CHAPTER 1: INTRODUCTION

Medicinal plants have been used in various human cultures mainly by the indigenous people around the world as the major component of traditional and herbal medicines due to their accessibility and faith of the people. The World Health Organization (WHO) estimated that more than 3.5 billion people in the developing countries rely on plants as components of their primary healthcare (Rasadah and Li, 2009). The reason why people choose herbals than modern drugs is the low cost of herbal products, hence making it more affordable to the lower income group market. Besides that, the general public impression that herbals are 'natural' and anything that is natural is considered as safe and less harmful, make them consume it without any doubt (Abas, 2000).

More than 35,000 plant species have been reported to be used for medical purposes and the number could be much higher as the knowledge on uses of plants were mostly undocumented but has been passed down orally from one generation to another (Jantan, 2004). The practice for traditional medicines in Malaysia is still common among various ethnic groups nowadays, as an alternative to the conventional therapeutic medicine or as nutritional and dietary supplements. Medicinal plants, mainly herbs, have a wide variety of uses other than to treat various ailments such as flavours and fragrances, dyes, biopesticides, cosmetics and detergents (Rasadah and Li, 2009).

Plant materials contain hundreds of chemicals that are present naturally in them called phytochemicals. Phytochemicals include essential oils, oleoresin, alkaloids, flavanoids, glycosides, phenolic compounds and other bioactive compounds. The herbal materials need to be processed in order to obtain full benefit from them in the most convenient

form. They can be in the form of dried and ground herbs, whole extracts and as pure phytochemicals substances (Ramlan and Mohamad Roji, 2000).

Essential oils and extracts of plants have been used for many thousands of years, and recently, have evoked interest as sources of natural products. Investigations concerning the antimicrobial and antioxidant activities of the plant, their extracts together with essential oils have been conducted in the search for new compounds to replace synthetic drugs. In particular, the antimicrobial activity of plant oils and extracts has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Shan *et al*., 2007).

As one of the components of traditional medicine, herbal medicine is regulated under the Control of Drugs and Cosmetics Regulation in Malaysia earlier, where registration of the product particularly based on their quality and safety profile. With increasing demand from the consumers, the license of Good Manufacturing Practices (GMP) was implemented in 1996 to manufacturers of herbal products (Ramli, 2000).

After all, although the number of medicinal plants being commercialized is undeniable, the importance of the conservation of plants should be noted. There should be a balance between consumption and conservation of medicinal plants to maintain their resources mainly for Zingiberaceae species where about 75 % of the 308 known species are endemic and about 25 % are non-endemic and widespread in South East Asia (Soepadmo, 2000). Thus the cooperation of all parties to improve awareness on conservation of medicinal plants resources will make the vision transforming Malaysia into a world centre in conservation, research and utilization of tropical biodiversity by the year 2020 will be accomplish.

1.1   Research objectives

In response to interest in the use of medicinal plants in Malaysia, Zingiberaceae species was one of the selected medicinal plants subjected to extensive chemical and pharmaceutical investigations under the 8th Malaysia plan (Jantan, 2004). Several reports have been published concerning the composition together with the biological properties such as antimicrobial, antioxidant and anti-inflammatory of these plant extracts (Habsah *et al*., 2000; Ficker *et al*., 2003; Chandarana *et al*., 2004; Chen *et al*., 2008).

Hence, this study is aimed at collecting new information and data on biological activities of two wild *Alpinia* species namely *Alpinia pahangensis* and *Alpinia mutica*. In this study, crude extracts of leaves and rhizomes extracted in solvents with a polarity sequence of hexane (non-polar), dichloromethane (medium polar) and methanol (polar) were screened for their biological activities. Thus, the objectives of this study are:

1.   To determine the antioxidant activity of the crude extracts of leaves and rhizomes of *Alpinia pahangensis* and *Alpinia mutica* using 2,2-diphenylpicrylhydrazyl (DPPH) assay.

2.   To investigate the antimicrobial activities of the crude extracts of rhizomes and leaves of *Alpinia pahangensis* and *Alpinia mutica*.

CHAPTER 2: LITERATURE REVIEW

2.1   An overview of the family Zingiberaceae

Zingiberaceae is the largest family of the order Zingiberales with 53 genera and over 1200 species. This family is widely distributed throughout the tropics and subtropics and also one of the largest families of the monocotyledons in the Malesian region which includes Malaysia, Indonesia, Brunei, Singapore, Philippines and Papua New Guinea (Larsen *et al*., 1999; Larsen, 2001).

Classification of Zingiberaceae based on vegetative and floral characteristics according to Holtum's (1950) comprises three tribes that are Hedychieae, Alpinieae and Globbeae as illustrated in Figure 2.1. The family Costaceae was included in the Zingiberaceae in earlier classifications, but with a number of distinctive characters; for example lack of aromatic oils, branched aerial stems, and spiral monostichous phyllotaxy, it is now accepted as the sister clade to the gingers (Larsen *et al.*, 1999; Kress *et al*., 2002).

Recently molecular analyses have been used to study the phylogenetic relationships among the genera of the Zingiberaceae. The results of these molecular data analyses propose a new classification of the Zingiberaceae that recognize four subfamilies and four tribes (Kress *et al*., 2002). Tribes of the new classification of the Zingiberaceae are shown in Figure 2.2.
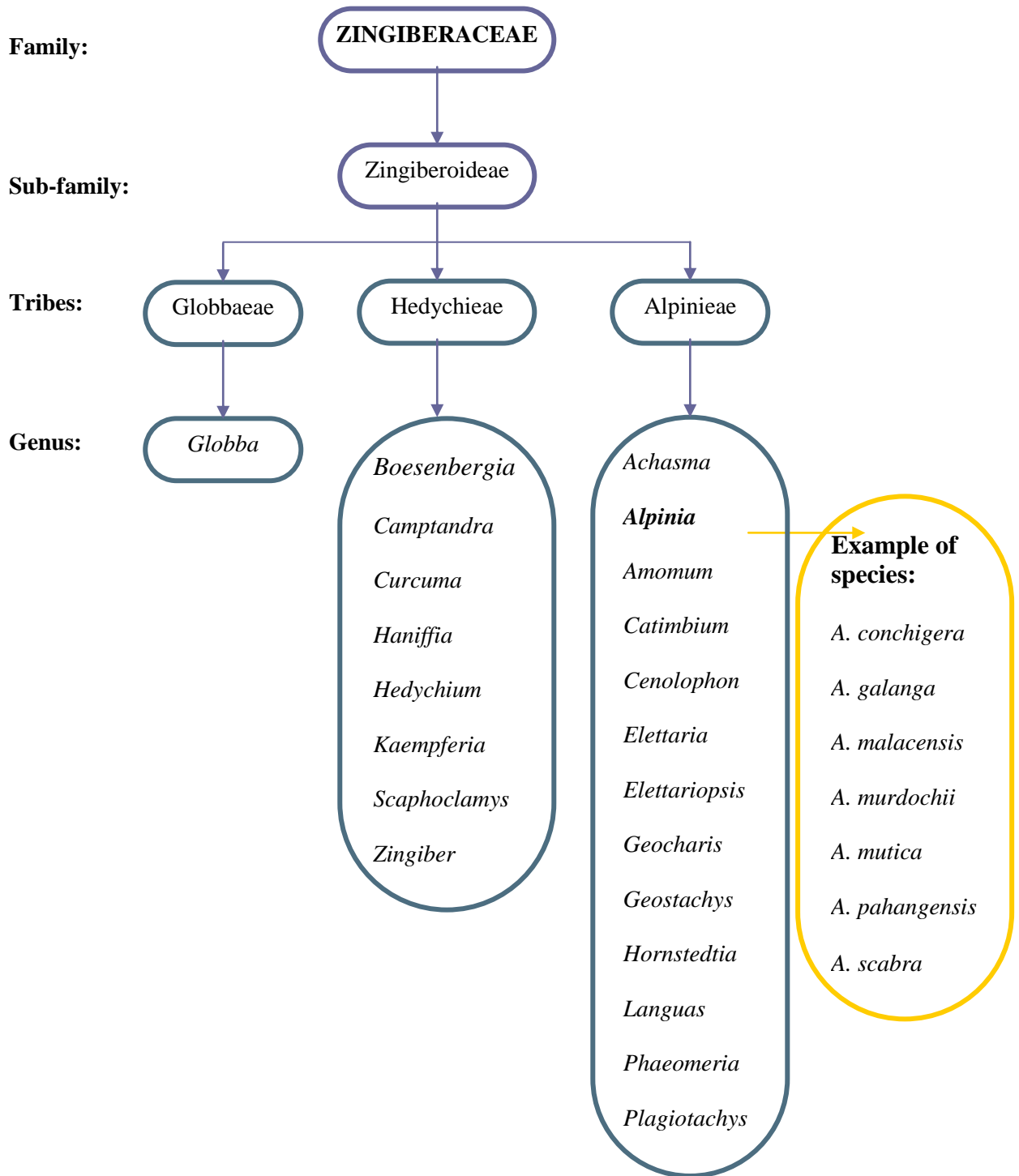
Family: ZINGIBERACEAE

Sub-family: Zingiberoideae

Tribes: Globbaeae | Hedychieae | Alpinieae

Genus:

Globba

Hedychieae genera:
Boesenbergia
Camptandra
Curcuma
Haniffia
Hedychium
Kaempferia
Scaphoclamys
Zingiber

Alpinieae genera:
Achasma
Alpinia
Amomum
Catimbium
Cenolophon
Elettaria
Elettariopsis
Geocharis
Geostachys
Hornstedtia
Languas
Phaeomeria
Plagiotachys

Example of species:
A. conchigera
A. galanga
A. malacensis
A. murdochii
A. mutica
A. pahangensis
A. scabra

Figure 2.1: Classification of Zingiberaceae according to Holtum's (1950)

Family: ZINGIBERACEAE

Sub-family; Siphonochiloideae | Tamijioideae | **Alpinioideae** | Zingiberoideae

Tribes: *Siphonochileae* | *Tamijieae* | ***Alpinieae*** (16 genera) | *Riedeliaea* (4 genera) | *Zingibereae* (25 genera) | *Globbeae* (4 genera)

Genera: *Siphonochilus* | *Tamija*

Examples:
Aframomum
Alpinia
Amomum
Elettaria
Elettariopsis
Etlingera
Geocharis
Hornstedtia

Examples:
Burbidgea
Pleuranthodium
Riedelia
Siamanthus

Examples:
Boesenbergia
Camptandra
Curcuma
Haniffia
Hedychium
Kaempferia
Roscoea
Zingiber

Examples:
Gagnepainia
Globba
Hemiorchis
Mantisia

Figure 2.2: Classification of Zingiberaceae based on molecular data (Kress *et al*., 2002).

Zingiberaceae species or can be generally referred to as gingers, grow naturally in damp, humid, shady areas with good light. Gingers have a wide range of habitats ranging from riverine to limestone rocks and from the lowlands to the upper montane regions. They are generally abundant in lowland to hill forests, notably between 200 m and 500 m above the sea level. They also vary in height and size from gigantic erect leafy shoots which can sometimes grow over 8 m high and others can be as small as 10 cm or less or prostrate near the ground (Larsen *et al.*, 1999).

Zingiberaceae is well known and have been cultivated for its medicinal and economic significance with many species that provide useful products for food such as fresh vegetables (commonly known as ulam in Malaysia), spices, medicine, flavoring agents, fragrance, coloring or natural dyes, condiments, ornamentals and recently as cut flowers (Burkill, 1996; Larsen *et al.*, 1999). The genera of *Alpinia*, *Amomum*, *Curcuma* and *Zingibe*r are among the main gingers used in traditional medicine (Table 2.1).

Table 2.1: Selected examples of uses of Zingiberaceae species

Zingiberaceae species	Local name	Uses	References
Alpinia galanga L. Willd.	*Lengkuas*	The rhizome is cut and dipped into kerosene and then rubbed onto skin infected with fungus as a cure.	Ong and Norzailina, 1998
Alpinia calcarata Rosc.	-	Used in treatment of rheumatism, inflammation, bronchial catarrh and asthma, stimulate digestion, purify the blood, prevent bad breath and improve the voice and as antifungal agent.	Arambewela *et al*., (2005)
Alpinia officinarum Hance	-	The root is boiled as tonic soups.	Guo *et al*., (2008)
Amomum (the genus) e.g: *Amomum subulatum*	*Buah pelaga* (inferior substitutes of cardamom)	Seeds as spices, plant parts used for curing toothache, dysentery, diarrhoea, rheumatism, vomiting, dyspepsia and lung diseases.	Sabulal *et al*., (2006)
Curcuma longa L. (*Curcuma domestica* Val.)	*Kunyit*	Antimicrobial, antioxidant, antiinflammatory, anticancer, hepatoprotective, antiallergic, wound healing, antitumour, antispasmodic and anti-HIV properties.	Chandarana *et al*., (2004)
Curcuma zedoaria Rosc	*Temu kuning*	Used to treat inflammation, as tonic and flavouring.	Souri *et al*., (2008)
Elettaria cardamomum L.	*Buah pelaga*	Used to treat skin condition and in digestion. The oil is used in cosmetics.	Khalaf *et al*., (2008)
Etlingera elatior	*Kantan*	Flavouring and medicine.	Faridahanim *et al*., (2007)
Zingiber zerumbet Smith	*Lempuyang*	Decoction of the rhizome or juice extracted from the fresh rhizome is taken to treat jaundice.	Ong and Norzailina, 1998)
Zingiber officinale Rosc.	*Halia*	Treatment of rheumatism, nervous diseases, gingivitis, toothache, asthma, stroke, constipation and diabetes	Lantz *et al*., (2007)

2.2 A brief description of the genus *Alpinia*

Alpinia from the tribe Alpinieae is the largest genus in the Zingiberaceae family with over 250 species in tropical Asia (Larsen *et al*., 1999). They are grown in humid and shady conditions with rich soils. They can be found in secondary vegetation, bamboo and teak forest, brushwood and ravines but rarely in primary forest and some are village plants which usually grow in the open and rich soil (Ibrahim, 2002). *Alpinias* range from medium to large forest plants with some species reaching a height of over three meter (Larsen *et al*., 1999).

Alpinia is propagated by vegetative propagation from a portion of rhizomes. This genus has a terminal inflorescence on the leafy shoot. The flowers are yellowish-green to creamy coloured or red. It has reduced staminodes to large teeth (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 of *Alpinia* are smooth, spherical or ellipsoid (Larsen *et al*., 1999).

2.3 *Alpinia* species studied

Selected wild *Alpinia* species used in this study are *Alpinia pahangensis* Ridl. and *Alpinia mutic*a Roxb. Their extracts from different solvent were obtained and their antimicrobial and antioxidant activity were examined. General descriptions on the botanical aspect of each species are as described below.

2.3.1 *Alpinia pahangensis* Ridl.

Figure 2.3:
The rhizome of *Alpinia pahangensis* Ridl.
(Photo: Devi R.S.)

Figure 2.4:
The flower of *Alpinia pahangensis*
Ridl. (Photo: Myriana Z.)

Botanical name   **:**   *Alpinia pahangensis* Ridl.

Synonym   **:**   *Alpinia burkillii* Hend. and *Languas pahangensis* Hend

General description :   The stem is about 2 to 3 meter tall. The leaf is commonly about 75 by 13 cm wide, light green with short hairy on both surface. Petiole is about 3.5 cm long and hairy. Inflorescence is about 20 to 30 cm long with a long sheath at the base. Rachis stout; densely short hairy, bearing 20 to 25 cincinni, each with 2 to 7 flowers. Primary bracts at base of inflorescence are very short, fringed with long hairs, towards apex of inflorescence much longer and the highest ones sometimes as long as flower bracts. Stalks of cincinni are velvet-hairy, commonly to 1 cm long, at bases of large inflorescences sometimes to 2.5 cm long. Secondary bracts are narrowly funnel shaped, obliquely truncate, thin and papery, short hairy or nearly glabrous on outer surface, fringed with rather long hairs, the outer ones commonly 2 to 3 cm long and cream in colour. Pedicels of flowers are 2 cm long and hairy. Ovary covered with spreading stiff hairs. Calyx with ovary about 2 cm long is tubular, not deeply split,

white, lobes almost equal, hairy, one or two of them with slender points up to 3 mm long. Corolla tube little shorter than calyx, slender; lobes densely hairy and cream in colour (Holttum, 1950). Ridley (1924) reported that this unexploited species can be found easily at lowland areas of Pahang, Peninsular Malaysia.

2.3.2 *Alpinia mutica* Roxb.

Figure 2.5: The flowers and fruits of *Alpinia mutica* Roxb. (Photo: Phang C.W.)

Botanical name : *Alpinia mutica* Roxb.

Synonym : *Catimbium muticum* (Roxb.) Holtt. and *Languas mutica* (Roxb.)

Local name : Chengkenam

General description :   The stem is about 1.2 to 1.7 m tall and rather close together. The leaf is glabrous except for hairy edges and apex and sometimes a few hairs on base of midrib beneath. Petiole is about 2 cm long. Inflorescence is about 15 cm long which is emerging from uppermost leaf sheath. Rachis stout; densely short hairy, about 12 flowering branches, each bearing with 2 or 3 flowers. Primary bracts do not occur. Hairy stalks of flowering branches are about 5mm long. Secondary bracts about 5 to 15 mm long in base of inflorescence rudimentary or apparently lacking are soon falling and present on upper cincinni only. Pedical of flowers are 5 mm long and hairy. Ovary covered with stout and hairy stalk commonly to 5 mm long at flowering. Calyx is about 1.7 to 2 cm long, funnel shape, three short lobes are hairy and toothed otherwise it can be sparsely hairy, white and split deeply at flowering. Corolla tube is shorter than calyx, lobes are 2.5 to 3.0 cm long with sparsely hairy on back and white in colour. The dorsal and laterals are 1 to 2 cm and 1.5 cm wide respectively (Holttum, 1950).

2.4   Extraction methods

Extraction is the preliminary step for separation of medicinally active constituents of plant tissues from the inactive components using selective solvents with appropriate extraction technique. Different plant parts like leaves (piper), fruits (citrus), rhizome (ginger) and flower (ylang ylang) can be used in the preparation of extracts. Depending upon the molecular structure, the components may be water soluble or water insoluble. Water insoluble compounds could be extracted using organic solvents such as pentene, hexane, chloroform, acetone and methanol (Sabulal and George, 2006; Ncube *et al*., 2008).

During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity into solvents (Ncube *et al*., 2008). The nature of the solvent, their concentration and polarity has the capacity to affect quantity and extract of different phytoconstituents in the solvent (Cowan, 1999; Doughari, 2006). Solvents in the order of increasing polarity are shown in Table 2.2.

Table 2.2: Solvents in the order of increasing polarity (Sabulal and George, 2006)

Solvents	Polarity (E°)
n-Pentene	0.00
Hexane	**0.00**
Isooctane	0.01
Petroleum ether	0.01
Cyclohexane	0.04
Xylene	0.26
Diisopropyl ether	0.28
Toluene	0.29
Diethyl ether	0.38
Chloroform	0.40
Dichloromethane	**0.42**
Tetrahydrofuran	0.45
Acetone	0.56
Dioxane	0.56
Ethyl acetate	0.58
Acetonitrile	0.65
Iso-and n-propanol	0.82
Ethanol	0.88
Methanol	**0.95**
Water	Highest

There are several methods used for preparing extracts depending on the type of substance to be isolated. Among the methods are steam distillation, volatile solvent extraction, supercritical fluid extraction (to obtain essential oils) and solvent extraction. Solvent extraction involves various techniques such as soxhlet extraction, maceration, homogenization and serial exhaustive extraction (Ncube *et al*., 2008).

Solvent extraction is most frequently used for isolation of plant antioxidant and antimicrobial compounds and the most commonly used solvents are methanol and ethanol (Ncube *et al*., 2008; Sultana *et al*., 2009). Dichloromethane and hexane have also been used by a number of researchers, especially in the successive extraction with solvents of increasing polarity (Ncube *et al*., 2008).

Serial exhaustive extraction is a technique of choice in this present study to obtain the crude extracts from leaves and rhizome of *Alpinia pahangensis* and *Alpinia mutica*. The successive extraction employed in this study started from a non-polar (hexane) to medium polar (dichloromethane) to a more polar solvent (methanol) to ensure that a wide polarity range compounds could be extracted (Ncube *et al*., 2008). Examples of phytochemicals extracted by different solvents are presented in Table 2.3.

Table 2.3: Types of phytochemicals extracted by different solvents (Ramlan and Mohamad Roji, 2000)

Polarity	Solvent	Class of chemical constituents extracted
Low	Light petroleum Toluene	Waxes, Fats, Fixed Oils, Volatile oils Alkaloids, Aglycones
Medium	Dichloromethane Acetone Ethanol Methanol	Alkaloids, Aglycones, Volatile oils Alkaloids, Aglycones, Glycosides Glycosides Sugars, Amino acids, Glycosides
High	Water Aqueous acid Aqueous alkali	Sugars, Amino acids, Glycosides Sugars, Amino acids, Bases Sugars, Amino acids, Acids

2.5   Extracts of *Alpinia* species

Chemical constituents of several *Alpinia* species have been studied. Hasnah and Md. Rizal (1995) reported that the rhizomes of *A. purpurata* and *A. oxymitra* yielded diterpene; labda 8 (17) and 12-diene-15, 16 dial as the major components while an amide (piperine) as the minor components only for *A. purpurata*. In addition, Victório *et al.*, (2009) verified that flavonoids (rutin and kaempferol-3-O-glucuronide) were present from the leaves of *A. zerumbet*.

Investigation done by Hasnah and Shajarahtunnur (1999) on rhizome crude extracts of several *Alpinia* species gave the following results: *A. javanica* yielded diterpene; labda 8 (17) and 12-diene-15, 16 dial as the major compounds while coronarin as the minor compound. The major compound of *A. mutica* is 5, 6-dehydrokawain while chalcone

flavokawin was the major compound for *A. rafflesiana*. Both extracts yielded 1, 7-diphenyl-5-hydroxy-6-hapten-3-one. Methyl cinnamate and pinocembrin were also isolated as the minor components from the rhizomes of *A. rafflesiana* and *A. mutica* respectively. Table 2.4 shows a summary of the chemical constituents obtained from extracts of *Alpinia* species from previous studies. However, it should be noted that identification of chemical constituents is not in the scope of this present study.

2.6   Antimicrobial activity

Extracts of some spices and herbs used today are valued for their antimicrobial activities and medicinal effects in addition to their flavor and fragrance qualities (Shan *et al*., 2007; Natta *et al*., 2008). Numerous studies have been published on the antimicrobial activities of extracts of Zingiberaceae species against different types of microbes by several researchers (Ficker *et al*., 2003; Chandarana *et al*., 2004; Habsah *et al*., 2004; Chan *et al*., 2007; Chen *et al*., 2008; Zahra *et al*., 2009).

The antimicrobial activities of plant extracts form the basis for many applications, including raw and processed food preservation, pharmaceuticals, alternative medicines and natural therapies (Shan *et al*., 2007). Hence, in this present study, the antimicrobial activity of the extracts have been tested using disc diffusion assay and minimum inhibition concentration (MIC) assay against six gram positive bacteria namely *Bacillus cereus, B. subtilis, Staphylococcus aureus, Streptococcus mutans, S. sanguis* and *S. mitis* (Streptococci species are oral bacteria), three gram negative bacteria namely *Proteus vulgaris, Pseudomonas aeruginosa* and *Escherichia coli* and three unicellular fungi namely *Candida parapsilosis, C. albican* and *Schizosaccharomyces pombe*.

Table 2.4: Summary of the chemical compounds of various extracts of *Alpinia* species from previous studies

Species	Locality	Parts	Compounds	References
Alpinia zerumbet	Brazil	Leaves	Flavaoids: Rutin and kaempferol-3-*O* glucuronide	Victório *et al*., (2009)
Alpinia malaccensis	Thailand	Rhizome	Kavalactone, labdane-type diterpenoids, Chalcone, flavanones	Nuntawong and Suksamrarn (2008)
Alpinia mutica	Johor, Malaysia	Fruit	Pinocembrin, alpinetin, cardamonin, 2',3',4',6'-tetrahydroxychalcone, 5,6-dehydrokawain,flavokawain B	Jantan *et al*., (2008)
Alpinia purpurata	Johor, Malaysia	Rhizome	Diterpene: labda 8 (17) and 12 diene-15, 16 dial amide (piperine)	Hasnah and Md. Rizal (1995)
Alpinia calcarata	India/ New York Botanical Garden	Leaves	Benzoids: Protocatechnic acid, vanillic acid, syringic acid, flavoniods, alkaloids	Merh *et al*., (1986)
Alpinia nutans	India/ New York Botanical Garden	Leaves	Tannis, saponins, alkaloids, ferulic acid, syringic acid, vanillic acid, kaempferol, quercetin, penodin	Merh *et al*., (1986)
Alpinia allhugas	India/ New York Botanical Garden	Leaves	Alkaloids, chlorogenic acid, 16-p-OH-benzoic acid, vanillic acid	Merh *et al*., (1986)
Alpinia bracteata	India/ New York Botanical Garden	Leaves	Tannins, alkaloids, p-OH-benzoic acid, protocatechuic acid, syringic acid, vanillic acid, quercetin	Merh *et al*., (1986)

2.7 Gram positive bacteria used in this study

2.7.1 *Bacillus cereus and Bacillus subtilis*

Bacillus cereus is an aerobic, facultative anaerobic, endospore forming rods and mesophilic bacterium. As a ubiquitous species it can be found in a wide range of habitats; as a soil saprophyte or possessing industrial and clinical significance. It can also be found in foods that originated from plants or animals such as dairy products, meats, infant's food, rice dishes, vegetables, spices and cereals (Drobniewski, 1993; McKillip, 2000; Vilas-Bôas *et al*., 2007).

Bacillus cereus clinical infections can be categorized into non-gastrointestinal infections and gastrointestinal infections. Gastrointestinal disease is more common because of their relation with food poisoning. Food contaminated from *B. cerues* can cause changes in their textures or taste because of the multiplication of the vegetative cells or the production of toxin. It may cause illness through an emetic toxin (vomit-inducing) produced from growing cells in the food; or diarrheal toxins (enterotoxins) produced during vegetative growth of *B. cereus* in small intestine (Vilas-Bôas *et al*., 2007).

In Asia, the emetic form of food poisoning is more commonly reported than the diarrheal type but vice versa in Europe and North America (McKillip, 2000). Apparently, this situation is closely related to ecological, economic and cultural factors. Strains producing emetic toxin grow well in rice dishes and other starchy food likes pasta and potato. Conversely, diarrhoeal toxin grows in a wide variety of foods from vegetables and salads to meat and casseroles.

Compared with *B. cereus* as the well known food poisoning organism, *B. subtilis* does cause human infections. There are reports implicating this bacterium can cause systemic and gastrointestinal disease (Logan, 1988; Drobniewski, 1993; Matarante *et al*., 2004). *Bacillus subtilis* systemic infections include endocarditis, fatal pneumonia, bacteremia, septicaemia, and infection of a necrotic axillary tumour. The use of laundry products containing proteolytic enzyme known as protease from this bacterium may act as an irritant such as dermatitis and respiratory ailments for example asthma (Logan, 1988; Drobniewski, 1993).

Although *B. subtilis* has rarely been linked to incidence of food borne illness, few cases have been reported as described by Kramer and Gilbert (1989). Above all, since the incidence of infection from *B. subtilis* is fairly low (Drobniewski, 1993; Matarante *et al*., 2004; Park *et al*., 2006), this bacterium is considered safe. In fact, *B. subtilis* is commonly used as probiotics in foods and in pharmaceutical preparations (Matarante *et al*., 2004).

2.7.2   *Staphylococcus aureus*

Staphylococcus aureus is a facultative anaerobic, spherical single or pair cocci or grape-like clusters, non-motile and catalase and coagulase positive. It is usually found in the nostrils, and on the skin and hair of warm-blooded animals (Loir *et al.*, 2003). *Staphylococcus aureus* can cause a variety of infections in human ranging from superficial skin lesions such as pimples and furuncles to toxic shock syndrome and sepsis such as pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, osteomyelitis and endocarditis. Infection acquired from hospital or known as

nosocomial infection can be found through surgical wounds and infections associated with indwelling medical devices (Loir *et al*., 2003; Todar, 2008).

Staphylococcal food poisoning is caused by staphylococcal enterotoxins. Staphylococcal enterotoxins are short proteins and highly stable, resist most proteolytic enzymes and keep their activity in the digestive tract after ingestion. Thus, abdominal cramps, nausea, vomiting, sometimes followed by diarrhea are among the symptoms of staphylococcal food poisoning. However, diarrhea alone is not specific for staphylococcal infection (Loir *et al*., 2003).

2.7.3   Oral bacteria used in this study

Clinical observations in human as well as animals indicate that plaque formation is a cause for both dental caries and periodontal diseases. Various microorganisms are essential in the pathogenesis of these two conditions and Streptococci are one of them.

Streptococci species are found in high numbers of the total bacteria population in human mouth mainly in plaque, and those that can be identified are *Streptococcus mutans, S. sanguis, S. mitior (mitis), S. salivarius* and *S. milleri* (Hamada and Slade, 1980). *S. mutans* is the best-defined species among the oral streptococci with a large amount of information on it but data on the other species are relatively few (Hamada and Slade, 1980; Bowden, 1990). *Streptococcus mutans, S. sanguis* and *S. mitis* are selected species used for this study.

Streptococcus mutans is acidophilic, flourishing at very low pH values, adheres to tooth surfaces by hydrophobic bond and can behave differently depending on where it is found in the mouth (Slavkin, 1999). *Streptococcus mutans* is highly cariogenic

pathogen in humans (Kuramitsu, 2001; Nostro *et al.*, 2004). It preferentially colonizes the human tooth surface and prosthetic devices, usually found when the teeth erupt (Hamada and Slade, 1980). It ferments mannitol, sorbitol and several of other sugar, and synthesize adherent water soluble glucan from sucrose metabolizes by the glucosetransferase. The presence of these glucans along with the microbial aggregation and accumulation of acids are critical in the development of dental caries and other oral diseases (Slavkin, 1999; Nostro *et al.*, 2004).

Similar to *S. mutans, S. sanguis* is also found to prefer to colonize the human tooth surfaces and prosthetic devices and usually found when the teeth erupt (Hamada and Slade, 1980). This bacterium is among the predominant streptococci in developing human dental plaque and first to adhere to salivary pellicle (Liljemark and Bloomquist, 1996). It was first isolated and identified by White and Niven in 1946 from the blood of patients with subacute endocarditis. However, a researcher named Carlsson (1968) found microorganism with similar colony morphology in dental plaque and classified it as *S. sanguis* (Hamada and Slade, 1980; Murray *et al.*, 1984).

Streptococcus mitior is frequently known as *S. mitis*. The name *S. mitis* has been proposed for a group of streptococcal strains which form a homologous cluster in numerical taxonomic study (Murray *et al.*, 1984). It has no preferred site in the oral cavity like *S. mutans* and *S. sanguis*. Studies by Nyvad and Killian (1987) and Killian *et al.*, (1989) have shown that *S. mitis* will colonize root surfaces and variation in colonization by different strains can occur as a result of diet or caries status. It is peroxidogenic but does not ferment inulin, sorbitol and monnitol. It also does not hydrolyze arginine and esculin (Hamada and Slade, 1980; Murray *et al.*, 1984).

Natural products have shown to be an alternative to synthetic chemical substances for decay prevention. In vitro study on antibacterial activity of plants in Mediterranean regions namely *Helichrysum italicum* against oral streptococci indicate that all streptococci were susceptible to its ethanolic extract (Nostro *et al.,* 2004). Besides that, plant based production of biologically active recombinant proteins, facilitates conventional oral delivery through the consumption of plant tissues. Potatoes, bananas and tomatoes are being investigated for vaccine production (Slavkin, 1999).

2.8   Gram negative bacteria used in this study

2.8.1   *Proteus vulgaris*

Proteus vulgaris is a facultative anaerobe, rod-shaped bacillus and grow at temperature 37°C. The bacterium is widely distributed in the natural environment such as in polluted water and in soil and manure (Różalski *et al*., 1997). It occurs naturally in the intestinal tracts of human; representing 5% in faeces of healthy person (Peerbooms *et al*., 1985). As a member of the *Proteus* species, one of its characteristics is to exhibit swarming growth which distinguishes them from other members of gram negative bacteria (Różalski *et al*., 1997). The swarm cell formation is induced by proteolytic enzymes detected from *Proteus* strains which also play as important virulence factor in this species (Senior, 1999).

Proteus vulgaris may cause infections of the urinary tract, blood, wounds and also a common cause of sinus and respiratory tissue infections especially in South East Asia. Although it can be dead if left untreated, this bacterium is extremely hard to eradicate. The bacterium reacts with antibiotics that have only an intermediate effect on it (Sampathkumar *et al*., 2008).

2.8.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an aerobic, motile, rod shaped and demonstrate the most consistent resistance to antibiotics. It can grow in almost any environment including in high salt content and over a wide temperature, range between 20 to 40°C. It used ammonia and carbon dioxide as sole nitrogen and carbon sources (Ryan and Ray, 2004).

Pseudomonas aeruginosa is an opportunistic human pathogen as a common cause of nonsocomial infections and responsible for persistent infections in immunocompromised individuals (those with burns, HIV or undergoing cancer chemotherapy) and for the chronic lung infections of patients with cystic fibrosis (Lau *et al*., 2004, Walker *et al*., 2004). It is also capable of causing serious infections in nonmammalian host species such as insects, nematodes and plants (Walker *et al*., 2004).

2.8.3 *Escherichia coli*

Escherichia coli's natural habitat is the gastrointestinal tract of warm-blooded animals, and in human is the most common facultative anaerobe in the gut. Although most strains exist as harmless symbionts, there are many pathogenic *E. coli* strains that can cause a variety of diseases in animals and human (Donnenberg and Whittam, 2001).

Pathogenic forms of *E. coli* associated with human and animal diseases are remarkably diverse. It is primarily regarded as the cause for food-borne diarrhoeal diseases with symptoms similar to cholera such as diarrhea to severe dysentery; other *E. coli* may

colonize the urinary tract, resulting in cystitis or pyelonephritis, or may cause other extraintestinal infections, such as urinary tract infections, surgical wound infections, septicemia and neonatal meningitis (Donnenberg and Whittam, 2001; Saeed *et al*., 2009).

2.9   Unicellular fungi used in this study

2.9.1   *Candida albicans*

Candida albicans is an ubiquitous, dimorphic fungus, commansel of warm blooded animal including human, usually live as part of an individual's normal mucosal (oral and vaginal cavities and the digestive tract) microflora, high-frequency phenotypic transition, aerobic, facultative anaerob and the most virulent of the *Candida* species (Scherar and Magee, 1990; Molero *et al*., 1998; Naglik *et al*., 2004).

Among the interesting properties of *C. albicans* is its ability to grow either by budding which form an ellipsoid bud or in hypal form. Hyphal form is a morphological transition of the usual budding yeast shape whereby the slipper shaped cells outgrowth on the surface of blastospore, elongated and form a *C. albicans* germ tube. Hyphae will then arise from the centre of the elongated cells and give rise to new mycelia or yeast like forms. These growing abilities may lead to change their phenotypes including differences in colony morphology, cell shape as well as their surface and permeability. Thus, phenotypic variations and ability to switch between the yeast and the hyphal mode of growth has lead to the intrinsic biological process interest and has been implicated in its pathogenecity (Scherar and Magee, 1990; Molero *et al*., 1998).

Infections caused by *C. albicans* and other *Candida* species are known as candidiasis and can be categorized into superficial and deep seated infection. Superficial infections include oral and vaginal thrush and chronic mucocutaneous candidiasis. Meanwhile, myocarditis, acute disseminated and septicaemia are among the major clinical problem caused by *C. albicans* (Molero *et al*., 1998).

2.9.2 *Candida parapsilosis*

Candida parapsilosis can be found as isolated yeast from nonhuman sources such as soil, fresh and marine water, insects and domestic animals. It can be oval, round or cylindrical in shape. It exists in either a yeast phase or a pseudohypal form. The pseudohypal formation is linked to a specific set of amino acids (Trofa *et al*., 2008; Nosek *et al.*, 2009).

It is also a normal human commensal and one of the most frequently isolated fungi from the blood, skin and nails and surfaces of medical plastics and prosthetic devices such as peritoneal dialysis catheters and prosthetic heart valves and maybe indwelling access devices for cancer patients (Kuhn *et al*., 2004; Nosek *et al*., 2009). Several reports indicate that *C. parapsilosis* is the most commonly isolated *Candida* species from blood cultures (Trofa *et al*., 2008).

Recently, an outbreak of *C. parapsilosis* infections in hospital (nosocomial infection) especially at intensive care unit had attracted some attention. Particularly, this yeast is isolated from hospitalized patients with serious underlying conditions from surgery and critically ill newborn baby in whom it is known to be associated with the use of central lines and parenteral nutrition (Kuhn *et al*., 2004; Trofa *et al*., 2008). In this situation,

the hands of healthcare workers could be the predominant environmental source for existence of this species in the ICU (Kuhn *et al.*, 2004).

2.9.3   *Schizosaccharomyces pombe*

Schizosaccharomyces pombe is a species of yeast also known as 'fission yeast' due to the reproducing character of their cells. It has an ability to grow at reduced water activities, in the presence of preservatives, and at 37°C (Pitt and Hocking, 1985). It may spoil foods due to their ability to resist the common food preservatives like sulphur dioxide (SO_2), sorbate or benzoate but the spoilage is relatively uncommon probably due to infrequent distribution of this species (Pitt and Hocking, 2009).

Food spoilage caused by this yeast is found only occasionally in soft drinks with sugar syrups, and juice concentrates are likely to be the source of infection. The yeast has also been isolated from grapes, apples, grapes juice, wine and palm wine. It is also found in high sugar products such as molasses, raisins, dried dates, cane sugar, sugar syrups and juice concentrates (Baekhout and Robert, 2003).

It is frequently the dominant yeast in the production of fermented beverages especially those produced from cereals such as maize and millet through natural fermentation process in the tropics (Adams and Moss, 2008). The yeast have been employed extensively as models for genetic analysis of a variety of complex pathways and processes, including cell division, secretion, transcription, and receptor-mediated signal transduction which makes it ideal for applying genetic approaches to many biological questions (Matsuyama *et al.*, 1999).

Table 2.5: Selected examples of microorganisms and their infection

Microbiological classifications	Species	Infection or diseases	References
Gram positive bacteria	*Bacillus cereus*	Non-gastrointestinal infections Gastrointestinal infections (more common) ▪ Food poisoning	Vilas-Bôas *et al.*, (2007)

	Bacillus subtilis	Systemic infection
		  ▪ Endocarditis, fatal pneumonia, bacterimia, septicemia, necrotic axillary tumor
		Irritant (use of laundry products containing enzyme from this bacteria)
		  ▪ Dermatitis
		  ▪ Respiratory ailments (asthma)
		Food poisoning
			Matarante *et al.*, (2004)
			Drobniewski *et al.*, (1993)
			Logan (1988)

	Staphylococcus aureus	Superficial skin lesions
		  ▪ Pimples, furuncles
		Toxic shock syndrome
		Sepsis
		  ▪ Pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, endocarditis
		Nosocomial infections
		  ▪ Surgical wounds, infections with indwelling medical devices
		Food poisoning
			Todar (2008)
			Loir *et al.*, (2003)

Gram positive bacteria (oral bacteria)	*Streptococcus mutans* *Streptococcus sanguis* *Streptococcus mitis*	*Streptococci* species are causes of dental caries and periodantal diseases	Hamada and Slade (1980)

Table 2.5: Selected examples of microorganisms and their infection - cont'

Microbiological classifications	Species	Infection or diseases	References
Gram negative bacteria	*Proteus vulgaris*	Urinary tract infections Wound infections Sinus and respiratory tissues infections	Sampathkumar *et al.*, (2008)
	Pseudomonas aeruginosa	Nosocomial infections Capable of infections in nonmammalian host species	Lau *et al.*, (2004) Walker *et al.*, (2004)

	Escherichia coli	Food borne diseases
		  • Diarrhea
		  • Dysentery
		Cystitis
		Pyelonephritis
		Extraintestinal infections
		  • Urinary tract infections
		  • Surgical wound infections
		  • Septicemia
		  • Meningitis (newborn baby)
			Saeed *et al.*, (2009) Donnenberg and Whittam (2001)

Unicellular fungi	*Candida albicans*	Candidiasis
		  • Superficial infection- oral and vaginal thrush, chronic mucocutaneous candidiasis
		  • myocarditis, acute disseminated, septicaemia
			Molero *et al.*, (1998)

	Candida parapsilosis	Candidiasis • Nosocomial infection- isolated from patients with serious surgery and critically ill newborn baby associated with the use of central lines and parenteral nutrition	Nosek *et al.*, (2009) Kuhn *et al.*, (2004)
	Schizosaccharomyces pombe	Food spoilage, presevative resistantant yeast	Pitt and Hocking (2009)

2.10   Antioxidant activity

Oxygen in its natural state has two oxygen atoms; can be easily broken down to singlet oxygen by heat and ultraviolet (UV). Singlet oxygen makes it a free radical agent known as Reactive Oxygen Species (ROS) with one or more unpaired electron. It can cause damage to cells, nucleic acids, protein and fatty tissues. In addition, damaging tissue and immune system occurs when it attack biological molecules. They also cause lipid peroxidation, which leads to aging symptoms and age dependent conditions (Lee *et al*., 2003; Vimala *et al*., 2003).

Antioxidants are substances that can fight and destroy excess free radicals and repair oxidative damage in biomolecules. Plants particularly in the tropical region absorb the sun's radiation and generate high levels of oxygen as secondary products of photosynthesis because they are exposed to long periods in intense sunlight. Thus, in order to survive from toxic ROS produced by oxygen, plants will produce various antioxidant compounds as part of their natural defense mechanism. Among natural antioxidant substances in plants are lignans, flavanoids, phenolic acids and phenol to name a few (Vimala *et al*., 2003).

There are several methods used for *in vitro* determination of antioxidant activity such as hydroxyl radical (HO$^{\bullet}$) scavenging assay, superoxide radical anion (O2$^{\bullet-}$) scavenging assay, peroxyl radical (ROO$^{\bullet}$) scavenging asay, 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging assay, ferric reducing antioxidant assay (FRAP) and many others (Magalhães *et al*., 2008). In the present study, DPPH free radical scavenging assay is chosen to determine the antioxidant activity of crude extracts of *Alpinia pahangensis* and *Alpinia mutica*.

DPPH radical scavenging assay is a simple, rapid and convenient method to evaluate the radical scavenging activity of extracts from plants, food material or on single compounds (Prakash, 2001; Lo Scalzo, 2008). The odd electron in the DPPH gives a maximum strong absorption at 517 nm and is purple in color. The color turns from purple to yellow when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant (Prakash, 2001).

CHAPTER 3: METHODOLOGY

3.1 Plant materials

Two *Alpinia* spesies namely *Alpinia pahangensis* and *Alpinia mutica* were used in this study for biological activities of their crude extracts. The parts examined were rhizomes and leaves. *Alpinia pahangensis* and *A. mutica* was collected from Tasik Chini, Pahang and Perak respectively.

3.2 Preparation of plant materials

Fresh samples of rhizomes and leaves were washed and sliced into small pieces. The samples were then oven dried at 40°C successively for three days. Afterwards, the dried samples were ground using the grinder.

3.3 Extraction of plant materials

Extraction was done successively using three solvents with different polarity namely hexane (non-polar), dichloromethane (medium polar) and methanol (polar). The ground rhizomes and leaves of *A. pahangensis* and *A. mutica* were extracted with hexane for 72 hours in a conical flask. The hexane suspension was filtered with Whatman filter paper. The filtrate was dried by using a rotary evaporator in order to get the crude extracts. The hexane solvent was replaced by dichloromethane and methanol respectively by applying the same extraction method as above. The crude extracts were labeled and kept at 4°C in vials until used. A summary of samples and crude extract preparations are shown in Figure 3.1.
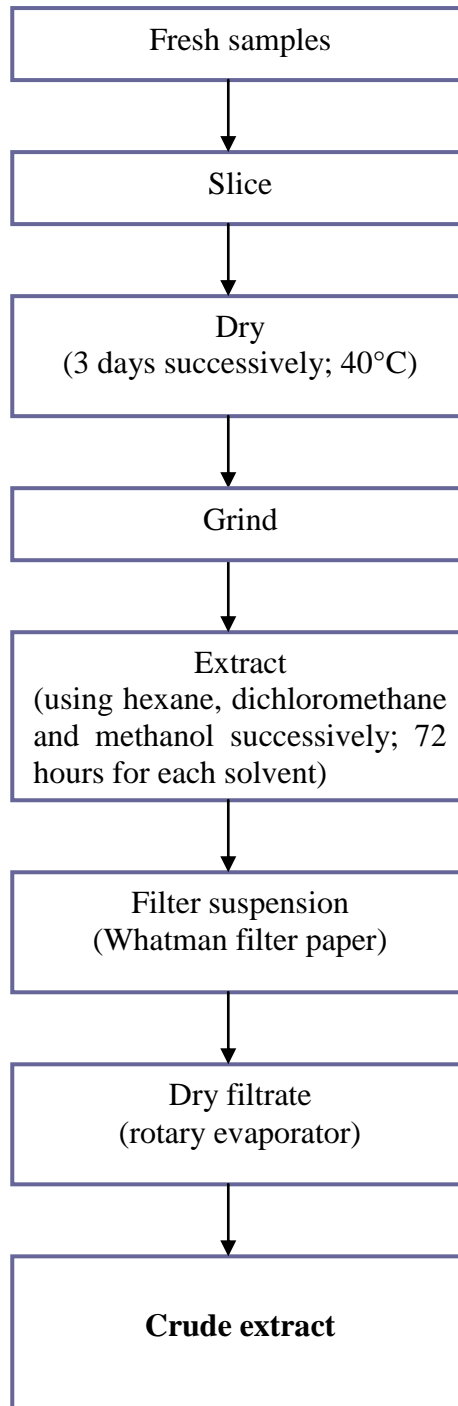
```
          ┌─────────────────────────────┐
          │        Fresh samples        │
          └─────────────────────────────┘
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                         ▼
          ┌─────────────────────────────┐
          │            Slice            │
          └─────────────────────────────┘
                         │
                         ▼
          ┌─────────────────────────────┐
          │             Dry             │
          │  (3 days successively; 40°C) │
          └─────────────────────────────┘
                         │
                         ▼
          ┌─────────────────────────────┐
          │            Grind            │
          └─────────────────────────────┘
                         │
                         ▼
          ┌─────────────────────────────┐
          │           Extract           │
          │ (using hexane, dichloromethane │
          │ and methanol successively; 72 │
          │   hours for each solvent)   │
          └─────────────────────────────┘
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                         ▼
          ┌─────────────────────────────┐
          │      Filter suspension      │
          │   (Whatman filter paper)    │
          └─────────────────────────────┘
                         │
                         ▼
          ┌─────────────────────────────┐
          │        Dry filtrate         │
          │     (rotary evaporator)     │
          └─────────────────────────────┘
                         │
                         ▼
          ┌─────────────────────────────┐
          │                             │
          │       Crude extract         │
          │                             │
          └─────────────────────────────┘
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Figure 3.1: Flow chart: preparations of samples and crude extracts

3.4   Determination of percentage yield of the crude extracts

The percentage yield of the crude extracts was calculated based on the weight of crude extract and dried material (ground samples) according to the formula using by Siddiq *et al.*, (2008) as below:

$$\% \text{ of crude extracts yield} = \frac{\text{Weight of crude extract (g)}}{\text{Weight of dried plant material (g)}} \times 100$$

3.5   Antimicrobial activity

3.5.1   Chemicals and microbial strain

Muller Hinton agar (MHA), Brain Heart Infusion agar (BHIA), Sabauroud Dextrose agar (SDA), Yeast Potato agar (YPA), Muller Hinton broth (MHB), Brain Heart Infusion broth (BHIB) and Yeast Potato broth (YPB) were purchased from Oxoid. Sabauroud Dextrose broth (SDB), Dimethyl sulfoxide (DMSO), Streptomycin and Nystatin were purchased from Sigma.

For antimicrobial activity, twelve test microorganisms were obtained from Mycology Laboratory, Institute of Biological Science, Faculty of Science, University of Malaya. The crude extracts were individually tested against 12 microorganisms as shown in Table 3.1

Table 3.1: Test organisms for antimicrobial activity assay

Microorganisms	Scientific name	Type of media/broth used
Bacteria gram positive	*Bacillus cereus*	MHA/ MHB
	Bacillus subtilis	MHA/ MHB
	Staphylococcus aureus	MHA/ MHB
Bacteria gram negative	*Escherichia coli*	MHA/ MHB
	Pseudomonas aeruginosa	MHA/ MHB
	Proteus vulgaris	MHA/ MHB
Oral bacteria	*Streptococcus mutans*	BHIA/BHIB
	Streptococcus mitis	BHIA/BHIB
	Streptococcus sanguis	BHIA/BHIB
Fungi	*Candida albicans*	SDA/SDB
	Candida parapsilosis	SDA/SDB
	Schizosaccharomyces pombe	YPA/YPB

Key:

MHA   :   Muller Hinton agar   MHB :   Muller Hinton broth

BHIA   :   Brain Heart Infusion agar   BHIB: Brain Heart Infusion broth

SDA   :   Sabauroud Dextrose agar   SDB :   Sabauroud Dextrose broth

YPA   :   Yeast Potato agar   YPB :   Yeast Potato broth

3.5.2 Preparation of inoculum

Media and broths were sterilized by autoclaving at 120°C for 15 minutes. All microorganisms were grown and maintained on appropriate agar as mentioned in Table 3.1 and then stored at 4°C under aerobic condition. For the purpose of antimicrobial assays, microorganisms were cultured in the broths as stated in Table 3.1 at 37°C overnight. Inocula consisting 1×10^8 cfu/ml were determined by absorbance value measured by spectrophotometer at wavelength 625 nm with absorbance of 0.08 to 0.12 to obtain turbidity comparable to that of the 0.5 McFarland standard (NCCLS, 2003).

3.5.3 Disc diffusion assay

Disc diffusion assay of the crude extracts were determined according to the method of Baeur *et al.*, (1966) with slight modifications. The sterile cotton buds were dipped into the microorganism liquid inoculum and the excess liquid were removed by turning the swab against the side of the culture tube. Then the inoculum was spread evenly on the surface of agar plates by swabbing in two directions.

The extracts of *A. pahangensis* and *A. mutica* were dissolved with 100% DMSO to give concentrations of 100 mg/ml, and 10µl of extracts were pipetted onto sterile discs (6 mm) for the purpose of screening. The discs were placed on the inoculated agar. For positive control, antibiotic Streptomycin and Nystatin were prepared at 100 mg/ml as control for antibacterial and antifungal tests, respectively. Dimethyl sulfoxide and broth were used as negative control in this study. The test plates were then inverted and incubated aerobically at 37°C for 24 hours. The antimicrobial activity was recorded by measuring the clear inhibition zone (in mm) around each disc. All analyses were carried

out in triplicate and the average values were recorded. The activities were categorized as weak ($\leq$ 9.5 mm), moderate (10 to 14.5 mm) and strong ($\geq$ 15 mm) according to Najihah *et al*., (2009).

3.5.4   Minimum inhibition concentration (MIC)

Extracts that showed strong antimicrobial activity using disc diffusion assay were used to determine their MIC values. MIC was defined as the lowest concentrations of extracts that kill the initial inoculum and resulting less than ten colonies growth (Hammer *et al*., 1998). The MIC assay was carried out using macrodilution (tube) broth method according to NCCLS (2003) with slight modification as proposed by Teoh, (2007) due to the insolubility of compounds in 100 % aqueous broths.

Experiment was carried out to determine the effect of DMSO ratio on the growth of bacteria and the result showed that incorporation of 5 % DMSO into broth did not inhibit the growth of microorganisms. Thus, 95 % of broth was used as a medium for the overnight microorganism cultures and a diluent for the initial stock of solution of the crude extracts.

Microorganisms were inoculated into 2 ml of broth containing 5 % DMSO and incubated overnight at 37°C. Inocula consisting of 1×10^8 cfu/ml were determined by absorbance value measured by spectrophotometer at wavelength 625 nm with absorbance of 0.08 to 0.12. The bacterial suspensions were subsequently diluted 100-fold to yield an inoculum of 1×10^6 cfu/ml.

Stock solutions of the crude extracts in DMSO (50 mg/ml) were prepared and diluted with 95 % broth to obtain concentrations of 25, 12.5, 6.25, 3.125, and 1.56 mg/ml. 1 ml of 100-fold inoculum with 1 ml of varying concentrations of extracts were added in autoclaved cultured tubes. This resulted in a 1:2 dilution of extract concentrations and inoculum. The final concentrations of extracts were 12.5, 6.25, 3.125, 1.56, and 0.78 mg/ml. The final inoculum density after 1:2 dilutions was 5×10^5 cfu/ml.

Tubes containing only the diluted crude extract solution without microorganism were used as negative control. Broth (95 %) served as the growth control to monitor the occurrence of contamination to other culture tubes as well as the spectrophotometer blank. The tubes were incubated at 37°C for 18 hours. All analyses were carried out in triplicate. Streptomycin at concentrations ranging from 10 - 0.313 mg/ml was used as positive control for antibacterial activity.

The minimum inhibition concentration (MIC) activity was determined photometrically. The concentration of plant extracts with microorganisms which gave a sharp decline in the absorbance value was considered as MIC (Devienne and Raddi, 2002). A loopful of broth was taken out from the tubes at this and higher concentration and immediately streaked on MHA. The plates were incubated aerobically at 37°C for 24 hours and the development of microorganism was checked. The activities were categorized as weak (MIC ≥ 5000 µg/ml), moderate (MIC: 1000-4900 µg/ml) and strong (MIC ≤ 1000 µg/ml).

3.6 DPPH radical scavenging assay

The DPPH assay was selected to evaluate the radical scavenging activity of the extracts because it has been largely used as a simple, rapid and convenient method to assess antioxidant activity from plants, food material or on single compounds extracts (Prakash, 2001; Lo Scalzo, 2008). The activity of the test samples can be observed from the degree of colour change. If the stable free radicals have been scavenged, DPPH which is purple in colour will turn to yellow because it was supplied with electrons or hydrogen ions from the test samples. The method used in this study was adopted from the work of Ashril *et al*., (1997).

For pre-screening, 250 µl of each sample (20 mg/ml) were individually mixed with methanol and 25 µl of DPPH solution (8 mg/ml) to get the final concentration of 5 mg/ml of sample. All samples and the control (methanol) were monitored for their absorbance values after an incubation period of 30 minutes at room temperature using Shimadzu double beam spectrophotometer (UV 2450) at 517 nm. All analyses were carried out in triplicate and the average values were recorded.

The positive extracts (50 or more percentage inhibition) were retested for their antioxidant activity at concentrations of 5 mg/ml to 0.5 mg/ml to determine the IC_{50} value. Ascorbic acid was used as positive control. Reaction mixtures of ascorbic acid, extracts, DPPH and methanol for DPPH assay at different concentrations are shown in Appendix II (a) and Appendix II (b). The percentage of inhibition of each of the test samples was calculated according to the formula:

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control- Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

```
┌─────────────────────────────────────────────┐
│              Plant samples                   │
│     Alpinia mutica (leaves and rhizomes)     │
│   Alpinia pahangensis (leaves and rhizomes)  │
└─────────────────────────────────────────────┘
                      │
                      ▼
┌─────────────────────────────────────────────┐
│              Crude extract                   │
└─────────────────────────────────────────────┘
                      │
                      ▼
┌─────────────────────────────────────────────┐
│            Percentage of yield               │
└─────────────────────────────────────────────┘
                      │
                      ▼
┌─────────────────────────────────────────────┐
│            Biological activites              │
│                                              │
│            Antioxidant activity              │
│   • DPPH free radical scavenging activity    │
│                                              │
│            Antimicrobial activity            │
│   • Disc diffusion assay                     │
│   • Minimum inhibition concentration (MIC)   │
└─────────────────────────────────────────────┘
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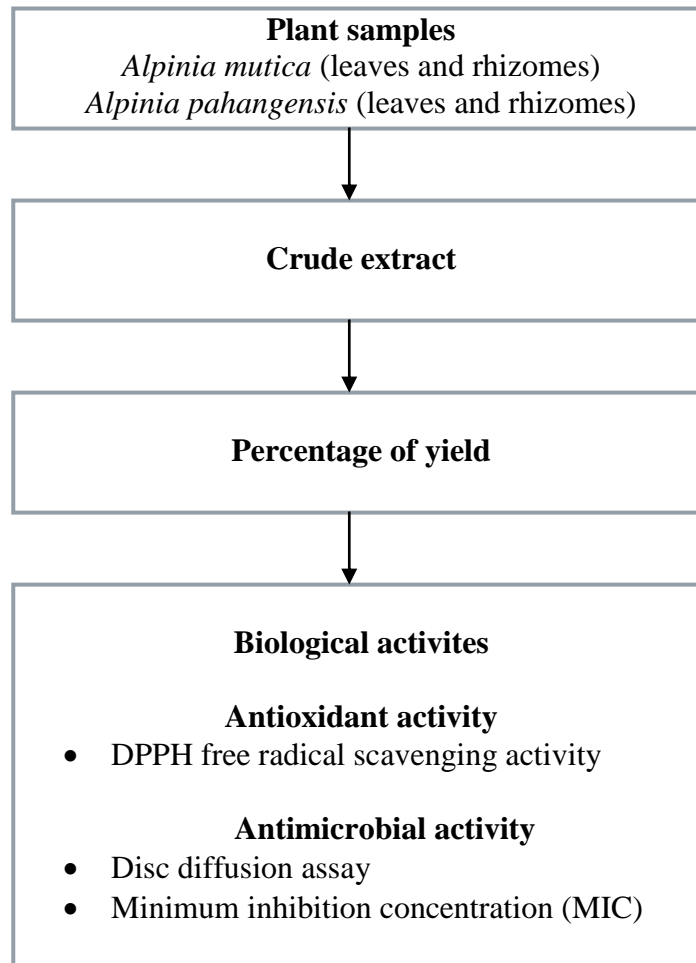
Figure 3.2: Flow chart: Brief methodology

CHAPTER 4: RESULTS AND DISCUSSION

4.1   Preparation of plant material, extraction and the yield of extract

The choice of plant material used in the extract preparation is usually guided by the traditional use of the plant and the ease of handling of the different plant parts like the leaf, stem, fruit and rhizome. In this study, once the plants had been authenticated as *Alpinia pahangensis* and *Alpinia mutica*, their rhizomes and leaves were collected and examined for contamination. At this stage, it is essential to ensure that the plant materials were free from contamination from other plants, insects as well as disease such as affected by viral, bacterial and fungal infection. Contamination with disease may cause not only microbial synthesis detected in plants but also alternation in plant metabolism which lead to formation of unexpected products during analysis (Harborne, 1998).

In order to separate medicinally active portion from plant tissues, fresh or dried plant material can be used. However, generally, plants which are used to treat illness were traditionally consumed in the dry form by the practitioner. Hence, dried plant material was chosen for this study. Plant materials need to be dried as quickly as possible after collection, by using low temperature in order to preserve the active phytochemicals in the plant and to avoid too many chemical changes (Harborne, 1998; Ramlan and Mohamad Roji, 2000).

Dried plant materials can be stored before analysis, for long periods of time, the reason being the differences in the water content in fresh plant materials may affect the solubility of subsequent separation particularly by liquid-liquid extraction, hence dried plant material is preferred instead of fresh material. Furthermore, the secondary

metabolic plant components should be relatively stable, especially if it is to be used as an antimicrobial agent (Ncube *et al*., 2008). Interestingly, a study done by Eloff (1999) revealed that extracts of herbarium specimens of leaves of *Combretum erythrophyllum* collected 96 to 120 years ago and *Helichrysum pedunculatum* aged more than 100 years old with light fungal infection did not show a decrease in their antibacterial activity.

The dried plant materials were subsequently ground to make it into powder form. The objective for powdering the plant material is to break up its organ, tissue and cell structures so that its medicinal ingredients are exposed to the extraction solvent. Furthermore, size reduction maximizes the surface area, which in turn enhances the mass transfer of active principle from plant material to the solvent (Handa, 2008). A study done by Gião *et al*., (2009) on effect of particle size upon the extent of extraction of antioxidant powder from three types of medicinal plants confirm that a smaller particle size led to a higher extraction amount.

Solvent extraction is the most commonly used procedure to prepare extracts from plant materials due to their ease of use, efficiency and wide applicability (Dai, 2010). Although the traditional practitioner primarily use water to extract the beneficial medicinal properties from plants, plant extracts from organic solvents have been found to give more consistent antimicrobial activity if compared to water extract (Parekh *et al*., 2005). In another study, antioxidant activity of six Zingiberaceae species extracted with organic solvent, propylene glycol, also verified a better antioxidant activity compared to the water extracts (Rozanida, 2007).

In this study, successive extraction of the rhizomes and leaves of *A. mutica* and *A. pahangensis* was carried out starting from a non-polar (hexane) to a medium polar

(dichloromethane) and finally a polar solvent (methanol) seem to be an ideal method as proposed by Ncube *et al*., (2008). A study done by Wojcikowski *et al*., (2007) on the antioxidant properties of 55 medicinal herbs; and Uthayarasa *et al*., (2010) on antibacterial activity of medicinal plants, revealed that optimum and effective effect of extraction and better antioxidant and antimicrobial activity can be provided using successive extraction. These studies indicate that the composition of extracts and their biological activities is influenced by the extraction procedures and the solvents used.

In this study, two wild *Alpinia* species; *A. pahangensis* and *A. mutica* were screened for the biological activities of their crude extracts from rhizomes and leaves. The percentage yield of the extracts is shown in Table 4.1. The yield varies with the extracting solvent, from non-polar (hexane) to more polar solvent (dichloromethane and methanol).

The results revealed that the yield of rhizome crude extracts for both species of *Alpinia* was higher than the leaf extracts. Among the extracts, the highest yield (7.16 %) was obtained from the rhizome of *A. mutica* (methanol extract) while the lowest yield (0.27 %) was from the leaf of *A. mutica* (dichloromethane extract). The colour of the leaf crude extracts was generally dark green while that of the rhizome was golden yellow (Table 4.1).

Factors like the maturity stage may affect the phytochemical content of the plant. To maximize it, it is suggested that the plant should be harvested just as the flower buds first appear especially for the production of aroma and fragrances from aromatic plants and flowers. It is because, at this stage of growth, the leaves contain the maximum amount of volatile oils (Ramlan and Mohamad Roji, 2000). A study on the

accumulation of bioactive compound from the rhizomes of *Curcuma mangga* (Zingiberaceae) during its growth and developmental stages, showed that bioactive components particularly phenolics vary with distinct development phases (Policegoudra *et al*., 2007).

The polarity of the solvent used may influence the yield of extract since the general principle in solvent extraction is, polar solvents will extract polar phytochemicals and non-polar solvents extract non-polar phytochemicals (Ramlan and Mohamad Roji, 2000; Handa, 2008; Dai, 2010). In comparing the effect of solvents on crude extract yield, it was found that the increase in yield correlated directly with the solvent polarity except for the leaves of *A. pahangensis* where the yield was higher (3.16 %) in dichloromethane extract if compared with the methanol extract (1.78 %). This indicated that the crude extracts from the rhizomes and leaves of the two *Alpinia* species contained a higher amount of polar fractions than non-polar fractions. Thus in this study, methanol seems to be the best solvent to bring out the highest yield for the plants under investigation.

Table 4.1: Percentage yield of *A. pahangensis* and *A. mutica*

Alpinia species	Plant part	Crude extract	Yield of crude extracts (%)	Description
Alpinia pahangensis	Leaves	Hexane	0.36	Dark green in colour
		Dichloromethane	3.16	Dark green in colour
		Methanol	1.78	Dark green in colour
	Rhizome	Hexane	0.89	Golden yellow in colour
		Dichloromethane	1.35	Golden yellow in colour
		Methanol	2.20	Golden yellow in colour
Alpinia mutica	Leaves	Hexane	0.44	Dark green in colour
		Dichloromethane	0.27	Dark green in colour
		Methanol	1.92	Dark green in colour
	Rhizome	Hexane	1.29	Golden yellow in colour
		Dichloromethane	1.75	Golden yellow in colour
		Methanol	7.16	Golden yellow in colour

$$\% \text{ of crude extracts yield} = \frac{\text{Weight of crude extract (g)}}{\text{Weight of dried plant material (g)}} \times 100$$

4.2   Antimicrobial activity

Antimicrobial assay is one of the most useful bioactivity because it forms the basis of many applications, including as food preservation, alternative medicine and natural therapies and in pharmaceutical industry especially for the treatment of dermatological diseases (Shan *et al*., 2007). Although plants from Zingiberaceae family are generally regarded as safe for human consumption, investigation on their antimicrobial activities particularly on the species of *Alpinia* in the literature are scarce. Screening of dichloromethane and methanol rhizome extracts for antimicrobial activity of Zingiberaceae species including *A. mutica* was carried out by Habsah *et al*., (2000) but no work was done on *A. pahangensis* extracts.

Lack of standardized method in determining antimicrobial activity may affect the outcome of susceptibility tests and lead to different results obtained by different authors even when using the same plant species. Several variables may influence the results and make it difficult to compare with others when dealing with plant extracts. Among the variables are the environmental and climatic conditions under which the plant grew, the choice of plant extract and extraction method, different antimicrobial test method and test microorganisms (Hammer *et al*., 1999; Ncube *et al*., 2008). A study done by Mitscher *et al*., (1972) on antimicrobial activity from higher plants found that extracts are generally richest in antibacterial agents after the flowering (sexual) stage of their growth is completed, and the plants taken from stressful environment were also found to be particularly active.

With regard to the method used in the evaluation of antimicrobial activity of plant extracts, Wilkinson (2006) suggested that for large-scale screening, disc and agar

diffusion methods offer a fast, cost-effective, low- technology, and generally reliable method of sorting extracts worthy of further investigation from those unlikely to be of value. Although more time and labor intensive are required to carry out broth dilution assay, this method is able to provide more information for the best use as a follow up to a large scale screening of plant extracts. Thus, for these reasons, disc diffusion assay and minimum inhibition concentration (MIC) by macrodilution (tube) broth were selected and carried out during this research.

The crude extracts were individually tested against 12 microorganisms namely six gram positive bacteria: *Bacillus cereus, B. subtilis, Staphylococcus aureus, Streptococcus mutans, S. sanguis* and *S. mitis*, three gram negative bacteria: *Proteus vulgaris, Pseudomonas aeruginosa* and *Escherichia coli* and three unicellular fungi: *Candida parapsilosis, C. albicans* and *Schizosaccharomyces pombe.* The microorganisms used in this study are commonly pathogenic to human except for the fungus *S. pombe*, which is better known as a cause of food spoilage (Pitt and Hocking, 2009) and have been employed extensively as models for genetic analysis of a variety of complex pathways and processes (Matsuyama *et al*., 1999).

4.2.1   Disc diffusion assay

Disc diffusion assay is usually used as the preliminary screening for antimicrobial activity. Throughout this study, Streptomycin was used as positive control for bacteria while Nystatin was used for the fungi, *Candida* spp. and *S. pombe.* As negative controls, the sterile broth and DMSO were used. The antimicrobial activity results from the disc diffusion assay at a concentration of 100 mg/ml are summarized in Table 4.2 to Table 4.5. The results are presented according to their microbiological classifications.

The results showed that Streptomycin (100mg/ml), positive control for bacteria gave strong activity against all the bacteria with inhibition zones ranging from 20.5 mm to 32.8 mm (Table 4.2 to Table 4.4) while Nystatin (100mg/ml) as positive control for unicellular fungi showed inhibition zone from 19.6 mm to 27.1 mm (Table 4.5). As for the negative control, there were no inhibition zones for all the antimicrobial tests (Table 4.2 to 4.5).

By classifying the antibacterial activity as gram positive or gram negative, frequently it would be expected that gram positive bacteria would be more active than gram negative bacteria. This kind of results is supported by many previous studies investigating on the antimicrobial activity of plant extracts and essential oils against pathogenic and food spoilage microorganisms (Hammer *et al*., 1999; Ficker *et al*., 2003; Chandarana *et al*., 2004; Habsah *et al*., 2004; Shan *et al*., 2007).

This phenomenon may be due to the features of gram negative organisms which possess an outer membrane composed of a lipopolysaccharide monolayer surrounding their cell wall. For that reason, the extracts were not able to penetrate and diffuse their compounds through the outer membrane of gram negative bacteria. Contrary to the gram negative bacteria, gram positive bacteria lack the outer membrane which makes them more susceptible to antibacterial agents (Chandarana *et al*., 2004).

However, in this study gram negative bacteria were found to be active particularly against *A. pahangensis*. All *A. pahangensis* rhizome extracts and for the leaf, hexane and dichloromethane extracts, showed weak to moderate inhibitory activity against all the gram negative bacteria tested (*Proteus vulgaris*, *Pseudomonas aeruginosa* and *Escherichia coli*) with inhibition zones ranging from 8.0 mm to 14.5 mm.

Table 4.2: Antibacterial activity of extracts of *Alpinia* species by disc diffusion assay against gram negative bacteria (*Proteus vulgaris*, *Pseudomonas aeruginosa* and *Escherichia coli*)

Species	Plant part	Crude extract	Zone of inhibition (mm)[a]		
			Gram negative bacteria		
			Proteus vulgaris	*Pseudomonas aeruginosa*	*Escherichia coli*
Alpinia pahangensis (100 mg/ml)	Rhizome	Hexane	14.3 ± 0.5	11.1 ± 0.2	14.5 ± 0.5
		Dichloromethane	14.5 ± 1.0	11.5 ± 0.5	14.5 ± 0.7
		Methanol	11.3 ± 0.5	8.0 ± 1.0	9.5 ± 1.3
	Leaf	Hexane	12.3 ± 0.2	8.3 ± 0.5	8.5 ± 0.5
		Dichloromethane	10.6 ± 1.2	9.00 ± 0	9.6 ± 0.5
		Methanol	na	na	na
Alpinia mutica (100 mg/ml)	Rhizome	Hexane	na	na	na
		Dichloromethane	10.8 ± 1.2	7.0 ± 0	na
		Methanol	10.0 ± 1.4	na	na
	Leaf	Hexane	10.2 ±1.0	na	na
		Dichloromethane	9.6 ± 1.1	7.3 ± 0.5	na
		Methanol	na	na	na
Streptomycin (100 mg/ml)			29.1 ± 0.2	29.6 ± 0.7	32.8 ± 1.2
Nystatin (100 mg/ml)			na	na	na
DMSO			na	na	na
Broth			na	na	na

Size of disc (6mm)

Key

[a] **:**   The results are expressed as mean ± standard deviation (n=3)

na :   no activity observed

Thus, for *A. pahangensis*, only the leaf methanol extract was found to be inactive against the three gram negative bacteria tested (Table 4.2).

As for *A. mutica*, the leaf or rhizome extracts failed to inhibit the growth of *E. coli*, while only a few extracts showed weak activity towards *P. vulgaris* and *P. aeruginosa*. The same results for *E. coli* was obtained by Habsah *et al*., (2000) but it was reported that the rhizome dichloromethane extracts of *A. mutica* showed the most potent inhibitory activity against *P. aeruginosa* (minimum inhibition dose: 125 µg per disc). However, in this study the rhizome and leaf dichloromethane extracts only showed weak activity with zone of inhibition of 7.0 mm and 7.3 mm respectively. The rhizome methanol and dichloromethane extracts showed moderate activity (10.0 mm and 10.8 mm respectively) towards *P. vulgaris*. As for the leaf, the zone of inhibition at 10.2 mm and 9.6 mm were obtained from hexane and dichloromethane extracts respectively against *P. vulgaris* (Table 4.2).

The rhizome extracts of *A. pahangensis* displayed strong to weak antibacterial activity with inhibition zones ranging from 8.6 mm to 16.5 mm against gram positive bacteria: *Bacillus cereus*, *B. subtilis* and *S.* aureus. The strongest inhibition (16.5 mm) was showed by dichloromethane extract. For the leaf, methanol extracts showed no activity against these three bacteria that generally cause food poisoning but showed moderate to weak inhibiton (9.0 mm to 11.5 mm) with hexane and dichloromethane extracts (Table 4.3).

For *A. mutica*, almost all of the rhizome and leaf extracts failed to inhibit the growth of the gram positive bacteria (*B. cereus*, *B. subtilis* and *S. aureus*) except for the weak activity demonstrated by dichloromethane rhizome extracts (8.1 mm to 8.3 mm). The

methanol rhizome extract and leaf dichloromethane extract also showed weak activity against *B. subtilis* (Table 4.3).

For the antimicrobial activity against *Streptococci* species (Table 4.4), almost all extracts failed to inhibit the growth of gram positive oral bacteria except for *A. pahangensis* rhizome extracts together with the leaf hexane and dichloromethane extracts against *S. mitis*. The rhizome hexane and dichloromethane extracts displayed moderate activity (14.5 mm) while the rhizome methanol extracts as well as leaf hexane and dichloromethane extracts showed weak activity against this bacterium (8.5 mm to 9.6 mm).

Table 4.3: Antibacterial activity of extracts of *Alpinia* species by disc diffusion assay against gram positive bacteria which generally cause food poisoning (*Bacillus cerues*, *Bacillus subtilis* and *Staphylococcus aureus*).

Species	Plant part	Crude extract	Zone of inhibition (mm)[a]		
			Gram positive bacteria		
			Bacillus cerues	*Bacillus subtilis*	*Staphylococcus aureus*
Alpinia pahangensis (100 mg/ml)	Rhizome	Hexane	15.8 ± 1.2	16.3 ± 0.5	12.6 ± 1.0
		Dichloromethane	16.5 ± 0.5	16.5 ± 0	12.5 ± 1.3
		Methanol	10.8 ± 1.0	12.1 ± 1.1	8.6 ± 0.5
	Leaf	Hexane	11.5 ± 2.2	10.1 ± 1.0	9.3 ± 0.5
		Dichloromethane	11.5 ± 1.3	10.8 ± 0.2	9.0 ± 0
		Methanol	na	na	na
Alpinia mutica (100 mg/ml)	Rhizome	Hexane	na	na	na
		Dichloromethane	8.1 ± 0.2	8.3 ± 0.5	8.3 ± 0.5
		Methanol	na	8.0 ± 1.4	na
	Leaf	Hexane	na	na	na
		Dichloromethane	na	8.0 ± 0	na
		Methanol	na	na	na
Streptomycin (100 mg/ml)			23.6 ± 1.2	27.3 ± 0.5	28.0 ± 1.0
Nystatin (100 mg/ml)			na	na	na
DMSO			na	na	na
Broth			na	na	na

Size of disc (6mm)

Key

[a] **:**   The results are expressed as mean $\pm$ standard deviation (n=3)

na :   no activity observed

Table 4.4: Antibacterial activity of extracts of *Alpinia* species by disc diffusion assay against gram positive oral bacteria (*Streptococus mutans*, *Streptococcus sanguis* and *Streptococus mitis*)

Species	Plant part	Crude extract	Zone of inhibition (mm)[a]		
			Gram positive oral bacteria		
			Streptococus mutans	*Streptococcus sanguis*	*Streptococus mitis*
Alpinia pahangensis (100 mg/ml)	Rhizome	Hexane	na	na	14.5 ± 0.5
		Dichloromethane	na	na	14.51± 0.7
		Methanol	na	na	9.5 ± 1.3
	Leaf	Hexane	na	na	8.5 ± 0.5
		Dichloromethane	na	na	9.6 ± 0.5
		Methanol	na	na	na
Alpinia mutica (100 mg/ml)	Rhizome	Hexane	na	na	na
		Dichloromethane	na	na	na
		Methanol	na	na	na
	Leaf	Hexane	na	na	na
		Dichloromethane	na	na	na
		Methanol	na	na	na
Streptomycin (100 mg/ml)			20.5 ± 0.8	23.0 ± 0.8	22.6 ± 0.7
Nystatin (100 mg/ml)			na	na	na
DMSO			na	na	na
Broth			na	na	na

Size of disc (6mm)

Key

[a] **:**   The results are expressed as mean ± standard deviation (n=3)

na :   no activity observed

As natural products have recently been shown to be an alternative to synthetic chemical substances for tooth decay prevention (Nostro *et al.,* 2004), the rhizome of *A. pahangensis* has the potential to be developed as one of the ingredients in products related to oral care products such as mouth rinse and toothpaste. However, further studies are required to evaluate the effect of the extracts in conditions similar to *in vivo* because it has been known that most oral streptococci have a high degree of surface hydrophobicity which plays as important factor in the initial bacterial adherence to tooth surfaces (Westergren and Olsson, 1983).

All solvent extracts of rhizomes and leaves of *A. pahangensis* and *A. mutica* showed no activity against the three fungi tested in this study (*Candida parapsilosis*, *C. albicans* and *Schizosaccharomyces pombe*; Table 4.5). For *A. mutica* this result supports the previous study by Habsah *et al*., (2000) that *A. mutica* dichloromethane and methanol rhizome extracts failed to exhibit inhibition against *C. albicans*. Similarly, Ficker *et al*., (2003) reported that the ethanol extract from the rhizome of this plant was completely inactive against a series of human pathogenic fungi including *C. albicans* while the extracts from the stalk showed weak inhibition against filamentous fungi, *Wangiellia dermatiditis*. A study by Srividya *et al*., (2010) revealed that none of the hydroalcoholic and methanol rhizome extracts of *A. officinarum* showed antifungal activity against *Aspergillus niger* and *C. albicans*. Despite of no activity for the extracts, however, the essential oils from rhizome and leaf of *A. pahangensis* exhibited moderate activity against *C. albicans* in MIC assay (Devi, 2009).

Table 4.5: Antifungal activity of extracts of *Alpinia* species by disc diffusion assay against three unicellular fungi (*Candida parapsilosis*, *Candida albican* and *Saccharomyces pombe*)

Species	Plant part	Crude extract	Zone of inhibition (mm)[a]		
			Unicellular fungi		
			Candida parapsilosis	*Candida albican*	*Schizosaccharo -myces pombe*
Alpinia pahangensis (100 mg/ml)	Rhizome	Hexane	na	na	na
		Dichloromethane	na	na	na
		Methanol	na	na	na
	Leaf	Hexane	na	na	na
		Dichloromethane	na	na	na
		Methanol	na	na	na
Alpinia mutica (100 mg/ml)	Rhizome	Hexane	na	na	na
		Dichloromethane	na	na	na
		Methanol	na	na	na
	Leaf	Hexane	na	na	na
		Dichloromethane	na	na	na
		Methanol	na	na	na
Streptomycin (100 mg/ml)			na	na	na
Nystatin (100 mg/ml)			24.3 ± 1.0	19.6 ± 1.2	27.1 ± 0.7
DMSO			na	na	na
Broth			na	na	na

Size of disc (6mm)

Key

[a] **:**   The results are expressed as mean ± standard deviation (n=3)

na :   no activity observed

The findings in this study supported the previous studies that plant extracts from organic solvents is a better solvent for more consistent antimicrobial activity from medicinal plants compared to other solvents like water (Eloff, 1998; Parekh *et al*., 2005).

The antimicrobial activity of plants relies on secondary metabolites synthesized by the plant itself. There are many useful antimicrobial compounds derived from plants. Among the major classes are phenolics, terpenoids and essential oils, alkaloids, lectins and polypeptides and polyacetylenes (Cowan, 1999). A study on the antibacterial activity of flavonoids and diarylheptanoids from *Alpinia katsumadai* seeds indicated that pinocembrin and alpinetin showed effects on the antibacterial activity against gram positive bacteria, *Staphylococcus aureus* and gram negative bacteria namely *Escherichia coli* and *Helicobacter pylori* (Huang *et al*., 2006). Hasnah and Shajarahtunnur (1999), and Malek *et al*., (2011) also found the same compounds (pinocembrin and alpinetin) in their study on the chemical constituents from the rhizome of *A. mutica*. Thus, the antibacterial activity showed by the rhizome of *A. mutica* extracts in the present study may be due to the presence of pinocembrin and alpinetin. However, it has to be noted that the mechanisms of antimicrobial action of plant secondary metabolites are not fully understood. A single compound may not be responsible for the antimicrobial activity but may be caused by a combination of compounds interacting in an additive or synergistic manner (Natta *et al*., 2008; Paiva *et al*., 2010; Ulukanli and Akkaya, 2010).

Based on the results obtained from the disc diffusion assay, it was observed that the non-polar fractions of the crude extracts were mostly able to inhibit microorganism activity. Investigation on the antimicrobial activity of *Alpinia kumatake* leaves

extracted using different organic solvents against six strains of bacteria, nine strains of fungi and five strains of yeasts showed strong inhibitory against bacteria but poor antimicrobial activity for yeast and fungi. Among the solvents investigated, leaves extracted with non-polar solvent, diethyl ether exhibited the highest antimicrobial activity (Sharma and Hashinaga, 2004).

The results from this study demonstrated that among the investigated rhizome extracts of *A. pahangensis*, the dichloromethane extracts exhibited the highest antibacterial effect followed by hexane and methanol, respectively. Apparently, the crude extracts particularly the dichloromethane extracts from leaves and rhizomes of *A. pahangensis* inhibit a distinct group of bacteria compared to *A. mutica*.

The strength of the disc diffusion assay was classified according to Najihah *et al*., (2009) as shown in Table 4.6. In this study, strong inhibition zones (more than 15.0 mm) showed by the hexane and dichloromethane extracts from the rhizomes of *A. pahangensis* tested with gram positivre bacteria namely *B. cereus* and *B. subtilis* were determined for their MIC values with macrobroth dilution method.

Table 4.6: Antimicrobial activity range by disc diffusion assay (Najihah *et al*., 2009)

Zone of inhibition (mm)	Activity status
≥ 15 mm	Strong
10 to 14.5 mm	Moderate
≤ 9.5 mm	Weak

4.2.2 Minimum Inhibition Concentration

The minimum inhibition concentration (MIC) is defined as the lowest concentrations of extracts that kill the initial inoculum and resulting less than ten colonies growth after streaking onto agar plates (James, 1990; Hammer *et al*., 1998; Poeta *et al*., 1998). Rios and Recio (2005) reported that the liquid dilution method is the best way to establish the real potency of a pure compound but solubility is an obvious requisite. Difficulty of achieving stability incorporating hydrophobic extracts into broth (aqueous media) occurs throughout this study. The extracts had separated out once pipetted into broth. Wilkinson (2006) stated that the problem can occur not just in the broth dilution but also in other microbial assay such as agar dilution assay. According to him, the situation happen because there is no solid phase to trap the compound and they will rapidly separate from the media and form a layer across the surface of the media.

In order to enhance the solubility of the extracts into broth, several researchers recommend the use of Tween or ethanol; however these compounds may interfere in the determination of MIC values and the growth of some test organisms (Wilkinson, 2006). To overcome this problem, an experiment was carried out to determine the effect of DMSO ratio as proposed by Teoh (2007) on the growth of bacteria and the result showed that incorporation of 5 % DMSO into broth did not inhibit the growth of microorganisms. Thus, 95 % of broth was used as a medium for the overnight microorganism cultures and a diluent for the initial stock of solution of the crude extracts.

The MIC values were tested at concentrations ranging from 780 µg/ml to 12,500 µg/ml and the results are presented in Table 4.7. The absorbance values of bacterial growth

incubated with various concentrations of the hexane and dichloromethane rhizome extracts of *A. pahangensis* are shown in Appendix I (d).

The hexane rhizome extracts of *A. pahangensis* tested against *Bacillus cereus* (gram positive) gave weak MIC value at 12,500 µg/ml while dichloromethane rhizome extracts showed moderate activity at concentration of 3120 µg/ml. Moderate MIC activity at 3120 µg/ml was also exhibited by the hexane and dichloromethane rhizome extracts of *A. pahangensis* against *Bacillus subtilis* (gram positive) which exhibit inhibition close to the Streptomycin as reference antibiotic (2500 µg/ml) (Table 4.7).

Table 4.7: The minimum inhibitory concentration of hexane and dichloromethane rhizome extracts of *A. pahangensis* against *B. cereus* and *B. subtilis*

Samples	Crude extract	MIC (µg/ml)	
		Gram positive bacteria	
		Bacillus cereus	*Bacillus subtilis*
A, pahangensis (Rhizome)	Dichloromethane	12,500	3120
	Hexane	3120	3120
Streptomycin		2500	2500

Table 4.8: Minimum inhibition concentration activity range (Devi, 2009)

MIC (µg/ml)	Activity status
1000 µg/ml	Strong
1000-4900 µg/ml	Moderate
$\geq$ 5000 µg/ml	Weak

There are few reports which have been published concerning the biological activities of *Alpinia* species in the literature. A study on antimicrobial activity of *Alpinia officinarum* rhizome extracted with hydroalcoholic and methanol showed moderate to strong MIC activity in the range of 31.25 μg/ml to above 1000 μg/ml against gram positive bacteria: *Bacillus cereus* and *Staphylococcus aureas* and gram negative bacteria namely *Pseudomonas aeruginosa* and *Escherichia coli*.

A previous study on the antimicrobial activity of Zingiberaceae species in Taiwan which include nine species of *Alpinia* namely *Alpinia japonica, A. kawakamii*, *A. pricei*, *A. mesanthera, A. kusshakuensis, A. officinarum*, *A. shimadai*, *A. uraiensis* and *A. zerumbet* was carried out by Chen *et al*., (2008). Rhizomes of these plants were extracted with methanol. The MIC results showed that most of the extracts had an ability to inhibit the growth of *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus* and *Vibrio parahaemolyticus* with strong MIC activity in the range of 50 μg/ml to 80 μg/ml.

There are quite a number of studies on antimicrobial activity of *Alpinia galanga* since it has been widely cultivated and used. It is reported that the oven-dried ethanol galangal flower extract showed the most effective activity against gram positive bacteria, *Staphylococcus aureus* strains with MIC ranging from 352 μg/ml to 547 μg/ml (Hsu *et al*., 2010). Oonmetta-aree *et al*., (2006) reported that the ethanol extracts from the galangal rhizomes had a strong inhibitory effect against *S. aureus*, with an inhibition zone of about 22 mm and MIC value of 352 μg/ml. In addition, Voravuthikunchai *et al*., (2006) also reported strong antimicrobial activity from galangal rhizome chloroform extract against *S. aureus* and methicillin-resistant *S. aureus* (MRSA), with MIC value of 390 μg/ml.

The study on the crude extracts of *A. pahangensis*, a wild ginger endemic to Pahang is being carried out for the first time, hence there are no available data on the chemical constituents of the extracts. However a recent study on the biological activities of the essential oils of *A. pahangensis* revealed that the rhizome oils showed strong MIC activity (MIC ≤ 1000 μg/ml) against five strains of gram positive bacteria, *S. aureus* (Sa 2, Sa 3, Sa 7, Sa VISA and Sa VRSA) while the leaf oils exhibited moderate activity (1000-4900 μg/ml) except against Sa VSRA which showed strong activity (Devi, 2009).

Devi (2009) also found that both rhizome and leaf oils exhibited moderate activity against four selected fungi (*Candida albicans*, *C. glabrata*, *Microsporum canis* and *Trycophyton rubrum*). The antimicrobial activity of the rhizome oil may due to the presence of monoterpenes, β-pinene as main component, as well as camphor, 4-terpineol and α-terpineol. Thus, it appears that the rhizome of *A. pahangensis* contain more antimicrobial compounds than the leaf.

4.3   Antioxidant activity

4.3.1   DPPH radical scavenging assay

Oxidant is a substance that can induce oxidative damage to various biological targets such as nucleic acids, lipid and proteins. Therefore, in order to reduce it efficiently, a substance called antioxidant is needed (Magalhães *et al*., 2008). Among many methods developed to evaluate the antioxidant activity *in vitro*, the 2, 2-diphenylpicrylhydrazyl (DPPH) free radical scavenging assay was selected in this study. As a rapid, simple and inexpensive method, the assay is widely used to assess the ability of compounds to act as free radical scavengers or hydrogen donors. Since antioxidant compounds might be water soluble, lipid soluble, insoluble or bound to cell walls, this method ensures a better comparison of the results and covers a wider range of possible applications because it has an ability to determine the activity of hydrophilic and lipophilic species (Prakash 2001; Sacchetti *et al*., 2005; Cheng *et al*., 2006). As a reliable parameter to evaluate antioxidant activity, natural phytochemicals that have been successfully screened are fruits, vegetables, herbs, tea, beans, oilseeds as well as in food (Prakash 2001; Cheng *et al*., 2006).

The scavenging activity can be verified by a decrease in absorbance at 517 nm due to the reduction of the stable DPPH radical in the presence of hydrogen donating antioxidants to form less reactive radicals. This assay is dependent on the ratio of antioxidant and DPPH in the testing reaction mixtures (Cheng *et al*., 2006; Maisuthisakul *et al*., 2007).

All tested extracts of *A. pahangensis* and *A. mutica* showed a dose dependent scavenging activity where it was observed that an increase in the concentration of the extracts resulted in an increase in antioxidant activities. Previous studies also

demonstrated similar results in rhizome extract of *Alpinia conchigera* (Rozanida, 2007) *A. officinarum* (Srividya *et al*., 2010), *A. galanga* and *A. allughas* (Vankar *et al*., 2006) to name a few. The same result can also be found from other plants such as cocoa (Azizah *et al*., 1999), Korean medicinal salad plants (Chon *et al*., 2008) and black soybean (Haiwei, 2010).

Primarily, pre screening was conducted at 5 mg/ml and any sample that show 50 or more percentage of inhibitions which is considered as active activity, will be further analyzed at concentration 5 mg/ml to 0.5 mg/ml to determine their IC_{50} value. Free radical scavenging activity of all crude extracts of *A. pahangensis* and *A. mutica* at 5 mg/ml are shown in Table 4.9. In general, at concentration 5 mg/ml the pattern of antioxidant activity showed that the highest antioxidant activity was observed in the rhizome methanol extracts and leaf hexane extracts for both *Alpinia* species studied.

In this study, IC_{50} value for the dichloromethane and methanol leaf extracts of *A. mutica* could not be determined since the samples showed percentage inhibition below than 50 % during pre-screening assay. Ascorbic acid (Vitamin C) was used as a reference to compare the antioxidant activity of the extracts (Table 4.9).

Among *A. pahangensis* rhizome extracts, methanol extract gave the highest antioxidant activity at 81.53 % followed by dichloromethane extract (80.54 %) while hexane extract showed moderate activity (57.42 %). Inversely, leaf hexane extracts of *A. pahangensis* showed the highest antioxidant activity (77.67 %) while moderate activity was shown by both dichloromethane and methanol extracts (60.76 and 59.98 % respectively).

Table 4.9: Percentage inhibition of DPPH radical scavenging activity of *A. pahangensis* and *A. mutica* extracts at the concentration of 5 mg/ml

Plant extracts	Inhibition (%)	
	Rhizome	**Leaves**
Alpinia pahangensis		
▪ Hexane	57.42 ± 3.33	77.67 ± 3.58
▪ Dichloromethane	80.54 ± 2.78	60.76 ± 3.08
▪ Methanol	81.53 ± 3.35	59.98 ± 2.08
Alpinia mutica		
▪ Hexane	55.31 ± 1.32	78.54 ± 0.42
▪ Dichloromethane	77.24 ± 0.83	47.02 ± 0.97
▪ Methanol	83.81 ± 0.28	47.00 ± 0.43
Ascorbic acid (200 µg/ml) (Standard reference)	93.74 ± 0.5	

Data are expressed as mean ± standard deviation (n=3)

Table 4.10: Antioxidant activity range (Vimala *et al*., 2003)

Activity range (%)	Activity status
70-100	Strong
40-69	Moderate
≥ 40	Weak

In the case of *A. mutica*; the highest antioxidant activity among the rhizome extracts was methanol (83.81 %) followed by dichloromethane extract (77.24 %) while hexane extract gave moderate activity (55.31 %). At the same concentration, leaf hexane extracts of *A. mutica* showed the highest antioxidant activity (78.54 %) but only moderate activity for both dichloromethane (47.02 %) and methanol extracts (47 %). Above all, the standard reference (ascorbic acid) showed the highest percentage inhibition of 93.74 %.

In summary, the antioxidant activity of the rhizome and leaf extracts for the different solvent decreased in the following order; *A. mutica* (methanol rhizome extract)> *A. pahangensis* (methanol rhizome extract)> *A. pahangensis* (dichloromethane rhizome extract)> *A. pahangensis* (dichloromethane leaf extract)> *A. mutica* (dichloromethane rhizome extract) > *A. pahangensis* (hexane leaf extract)> *A. pahangensis* (hexane rhizome extract)> *A. mutica* (hexane leaf extract) > *A. pahangensis* (methanol leaf extract)> *A. mutica* (hexane rhizome extract) > *A. mutica* (dichloromethane leaf extract) > *A. mutica* (methanol leaf extract).

A parameter used to measure antioxidant activity is the IC_{50}, which is defined as the concentration (in mg/ml) of extract required to inhibit the formation of DPPH radicals by 50%. Determination of IC_{50} value of extracts in this study was calculated using linear regression equation of the graph plotting between the percentages of antioxidant inhibition against the tested concentrations as shown in Figure 4.1a to Figure 4.1j. In addition, the activity for ascorbic acid as standard reference is shown in Figure 4.2. All the plots showed R^2 value range from 0.9686 to 0.8156. The smaller IC_{50} value indicates the higher antioxidant activity of the plant extract (Zhang *et al*., 2010). Table 4.11 shows the IC_{50} value of plant extracts and ascorbic acid as positive reference.
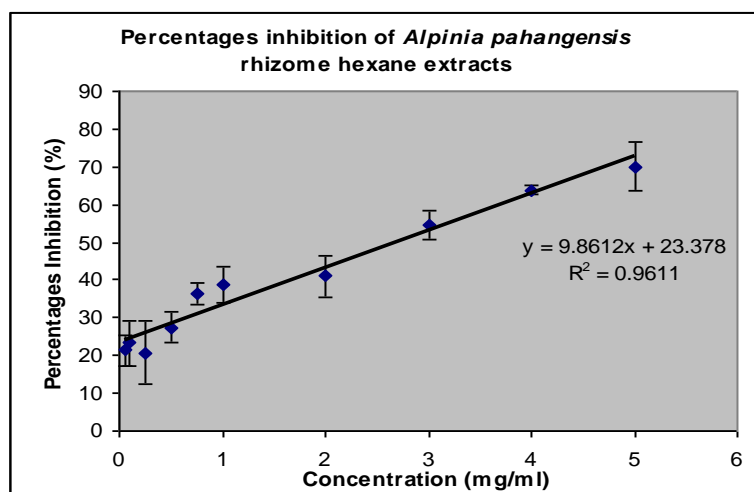
Figure 4.1a: The DPPH radical scavenging activity of *A. pahangensis* rhizome hexane extracts
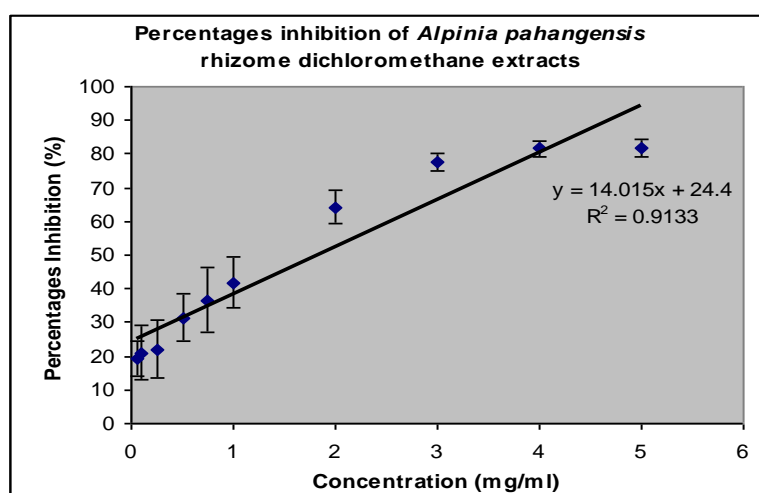
Figure 4.1b: The DPPH radical scavenging activity of *A. pahangensis* rhizome dichloromethane extracts
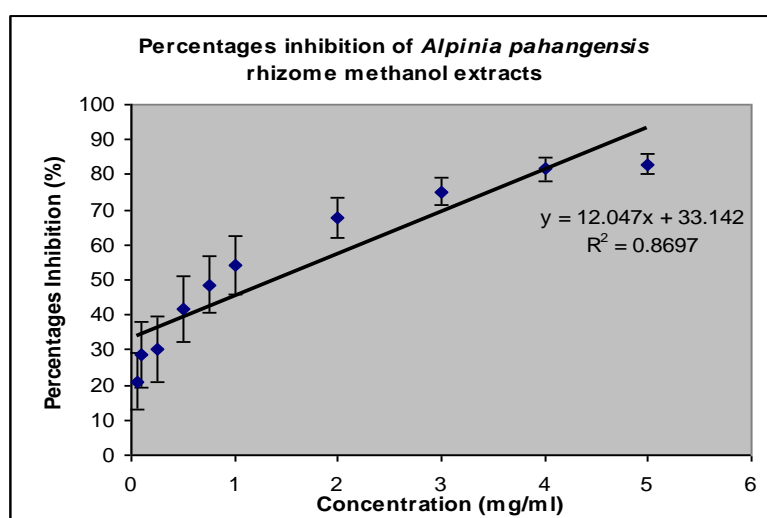
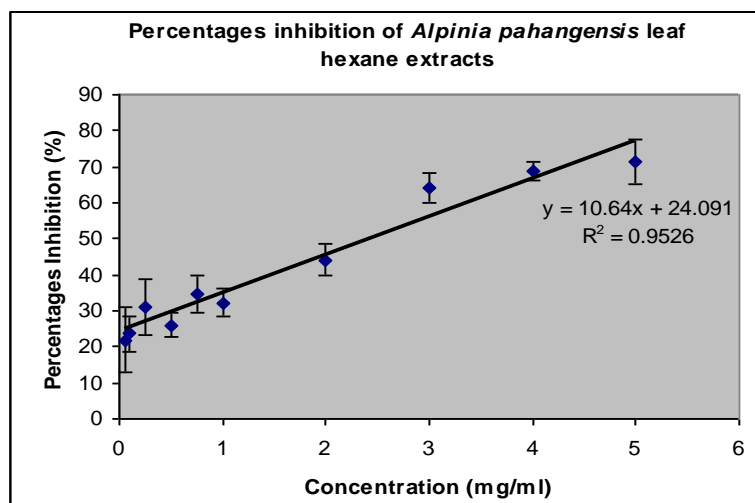**Figure 4.1c:** The DPPH radical scavenging activity of *A. pahangensis* rhizome methanol extracts

Figure 4.1d: The DPPH radical scavenging activity of *A. pahangensis* leaf hexane extracts
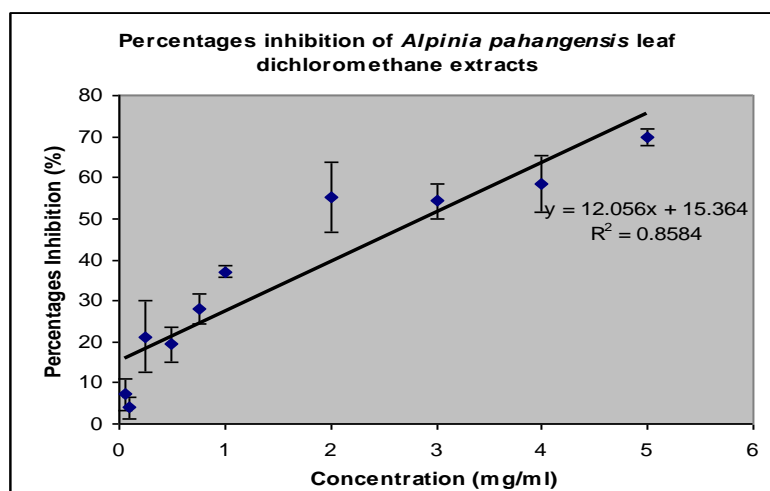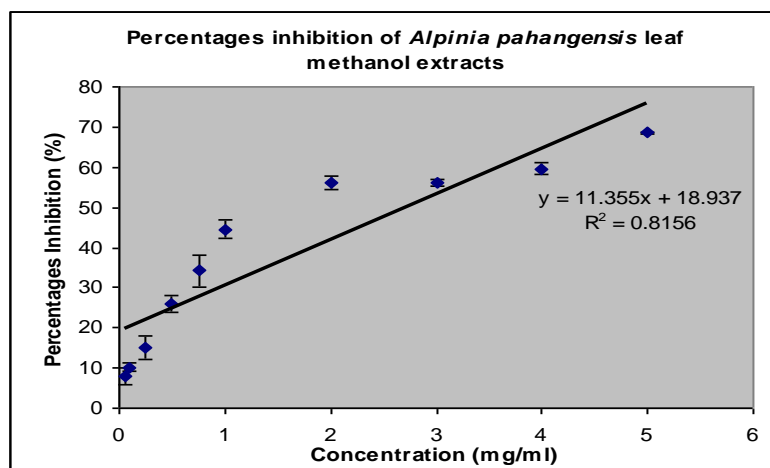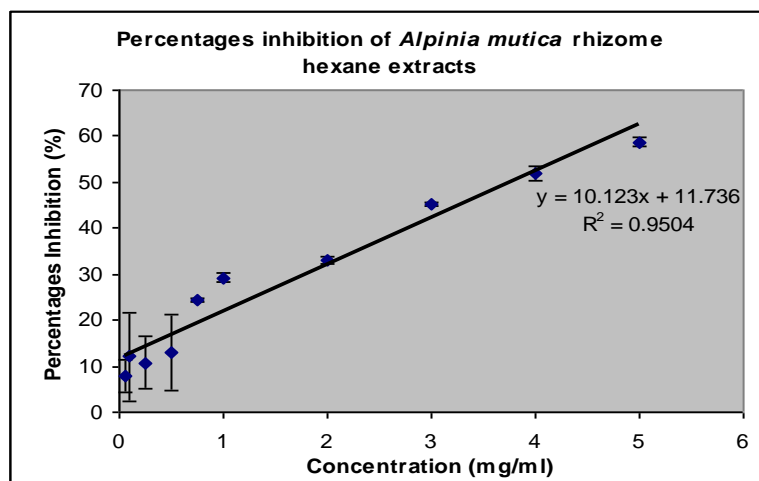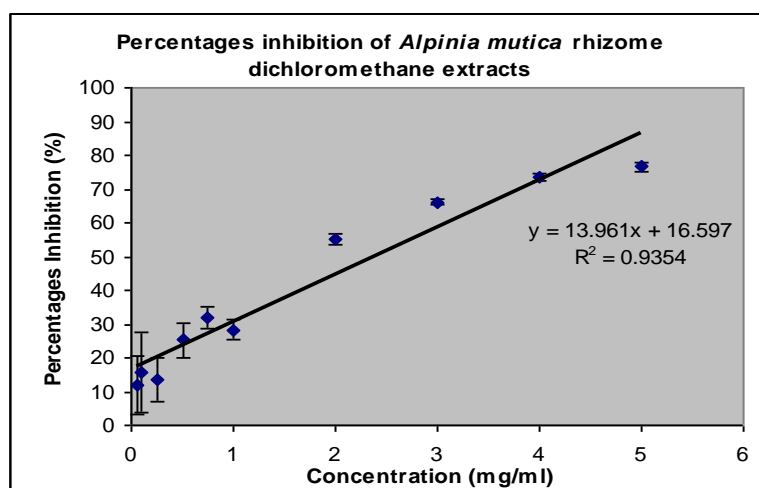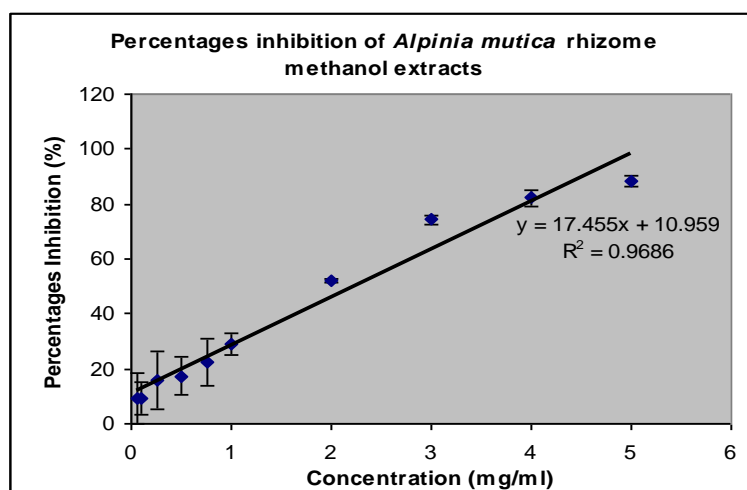

Figure 4.1e: The DPPH radical scavenging activity of *A. pahangensis* leaf dichloromethane extracts

Figure 4.1f: The DPPH radical scavenging activity of *A. pahangensis* leaf methanol extracts

Figure 4.1g: The DPPH radical scavenging activity of *A. mutica* rhizome hexane extracts

Figure 4.1h: The DPPH radical scavenging activity of *A. mutica* rhizome dichloromethane extracts

Figure 4.1i: The DPPH radical scavenging activity of *A. mutica* rhizome hexane extracts
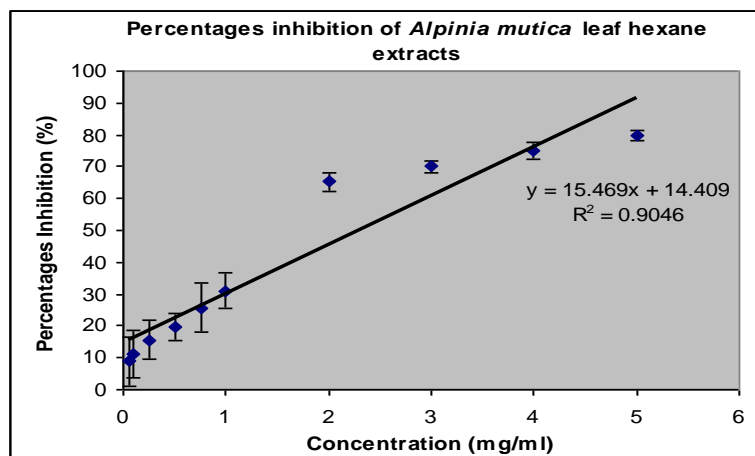
Figure 4.1j: The DPPH radical scavenging activity of *A. mutica* leaf hexane extracts
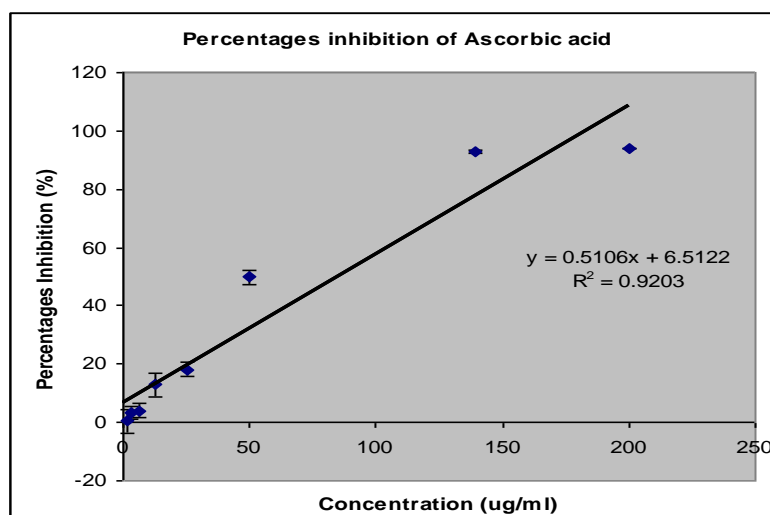

Figure 4.2: The DPPH radical scavenging activity of ascorbic acid (standard reference)

Generally, between the two *Alpinia* species studied, *A. pahangensis* showed higher antioxidant activity compared to *A. mutica*. Among all extracts examined as well as within *A. pahangensis* rhizome extracts, methanol extract of the rhizome of *A. pahangensis* demonstrated the highest DPPH free radical scavenging activity with IC_{50} value of 1.71 mg/ml followed by dichloromethane extract (1.85 mg/ml) and hexane extract (2.66 mg/ml). As for the leaf of *A. pahangensis*, the hexane extracts showed the highest antioxidant activity with IC_{50} value of 2.41 mg/ml while dichloromethane (2.88 mg/ml) and methanol extract (3.45 mg/ml).

Table 4.11: The IC_{50} value of plant extracts and ascorbic acid

Plant extracts	IC_{50}^{a}	
	Rhizome	Leaves
Alpinia pahangensis		
▪ Hexane	2.66	2.41
▪ Dichloromethane	1.85	2.88
▪ Methanol	1.71	3.45
Alpinia mutica		
▪ Hexane	3.82	2.49
▪ Dichloromethane	2.39	nd
▪ Methanol	2.25	nd
Ascorbic acid (200 µg/ml) (Standard reference)	0.085	

Key

^{a} : Concentration (mg/ml) for a 50 % inhibition

nd: not determined, IC_{50} not achieved

The highest DPPH free radical scavenging activity among rhizome extracts for *A. mutica* was methanol with IC_{50} value of 2.25 mg/ml followed by dichloromethane extract (2.39 mg/ml) and hexane extract (3.82 mg/ml). Meanwhile, leaf hexane extracts of *A. mutica* showed the IC_{50} value of 2.49 mg/ml. All extracts showed antioxidant activity with IC_{50} value higher than ascorbic acid (0.085 mg/ml) which indicate lower antioxidant activity.

Phytochemical components including phenolic compounds have been reported to be related with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals. Hence, the antioxidant activity of rhizome of *A. mutica* may

be due to the presence of phenolic compounds (5, 6-dehydrokawain, pinocembrin, flavokawin B) and diarylheptanoid isolated from their rhizome as reported by Hasnah and Shajarahtunnur (1999). Diarylheptanoids isolated from a member from the same genus, *A. officinarum* have been shown to exhibit various biological activities including antioxidant (Ly *et al*., 2003; Sawamura *et al*., 2010). Nonetheless no reports have been found for their leaf compounds as well as for *A. pahangensis*.

Hence, it can be summarized that the methanol extracts of rhizomes of *A. pahangensis* and *A. mutica* exhibited a higher antioxidant activity than the other solvent extracts. As for the leaves, the hexane extracts appear to be showing a better antioxidant activity than the rest.

CHAPTER 5: CONCLUSION

The yield of crude extracts extracted with non-polar (hexane) to a medium polar (dichloromethane) and finally a polar solvent (methanol) of both leaf and rhizomes were found to be correlated with the degree of the solvent polarity. The yield was found to increase with increase in solvent polarity except for the leaf of *A. pahangensis* where the yield of the dichloromethane extract was higher compared to the methanol extract. The results suggest that the species investigated may contain a higher amount of polar fraction than non-polar fraction. Higher yield of crude extracts were obtained from the rhizomes rather than the leaves of both species. As the yields of extracts were unpurified and taken as total extracts, it is important to regard that the extracts may still contain the mixture constituents from both polar and non-polar compounds.

In the disc diffusion assay, the hexane and dichloromethane rhizome extracts of *A. pahangensis* possessed strong antibacterial activity against gram positive bacteria: *Bacillus cereus* and *B. subtilis* and moderate activity against *Staphylococcus aureus* and oral bacteria, *Streptococcus mitis*. However, the methanol rhizome extracts of *A. pahangensis* were less active by displaying only moderate inhibition towards *B. cereus* and *B. subtilis* and weak activity once tested with *S. aureus* and *S. mitis*. As for gram negative bacteria, moderate activity against *Proteus vulgaris*, *P. aeruginosa* and *Escherichia coli* were shown when tested with *A. pahangensis* rhizome hexane and dichloromethane extracts as well as methanol extract against *P. vulgaris* only. In addition, methanol rhizome extract showed weak activity against *P. aeruginosa* and *E. coli*. As for *A. mutica*, the dichloromethane rhizome extracts showed weak activity against gram positive bacteria *B. cereus*, *B. subtilis* and *S. aureus* as well as towards gram negative bacteria namely *P. vulgaris* and *P. aeruginosa*. The methanol rhizome

extract also showed weak activity towards two bacteria that is *B. subtilis* (gram positive) and *P. aeruginosa* (gram negative).

As for the leaves of *A. pahangensis*, the hexane and dichloromethane extracts showed moderate activity against gram positive bacteria: *B. cereus* and *B. subtilis* and *P. vulgaris* (gram negative) and weak inhibition against *P. aeruginosa* (gram negative) and *S. aureus* (gram positive). The hexane leaf extracts exhibit weak activity when tested with *S. mitis* (gram positive) and *E. coli* (gram negative). Moderate activity of inhibition was obtained for the hexane and dichloromethane leaf extracts of *A. mutica* against gram negative bacteria namely *P. vulgaris* while dichloromethane extracts showed weak activity towards *P. aeruginosa*. (gram negative) and *B. subtilis* (gram positive). The results demonstrated that all leaf and rhizome extracts of *A. pahangensis* and *A. mutica* failed to exhibit any inhibition against all tested unicellular fungi: *Candida albicans*, *C. parapsilosis* and *Schizosaccharomyces pombe* and two gram positive oral bacteria namely *Streptococcus mutans* and *S. sanguis*.

Determination of MIC values for antibacterial activity against gram positive bacteria: *B. cereus* and *B. subtilis* from disc diffusion assay of hexane and dichloromethane rhizome extracts of *A. pahangensis* resulted in moderate MIC activity which gave inhibition value close to the standard reference, Streptomycin. The overall results for the antimicrobial activity indicated that the rhizome extract was more active and *A. pahangensis* exhibit more activity against microorganism tested compared to *A. mutica*.

The antioxidant activity of the leaf and rhizome extracts of *A. pahangensis* and *A.mutica* were evaluated by DPPH radical scavenging assay. At the concentration of

5 mg/ml, the antioxidant activity of the dichloromethane and methanol rhizome extracts of *A. mutica* and *A. pahangensis* was quite high while the hexane extracts showed moderate activity. However, for the leaf extracts, the situation is the reverse, that is, the hexane extracts scavenged strong activity whereas dichloromethane and methanol extracts showed moderate activity. As for the IC_{50} value, among the rhizome extracts, the rhizome of *A. pahangensis* extracted with methanol allocate greater antioxidant activity than that of the *A. mutica*. Meanwhile, for the leaf extracts, the highest antioxidant activity was shown by the hexane extract of *A. pahangensis*.

Generally, of the two *Alpinia* species studied, *A. pahangensis* showed greater response and potent activities compared to *A. mutica* in terms of the present research analysis which may due to the synergistic effects of compounds present in this unexploited species. Comparatively, although polar solvent (methanol) is found to obtain the highest yield of crude extracts, they had shown less biological activities if compared to hexane and dichloromethane extracts. It reveals that the amount of yield does not influence their biological activities whereas the active ingredients in the extracts play the major role.

In conclusion, *A. pahangensis* is likely to be an interesting species to be explored thoroughly. For further investigation, isolation and purification of active compounds from active extracts is required to identify the chemical constituents responsible for their antioxidant and antimicrobial activities.