al*., 2010; Janssen & Scheffer, 1985; Pornpimon & Sakaman, 2008; Kusirisin *et al*., 2009; Wannissorn *et al*., 2005).

In an effort to expand the spectrum of antifungal agents from natural resources, *Alpinia conchigera* belonging to Zingiberaceae family has been selected. In some literature, this plant has been described to be useful against skin diseases, carminative, rheumatism and it is used in traditional medications for post-partum (Athamaprasangsa *et al*., 1994; Aun *et al*.,2011; Awang *et al*., 2010; Aziz *et al*., 2013; Bhuiyan *et al*., 2010; Burkill, 1966; Holttum, 1950; Ibrahim *et al*., 2009; Ong & Nordiana, 1999; Wannissorn *et al*., 2005).

In Thailand, the rhizomes are used in traditional medicine to relieve the gastro-intestinal disorders and in the preparations of food dishes (Matsuda *et al*., 2005). *Alpinia conchigera* also used as food condiment in northern states of Peninsular Malaysia and

sporadically in traditional medicine in the east coast to treat fungal infections (Aziz *et al*., 2013; Hasima *et al*., 2010; Ibrahim *et al*., 2009).

Hasima *et al.* (2010) studied 1'S-1'-acetoxyeugenol acetate which isolated from the rhizomes of *Alpina conchigera* represented as a potential chemotherapeutic agent against human breast cancer cells with higher cytotoxicity potency. It inhibited the growth of human breast adenocarcinoma (MCF-7) cells with IC50 values of 14.0 μm and the cytotoxicity induced to be dose and time-dependent 100% inhibition achieved after 36 h of treatment. Induces cell cycle of 1'S-1'-acetoxyeugenol acetate were consistent for human mammary epithelial cells (HMEC) normal breast cell control. Their findings indicate that 1'S-1'-acetoxyeugenol acetate possesses anti-cancer properties through the induction of cell arrest and apoptosis in MCF-7 human breast carcinoma (Hasima *et al*., 2010).

Moreover, 1'- acetoxychavicol acetate extracted from the rhizomes of *Alpinia conchigera* also inhibited the growth of human oral squamous cell carcinoma and it was suggested for further potentiate the effect of standard in combination of cisplatin treatment by modulation of proinflammatory microenvironment (In *et al.*, 2012). The antifungal activity study of the essential oil of *Alpinia conchigera* have been done by Halijah *et al*. (2009), the results showed weak inhibition against *Microsporum canis* (ATCC 36299), *Tricophyton mentagrophytes* (ATCC 18748) and *Trichophyton rubrum* (ATCC 28188).

The isolated compounds from the dichloromethane crude extract of the rhizomes *Alpinia conchigera* namely, t*rans*-*p*-coumaryl diacetate, 1'S-1'-acetoxyeugenol acetate, 4-hydroxybenzaldehyde and the dichloromethane crude extract itself indicated a strong inhibition against *Microsporum canis* (ATCC 36299), *Trichophyton rubrum* (ATCC 28188) and *Candida albicans* (ATCC 10231) (Aziz *et al*., 2013). These findings revealed that the

presence of an active compound will enhance the uses of the plant. In this study, the antifungal activity of the SFE extract shall be reported.

4.2 Methodology of Antifungal activity

The antidermatophyte activity was quantified in potato dextrose broth (DIFCO) using the microdilution method using flat bottom 96 wells microtitre plates. A serial twofold dilutions of the test compounds dissolved in dimethyl sulfoxide (DMSO) from Ajax Finechem (Seven Hills, NSW, Australia) were prepared prior to the addition of 100 µl overnight dermatophytic suspension (108 cfu/ml) followed by incubation at 25°C for 24 h. The highest concentration of DMSO remaining after dilution (5%, v/v) caused no inhibition of dermatophytes growth. Positive control includes wells with antibiotic Nystatin (Sigma-Aldrich) as comparison, a set of wells containing standardize dermatophytic inoculum to ensure their viability and broth as indication of aseptic condition. The target dermatophytics and the extracts used in this experiment are as below:

A1 : the SFE leaf extract of *Alpinia conchigera* at 40°C and 1500 psi

A2 : the SFE leaf extract of *Alpinia conchigera* at 40°C and 5000 psi

B1 : the SFE rhizome extract of *Alpinia conchigera* at 40°C and 1500 psi

B2 : the SFE rhizome extract of *Alpinia conchigera* at 40°C and 5000 psi

Microsporum canis (ATCC 36299)

Trichophyton mentagrophytes (ATCC 18748)

Trichophyton rubrum (ATCC 28188)

The Minimum Inhibitory Concentration (MIC) value was defined as the lowest concentration producing no visible growth (absence of turbidity and or precipitation) as observed through the naked eye and MFC is the concentration of antifungal agent at which the number of colony forming units is zero. The MIC value was recorded as the average of triplicates in a single experiment. For further inhibitory type (cidal /static) confirmation 20 µl (1 mg/ml) of 3-(4,5-Dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich) was added to the suspension in the selected wells, followed by aerobic incubation at 25°C up to 24 hour. The colour of the reagent-suspension will remain clear/yellowish indicative of cidal activity as opposed to dark blue indicating growth.

4.3 Results and discussion

4.3.1 Antifungal activity (Dermatophyte)

The crude SFE extracts obtained was evaluated for their inhibitory potential by comparing their respective minimum inhibitory concentration values against the three dermatophytic fungi. The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values were recorded as the average of triplicates. An antibiotic nystatin was used as a positive control for the dermatopytes and as well as for the comparison of the anti-dermatophytic potentials.

The results of the antifungal assay (MIC) are given in Table 4.1 and the strength of activity was classified as strong (MIC $\leq$ 1000 µg/ml), moderate (1000 µg/ml < MIC< 4900 µg/ml) and weak (MIC $\geq$ 5000 µg/ml) (Khalijah *et al*., 2011). From the table, the supercritical fluid extract from the rhizomes of *Alpinia conchigera* (B2) showed the

strongest inhibition (the best activity) against *Trichophyton mentagrophytes* (ATCC 18748) compared to the rhizomes of *Alpinia conchigera* (B1) but the rhizomes of the SFE extract, B1 indicate a good inhibition against *Microsporum canis* (ATCC 36299) and *Trichophyton rubrum* (ATCC 28188) compared to the rhizomes of the SFE extracts, B2. The MIC value of B2 against *Trichophyton mentagrophytes* is 154 μg/ml whereas its MFC value is 309 μg/ml. This result showed that the crude extract of rhizomes at high pressure of SFE indicated good inhibition against those dermatophytes compared to the crude extract of the rhizomes at low pressure of SFE.

On the other hand, the SFE extract from the leaves of *Alpinia conchigera* (A1 and A2) exhibited MIC values of 1258 μg/ml which were considered moderate against *Microsporum canis*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*.

From the GCMS analysis all extract A1, A2, B1 and B2 showed the presence of acetoxychavicol acetate, *trans-p*-coumaryl diacetate and acetoxyeugenol acetate however their amount differed from each other. It was reported by Aziz *et al*. (2013) that acetoxychavicol acetate and *trans-p*-coumaryl diacetate were the active ingredients for the antifungal activity. It was found that in both extract, the most potent extract B1 and B2, the amount of these two compounds were more than in A1 and A2 which showed weaker activities (Table 4.1). Therefore, the results obtained support the traditional uses of *Alpinia conchigera* rhizome in the treatment of skin infection (Ibrahim *et al.*, 2009; Ong & Nordiana, 1999).

Table 4.1 : The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) values (µg/ml) of the crude of leaves and rhizomes *Alpinia conchigera* against selected dermatophyte.

Microbes / **Samples**	**MIC (MFC) : µg/ml**		
	Microsporum canis **ATCC 36299**	***Trichophyton mentagrophytes*** **ATCC 18748**	***Trichophyton rubrum*** **ATCC 28188**
A1	1258 (>1258)	1258 (>1258)	1258 (>1258)
A2	>1250 (>1250)	nd	nd
B1	187 (187)	375 (375)	187 (187)
B2	309 (309)	154 (309)	309 (309)
Nystatin	<39 (<39)	<39 (<39)	<39 (<39)

*All the samples were run in triplicate (n=3); nd= not detected

MIC (µg/ml)	**Activity status**
≤ 1000	Strong
1000-4900	Moderate
≥ 5000	Weak

CHAPTER 5: CONCLUSION

5.1 Conclusion

The total compositions of SFE leaves extract (A1 and A2) were 94.96% and 90.31%, respectively. The rhizomes SFE extract (B1 and B2) comprise about 94.59% and 98.07% of the total compositions of the rhizomes extract. The chemical constituents of crude of all 4 samples were determined by GCMS which indicated that acetoxychavicol acetate as the most abundant constituent in both leaves and rhizomes of *Alpinia conchigera*. Chavicol, eugenol, *β*-bisabolene, *n*-pentadecane, *n*-heptadecane, *n*-hexadecanoic acid, acetoxyeugenol acetate and phytol were detected in all extracts. Some of the compounds reported to be extracted from hydrodistillation technique are *β*-bisabolene, *β*-pinene, *β*-sesquiphallandrene, *β*-elemene, *β*-caryophyllene and chavicol in which *β*-bisabolene was the major compounds (Ibrahim *et al*., 2009). Acetoxychavicol acetate was not extracted with hydrodistillation technique due to the boiling point is 325.4°C. However, in the presence of high pressure as applied in the SFE technique, acetoxychavicol acetate was then extracted.

These SFE extracts exhibited antifungal activities against *Microsporum canis* (ATCC 36299), *Trichophyton mentagrophytes* (ATCC 18748) and *Trichophyton rubrum* (ATCC 28188). The SFE rhizomes extracts (B1 and B2) showed lower MIC value as compared to the SFE leaves extracts (A1 and A2). Amongst the extracts, B2 showed the strongest inhibition against *Trichophyton mentagrophytes* (ATCC 18748) with MIC value of 154 μg/ml. *Trans-p*-coumaryl diacetate and 1'S-1'-acetoxyeugenol acetate were the antifungal components previously reported by Aziz *et al*. (2013). Both compounds were

detected in all extracts and as predicted the two most potent extract, B1 and B2, possess the highest amount of both *trans-p*-coumaryl diacetate and acetocyeugenol acetate. This supports the use of *Alpinia conchigera* as traditional antifungal agent in the Northern of Malaysia (Ibrahim *et al*., 2009; Ong & Nordiana, 1999).

In addition, this medicinal plant was found to be rich in macronutrient; Mg, Ca and Mn which are crucial for human health. The presence of Mg, Ca and Mn in *Alpinia conchigera* was found to be within the range of permissible limit for the agricultural products. For example, Mg is capable in preventing and managing deadly diseases such as high blood pressure, heart disease and diabetes whereas Ca will prevent the malaria's patient from a hypocalcaemia during the malaria disease. Consequently, *Alpinia conchigera* can be considered as a potential source of the mentioned macronutrients and has a good prospect to be developed into a health supplement owing to its richness in essential minerals together with its medicinal properties (Ibrahim *et al*., 2009; Aziz *et al*., 2013; Bhuiyan *et al*., 2010; Athamaprasangsa *et al*., 1994).

Hence the medicinal plant, *Alpinia conchigera*, has been shown to possess many bioactive compounds (acetoxyeugenol acetate, acetoxychavicol acetate and *trans-p*-coumaryl diacetate) and it is also shown to be rich in macronutrients; Mg and Ca therefore support its use as a medicinal plant and also as food condiment.

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Figure 2.2 : SFE extractor

Figure 2.3 : Co-solvent for SFE

Figure 2.4 : The crude oil SFE extract of the rhizomes of *Alpinia conchigera*.

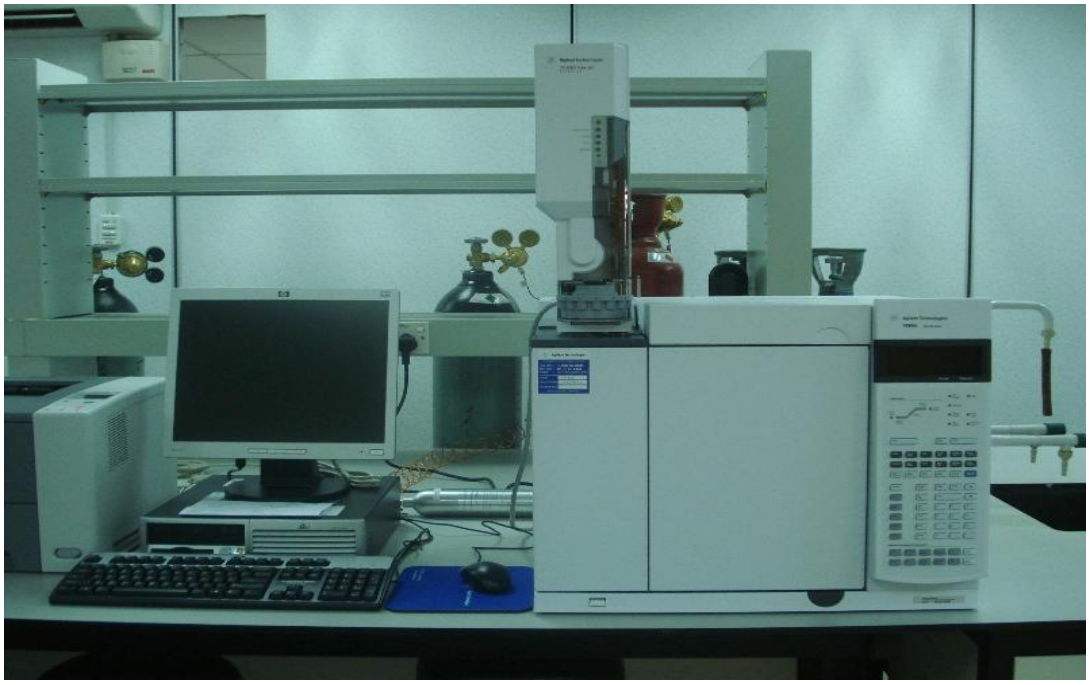


Figure 2.7 : The Gas Chromatography Flame Ionization Detector (GC-FID)

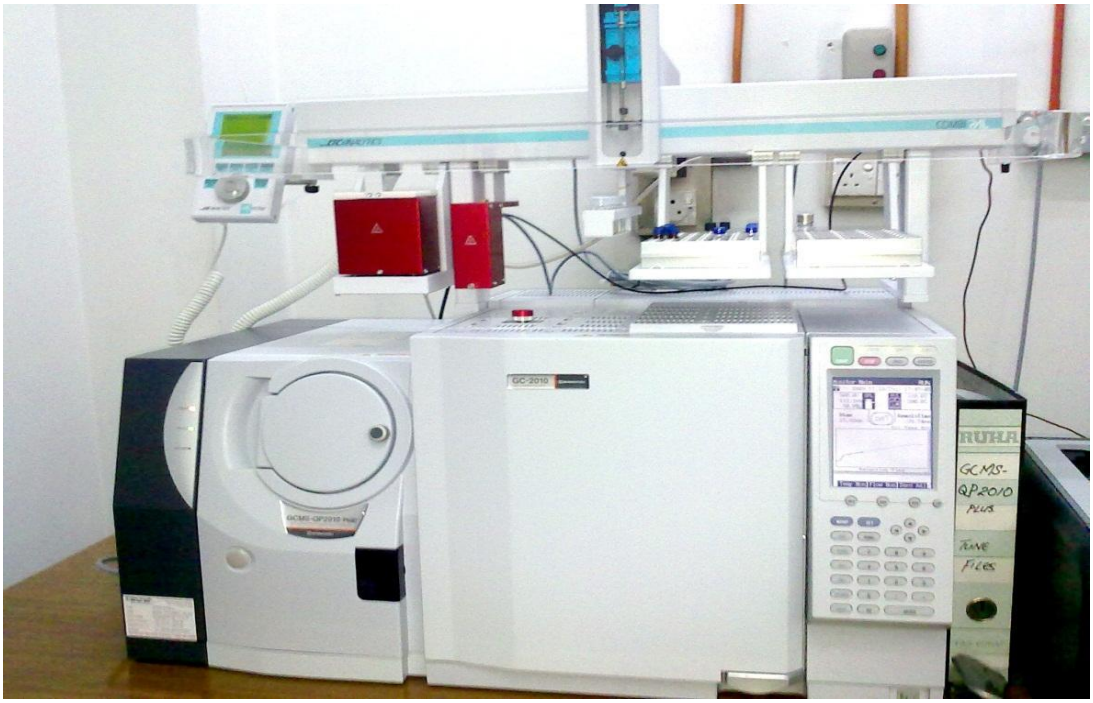


Figure 2.8 : The Gas Chromatography Mass Spectrometry (GC-MS)

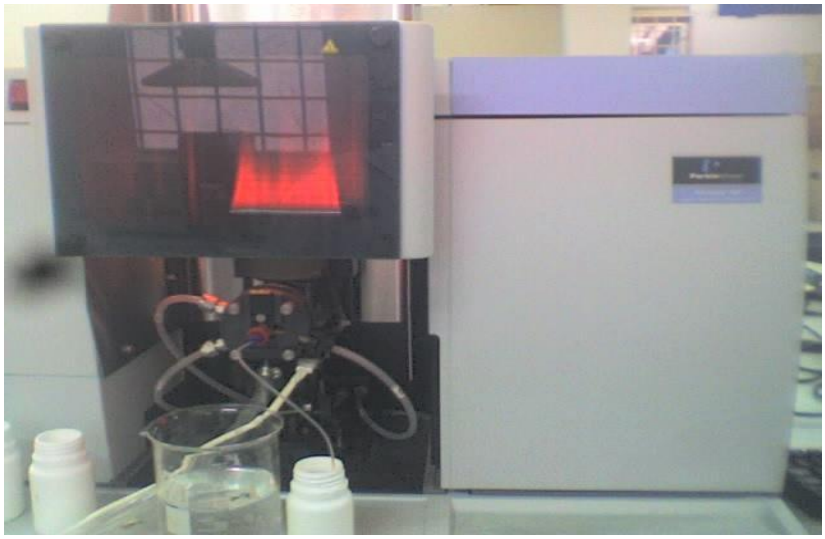


Figure 3.1 : The Flame Atomic Absorption Spectrometry (FAAS)

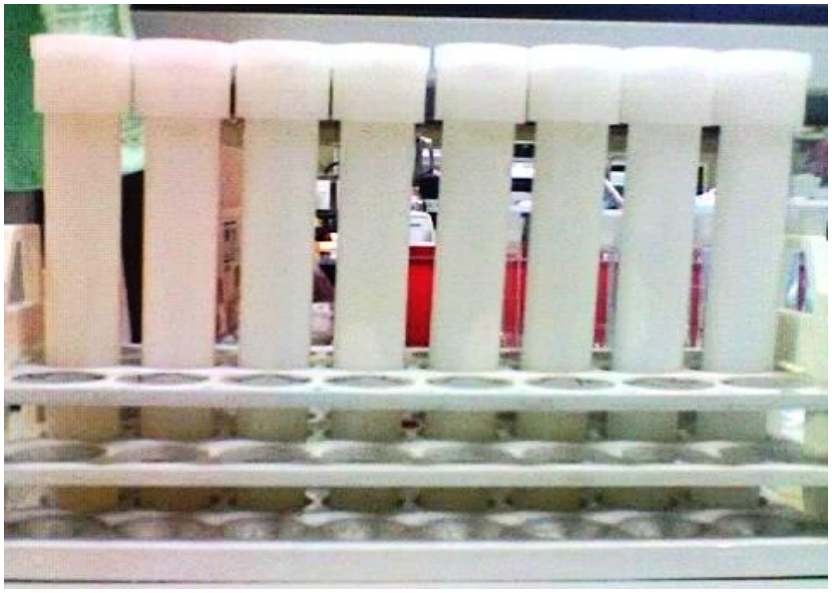
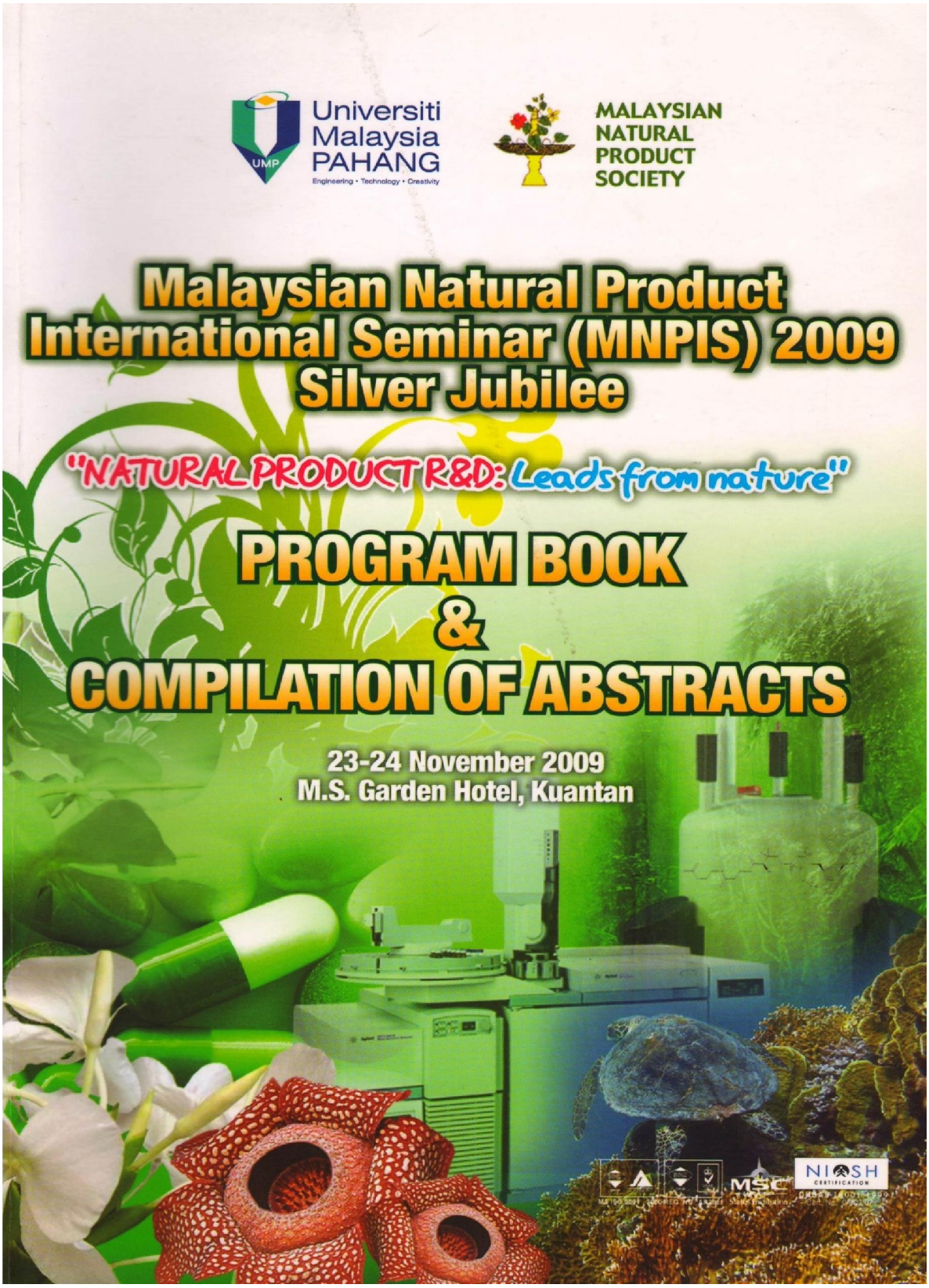


Figure 3.2 : Sample in vessel after complete digestion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Preliminary studies on supercritical fluid extraction of *Alpinia conchigera* Griff.

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Keywords: supercritical fluid extraction, *Alpinia conchigera*, gas chromatography mass spectrometry.

Abstract

The extracts oil from dried rhizomes, leaves and pseudostems of *Alpinia conchigera* Griff. collected from Jeli, Kelantan were extracted by supercritical fluid extraction technique (SFE). All the extracts were analyzed by using capillary gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS). Carbon dioxide was used at different pressure and temperature. The optimum condition with varying pressure and temperature of SFE will be investigated and discussed.

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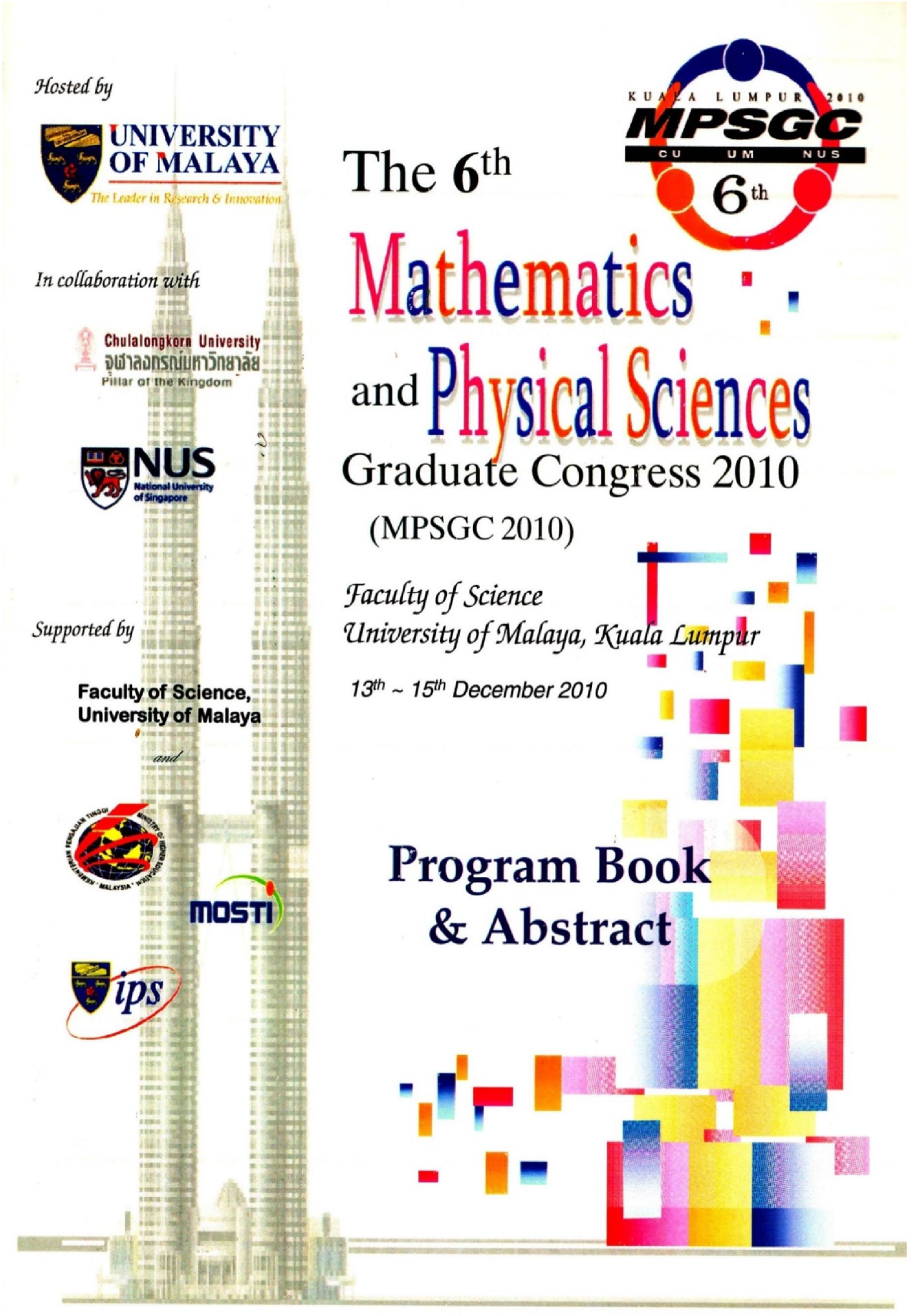
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& Abstract

Studies on Supercritical Fluid Extraction of *Alpinia Conchigera* Griff.

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Abstract: *Alpinia conchigera Griff.* is one of the species of the genus *Alpinia* which belongs to the family Zingiberaceae. *A. conchigera* is a perennial herb commonly found growing in damp and open spaces area. In the east coast Peninsular Malaysia, it is used to treat the skin fungal infection. Therefore, our group has initiated a chemical investigation on the dried rhizomes and leaves of *A. conchigera* Griff. using supercritical fluid extraction technique (SFE). Carbon dioxide as a solvent was used at different pressure and temperature. The volatile constituents for both rhizomes and leaves were identified by using gas chromatography mass spectrometry (GC-MS). 1'-acetoxychavicol acetate was defined as a major compound in the rhizomes while phytol was the major compound in the leaves of *A. conchigera* Griff.

Keywords: Supercritical fluid extraction, *Alpinia conchigera*, gas chromatography mass spectrometry.

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