CHAPTER 1

INTRODUCTION

Introduction

Plants are well known for their medicinal importance in both western type pharmaceuticals as well as traditional or indigenous preparations. Plants have been used in the treatment of various human ailments dating back to prehistoric times (Cowan, 1999). A report by World Health Organisation (WHO) states that 80% of the world's population relies on traditional medicines to meet their daily health requirements (Carlson, 2004). However, from the estimated 250,000 species of higher plants described, only 5-15% has been studied for their potential therapeutic value (Rojas *et al*., 2003; Steep, 2004).

Herbal remedies used in traditional medicines provide an interesting source for development of new drugs. Besides that it also plays an important role for basic health needs in the developing countries. According to WHO, a medicinal plant is defined as any plant which contains substances that can be used for therapeutic purposes or which are precursors of new pharmaceutical semi synthetic drugs (Berkarda, 1978; Wijesekera, 1991; Khafagi and Dewedar, 2000).

Medicinal plants have been extensively used worldwide for many purposes for decades. Extracts and oils of medicinal plants form the basis for many applications such as in the preservation of raw and processed foods, drugs, alternative medicines and natural therapies. Recently their antimicrobial activities have been studied against many microorganisms (Hamburger and Hostettmann, 1991). WHO has described medicinal plants as the best sources for obtaining a wide variety of drugs (Santos *et al*., 1995). Therefore such plants need to be thoroughly investigated to determine their structural and functional properties as well as their efficiency (Eloff, 1998). Recently there was a huge demand for new antifungal compounds due to lack of efficacy, side effects and / or

resistance associated with some of the existing drugs (Aperis *et al*., 2006; Barker and Rogers, 2006; Klepser, 2006; Mondello *et al*., 2006). Natural products obtained from many plants have been attracting scientific interest (Ledezme and Apitz, 2006; Pyun and Shin, 2006).

Some of the commonly known drugs derived from plants are morphine, codeine, cocaine and quinine. Besides that catharanthus alkaloids, belladonna alkaloids, colchicines, phytostigminine, pilocarpine, reserpine are plant derived anticancer drugs. Many of these drugs are still used widely nowadays and there are yet any synthetic substitutes that are found to possess the same efficacy and pharmacological specificity (Balandrin *et al*., 1985).

It was estimated that about 25% of the drugs prescribed in the industrialized countries directly or indirectly via semi-synthesis contain compounds derived from plants. Flowering plants play a major role in drug development as well as 11% of the 252 drugs considered as basic and essential by WHO are exclusively derived from them (Rates, 2001). There is a huge market for plant derived drugs in the western countries as the prescription drugs containing phytochemicals were valued at more than US$30 billion in 2002 in the USA alone (Raskin *et al*., 2002). The use of plant extracts and phytochemicals with known antibacterial properties will continue to play a major role in developing novel drugs (Artizzu *et al*., 1995; Izzo *et al*., 1995; Shapoval *et al*., 1994).

Lactic acid bacteria are widely used in the industry as a starter culture for food fermentation. It could contribute to flavours as well as extend the shelf-life of the food (Liepe, 1983). Lactic acid bacteria produce various compounds such as organic acids, hydrogen peroxide, lactoperoxidase, diacetyl, bacteriocins or bactericidal proteins

during lactic acid fermentations (Oyetayo *et al*., 2003; Yang, 2000). In many cases the antibacterial properties of lactic acid bacteria is probably the combination of these factors.

Some food-grade lactic acid bacterial strains could produce antimicrobial proteins that will inhibit the growth of other bacteria, which are associated with food spoilage and health hazards of food origin (Daeschell and Klaenhammer, 1985). It appears that fermentation of food could produce an environment that is inhibitory to a variety of bacteria although the substrate for fermentation may vary. Reports indicates that these antimicrobial compounds differ greatly in their molecular weights ranging from 3500 (Bailey and Hurst, 1971) to 100 000 (Barefoot and Klaenhammer, 1983). If the antimicrobial effect is a property of fermented foods, it could be an important strategy for the reduction of the high levels of fecal bacteria in weaning foods in developing countries.

In contrast to antibiotics, bacteriocins or bacteriocin producing strains and bacteriocin like inhibitory substances (BLIS) may be used as natural food preservatives (West and Warner, 1988) to improve the safety of some products such as dairy and meat products. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria. These proteins exhibit quite specific range of antimicrobial properties. Bacteriocins are commonly found in Gram-positive and Gram-negative bacteria. Those that are produced by lactic acid bacteria are of particular interest due to their potential application in the food industry as natural preservatives (Ross *et al*., 2002).

The objectives of this study are:

1. To isolate protein/peptide and secondary metabolites from plant and fermented extract sources.
2. To test for antimicrobial properties of the samples using test microorganisms with identified species *in vitro*.
3. To determine the antioxidant properties of the samples.
4. To separate and detect the compounds in crude samples that showed antimicrobial and antioxidant activities using high performance liquid chromatography (HPLC) and matrix-assisted laser desortion/ionization (MALDI) Time of flight (TOF)/TOF.
5. To determine acute toxicity if any by using Institute of Cancer Research (ICR) mice by using force feed method.

CHAPTER 2

LITERATURE REVIEW

Literature review

Literature review

2.1 Peptide

Peptides can be divided into several major classes such as ribosomal peptides, non ribosomal peptides, digested peptides, peptones and peptide fragments. Based on amino acid sequences, secondary structures, and functional similarities, antimicrobial peptides (AMPs) are tentatively classified into four distinct groups: the linear basic peptides forming amphipathic α-helices conformation with no cysteine residues (Zasloff, 2002); peptides containing cysteine residues with one to four intra-molecular disulfide bonds (Terras *et al*., 1993); peptides with an over-representation of specific amino acids, such as proline, arginine, and glycine residues (Mor and Nicolas, 1994) or tryptophan-rich peptides (Lee *et al*., 2003); and the peptides produced by the hydrolysis of large inactive or little active proteins (Casteels-Josson *et al*., 1994).

A variety of peptides and proteins have been used to produce biopesticides, biopesticidal microbes, and pest-resistant crops. These compounds are derived from a number of sources including the venoms of predatory or parasitoid animals (Gershburg *et al*., 1998; Harrison and Bonning, 2001; Taniai *et al*., 2002; Volynski *et al*., 1999), arthropod-pathogenic microbes including bacterial symbiotes of entomopathogenic nematodes (Beard *et al*., 2001), plant lectins, protease inhibitors (Brunelle *et al*., 2005; Cheng and Xue, 2003; Slack *et al*., 1995) or ribosome inactivating proteins (Sharma *et al*., 2004), arthropod hormones and neuropeptides (Altstein *et al*., 2000; Altstein, 2001, 2004; Borovsky, 2003a, b; Borovsky *et al*., 1990, 1993; Ma *et al*., 1998; Menn and Borkovec, 1989), biotin-binding proteins (Burgess *et al*., 2002), chitinases (Gopalakrishnan *et al*., 1995; Kramer and Muthukrishnan, 1997), enzymes controlling aromatic aldehyde synthesis (O'Callaghan *et al*., 2005), viral enhancins (Cao *et al*.,

2002; Granados *et al*., 2001; Lepore *et al*., 1996), plant defensins (Lay and Anderson, 2005), and plant hormones (Dinan, 2001).

A huge number of insect selective fungi and fungal-derived toxins that are both peptidic and nonpeptidic are known. A great many fungi and yeasts have been used as antimicrobial agents to manage crop diseases (Punja and Utkhede, 2003; Benitez *et al*., 2004), but there are also many fungi currently being utilized in insect control (Scholte *et al*., 2005). Examples include *Beauvaria bassiana* for the control of numerous insects such as sand flies (Warburg, 1991), *Metarhizium anisopliae*, first used in 1888 (Taborsky and Taborsky, 1992) against Clones punctiventris and more recently against a variety of pests including the mosquito *Anopheles gambiae* (Scholte *et al*., 2005), *Lagenidium giganteum* against mosquitoes (Kerwin and Washino, 1987; Kerwin *et al*., 1994), and *Verticillium lecanii* as an aphid control (Ashouri *et al*., 2004; Kim *et al*., 2005). Other lesser known fungi have also been identified as pathogenic and considered for biological control of mites like *Varroa destructor*, a honey bee ectoparasite (Hastings, 1994; Peng *et al*., 2002; Umina *et al*., 2004).

Like fungi, many bacterial species produce insecticidal toxins of tremendous biotechnological, agricultural, and economic importance. Although Bt currently accounts for at least 80% of the sprayable bioinsecticide market and is the only sanctioned source of insect-resistant genes for use within GM plants, the toxins of the bacteria *Serratia marcescens* (Downing *et al*., 2000; Downing and Thomson, 2000; Inglis and Lawrence, 2001), *Photorhabdus luminescens* and *Xenorhabdus nematophilus* may also provide useful alternatives (Chattopadhyay *et al*., 2004; French-Constant and Bowen, 2000). Of the more than 20 known groups of insect pathogenic viruses,

classified into 12 families, only a modest number of viruses have been explored for their insecticidal potential (Tanada and Kaya, 1993).

In addition to baculoviruses, insect parvoviruses have demonstrated insecticidal power (Tal and Attathom, 1993), and the introduction of Oryctes virus into outbreak areas of the rhinoceros beetle, which led to a dramatic reduction in palm damage in many areas of the Asia-Pacific region, was touted as a major success for viral biocontrol (Caltagirone, 1981; Jackson *et al*., 2005). Tetraviruses such as the cotton bollworm (*Helicoverpa armigera*) stunt virus have been isolated from a number of pest species and may also find utility as direct control agents, by transgenic generation in plants or other organisms, or as gene delivery vehicles (Hanzlik *et al*., 1995; Pringle *et al*., 2003; Yi *et al*., 2005; Bothner *et al*., 2005). However, among insect pathogenic viruses, members of the family Baculoviridae are the most commonly found, studied and used (Kamita *et al*., 2005). Due to inherent insecticidal activities, natural baculoviruses have been used as safe and effective biopesticides for the protection of field and orchard crops, and forests in the Americas, Europe, and Asia (Moscardi, 1999; Vail *et al*., 1999; Copping and Menn, 2000; Lacey *et al*., 2001).

2.2 Secondary metabolites

Plants produce primary and secondary metabolites which encompass a wide array of functions (Croteau *et al*., 2000). Primary metabolites, which include amino acids, simple sugars, nucleic acids, and lipids, are compounds that are necessary for cellular processes. Higher plants are capable to produce a wide variety of low molecular weight compounds which is known as secondary metabolites. Secondary metabolites have no recognized role in the maintenance of fundamental life processes in the plant that synthesize them (Namdeo, 2007). Secondary metabolites are organic compounds that

are not directly involved in the normal growth, development, or reproduction of the plant. Unlike primary metabolites, absence of secondary metabolites does not result in immediate death. Secondary metabolites production is normally less than 1% of the dry weight and it depends greatly on the physiological and developmental stage of the plant (Oksman and Inze, 2004). Secondary metabolites include compounds produced in response to stress, such as the case when acting as a deterrent against herbivores (Keeling and Bohlmann, 2006). Plants can manufacture many different types of secondary metabolites, which have been subsequently exploited by humans for their beneficial role in a diverse array of applications (Balandrin *et al*., 1985).

Plants could produce a diverse array of secondary metabolites as natural protection against microbial and insect attack. Some of these compounds are constitutive, existing in healthy plants in their biologically active forms. Others, such as cyanogenic glycosides and glucosinolates, occur as inactive precursors and are activated in response to tissue damage or pathogen attack. This activation often involves plant enzymes, which are released as a result of breakdown in cell integrity. Some of these compounds might also be toxic to animals. Indeed, many of these compounds have been used in the form of whole plants or plant extracts for food or medical applications in man (Wallace, 2004). Secondary metabolites have been exploited for many years for a wide variety of purposes including their use as food, fragrances, pigments, insecticides and medicines. Historically plants have served as the major source of useful natural product with pharmaceutical properties (Carte, 1996). According to Joffe and Thomas (1989) about 25% of all pharmaceutical sales are drugs derived from plant natural products and an additional 12% are based on microbial produced natural products.

2.3 Antimicrobial

Clinical microbiologists have two reasons to be interested in antimicrobial plant extracts. First, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians and it is reported that, on average, two or three antibiotics derived from microorganisms are launched each year (Clark, 1996). After a downturn in the recent decades, current research on new drugs is again increasing as scientists realize that the effective life span of any antibiotic is limited. Globally billions are invested on finding new anti-infective agents which includes vaccines and it is expected to increase 60% from the spending levels in 1993 (Alper, 1998). Moreover the public is becoming increasingly aware of the problems resulting from over-prescription and misuse of traditional antibiotics.

Resistance to antimicrobial agents has resulted in morbidity and mortality from treatment failures and increased health care costs. Resistance to antibiotics is rising worldwide and is becoming a serious global problem (Ekpendu *et al*., 1994, Westh *et al*., 2004). In many countries, national surveillance and research programmes have been initiated to monitor resistance in bacteria isolated from human and food animals (Mevius *et al*., 1999). The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens (Bandow *et al*., 2003).

Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance. The association between increased rate of antimicrobial use and resistance has been documented for nosocomial infections as well as for resistant community acquired infections. Antibiotic resistance particularly on mobile elements can spread rapidly within human and animal populations. Antimicrobial resistance genes are shown to be capable of transferring between bacteria

of diverse origin under natural environment conditions (Kruse and Sorum, 1994). Due to the increasing resistance of bacterial isolates for antibiotics, more intense efforts are being made to find alternative antimicrobial components (Berkowitz, 1995).

Multidrug-resistant pathogens travel not only locally but also globally, with newly evolved pathogens capable of spreading rapidly into susceptible hosts. Because of the emergence of resistance to frequently prescribed antibiotics emerging in clinics over the past decade, it is necessary to discover and introduce new drugs. The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes (Afolayan, 2003). Numerous studies have identified compounds within herbal plants that are effective antibiotics (Basile *et al*., 2000). According to a survey in 1967, 76% of antibiotics originated from fungi, 12% from higher plants, 9% from bacteria and 3% from algae, lichens and animals (Edwards, 1980).

Despite the emergence of pharmaceutical producers of new antibiotics over the last three decades resistance to these drugs by microorganisms has developed. Microorganisms have the capability to replicate and acquire resistance to drugs (Cohen, 1992). There have been increasing reports of penicillin resistant pneumococci in many countries since 1976. The penicillin resistant pneumococci are common in countries such as South Africa, Spain, Eastern Europe, and the Asia Pacific region (Felmingham *et al*., 2002). The situation gets worse by the ability of the penicillin resistant clones to spread easily from continent to continent. During the past few decades, there have also been a widespread emergence of macrolide resistance among clinical pneumococcal isolates and in some parts of the world has exceeded resistance to β lactams (Felmingham *et al*., 2002).

The emergence of antimicrobial resistance has its roots and the subsequent transfer of bacteria with resistance genes among animals, animal products and the environment (McEwen and Fedorka-Cray, 2002). Extra-chromosomal genes were found to be responsible for these antimicrobial resistant phenotypes that could impart resistance to an entire antimicrobial class. These resistance genes have been associated with plasmids which are large, transferable and have extra-chromosomal DNA elements. Other DNA mobile elements such as transposons and integrons are present on plasmids. These DNA mobile elements transmit genetic determinants for antimicrobial resistance mechanisms and may cause rapid dissemination of resistance genes among different bacteria (McDermott *et al.*, 2002). The emergence of multiresistant bacteria to antimicrobial drugs has increased the need for new antibiotics or modifications of older antibiotics (Tollefson and Miller, 2000).

The antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in treatment of resistant microbial strains. Much effort went into screening plants used medicinally in different regions such as Rwanda (Vlietinck *et al.*, 1995), Nepal (Taylor *et al.*, 1995), and southern Mexico (Meckes *et al.*, 1995). There has also been a substantial increase in the number of papers where authors screen plants for antimicrobial properties. It has been estimated that 14–28% of higher plant species are used medicinally, that only 15% of all angiosperms have been investigated chemically, and that 74% of pharmacologically active plant-derived components were discovered after following up on ethnomedical use of the plant (Kaneda *et al.*, 1991).

The gene-encoded cationic antimicrobial peptides (AMPs) are important mediators in the primary host defense system against pathogenic microorganisms, which are widely

distributed in nature. In the last few years, the burgeoning reports of the occurrence and characterization of low-molecular-mass AMPs from a wide variety of organisms have been accumulating at a rapid rate because of their biochemical diversity, broad specificity against bacteria or fungi (Casteels-Josson *et al*., 1994; Mor and Nicolas, 1994; Sitaram and Nagaraj, 2002; Storici *et al*., 1996; Terras *et al*., 1993; Zasloff, 2002), and also because some of them have anti-viral (Zhang *et al*., 1992; Frechet *et al*., 1994; Wachinger *et al*., 1998; Rozek *et al*., 2000a; Sitaram and Nagaraj, 2002), anti-tumoral (Gesell *et al*., 1997; Rozek *et al*., 2000b; Winder *et al*., 1998) or wound-healing effects (Fernandes *et al*., 2002; Murphy *et al*., 1993).

2.4 Antioxidant

Even though all organisms have evolved and have the capability to protect them against oxidative damage, by possessing antioxidant defense and repair systems but these system is not sufficient to prevent their damage entirely (Simic, 1988).Once the capability of preventing oxidative damage become unbalanced by factors such as aging, deterioration of physiological functions may occur, which results in diseases and accelerating aging (Jeng *et al*., 2002).

Antioxidants are substances that can fight and destroy excess free radicals and repair oxidative damage in biomolecules. Antioxidant compounds could neutralize excess unstable free radicals and harmful chemicals formed in the body in response to environmental toxins like air pollution (Turner, 1997). Antioxidant prevents free radicals from damaging DNA which is the cell blue print. This could give antioxidant an important role and potential to reduce the genetic instability of cancer cells and thus may be useful in treatment (Maynard, 2001; Reddy *et al*., 2003).

Foods are the main source of antioxidants for the human body. Vitamin C, E, minerals like selenium, zinc and betacarotene are examples of food antioxidants. Consumption of more fruits and vegetables in our daily diet is the best way to get high antioxidant level in our body. There are many plants and herbs that are good sources of antioxidant. The number of antioxidant compounds synthesized by plants as secondary products, mainly phenolics, serving in plant defence mechanisms to counteract reactive oxygen species (ROS) in order to survive, is currently estimated to be between 4000 and 6000 (Havsteen, 2002; Robards *et al*., 1999; Wollgast & Anklam, 2000).

There are a lot of natural antioxidant compounds that have been isolated from different kinds of plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs (Ramarathnam *et al*., 1995). Demands for natural antioxidant from the botanical sources, especially from edible plants increased because of the long term safety and negative consumer perception of synthetic antioxidants (Amarowicz *et al*., 2004; Sakanaka *et al*., 2004).

The main characteristic of antioxidant is in its ability to scavenge free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Prakash *et al*., 2001).

Free radicals were a major interest for early physicists and radiologists and much later found to be a product of normal metabolism. All of us know well that radicals could

cause molecular transformations and gene mutations in many types of organisms. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. In healthy individuals, free radical production is continuously balanced by natural antioxidative defence systems (Gutteridge, 1993; Knight, 1995). Antioxidant can slowdown or stop the chain reaction of oxidation by giving away electrons without changing its stability (Kagan *et al*., 2002). It could inhibit oxidation by giving away an oxygen molecule without requiring much energy. Oxidation is the addition of oxygen or the removal of hydrogen which is caused by the free radicals.

Disruption of the balance between reactive oxygen species (ROS) production leads to the process called oxidative stress. As a consequence, ROS are known to be implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, atherosclerosis, cataracts, chronic inflammation, and neurodegenerative diseases, such as Alzheimer's or Parkinson's disease (Gutteridge, 1993; Knight, 1995). Besides that free radical damage to the skin contributes greatly to the aged appearance of the skin (Kagan *et al*., 2002). ROS and free radicals are also considered as inducers of lipid peroxidation and cause the deterioration of foods (Rechner *et al*., 2002a). There has been a growing interest in natural antioxidant because they have greater application in food industry for increasing the stability and shelf-life of food products (Suja *et al*., 2004). Although organisms have endogenous antioxidant defences produced during normal cell aerobic respiration against ROS, other antioxidants are taken from the diet, both from natural and synthetic origin (Rechner *et al*., 2002a).

Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important in the prevention of many diseases (Halliwell *et al*., 1992). Consumption of high antioxidant foods is associated with reduced risk of cancer and cardiovascular diseases. Antioxidant is also believed to reduce the risk of Alzheimer's (Engelhart *et al*., 2002). Increased consumption of fruits and vegetables containing high levels of phytochemicals has been recommended to prevent chronic diseases related to oxidative stress in the human body (Chu *et al*., 2002). Thus, synthetic antioxidants are widely used in the food industry. However, because of their toxic and carcinogenic effects, their use is being restricted. Thereby, interest in finding natural antioxidants, without undesirable side effects, has increased greatly (Rechner *et al*., 2002b).

The antioxidant activities of phenolics are related to a number of different mechanisms, such as free radical-scavenging, hydrogen donation, singlet oxygen quenching, metal ion chelation, and acting as a substrate for radicals such as superoxide and hydroxyl. A direct relationship has been found between the content of total phenolics and antioxidant capacity of plants (Ferreira *et al*., 2007; Robards *et al*., 1999). In fact, to counteract deleterious action of ROS, phenolic compounds, naturally distributed in plants, are effective (Ferreira *et al*., 2007).

2.5 Functional food

Functional foods continue to attract a great deal of attention among the scientific community as well as the public. Functional foods are a class of foods claimed to have health promoting or disease preventing property beyond the basic function of supplying nutrients. Fermented foods with live cultures are considered as functional foods with

probiotic benefits. Functional foods are an emerging field in food science due to their increasing popularity with health-conscious consumers.

According to the Functional Food Science in Europe project (FUFOSE), food can be regarded as functional if it satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects in a way which is relevant to either an improved state of health and well-being and/or the reduction of risk of disease. FUFOSE suggested that any claim for enhanced function and reduced risk of disease should be scientifically justified (Diplock *et al.*, 1999). New scientific developments offer a range of possibilities to increase the health-promoting properties of food products, by adapting their micronutrient content as well as the balance and composition of macronutrients.

International Life Sciences Institute (ILSI) Southeast Asia had organised a number of consultations among Asian nutrition scientists on the definition of functional foods. ILSI states that functional food should possess functional benefits that can be scientifically proven and the functional benefits should be obtained by consuming normal amounts of these foods. Besides that the food should contain functional nutrients and/or other substances that may be naturally present or be added to the food provided to be safe over long term usage for the intended target population based on existing science. This definition is generally similar to the definition that was given by FUFOSE. ILSI is a non-profit, worldwide foundation established in 1978 to advance the understanding of scientific issues relating to nutrition, food safety, toxicology, risk assessment and the environment. ILSI is headquartered in Washington DC, USA.

Under the Japanese nutrition improvement law, there are five categories of foods for special dietary uses, including foods for specified health use (FOHSU). Foods that contain beneficial ingredients such as dietary fibre, sugar alcohols, oligosaccharides, proteins, polyphenols, lacto- or bifido-bacilli, chitosan and sodium alginate are considered to help maintain good health. The FOSHU law will regulate the marketing and labelling of the products containing these ingredients.

2.6 Fermented products

Traditional fermentation products, such as foods containing probiotics, black rice vinegar, soy sauce (shoyu), miso, natto and tempeh, are quite popular foods in Japan. These fermented foods have attracted attention all over the world. These variety of traditional fermented foods consumed in Japan probably contributes to Japanese longevity, which is the world's greatest. In Ghana the traditionally recommended weaning food is prepared from fermented maize dough. The soaking process, which involves the addition of a large volume of water to whole maize grains, allows the selection of desirable organisms, such as lactic acid producing bacteria, yeasts and moulds (Akinrele & Bassir, 1967).

The metabolic activities of these organisms reduce the pH and increase the titratable acidity. A number of fatty acids are also produced (Akinrele, 1970). These have been shown *in vitro* to inhibit a wide variety of bacteria (Adams & Hall, 1988). The present investigation has shown that maize fermentation from raw material to final product involves different microenvironments with strong antimicrobial activities, determining the composition of the microflora of the final product.

For several centuries, fermented products derived from plant or animal materials have been an acceptable and essential part of the diet in most parts of the world. Health benefits have also often been associated with them. Probiotics can be defined as fermented food containing specific live microorganisms or a live microbial food or feed supplement, which beneficially affects the human or the host animal by improving its intestinal microbial balance (Kalantzopoulos, 1997).

2.7 Various plants

Figure 2.1: *Andrographis paniculata*

Andrographis paniculata which is commonly known as “king of bitters” is a member of the plant family *Acanthaceae*. In Malaysia this plant is locally known as “hempedu bumi” or “akar cerita”. *Andrographis paniculata* is an important medicinal plant, occurring wild which is used both in Ayurevda and Unani system of medicine (Chadha, 1985). For centuries, it has been widely prescribed for the treatments of various diseases, such as respiratory tract infection, fever, bacterial dysentery and diarrhea (Poolsup *et al.*, 2004).

Figure 2.2: *Curcuma mangga*

Curcuma mangga is an Asian spice known as white turmeric. Both the common turmeric and white turmeric are members of the ginger family. *Curcuma mangga* is normally eaten as a vegetable. White turmeric is a frequent ingredient in chutneys and relishes and can also be used in salads. Researchers have found anti-tumour activities from extracts of the rhizomes. Medicinally, the rhizomes are used as a stomachic and for chest pains, fever, and general debility (Tewtrakul and Subhadhirasakul, 2007).

Figure 2.3: *Carica papaya* leaf

Papaya Leaf with the Latin name *Carica papaya* contains the powerful proteolytic enzymes, papain and chymopapain, which digest proteins, small peptides, amides and

esters. The primary use of papaya leaf in herbal medicine today is as a vermifuge, that is, to remove intestinal worms. Traditionally, the leaf extract was used as a tonic for the heart, analgesia and treatment for stomach ache (Indran *et al.*, 2008).

Figure 2.4: *Allium sativum*

Garlic (*Allium sativum*) has been used as both food and medicine in many cultures for thousands of years. Garlic has long been considered as herbal "wonder drug", with a reputation in folklore for preventing everything from the common cold and flu to the plague. It has been used extensively in herbal medicine. Studies have shown that garlic can have a powerful antioxidant effect. Antioxidants can help to protect the body against damaging free radicals (Ankri and Mirelman, 1999).

Figure 2.5: *Cymbopogon citratus*

Cymbopogon citratus is commonly known as lemon grass. It is native to India but widely used as herb in Asian cuisine. In Malaysia this plant is locally known as "serai". It has a citrus flavor and can be dried and powdered or used fresh. *Cymbopogon citratus* is used as a pesticide and a preservative. Research shows that lemongrass oil has anti fungal properties (Shadap *et al.*, 1992). *Cymbopogon citratus* is consumed as a tea for anxiety in Brazilian folk medicine (Blanco *et al.*, 2009).

Figure 2.6: *Zingiber officinale*

Zingiber officinale with its common name ginger is a tuber that is consumed whole as a delicacy, medicine, or spice. Ginger is carminative, pungent, stimulant, used widely for

indigestion, stomachache, malaria and fevers. Many people like raw ginger, and this is the form most popular in South East Asia: Fresh ginger is grated or finely chopped and added to the dish before serving (Grant and Lutz, 2000).

Figure 2.7: *Beta vulgaris*

Beta vulgaris, the Latin name for the beet species. The roots and leaves of the beet have been used in folk medicine to treat a wide variety of ailments. Packed full of nutrients, it provides a rich source of carbohydrates, protein, and has high levels of important vitamins, minerals and micronutrients. It has been suggested the pigment molecule betanin in the root of red beets may protect against oxidative stress and has been used for this purpose in Europe for centuries, though it is not known if the effect is meaningful in the human body (Fisher, 1989)

Figure 2.8: *Momordica charantia*

Momordica charantia commonly referred to as bitter gourd. It belongs to the cucurbitaceae family and is commonly consumed as a vegetable. It contains an array of novel and biologically active phytochemicals including triterpenes, proteins and steroids. In numerous studies, at least three different groups of constituents found in all parts of bitter melon have clinically demonstrated hypoglycemic properties (blood sugar lowering) or other actions of potential benefit against diabetes (Lee *et al.*, 2009).

Figure 2.9: *Agaricus bisporus*

Agaricus bisporus is commonly known as button mushroom. It is cultivated in more than 70 countries and is one of the most widely consumed mushrooms in the world. *In vivo* research conducted on mice suggests the white button mushroom may enhance aspects of the immune system. *In vitro* testing has shown a compound present in the

white button mushroom has anticancer properties, inhibiting the growth of various cancer cell lines. The mushroom has also been shown to possess possible immune system enhancing properties (Ren *et al*., 2008; Yu *et al.*, 1993).

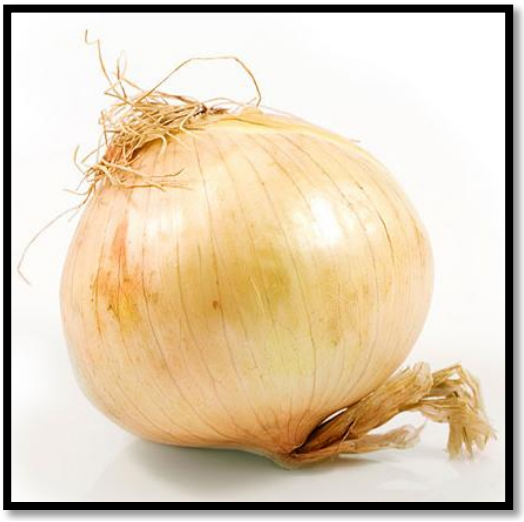

Figure 2.10: *Allium cepa*

Allium cepa are commonly used in our daily diet. They are known by the common name onion. Wide-ranging claims have been made for the effectiveness of onions against conditions ranging from the common cold to heart disease, diabetes, and osteoporosis. It also has flavonoids, such as quercetin and quercetrin, which have antioxidant and anticancer effects (Carson, 1987). Onion oil has been reported to effectively decrease the lipid levels in experimental animals (Bordia *et al.*, 1977).

CHAPTER 3

METHODOLOGY

Chapter 3: Methodology

Methodology

3.1 Preparation of fermented Samples

Fermented samples were prepared by solid state fermentation using lactic acid bacteria namely *Lactobacillus brevis, Lactobacillus fermentum, Leuconostoc cremoris, Lactobacillus lactis, Lactobacillus casei, Lactobacillus plantarum* and *Lactobacillus acidophilus* at 20% moisture and room temperature of 25^0C ± 2^0C for 72 hours of fermentation process with cherries, grapes and Brassica as the substrates. The process is similar to sauerkraut fermentation. The end products of the fermentation were tested for the presence of lactic acid bacteria, protein content and organic acids to evaluate the quality. These test samples were prepared in triplicates and also were used for the subsequent part of this study.

3.2 Secondary metabolite extraction

3.2.1 Source of samples

Fresh plant samples were purchased from local wet market at Petaling Jaya, Selangor, Malaysia. *Andrographis paniculata* plant was obtained from Taiping, Perak, Malaysia. This plant was confirmed by the previous research done at AIMST University.

3.2.2 Preparation of plant extracts

The fresh plant samples were washed under running tap water. They were chopped into small pieces and dried in the oven (Memmert, Germany) at temperatures not exceeding 40^oC for three days. Once all the plant samples were dried, they were pulverised to fine powder using an electrical blender (Super Blender National®, Japan). The plant parts that were used for extraction are the leaves for *Andrographis paniculata*, *Carica papaya* and *Cymbopogon citratus*. *Curcuma mangga, Allium sativum, Zingiber officinale, Beta*

vulgaris, *Allium cepa*, *Momordica charantia* and *Agaricus bisporus* fruiting body was used for the extraction.

3.2.3 Ethanolic extraction

The fine powder was soaked in ethyl alcohol 95% (v/v) (Systerm) for 3 days and filtered using Whatman® 5 filter paper (Whatman International Ltd., England). The filtered extract was evaporated to dryness under reduced pressure at temperatures below 40°C in rotary evaporator (Heidolph WB2000, Germany). The concentrated extracts were weighed individually and the yields were calculated according to the formula in figure 3.1.

$$\text{Yield (\%)} = \frac{\text{Weight of concentrated extract}}{\text{Weight of dried sample}} \times 100$$

Figure 3.1: Formula to calculate the yields of ethanolic extract in percentage (%)

3.2.4 Storage of concentrated extracts

The concentrated extracts were then transferred into 2ml eppendorf tubes and kept in refrigerator at 4°C for subsequent experiments (Ahmad *et al*., 2007).

3.3 Protein/peptide extraction

3.3.1 Preparation of stock solution

Stock solution of 2M potassium phosphate (KPO_4) (pH 7.8), 0.5M ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 10N sodium hydroxide (NaOH) and 80% glycerol for extraction buffer preparation were prepared.

3.3.1.1 Preparation of 2M potassium phosphate (KPO_4) (pH 7.8)

200ml of 2M KPO_4 stock solution was prepared by adding 63.2g of dipotassium phosphate (K_2HPO_4) and 5.0g of monopotassium phosphate (KH_2PO_4). It was topped up to 200ml with distilled water and then the 2M KPO_4 stock solution was adjusted to pH 7.8 using NaOH.

3.3.1.2 Preparation of 0.5M ethylenediaminetetraacetic acid (EDTA) (pH 8.0)

0.5M EDTA (pH 8.0) was prepared by adding 46.52g of EDTA and topped up to 250ml. The EDTA will not completely dissolved into solution until the pH approaches 8.0 and the distilled water amount almost the final volume. The pH needs to be continuously adjusted to pH 8.0 using NaOH as the EDTA dissolves.

3.3.1.3 Preparation of 10N sodium hydroxide (NaOH)

10N NaOH was prepared by adding 100g of NaOH to distilled water and then topped up to a final volume of 250ml.

3.3.2 Preparation of extraction buffer

The extraction buffer was prepared according to Table 3.1.

Table 3.1: Amount of solution for the preparation of 100ml protein extraction buffer

Extraction Buffer	Total 100ml
2M KPO_4 (pH 7.8)	5ml
0.5M EDTA	200µL
Triton X-100	1ml
80% Glycerol	12.5ml
dH_2O	81.1ml
Dithiothreitol (DTT) (1.0 M)	15.4mg

DTT was added immediately before using. It was stored at -20^{o}C.

3.3.3 Extraction of protein/peptides

The fresh plant samples were washed under running tap water. Non latex gloves were worn all the time during the extraction step because keratin and latex proteins are potential sources of contamination. The fresh plants were chopped into small pieces just before extraction. Chopped plant samples were placed in a cold mortar and pestle which was placed in an ice box to prevent protein denaturation. Approximately 2ml of extraction buffer was added for every gram of tissue. The tissue from different samples was grounded till there were no more visible tissues. The grounded tissue were transferred into a centrifuge tube and kept in the fridge prior to centrifugation.

The tissues were centrifuged at 12,000 rpm for 15 minutes at 4^{o}C using RC5C Sorvall Instrument High Speed centrifuge with SS-34 Rotor. The pellet was discarded and the supernatant was collected and stored at -20^{o}C. Next, the samples were transferred into 100ml beaker and freeze dried prior to protein quantification (Seveno *et al*., 2008) and reconstituted in 5ml Phosphate buffer saline before protein determination.

3.4 Protein quantification

3.4.1 Principles of Bradford method

Bradford assay is a protein determination method that involves the binding of acidic Coomassie Brilliant Blue G-250 dye to protein samples (Bradford 1976). The dye exists in three forms as described in figure 3.2 (Compton and Jones 1985). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form. However, when the dye binds to protein, it is converted to a stable unprotonated blue form. It is this blue protein-dye form that is detected at 595nm in the assay using a spectrophotometer or a microplate reader. This assay is useful as the extinction

coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

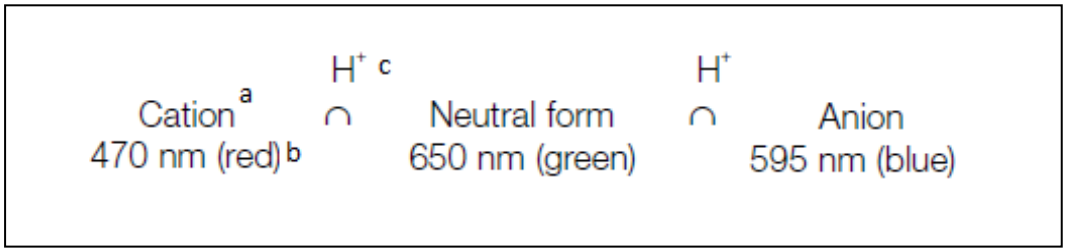

Figure 3.2: Three different form of Coomassie Brilliant Blue G-250 dye
a = the form that the dye exist
b= the wavelength that the dye absorbance is maximum
c= proton removal

3.4.2 Protein standard preparation

This assay was performed in test tubes. The assay used 0.1ml of the protein sample and 3ml of the Bradford Reagent (Sigma-Aldrich Inc, Saint Louis) per tube. The Bradford reagent was gently mixed in the bottle and brought to room temperature prior to use. BSA (bovine serum albumin) (Sigma-Aldrich Inc, Saint Louis) was used as the standard protein to measure the linear concentration ranging from 0.1 to 1.4mg/ml. Protein standards of appropriate concentration were dissolved in phosphate buffer saline (PBS) as the sample. The standard protein BSA was prepared according to table 3.2. After adding 3ml of Bradford Reagent to each tube, these were vortexed gently for thorough mixing. The total volume in each tube added up to 3.1ml that included 0.1ml of each sample. The samples and standards were incubated for 30 minutes at room temperature.

Table 3.2: Amount of sample, BSA and Bradford reagents to be added for preparation of the standard for protein determination

Tube No.	Sample (ml)	[BSA] protein standard (mg/ml)	Bradford Reagent (ml)
1[a]	0.1	0	3
2	0.1	0.25	3
3	0.1	0.5	3
4	0.1	1.0	3
5	0.1	1.4	3
6	0.1	(unknown)[b]	3

a= Tube number 1 will be the blank for the protein concentration determination. It contains 0.1ml of the buffer or solution used in the protein samples.

b= Tube number 6 (labeled unknown in table 3.2) contains 0.1ml of the samples which the protein concentration need to be determine.

3.4.3 Determination of protein concentration

The tubes content were transferred into disposable cuvettes and the absorbance of each protein samples were measured at the wavelength of 595nm using Beckman DU 7500i spectrophotometer, USA. The absorbance results obtained from the assay was tabulated. Next a standard curve was plotted for the net absorbance at 595nm versus the protein standard concentrations. Standard curve is enclosed in appendix J. The concentration of each protein was determined by comparing the absorbance to the standard curve prepared using the protein standards (Bradford Reagent Product Manual, Sigma-Aldrich, Inc, Saint Louis, 1976).

3.5 Identification of Test Microorganisms

3.5.1 Bacteria DNA extraction

The test microorganisms were obtained from the Microbiology Department, Faculty of Science, University of Malaya. The microbial DNA was extracted using i-genomic BYF DNA extraction kit for gram positive bacteria and i-genomic CTB DNA extraction kit for gram negative bacteria, (iNtRON Biotechnology, Seongnam). The extracted genomic DNA was examined by electrophoresis in a 1% agarose gel.

3.5.1.1 Sample treatment for both gram positive and negative bacteria

Bacteria were grown on agar plate and incubated at 37^{o}C for 14-16 hours. A single colony was picked up from the agar plate and inoculated into a 5ml broth medium. The broth medium was incubated overnight in a shaking incubator at 37^{o}C. The absorbance were adjusted to the value of 0.8 ~ 1.0 on a spectrophotometer at optical density 600. 2ml of the cultured bacteria cells were transferred into a 2ml tube and were centrifuged at 13000 rpm for 1 minute. Finally the supernatant was discarded and the bacteria pellet was resuspended with the remnant supernatant by vortexing vigorously.

3.5.1.2 DNA extraction for gram positive bacteria (BYF DNA extraction kit)

3.5.1.2.1 Pre-lysis step

100µl of patented buffer denoted as MP and 3µl of lysozyme solution was added into sample tube, and mixed well by vortex for 30 seconds. The lysate was incubated at 37^{o}C for 15 minutes. Next the pre-lysate was centrifuged at 13,000 rpm at room temperature for 1 minute. The supernatant was discarded and the cell pellet was resuspended by tapping before adding Buffer MPG.

3.5.1.2.2 Lysis step

200µl of patented buffer denoted MG, 10µl Proteinase K, and 5µl RNase A solution were added into a sample tube, and mixed by vortexing vigorously for 10 seconds. The lysate was then incubated for 15 minutes at 65^{o}C (the tube was shaken every 5 minutes).

3.5.1.2.3 DNA binding step

After the process of lysis completed, 250µl of patented buffer denoted as MB was added to the lysate and mixed by gently inverting 5~6 times. Next the lysate was spun down for 10 seconds to remove drops from inside the lid. 250µl of 80% ethanol was

added to the lysate and mixed by gently inverting 5~6 times. After mixing, the lysate was spun down 10 seconds again to remove drops from inside the lid. 750µl of the mixture was pipetted into spin column inserted in a 2ml collection tube. It was centrifuged at 13,000rpm at room temperature for 1 minute. Lastly the flow through and collection tube was discarded.

3.5.1.2.4 Washing step

Spin column was placed into a new 2.0ml collection tube. 700µl of patented buffer denoted as MW was added to the spin column and centrifuged at 13,000rpm for 1 minute. The flow through was discarded and it was centrifuged again for an additional 1 minute to dry the membrane. The flow through and collection tube were all together discarded.

3.5.1.2.5 Elution step

The spin column was placed into a new 1.5ml microcentrifuge tube and 50µl of patented buffer denoted as ME was added directly onto the membrane. Then it was incubated for 1 minute at room temperature and then centrifuged at 13,000rpm for 1 minute to elute. The spin column was discarded. The flow through in the 1.5ml microcentrifuge tube was stored at -20^{o}C.

3.5.1.3 DNA extraction for gram negative bacteria (CTB DNA extraction kit)

3.5.1.3.1 Lysis step

200µl of patented buffer denoted as CG, 10µl proteinase K and 3µl RNase A solution were added into sample tube and mixed by vortexing vigorously. The lysate were incubated at 65^{o}C for 30 minutes (the tubes were gently shaken every 5 minutes).

3.5.1.3.2 DNA binding step

After lysis was completed, 250µl patented buffer denoted as CB was added to the lysate and mixed by inverting a few times. After mixing it was spun down for 10 seconds to remove drops from inside the lid. Next, 250µl of 80% ethanol was added to the lysate and it was gently inverted a few times. After mixing, the lysate was spun down again for 10 seconds to remove drops from inside the lid. The whole mixture was carefully pipetted to the spin column inserted into a 2ml collection tube without wetting the rim. It was centrifuged at 13,000rpm for 1 minute. The flow through and collection tube altogether were discarded.

3.5.1.3.3 Washing step

The spin column was placed into a new 2.0ml collection tube and 700µl of patented buffer denoted as CW was added to the spin column. It was centrifuged at 13,000rpm for 1 minute. The flow through was discarded and centrifuged again at 13,000rpm for 1 minute to dry the membrane. The flow through and collection tube altogether were discarded.

3.5.1.3.4 Elution step

The spin column was placed into a new 1.5ml tube and 100µl patented buffer denoted as CE was added directly onto the membrane. It was incubated at room temperature for 5 minutes and then centrifuged at 13,000rpm for 1 minute to elute. The spin column was discarded. The flow through in the 1.5ml microcentrifuge tube was stored at -20^{o}C.

3.5.2 Polymerase chain reaction (PCR) condition

3.5.2.1 Reaction mixture preparation

Reaction mixtures of 20µL in total contained 2µL of 10 x PCR buffer, 2µL of dNTP mix (2.5mM each), 1µL of each primer (10 pmoles), 50ng of DNA template, 0.5µL of i-TaqTM DNA polymerase (5U/µL) (iNtRON Biotechnology, Seongnam).

3.5.2.2 PCR cycle condition

The Universal primers used to amplify 16S rRNA gene were (27f : 5'-AGA GTT TGA TCA TGG CTC AG and 1492r : 5'-TAC GGC TAC CTT GTT ACG ACTT) (Bioneer Corporation, Daejeon). The PCR conditions used were: initial denaturation at 94^{o}C for 5 minutes followed by denaturation at 94^{o}C for 1 minute, annealing at 52^{o}C for 1 minute, extension at 72^{o}C for 1.5 minutes and a final extension at 72^{o}C for 10 minutes. The amplification was done by Eppendorf Mastercycler personal (Eppendorf, Hamburg, Germany).

3.5.3 Agarose gel electrophoresis

3.5.3.1 Preparation of 0.5 X tris-borate-EDTA (TBE) buffer

0.5 X TBE buffer was prepared by dissolving 2.7g of Tris-base (Sigma, Steinheim, Germany), 1.38g of boric acid (BDH, Poole, England), and 0.15g of EDTA topped up to 500ml with distilled water. The pH needs to be continuously adjusted to pH 8.3 as the EDTA dissolves. The pH of the buffer was adjusted with 1M HCl or 1M NaOH. The buffer was autoclaved.

3.5.3.2 Preparation of 1.5% agarose gel

0.6g of agarose (Sigma, Steinheim, Germany) was added into 40ml of 0.5X TBE buffer. It was heated in microwave oven for 2 minutes. The gel solution was cooled down under running tap water. 2µl of ethidium bromide (Sigma, Steinheim, Germany) was added into the gel solution and mixed to facilitate visualization of DNA after electrophoresis.

3.5.3.3 Loading of melted gel

The gel solution was loaded into an electrophoresis tray containing gel combs. It was ensured that no bubbles were trapped underneath the comb and all bubbles on the surface of the agarose were removed before the gel sets. The gel was allowed to solidify at room temperature for 30 minutes. Once the gel had set, the comb was gently removed by pulling it straight up and out of the agarose gel. The tray along with the solidified gel was placed into the RunOne ™ System DNA electrophoresis equipment (KomaBiotech, Korea). Sufficient 0.5 X TBE buffer was added to cover the gel to a depth of about 1mm and was made sure no air pockets were trapped within the wells.

3.5.3.4 Loading the sample and running the gel

1µl of 6 x loading dye (Sigma, Steinheim, Germany) was dropped onto a clean parafilm. 2µl of the PCR product was mixed with the loading dye by pipetting and loaded into the sample well. 1µl of 1 Kb ladder marker (Bioneer, Daejeon, Korea) was loaded into separate wells of the gel for 16S rDNA PCR product. The electrophoresis gel was allowed to run at 100V for 30 minutes. The DNA was visualized under ultra violet (UV) light.

3.5.4 Purification of PCR product

The PCR product was purified using PCRquick-spinTM. (iNtRON Biotechnology, Seongnam). 20µl of the PCR product were added with 100µl of BNL buffer. The mixture was spun down briefly to ensure the contents were at the bottom of the tube. Spin column was placed in a collection tube for each PCR reaction product. The PCR product was transferred to the spin column and centrifuged at 13,000rpm for 1 minute. The flow through was discarded and spin column was placed into the same 2ml collection tube. 700µl of washing buffer was added to the column and centrifuged at 13,000rpm for 1 minute. The flow through was discarded and spin column was placed back into the same 2ml collection tube. It was centrifuged for 1 minute at 13,000rpm to dry the spin membrane. The spin column was placed into a clean 1.5ml microcentrifuge tube. 40µl of elution buffer was applied directly to the center of the column without touching the membrane with the pipette tip. It was incubated at room temperature for 1 minute and centrifuged at 13,000rpm for another 1 minute. The spin column was discarded. The flow-through in the 1.5ml microcentrifuge tube was stored at -20^{o}C.

3.5.5 Sequencing and results analysis

The 16S rDNA gene sequencing was done by Macrogen Inc. (Seoul) which uses ABI 3730xl DNA analyzer. The sequences result obtained from Macrogen Inc were analysed by using Qual Trace program (www.nucleics.com/qualtrace-dna-sequencing-demo/index.html). Only sequences which passed the analysis were used to generate the full 1.4 kilo bases 16S rDNA sequence for bacteria identification. The obtained reverse sequence were converted to antisense reverse sequence by FastPCR© program (www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm). The full length sequence was blasted at National Center for Biotechnology Information (NCBI) nucleotide collection

databases using megablast tool. The bacterial species were identified on the basis of at least 98% similarity to 16S rDNA sequences database.

3.6 Antibacterial activity

3.6.1 Disk diffusion assay

3.6.1.1 Principles of disk diffusion assay

In the disk-diffusion susceptibility test, paper discs impregnated with a defined quantity of the antimicrobial agent are placed on agar medium uniformly seeded with a suspension of the test microorganism. The antimicrobial agent diffuses into the medium forming zones of inhibition on the microbial growth around the disk corresponding to the susceptibility of the microorganism to the antimicrobial agent.

3.6.1.2 Preparation of Mueller-Hinton media

Mueller-Hinton agar was prepared by adding 8.5g of Bacto Agar (Difco, France) for every 11g of Mueller-Hinton broth (Becton, Dickinson and Company, Franklin Lakes, USA). The mixture was topped up with distilled water to 500ml using a volumetric flask. Next it was mixed thoroughly using a magnetic stirrer (Cole Parmer, Vernon Hills, USA) and transferred into 500ml Schott bottles. The bottles were loosely fastened to allow penetration and escape of steam during the sterilization cycle. Next it was autoclaved for 20 minutes at 121^{o}C and 15psi using TOMY autoclave SS-325, Japan. After autoclaving, the loose caps were fastened immediately. The medium was poured at the rate of approximately 20ml per plate. The medium in the plates were allowed to set at room temperature. All steps were conducted aseptically under a laminar hood (LABWIT, Shanghai).

3.6.1.3 Preparation of inoculum

Cultures to be tested were streaked onto highly nutritious agar medium such as brain heart infusion agar to obtain isolated colonies. After incubation at 37^{o}C overnight three to five well isolated colonies of the same morphological and molecular (16S rDNA) type of bacteria was selected from the agar plate culture. The top of each colony was touched with a wire loop and transferred to a 15ml centrifuge tube containing 5ml of Mueller-Hinton broth (Becton, Dickinson and Company, Franklin Lakes, USA). After inoculating, the broth was incubated at 37^{o}C on a shaker at 100 rpm for 12 hours.

3.6.1.4 Inoculum turbidity adjustment

The inoculum density was adjusted between 0.08 to 0.1 OD units at the wavelength of 625nm using spectrophotometer (Spectronic 20D+, USA) using a 1cm light path and matched cuvettes to determine the absorbance. The turbidity of the actively growing broth culture was adjusted with 0.85% sterile sodium chloride (BDH Analar, USA). Before inoculation it was diluted 10 times using 0.85% sodium chloride again to make it approximately 1 x 10^{8} colony forming units (CFU) per ml.

3.6.1.5 Inoculation of the plate

Mueller-Hinton agar plates were prepared in advance. Sterile cotton swab was used to streak the agar plate with the inoculum suspension. The swab was streaked over the entire surface three times, rotating the plate approximately 60 degrees after each application to ensure even distribution of the inoculum suspension.

3.6.1.6 Antimicrobial discs

Sterile 6mm filter paper discs (Whatman International Ltd, Maidstone) were used to place the samples on agar plates. The samples' concentrations were adjusted to 1 mg/ml for protein/peptide extracts and 50mg/ml for ethanolic extracts. All steps were performed aseptically under the laminar hood. All the plates were inverted and incubated at 37^{o}C for 16 hours. The positive control used was Tetracycline (30µg/disc) (Oxoid, Basingstoke). Antimicrobial tests were performed based on the guidelines of the "British Society for Antimicrobial Chemotheraphy" and "National Committee for Clinical Laboratory Standards, 2005".

3.6.1.7 Recording of results

After incubation, the diameter of the entire zone of inhibition (including the diameter of the disk) were measured and recorded in millimeters (mm) using vernier calipers. The measurement was made on the back of the inverted plate without opening the lid. The petri plate was held a few inches above a black, nonreflecting background and illuminated with reflected light. The diameter of clear zone was taken as the area showing no obvious, visible growth as detected with the unaided eye.

3.6.2 Minimal inhibitory concentration (MIC)

3.6.2.1 Principles of MIC

The MIC Assay is a technique used to determine the lowest concentration of a particular antimicrobial agent needed to inhibit the growth of a microorganism.

3.6.2.2 Preparation of Mueller Hinton broth

Mueller-Hinton broth was prepared and the pH was adjusted to the range of 7.2 to 7.4 at room temperature prior to autoclaving. It was autoclaved for 20 minutes at 121^{o}C and 15psi using the TOMY autoclave SS-325 Japan.

3.6.2.3 Preparation of antimicrobial agent

Stock solutions of 200mg/ml extract were prepared for all the samples. Stock solutions were kept in the refrigerator at 4^{o}C. The plant extracts were diluted with Mueller-Hinton broth to give the final concentrations of 50mg/ml, 40mg/ml, 30mg/ml, 20mg/ml and 10mg/ml. It was made up to a final volume of 3ml in sterile 15ml centrifuge tubes. Four replicates were prepared for every concentration of the extract. Out of four replicates one of the tubes was not inoculated with the microbial agent and was used as a positive control to compare with the inoculated tubes. All steps were conducted aseptically under the laminar hood.

3.6.2.4 Inoculum preparation

Three to five well isolated colonies of the same bacteria type was selected from an agar plate culture. The top of each colony was picked with a wire loop and transferred to a 15ml centrifuge tube containing 5ml of Mueller-Hinton Broth. After inoculating, the broth was incubated at 37^{o}C on a shaker at 100rpm for 12 hours. The turbidity of the actively growing broth culture was adjusted between 0.08 to 0.1OD units with 0.85% sterile sodium chloride at the wavelength of 625nm with the spectrophotometer using a 1cm light path and matched cuvettes to determine the absorbance.

3.6.2.5 Adding inoculum suspensions to antimicrobial agent

50µl of the final inoculum suspensions were added to the earlier prepared plant extracts in tubes within 15 minutes. The cap was carefully fastened after adding the inoculum suspension. All steps were conducted aseptically under the laminar hood.

3.6.3 Determination of minimum bacteriocidal concentration (MBC)

The 15ml centrifuge tubes containing the test antimicrobial agent for the MIC assay were vortexed and 10µl of the content from each tube was pipeted out and plated onto Mueller-Hinton Agar media. The lowest concentration of the extract that gave complete inhibition of the colony formation of the test bacteria cultured in petri plates was recorded as MBC.

3.7 Antioxidant Activity

3.7.1 Superoxide Dismutase (SOD) Activity assay

3.7.1.1 Principles of SOD

SOD kit utilizes a water-soluble tetrazolium salt, WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt] that produces a highly water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with superoxide anion (O_2^-) is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined by measuring superoxide anion-caused formation of water-soluble dye (color intensity). Higher SOD activity will reduce the formation of formazan and indirectly lower the OD value as well. This process is shown in figure 3.3 (Ukeda *et al*., 1999).

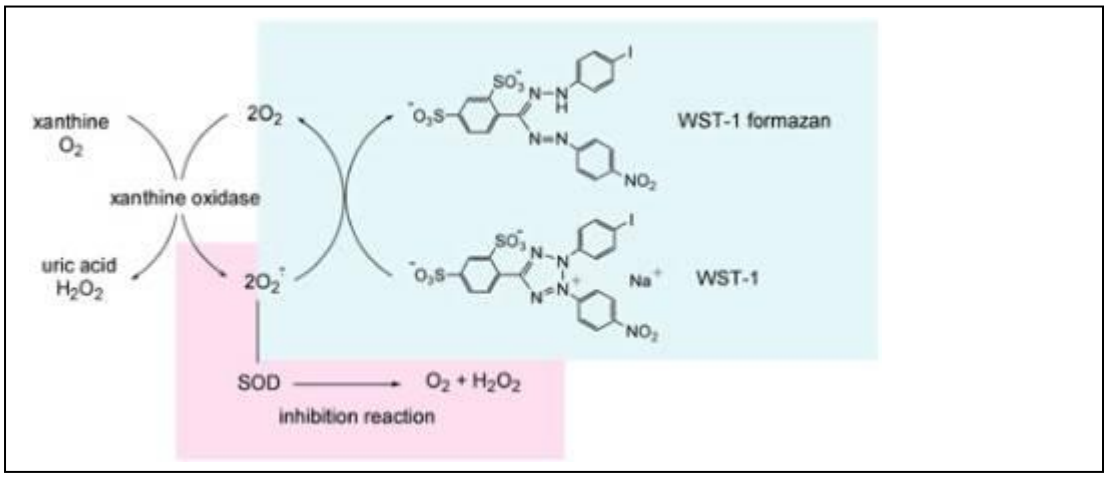

Figure 3.3: Superoxide Dismutase (SOD) reaction scheme

The figure above illustrating the coupled reaction between Xanthine Oxidase (XO), SOD, the generation of superoxide radical, and the reduction of the dye WST-1 by superoxide anion to a colored WST-1 formazan product that absorbs light at 450nm. SOD scavenges superoxide anion and thereby reduces the rate of formation of WST-1 formazan product.

3.7.1.2 Preparation of working solution

3.7.1.2.1 WST working solution

WST working solution was prepared by diluting 1ml of WST solution with 19ml of buffer solution provided in the SOD determination kit. WST working solution is stable for 3 weeks at 4^{o}C.

3.7.1.2.2 Enzyme working solution

The enzyme solution tube was centrifuged for 5 seconds and was mixed by pipeting. 15µl of the enzyme solution was then diluted using 2.5ml of dilution buffer. Enzyme working solution is stable for 3 weeks at 4^{o}C.

3.7.1.3 Preparation of 96 well microplate

A clean and sterile 96 well microplate was used for this experiment and table 3.3 shows the amount of solution to be added into each well. 20µl of sample solution was added to each sample and the blank 2 well. This means for every sample there will be a blank 2

as well. 20µl of double distilled water (ddH_2O) were added to blank 1 and blank 3 wells. 200µl of the prepared WST working solution were added to the entire well and mixed by gently shaking the plate. Dilution buffer was added to blank 2 and blank 3 wells. 20µl of Enzyme working solution were added to each sample and blank 1 well. These were gently mixed by shaking the plate without spilling out the content. The plate was incubated at 37^{o}C for 20 minutes.

Table 3.3: Amount of each solution need to be added for sample, blank 1, 2 and 3

	Sample	Blank 1	Blank 2	Blank 3
Sample solution	20µl		20µl	
ddH_2O		20µl		20µl
WST working solution	200µl	200µl	200µl	200µl
Enzyme working solution	20µl	20µl		
Dilution Buffer			20µl	20µl

i) If sample solution has visible colour a separate blank 2 lane was set.
ii) For an accurate measurement, multiple wells per sample were used.
iii) Usage of multi-channel pipette is recommended to avoid the reaction time lag of each well as superoxide will be released immediately after the addition of enzyme working solution.
iv) Samples were kept in an ice box to maintain its enzyme activity.

3.7.1.4 Measurement of absorbance value and results interpretation

The absorbance of the mixture was read at 450nm using Biorad Model microplate reader located in the Immunochemistry Lab, Department of Molecular Medicine, Faculty of Medicine, University of Malaya after the incubation. The positive control in this study was prepared by adding 20µl of vitamin C at concentration of 10mg/ml instead of sample working solution. All inhibition rates were stated in percentage. The SOD activity (inhibition rate %) was calculated using the equation in figure 3.4. (Fluka SOD Assay kit manual)

SOD activity (inhibition rate %) = {[(B1 – B3) - (S – B2)]/ (B1 – B3)} x 100

Figure 3.4: Formula for the calculation of SOD inhibition rate in percentage
B1= Blank 1
B2= Blank 2
B3= Blank 3
S = Sample

3.7.2 Scavenging activity of 2,2-Dipheny-1-Picrylhydrazyl (DPPH) Radical

950µl of 90µM DPPH solution was added to 50µl of the working samples. The volume was made up to 4ml with 95% ethanol. The solution was vortexed vigorously and they were incubated in the dark at room temperature for 2 hours. 95% ethanol was used as the blank and the colour intensity reduction caused by scavenging of the free radicals DPPH was measured at 515nm using Spectronic 20D+ spectrophotometer (USA). The positive control was prepared using vitamin C at a concentration of 10mg/ml. All samples were assayed in triplicate. The scavenging activity of the DPPH radicals in % is calculated using the formula shown in figure 3.5 (Lim and Murtijaya, 2007).

Scavenging activity of DPPH radicals (%) =

$$[(A515_{blank} - A515_{sample}) / A515_{blank}] \times 100$$

Figure 3.5: Formula to calculate the scavenging activity of DPPH radicals in percentage value
A515blank =Absorbance of blank value at 515 nm wavelength
A515sample =Absorvance of sample value at 515 nm wavelength

3.8 Hemolysis assay

3.8.1 Blood Sampling

Blood was withdrawn from the marginal veins of healthy New Zealand white rabbits ear using a 26Gx1/2" needle (TERUMO®, Belgium) and aspirated into 15ml Silicone Coated Blood Collection Tube (Vacutainer®, BECTON DICKINSON, USA). These

rabbits were maintained at the facility of the Microbiology Division, Institute of Biological Sciences, University of Malaya's animal house. This experimental procedure was approved by the University of Malaya Animal Experimental Ethnics Committee prior to commencing the study [Ethical number: ISB/05/08/2009/TWY (R)]. The ethical clearance certificate is enclosed in the appendix L.

3.8.2 Preparation of rabbit erythrocyte

The blood were pooled together in 50ml centrifuge tubes with flat top caps (Biologix, Kansas, USA) and centrifuged at 1000g for 20 minutes at 4^{o}C using high speed centrifuge (Model RC5C, USA) fitted with SS-34 fixed angel rotor. The top layer of buffy coat and plasma were carefully removed using a pipette without aspirating out the erythrocytes. The final erythrocyte suspension was produced by adding an equal volume of sterile isotonic phosphate buffer (IPB) after washing the cells thrice with sterile IPB adjusted to pH 7.4. The sterile IPB was prepared and autoclaved in advance. 500µl of final erythrocytes suspension was added into labeled sterile 15ml centrifuge tubes (MS® Centrifuge Tubes, USA). Samples of 10mg/ml concentration were prepared and 1ml of it was added into the centrifuge tubes containing erythrocytes suspension. 10mg/ml of Vitamin C was used as the positive control and IPB was used as the negative control.

3.8.3 Inducing oxidative stress

The centrifuge tubes were well capped and gently shaken prior to incubation. They were incubated at 37°C for 40 minutes. After 40 minutes all the tubes were added with IPB to make it up to 9ml. Oxidative stress was induced by adding 1ml of 10mM hydrogen peroxide and incubated at 37^{o}C for 150 minutes.

3.8.4 Measurement of absorbance value and results interpretation

After incubation, the tubes were gently removed from the incubator and the top layer of hemoglobin released in the supernatant was measured using Spectronic 20D+ spectrophotometer (USA) at 540nm. Erythrocyte hemolysis in ultrapure water was considered as complete hemolysis (100%). Hemolysis activity was calculated as in the formula shown in figure 3.6. (Reddy *et al*., 2007)

Hemolytic activity (Erythrocyte hemolysis %) = [(S – B) / H] x 100

Figure 3.6: Formula to calculate the erythrocyte hemolysis rate in percentage
S= Sample absorbance value
B= Blank absorbance value
H= Absorbance value of ultra pure water

3.9 Toxicity studies

3.9.1 Test animals

Toxicity studies were performed on 5 male and 5 nulliparous female Institute of Cancer Research (ICR) mice for each test group. The mice which are of 5 weeks age were purchased from Laboratory Animal Science Centre, Faculty of Medicine, University of Malaya. Mice were kept at Microbiology Division, Institute of Biological Sciences, Faculty of Science, University of Malaya animal house with well ventilated room for one week for acclimatisation prior to the experiment.

3.9.2 Acclimatisation

They were separated by sexes to prevent any pregnancy and changes in hormonal levels. Mice were provided with standard rodent pellet diet and fresh tap water daily. Their bedding was changed once in every two days. They were housed in a controlled room with 12 hours of light and darkness cycles. Twelve hours prior to experiment the food was withdrawn but they still had free access to fresh tap water. All the mice were

weighed and their weight was well tabulated according to their test group. The average weight variation for males and females mice was ensured between ±20% ranges. The mice were marked to identify and differentiate them within and between the test groups.

3.9.3 Preparation of test substance

The samples were prepared a day prior to the experiment day. The samples with the concentration of 100mg/ml were prepared by dissolving it in 10% Tween-80 (Hopkin & Williams, England). Samples were stirred for a few hours using magnetic stirrer with 100% Tween-80 so that it could be well dissolved. Later on the concentration of Tween-80 was adjusted to 10% by adding distilled water. A set of 5 female and 5 male mice were used as negative control. The negative control mice were administrated with 10% Tween-80 in distilled water.

3.9.4 Test substance administration

The mice were administrated with samples by forced oral administration method. Five male and five female mice within each test group were fed with five fixed-dose levels (500, 1000, 1500, 2000 and 2500mg/kg). The amount of samples fed to the mice was based on the weights of the mice. After oral administration the mice were fasted for 4 hours.

3.9.5 Observation and examination

Clinical symptoms including mortality, clinical signs such as mobility, feeding and gross findings were observed throughout the day after administration. Later on, the mice were observed thrice a day continuously for 14 days. This experimental procedure was approved by the University of Malaya Animal Experimental Ethnics Committee prior to

commencing the study [Ethical number: ISB/05/08/2009/SKS (R)]. The ethical clearance certificate is enclosed in the appendix K.

3.10 High performance Liquid Chromatography (HPLC) analysis

3.10.1 Principles of HPLC

An HPLC system consists of a pumping unit, sample-injection unit, separation unit, detection unit, and data-processing unit. Chromatography is a physical and chemical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase) while the other (the mobile phase) moves in a definite direction. The basis of HPLC is to separate compounds by charge, hydrophobicity, affinity, solubility, stability and molecular weight.

3.10.2 Equipment and material

The samples were analysed on Waters HPLC equipment equipped with a 200µl loop injection valve (Waters TM 717 plus Autosampler, USA) and connected to a (Waters TM 486 detector, USA) UV detector. The peptide/protein samples were detected at a wavelength of 254nm. A Chromolith® Performance Reversed-Phase-18 encapped monolithic HPLC column 100-4.6mm (Merck, Darmstadt, Germany) coupled with Chromolith® guard cartridge kit 5-4.6mm (Merck, Darmstadt, Germany) was used at ambient temperature to separate the compounds in the sample.

3.10.3 Experimental conditions for HPLC analysis

10µL of each sample were injected and run through the HPLC column at a flow rate of 1ml/min. The mobile phase used was ultrapure water added with 0.1% Trifluoroacetic acid (Merck, Darmstadt, Germany) for protein sequence analysis. The mobile phase was prepared fresh before the experiment and was filtered using vacuum filtration system

with 0.45µm membrane filter (Sartorius, Goettingen, Germany). All the samples were filtered with 0.20µm sterile single use syringe filter (Sartorius, Goettingen, Germany) before running the samples into the system. The system was primed and purged every time before running the samples and it was ensured to be free of air bubbles. The mobile phases were allowed to flush through the system to get a stable baseline prior to running the samples. The samples were placed in 1ml autosampler vials (Kimble Glass Inc, USA). Data were analysed using Empower software.

3.11 Matrix-assisted laser desorption/ionization (MALDI) Time of flight (TOF)

3.11.1 Principle of MALDI

The MALDI TOF/TOF Analyzer is a floor-standing mass spectrometer (MS) that delivers high resolution and accurate mass determination for the MS analysis of biomolecules including protein digests, intact proteins, lipids, and carbohydrates. In this MS there are three essential components namely ionization, separation and detection. A small amount of sample is mixed with a light absorbing matrix molecule and allowed to co-crystallize directly on MALDI plates. This is done easiest by room temperature evaporation. The MALDI plate is then placed in the high vacuum chamber of the machine. Nanosecond duration of laser pulses is focused to hit the sample causing desorption of the crystal and the formation of ionized protein molecules. Ionized molecules are then accelerated in an electromagnetic field and eventually reach the detector. It only requires millisecond timescale to analyze ions produced by a single laser pulse. Therefore, the technique lends itself to high throughput analysis.

3.11.2 Materials preparation

3.11.2.1Trypsin stock

Trypsin (Promega, USA) stock solution was prepared in a concentration of 0.1µg/µL by dissolving 20µg Trypsin into 200µL Trypsin suspension buffer. This solution was then stored at -20^{o}C for up to two months.

3.11.2.2Digestion buffer

10mg of the ammonium bicarbonate (Merck, Germany) was weighed and dissolved in 2.5mL of ultrapure water for a final concentration of 50mM. This solution can be stored at 4^{o}C for up to two months.

3.11.2.3Reducing buffer

8mg of dithiothreitol (DTT) (Sigma, Germany) was weighed and dissolved into 500µl of ultrapure water for a final concentration of 100mM. Reducing buffer was stored at -20^{o}C.

3.11.2.4Alkylation Buffer

Alkylation buffer was prepared just before use. 9mg of iodoacetamide was weighed and added to a foil wrapped tube to avoid exposure to light. 500µl of ultrapure water was added to make up to a final concentration of 100mM. No excess buffer was stored.

3.11.3 Reduction and Alkylation

15µl of digestion buffer and 1.5µl of reducing buffer were added to a 0.5ml microcentrifuge tube. 10µl of protein solution was added and the amount was topped up to 27µl with ultrapure water. Samples were incubated at 95^{o}C for 5 minutes and allowed

to cool down. 3µl of alkylation buffer was added to the tube and incubated in the dark at room temperature for 20 minutes.

3.11.4 Digestion

1µl of trypsin enzyme was added to reaction tube and incubated at 37^{o}C for 3 hours. An additional 1µl of trypsin was added and incubated at 30^{o}C overnight. The samples were vortex briefly and spun down at 1000 rpm for 1 minute.

3.11.5 Extraction

The samples were vortexed with 50µl of 50% acetonitrile for 15 minutes. All liquid were transferred to a new tube. Next the samples were vortexed again with 50µl of 100% acetonitrile for 15 minutes. All liquid were transferred to the same tube as the previous step. The samples collected in the new tube were speed vacuumed to remove acetonitrile. This step takes approximately an hour.

3.11.6 Cleaning of MALDI plates

MALDI plate was cleaned with detergent to remove matrix from spots. Gloved fingers were used to rub the plate. It was rinsed with MiliQ water. The plate was dried with Kims Wipes. A little polish was placed onto the plate and Kims Wipes was used to polish in circular motions until the whole plate is covered and no visible white polish is observed. The plate was rinsed with acetone and methanol. It was air dried and covered to prevent dust from falling onto the surface.

3.11.7 Desalting of sample

3.11.7.1 Preparation of desalting solution

Wetting solution, sample preparation, equilibration solution, wash solution and elution solution was prepared in advance as shown in table 3.4.

Table 3.4: Solution need to be prepared in advance for the desalting process

Solution	Preparation
Wetting solution	50% Acetonitrile (in ddH_2O)
Sample preparation	0.1% TFA (in ddH_2O)
Equilibration solution	0.1% TFA (in ddH_2O)
Wash solution	0.1% TFA (in ddH_2O)
Elution solution	0.1% TFA/50% CAN

TFA= Trifluoroacetic acid (Merck, Germany)
ddH_2O= Double distilled water
ACN= Acetonitrile (Merck, Germany)

3.11.7.2 Desalting procedure

Air dried samples were desalted prior to plating them onto the plates. The desalting procedure was done using Zip-Tip C 18 (Milipore, USA).

3.11.7.2.1 Equilibrating the ZipTip pipette tip for sample binding

The pipette plunger was depressed to a dead stop. Using the maximum volume setting of 10µL, wetting solution was aspirated into the tip. Wetting solution was dispensed to waste and the step was repeated thrice. Next, equilibration solution was aspirated and dispensed to waste thrice.

3.11.7.2.2 Bind and wash the peptides and proteins

Peptides and/or proteins were bind to ZipTip pipette tip by fully depressing the pipette plunger to a dead stop. The sample was aspirated and dispensed 7-10 cycles for maximum binding of complex mixtures. Wash solution was aspirated into the tip and dispensed to waste. This step was repeated three times.

3.11.7.2.3 Elution of the peptides and proteins

Using a standard pipette tip, 2µL of elution solution was dispensed into a new tube. The solution was carefully aspirated and eluant was dispensed through ZipTip pipette tip at least three times without introducing air into the samples.

3.11.7.3 Preparation of Matrix

Matrix was prepared by dissolving 5mg of α-cyano-4-hydroxycinnamic acid (ACHCA) (Sigma, Germany) to 500µL of buffer A. Buffer A was prepared as shown in table 3.5.

Table 3.5: Amount of the materials need to be added to prepare buffer A

Materials	Amount
100% Acetonitrile	250µL
100% Trifluoro Acetic Acid	2.5µL
Ultra Pure Water	247.5µL
Total	**500µL**

3.11.7.4 MALDI plate spotting

2µL of Matrix and 2µL of the desalted sample were mixed in a separate tube. They were mixed by pipetting up and down 4 times. 1µL was placed onto the indicated spot of the MALDI plate. Unused sample was frozen at -20^{o}C in case of further analyses were required. Spots were allowed to dry completely and MALDI TOF/TOF data collection was performed as soon as possible.

3.12 Statistical Analysis

All the analyses were performed in triplicates. The means of the data were subjected to a one-way analysis of variance (ANOVA) and the significance variation between the means was determined by Duncan's multiple range tests. A value of ($p< 0.05$) was set to determine statistical significance. Statistical analysis was done using SPSS Statistics v17.0 software.

CHAPTER 4

RESULTS AND

DATA ANALYSIS

Results and Data Analysis

Results and Data Analysis

4.1 Yields of ethanolic extracts

The bar chart in figure 4.1 shows the ethanolic extract yields from various samples which were extracted using 95% ethanol. The yields from various samples were presented in percentage form. The fermented extract yields the highest extract at 44.65%. The list was followed by functional food paste at 29.81%, fermented vinegar with lactic acid bacteria at 19.81% and fermented bean at 14.62%. Next the list was followed by *Curcuma mangga*, *Andrographis paniculata*, *Allium sativum* and *Cymbopogan citratus* with the extract yields of 12.75%, 11.28%, 11.05% and 5.1% respectively. The lowest ethanolic extract yield was from *Carica papaya* leaf at 3.85%.

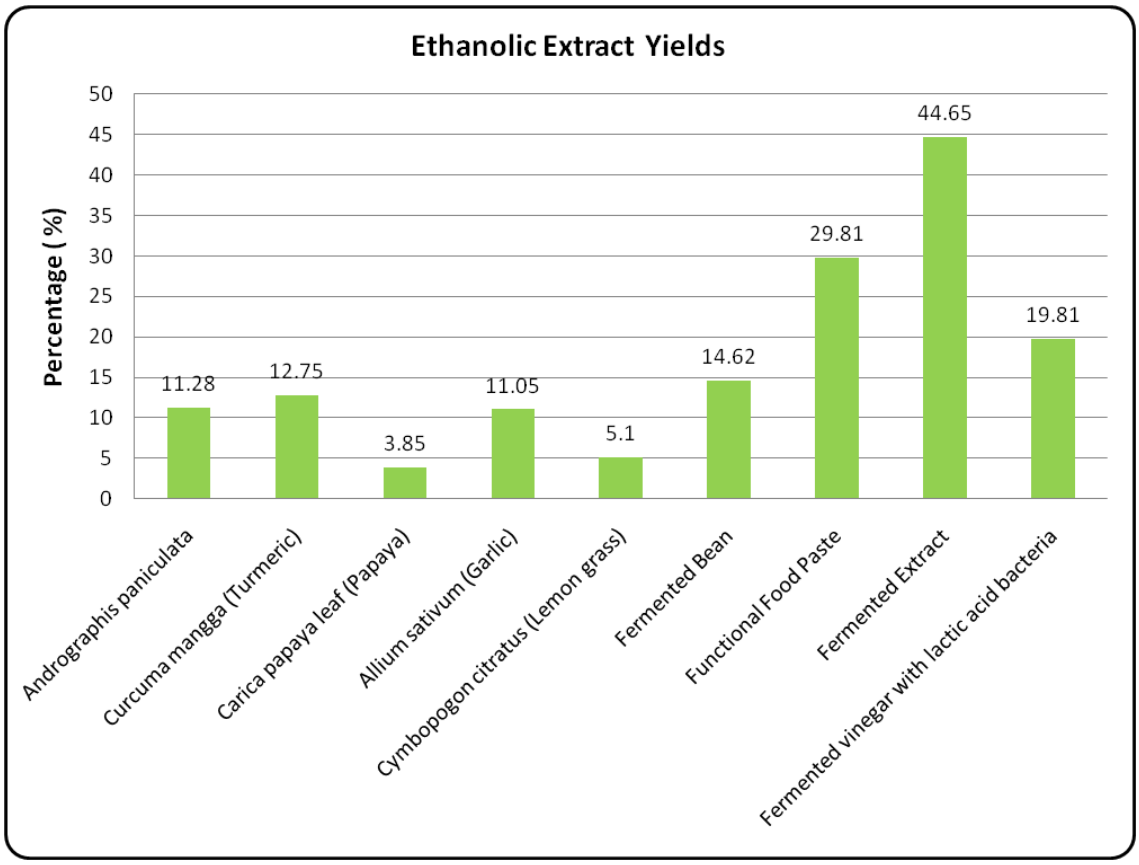

Figure 4.1: Yields of ethanolic extract from various plant and fermented extracts

The extracts were concentrated under reduced pressure using rotary evaporator and the concentrations were measured by weighing the sample yield. Percentage (%) (w/w) yield refers to gravimetric determination of total extractable compounds expressed as a percentage of the sample weight.

4.2 Yields of protein/peptide extracts

The bar chart in figure 4.2 shows the concentration of protein/peptide extracts obtained from various samples. The samples were extracted with the extraction buffer and centrifuged. The supernatant was collected and sent for freeze drying. The freeze dried samples were reconstituted and the protein concentration was measured using Bradford assay. The bar chart in the figure 4.2 shows that *Allium sativum* yielded the highest amount of protein/peptide extract at 1.82mg/g followed by *Andrographis paniculata* at 1.77mg/g. The amounts of protein/peptide extracted from both samples are significantly the same. This was determined by Duncan's multiple range tests with the value of $p<0.05$ was set to determine statistical significance.

The result was followed by *Zingiber officinale* protein/peptide extract at 1.56mg/g. *Zingiber officinale* protein/peptide extract were significantly lower compared to *Andrographis paniculata* and *Allium sativum* protein/peptide extract. Next the list was followed by *Curcuma mangga* and *Cybopogan citratus* protein/peptide extract at 1.36 mg/g. Statistical analysis between the latter two quantities are significantly the same. The lowest total extractable protein/peptide was obtained from *Momordica charantia* seeds at 0.49mg/g.

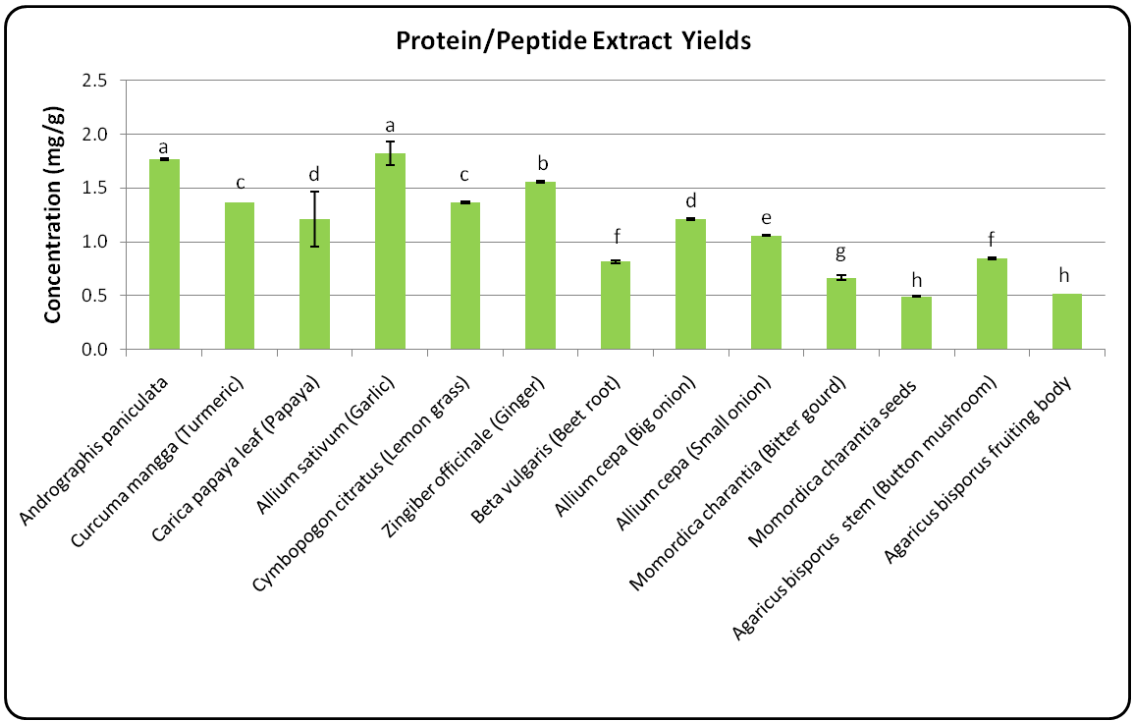

Figure 4.2: Yields of protein/peptide extracts from various samples

The protein/peptide were extracted from various plant and freeze dried. The concentrations of the protein/peptide were measured using Bradford method. Concentration refers to protein/peptide concentration in mg/g after reconstituting in 5ml of buffer. Letters in lower case show significant difference ($p<0.05$) determined by Duncan's multiple range tests.

4.3 Identification of test microorganisms

Figures 4.3, 4.4, 4.5 and 4.6 show the Basic Local Alignment Search Tool (BLAST) results of the various test microorganisms identified. The bacteria species were confirmed based on their 16S rDNA sequences. These were compared with the NCBI database using the BLAST program to identify the microbial species. The bacteria with highest score (bits) and lowest E value were chosen as the bacteria species. Microorganisms identified are *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

***Bacillus cereus* ATCC14579**

Sequences producing significant alignments:		Score (Bits)	E Value
1.	>gi|30018278|ref|NC_004722.1| Bacillus cereus ATCC 14579, complete genome	2706.3	0.0
2.	>gi|52140164|ref|NC_006274.1| Bacillus cereus E33L, complete genome	2698.4	0.0
3.	>gi|118475778|ref|NC_008600.1| Bacillus thuringiensis str. Al Hakam, complete ...	2694.4	0.0
4.	>gi|42779081|ref|NC_003909.8| Bacillus cereus ATCC 10987, complete genome	2694.4	0.0
5.	>gi|49476684|ref|NC_005957.1| Bacillus thuringiensis serovar konkukian str. 97...	2690.4	0.0

Figure 4.3: The blast result establishes the identity of the bacteria as *Bacillus cereus*
Identity of the microorganism was confirmed with highest score (bits) and lowest E value. The 16S rRNA sequences obtained from gene sequencing is enclosed in appendix C.

***Staphylococcus aureus* RF122**

Sequences producing significant alignments:		Score (Bits)	E Value
1.	>gi|82749777|ref|NC_007622.1| Staphylococcus aureus RF122, complete genome	2714.2	0.0
2.	>gi|151220212|ref|NC_009641.1| Staphylococcus aureus subsp. aureus str. Newman...	2714.2	0.0
3.	>gi|148266447|ref|NC_009487.1| Staphylococcus aureus subsp. aureus JH9, comple...	2714.2	0.0
4.	>gi|150392480|ref|NC_009632.1| Staphylococcus aureus subsp. aureus JH1, comple...	2714.2	0.0
5.	>gi|87159884|ref|NC_007793.1| Staphylococcus aureus subsp. aureus USA300, comp...	2714.2	0.0

Figure 4.4: The blast result establishes the identity of the bacteria as *Staphylococcus aureus*
Identity of the microorganism was confirmed with highest score (bits) and lowest E value. The 16S rRNA sequences obtained from gene sequencing is enclosed in appendix C.

***Escherichia coli* UT189**

Sequences producing significant alignments:		Score (Bits)	E Value
1.	>gi|91209055|ref|NC_007946.1| Escherichia coli UTI89, complete genome	2662.8	0.0
2.	>gi|26245917|ref|NC_004431.1| Escherichia coli CFT073, complete genome	2662.8	0.0
3.	>gi|89106884|ref|AC_000091.1| Escherichia coli W3110 DNA, complete genome	2660.8	0.0
4.	>gi|110640213|ref|NC_008253.1| Escherichia coli 536, complete genome	2660.8	0.0
5.	>gi|49175990|ref|NC_000913.2| Escherichia coli K12, complete genome	2660.8	0.0

Figure 4.5: The blast result establishes the identity of the bacteria as *Escherichia coli*
Identity of the microorganism was confirmed with highest score (bits) and lowest E value. The 16S rRNA sequences obtained from gene sequencing is enclosed in appendix C.

***Pseudomonas aeruginosa* PA7**

Sequences producing significant alignments:		Score (Bits)	E Value
1. >gi|152983466|ref|NC_009656.1|	Pseudomonas aeruginosa PA7, complete genome	2704.3	0.0
2. >gi|116048575|ref|NC_008463.1|	Pseudomonas aeruginosa UCBPP-PA14, complete gen...	2704.3	0.0
3. >gi|110645304|ref|NC_002516.2|	Pseudomonas aeruginosa PAO1, complete genome	2704.3	0.0
4. >gi|146280397|ref|NC_009434.1|	Pseudomonas stutzeri A1501, complete genome	2411.8	0.0
5. >gi|146305042|ref|NC_009439.1|	Pseudomonas mendocina ymp, complete genome	2386.1	0.0

Figure 4.6: The blast result establishes the identity of the bacteria as *Pseudomonas aeruginosa*

Identity of the microorganism was confirmed with highest score (bits) and lowest E value. The 16S rRNA sequences obtained from gene sequencing is enclosed in appendix C.

4.4 Antimicrobial activity

4.4.1 Ethanolic extracts

4.4.1.1 Disk diffusion assay of ethanolic extracts

The result in table 4.1 shows the inhibition of various plant and fermented samples that were tested against *Escherichia coli* and *Pseudomonas aeruginosa* (Gram negative bacteria) and *Bacillus cereus* and *Staphylococcus aureus* (Gram positive bacteria). The most pronounced inhibition zone for ethanolic extracts was obtained with *Andrographis paniculata* extract producing inhibition zones of 11mm against *S. aureus*, 14mm against *B. cereus* and 11.5mm against *P. aeruginosa*. This was followed by *Curcuma mangga* extract which produced inhibition zones of 8.5mm against *S. aureus*, 10.5mm against *B. cereus* and 9.5mm against *P. aeruginosa*. *Allium sativum* extract inhibited both *B. cereus* and *P. aeruginosa* respectively with 7mm inhibition. Fermented bean and fermented plant extracts only inhibited *P. aeruginosa* at 7mm and 8mm inhibition zones respectively. The plant and fermented extracts that showed some inhibition were selected to test for minimum inhibitory concentration. Minimum inhibitory concentration was determined by doing broth dilution assay.

Table 4.1: Inhibition zones of various plants and fermented ethanolic extracts by disk diffusion test

Ethanolic extracts	**Inhibition zones in (mm) against**			
	E. coli	***S. aureus***	***B. cereus***	***P. aeruginosa***
Andrographis paniculata	no inhibition	11 ± 1.0	14 ± 1.0	11.5 ± 1.5
Curcuma mangga (Turmeric)	no inhibition	8.5 ± 1.5	10.5 ± 0.5	9.5 ± 0
Carica papaya leaf (Papaya)	no inhibition	no inhibition	no inhibition	no inhibition
Allium sativum (Garlic)	no inhibition	no inhibition	7.0 ± 1.0	7.0 ± 1.0
Cymbopogon citratus (Lemon grass)	no inhibition	no inhibition	no inhibition	no inhibition
Fermented bean	no inhibition	no inhibition	no inhibition	7.0 ± 1.0
Functional food paste	no inhibition	no inhibition	no inhibition	no inhibition
Fermented extract	no inhibition	no inhibition	no inhibition	8.0 ± 1.0
Fermented vinegar with lactic acid bacteria	no inhibition	no inhibition	no inhibition	no inhibition
Positive control	15 ± 1.0	16 ± 0	19 ± 1.0	16.5 ± 0.5
Negative control	no inhibition	no inhibition	no inhibition	no inhibition

Inhibition zones were measured in mm and the diameter of inhibition zones reported includes the diameter of the disk which is 6mm. All tests were done in triplicates.

4.4.1.2 MIC and MBC of ethanolic extracts

For minimum inhibitory concentration (MIC) the plant and fermented ethanolic extracts' bacteriostatic values were determined at concentrations of 10mg/ml, 20mg/ml, 30mg/ml, 40mg/ml and 50mg/ml. Table 4.2 shows ethanolic extract of *Andrographis paniculata* exhibited notable antibacterial activity against both *S. aureus* and *B. cereus*. MIC value for *Andrographis paniculata* extract was below 10mg/ml for both *S. aureus* and *B. cereus* and was 20mg/ml for *P. aeruginosa*. Minimum bacteriocidal concentration (MBC) was determined to evaluate the bacteriocidal activities. Samples that showed MIC values were selected to perform MBC to determine whether the samples are just bacteriostatic or bacteriocidal as well. *Andrographis paniculata's* extract MBC value for both *S. aureus* and *B. cereus* was below 10mg/ml, which means at 10mg/ml concentration it gives complete bacteriocidal activity. For *P. aeruginosa*, MIC value for *Andrographis paniculata* extract was 20mg/ml but its MBC value was more than 50mg/ml. *Curcuma mangga* extract tested against both *S. aureus* and *P. aeruginosa* showed MIC value of 40mg/ml whereas MIC value for *B. cereus* was 30mg/ml. Fermented extract yielded an MIC value of 40mg/ml and MBC value of 50mg/ml. The rest of the ethanolic extract samples as presented in table 4.2 generated MIC and MBC values above 50mg/ml.

Table 4.2: Minimum inhibitory concentration and minimum bacterial concentration values obtained with various plants and fermented ethanolic extracts

Ethanolic Extract (mg/ml)	MIC and MBC against							
	E. coli		***S. aureus***		***B. cereus***		***P. aeruginosa***	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Andrographis paniculata	-	-	<10	<10	<10	<10	20	>50
Curcuma mangga (Turmeric)	-	-	40	>50	30	>50	40	>50
Carica papaya leaf (Papaya)	-	-	-	-	-	-	-	-
Allium sativum (Garlic)	-	-	-	-	>50	>50	>50	>50
Cymbopogon citratus (Lemon grass)	-	-	-	-	-	-	-	-
Fermented bean	-	-	-	-	-	-	>50	>50
Functional food paste	-	-	-	-	-	-	-	-
Fermented extract	-	-	-	-	-	-	40	50
Fermented vinegar with lactic acid bacteria	-	-	-	-	-	-	-	-

All tests were done in triplicates

ii) MIC = minimum inhibitory concentration and
MBC = minimum bacterial concentration

iii) < = less than

iv) > = more than

v) - = not tested for MIC or MBC

4.4.2 Protein/peptide extracts

4.4.2.1 Disk diffusion assay of protein/peptide extracts

Table 4.3 shows the results for disk diffusion assay of protein/peptide extracts. *Allium sativum* protein/peptide extract shows strong antimicrobial activities against all four bacteria tested with 15mm against *E. coli*, 28mm against *S. aureus*, 16mm against *B. cereus* and lastly 9mm against *P. aeruginosa*. *Momordica charantia* extract shows inhibition zones of 9mm against *B. cereus* and 10mm against *P. aeruginosa*. This is followed by *Momordica charantia* seeds extract that show inhibition zones of 10mm against *S. aureus* and 15mm against *B. cereus*. The above protein/peptide extracts that showed some inhibition were selected to test for minimum inhibitory concentration (MIC). MIC was determined by doing broth dilution assay.

Table 4.3: Inhibition zones of various protein/peptide extracts by disk diffusion test

Protein/peptide Extracts	Inhibition zones in (mm) against			
	E. coli	***S. aureus***	***B. cereus***	***P. aeruginosa***
Andrographis paniculata	no inhibition	no inhibition	no inhibition	no inhibition
Curcuma mangga (Turmeric)	no inhibition	no inhibition	no inhibition	no inhibition
Carica papaya leaf(Papaya)	no inhibition	no inhibition	no inhibition	no inhibition
Allium sativum (Garlic)	15 ± 1.5	28 ± 2.1	16.3 ± 1.5	9 ± 0.9
Cymbopogon citratus (Lemon grass)	no inhibition	no inhibition	no inhibition	no inhibition
Zingiber officinale (Ginger)	no inhibition	no inhibition	no inhibition	no inhibition
Beta vulgaris (Beet root)	no inhibition	no inhibition	no inhibition	no inhibition
Allium cepa (Big onion)	no inhibition	no inhibition	no inhibition	no inhibition
Allium cepa (Small onion)	no inhibition	no inhibition	no inhibition	no inhibition
Momordica charantia (Bitter gourd)	no inhibition	no inhibition	9 ± 0.3	10.0 + 0.9
Momordica charantia seeds	no inhibition	10 ± 0.5	15 ± 1.5	no inhibition
Agaricus bisporus stem (Button mushroom)	no inhibition	no inhibition	no inhibition	no inhibition
Agaricus bisporus fruiting body	no inhibition	no inhibition	no inhibition	no inhibition
Positive Control	18 ± 0.6	30 ± 0.0	34 ± 0.0	26 ± 0.0
Negative Control	no inhibition	no inhibition	no inhibition	no inhibition

Inhibition zones were measured in mm and the diameter of inhibition zones reported includes the diameter of the disk which is 6mm. All tests were done in triplicates.

4.4.2.2 MIC and MBC of protein/peptide extracts

As shown in table 4.4, *Allium sativum* protein/peptide extract produced MIC values of 200µg/ml against *E. coli* and *B. cereus* and 100µg/ml against *S. aureus* but the MIC value was above 500µg/ml for *P. aeruginosa*. Protein/peptide extracts in this study that showed their MIC values above 500µg/ml was not selected for MBC assay. This is because only those samples that exhibited some degree of bacteriostatic activity were selected to determine whether they are just bacteriostatic or bacteriocidal as well. As shown in table 4.4, all the MBC values were above 500µg/ml which means even some protein/peptide samples could inhibit the bacteria growth at the concentration of 500µg/ml or below but it could not kill the bacteria at the concentration of 500µg/ml.

Table 4.4: Minimum inhibitory concentration and minimum bacterial concentration of various protein/peptide extracts

Protein/Peptide Extract (µg/ml)	MIC and MBC against							
	E. coli		***S. aureus***		***B. cereus***		***P. aeruginosa***	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Andrographis paniculata	-	-	-	-	-	-	-	-
Curcuma mangga (Turmeric)	-	-	-	-	-	-	-	-
Carica papaya(Papaya leaf)	-	-	-	-	-	-	-	-
Allium sativum (Garlic)	200	>500	100	>500	200	>500	>500	-
Cymbopogon citratus (Lemon grass)	-	-	-	-	-	-	-	-
Zingiber officinale (Ginger)	-	-	-	-	-	-	-	-
Beta vulgaris (Beet root)	-	-	-	-	-	-	-	-
Allium cepa (Big onion)	-	-	-	-	-	-	-	-
Allium cepa (Small onion)	-	-	-	-	-	-	-	-
Momordica charantia (Bitter gourd)	-	-	-	-	500	>500	500	>500
Momordica charantia seeds	-	-	500	>500	500	>500	-	-
Agaricus bisporus stem (Button mushroom)	-	-	-	-	-	-	-	-
Agaricus bisporus fruiting body	-	-	-	-	-	-	-	-

i) All tests were done in triplicates.
ii) MIC = minimum inhibitory concentration
MBC = minimum bacterial concentration
iii) < = less than
iv) > = more than
v) - = not tested for MIC or MBC

4.5 Antioxidant activity

4.5.1 Superoxide Dismutase (SOD)

4.5.1.1 SOD activity of ethanolic extracts

Figure 4.7 shows the SOD activity for various plant and fermented extracts compared to 1mg/ml and 10mg/ml vitamin C as the positive controls. *Allium sativum* extract showed the highest inhibition rate at 93.00% followed by *Cymbopogon citratus* extract at 91.50%. Both of the samples are significantly lower compared to vitamin C. As shown in figure 4.7, the inhibition rates range from the highest inhibition rate by *Allium sativum* extract with a value of 93.00% to the lowest inhibition rate by functional food paste with a value of 35.70%. All the samples of plant and fermented extracts exhibited inhibition rates of above 60% except for functional food paste.

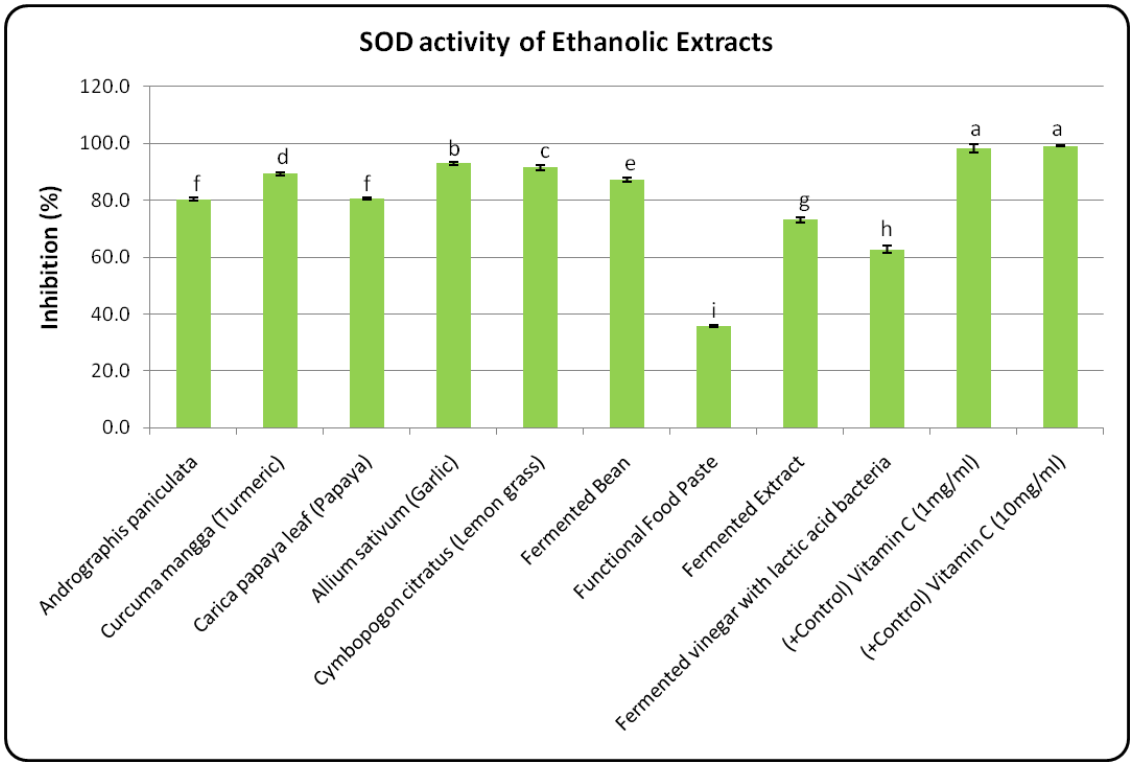

Figure 4.7: Inhibition rate of various plants and fermented ethanolic extracts
The antioxidant activities of the extracts were tested by superoxide dismutase kit. All tests were done in triplicates. Letters in lower case show significant difference ($p<0.05$) determined by Duncan's multiple range tests.

4.5.1.2 SOD activity of protein/peptide extracts

Figure 4.8 shows the SOD activity for various protein/peptide extracts compared to 1mg/ml and 10mg/ml vitamin C used as the positive controls in all the antioxidant tests. Both of the vitamin C samples at concentrations of 1mg/ml and 10mg/ml was not significantly different with inhibition values of 98.35% and 99.20% respectively. Protein/peptide extracts of *Momordica charantia* seeds and *Allium sativum* showed the strongest SOD activity compared to other extract samples. Protein/peptide extracts of both samples showed no significant difference at values of 93.61% and 92.73% respectively but was significantly lower compared to vitamin C. The list is followed by *Momordica charantia*, *Curcuma mangga* and *Andrographis paniculata* protein/peptide extract with inhibition rates of 86.39%, 84.4% and 84.33% respectively. As shown in figure 4.8, the inhibition rates ranged from the highest for *Momordica charantia* seeds protein/peptide extract with value of 93.61% to the lowest inhibition rate for *Agaricus bisporus* fruiting body protein/peptide extract with a value of 62.47%.

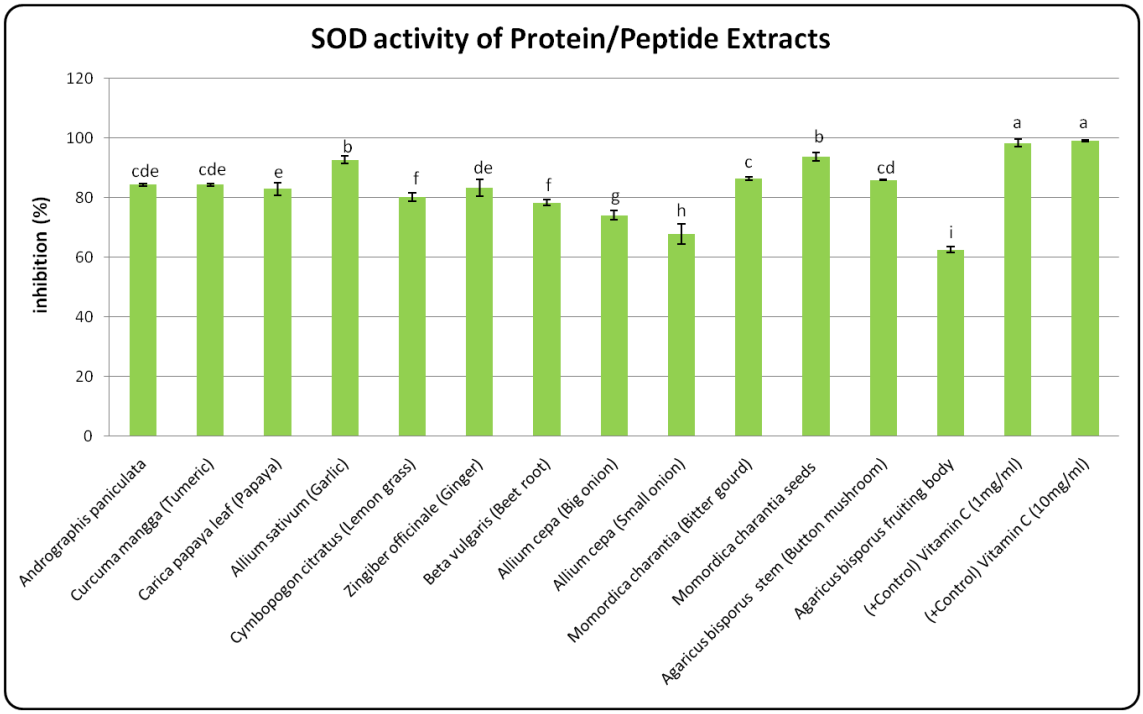

Figure 4.8: Inhibition rate of various protein/peptide extracts

The antioxidant activities of the extracts were tested by superoxide dismutase kit. All tests were done in triplicates. Letters in lower case show significant difference ($p<0.05$) determined by Duncan's multiple range tests.

4.5.2 DPPH assay

4.5.2.1 DPPH radical scavenging activities of ethanolic extracts

Figure 4.9 shows the scavenging activity of DPPH radicals' of various ethanolic extracts. Vitamin C with the concentration of 10mg/ml was used as the positive control. All the ethanolic extracts used were at the concentration of 10mg/ml. Vitamin C showed the highest scavenging activity with the value of 88.54%. *Curcuma mangga* extract followed the list with the value of 86.46%. The scavenging activity of *Curcuma mangga* extract and vitamin C are not significantly different. The list was followed by fermented beans extract with the value of 80.73%. Fermented beans extract are significantly lower compared to vitamin C and *Curcuma mangga* extract. The lowest value was showed by functional food paste extract with 11.46% and significantly lower compared to other ethanolic extracts tested.

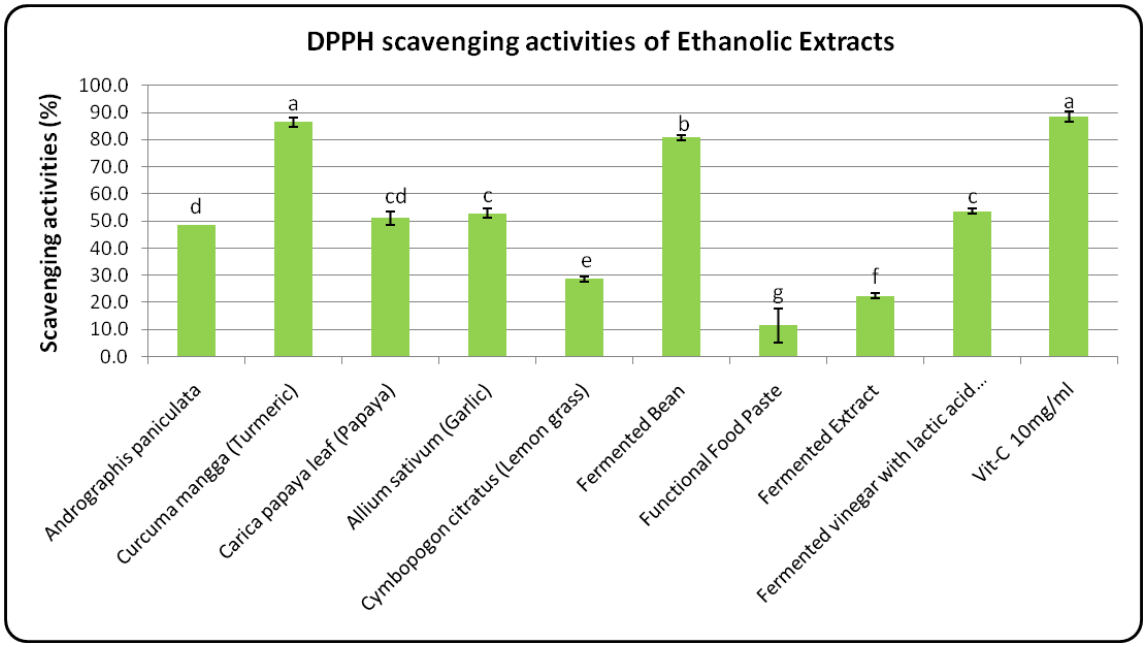

Figure 4.9: Percentage of DPPH scavenging activities of various plant and fermented extracts

All tests were done in triplicates. Letters in lower case show significant difference ($p<0.05$) determined by Duncan's multiple range tests.

4.5.2.2 DPPH radical scavenging activities of protein/peptide extracts

Figure 4.10 shows the scavenging activity of DPPH radicals of various protein/peptide extracts. Vitamin C at a concentration of 1mg/ml was used as the positive control. It showed the highest scavenging activity with 73.44%. Vitamin C is significantly higher compared to all the samples. All the protein/peptide extracts used were at concentrations of 1mg/ml. The list is followed by *Carica papaya* leaf and *Allium cepa* (big onion) protein/peptide extract with 27.30% and 25.40% respectively. Both of the samples are not significantly different but they are significantly lower compared to vitamin C. *Zingiber officinale*, *Cymbopogon citratus* and *Curcuma mangga* protein/peptide extract come next in the list with value of 18.41 %, 18.41% and 17.46% respectively. All three samples are significantly the same but significantly lower to *Carica papaya* leaf and *Allium cepa* (big onion) protein/peptide extract. The lowest positive value is showed by *Beta vulgaris* protein/peptide extract at 1.97%. *Allium*

sativum protein/peptide extract showed negative value at -169.84% despite repeated trials.

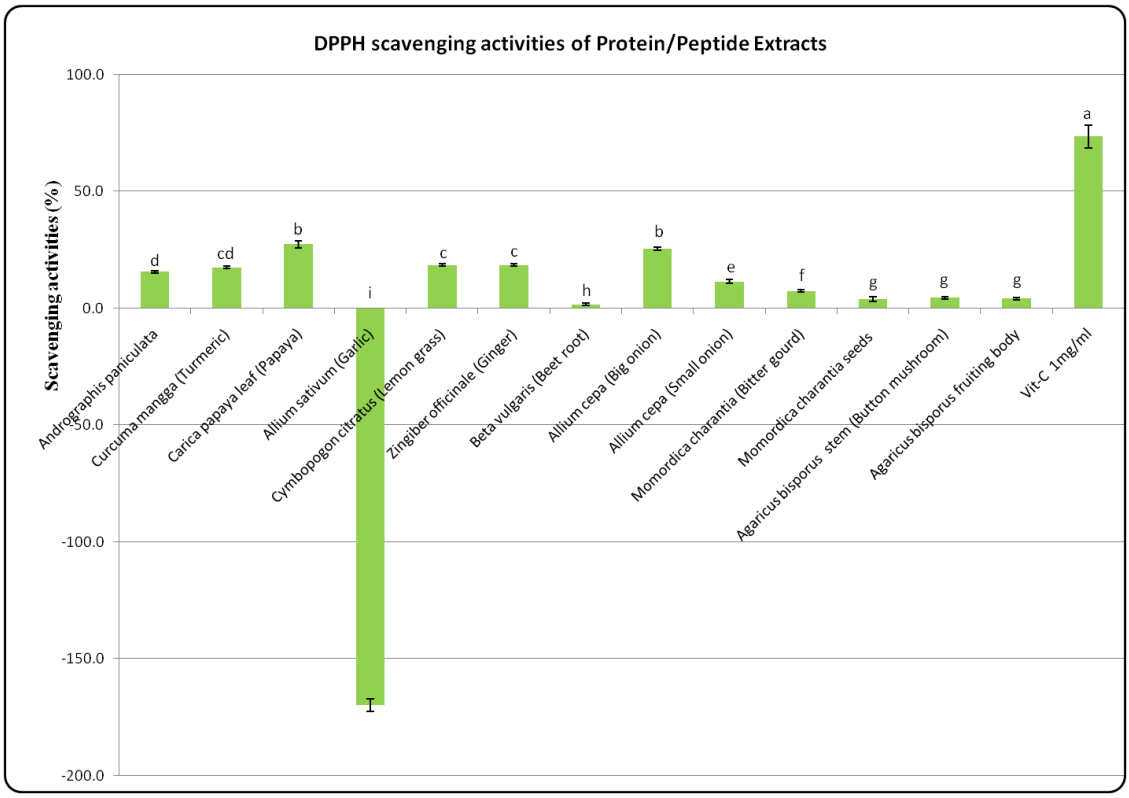

Figure 4.10: Percentage of DPPH scavenging activities of various protein/peptide extracts

All tests were done in triplicates. Letters in lower case show significant difference ($p<0.05$) determined by Duncan's multiple range tests.

4.6 Hemolysis assay

4.6.1 Anti hemolytic activity of ethanolic extracts

Figure 4.11 showed anti hemolytic activity of various plant and fermented ethanolic extracts. Ethanolic extracts exhibited lower hemolytic activity compared to the non-pretreated samples except for fermented vinegar with lactic acid bacteria which exhibited value of 97.64% compared to non-pretreated which was 58.90%. The rest of the ethanolic extracts showed hemolytic activities below 40%. *Cymbopogon citratus* extract exhibited the lowest hemolytic activity at 17.09% followed by *Andrographis paniculata* extract at 19.45%. Both samples showed significantly same hemolytic

activity but was significantly lower compared to the non-pretreated sample. Next the list was followed by *Curcuma mangga*, *Allium sativum*, fermented bean and fermented extract at 21.45%, 28.42%, 28.90% and 29.81% respectively.

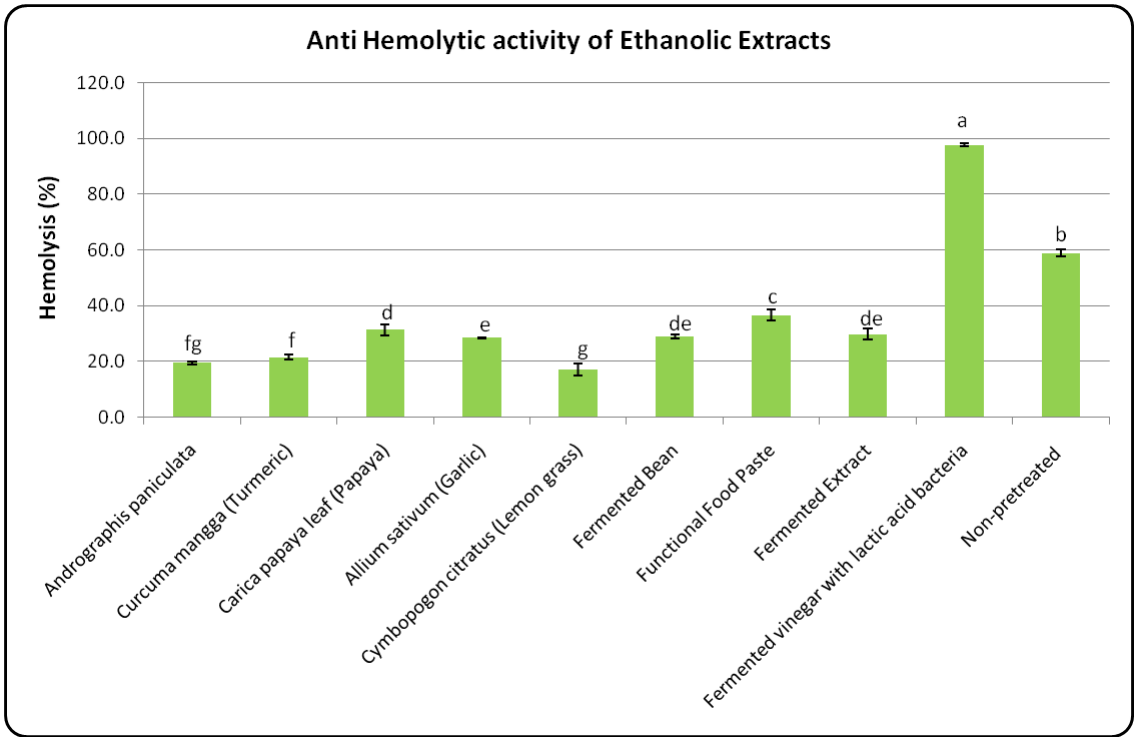

Figure 4.11: Percentage of anti hemolytic activity of various plant and fermented extracts

All tests were done in triplicates. Letters in lower case show significant difference ($p<0.05$) determined by Duncan's multiple range tests.

4.6.2 Anti hemolytic activity of protein/peptide extracts

Figure 4.12 shows the anti hemolytic activity of the various protein/peptide extracts. Erythrocyte hemolysis in pure water was considered as complete hemolysis (100%) while hemolysis of the pretreated and non-pretreated erythrocytes was expressed as a percentage of this value. The non pretreated sample exhibited hemolytic activity of 58.90%. Protein/peptide extracts from the fruiting body of *Momordica charantia* and the stem of *Agaricus bisporus* exhibited the lowest hemolytic activity with 13.87% and 13.60% respectively. Both of the protein/peptide extracts were not significantly different but they were significantly lower compared to the non-pretreated sample. Next

the list was followed by *Zingiber officinale*, *Allium cepa* (small onion), *Allium cepa* (big onion) and *Momordica charantia* seeds protein/peptide extract with hemolytic activity of 18.85%, 19.93%, 20.61% and 20.61% respectively. All the protein/peptide extracts exhibited hemolytic activity significantly lower compared to the non-pretreated samples. The hemolysis for protein/peptide extracts ranged from the lowest at 13.60% with *Agaricus bisporus* stem protein/peptide extract to the highest hemolysis at 44.85% with *Carica papaya* leaf protein/peptide extract.

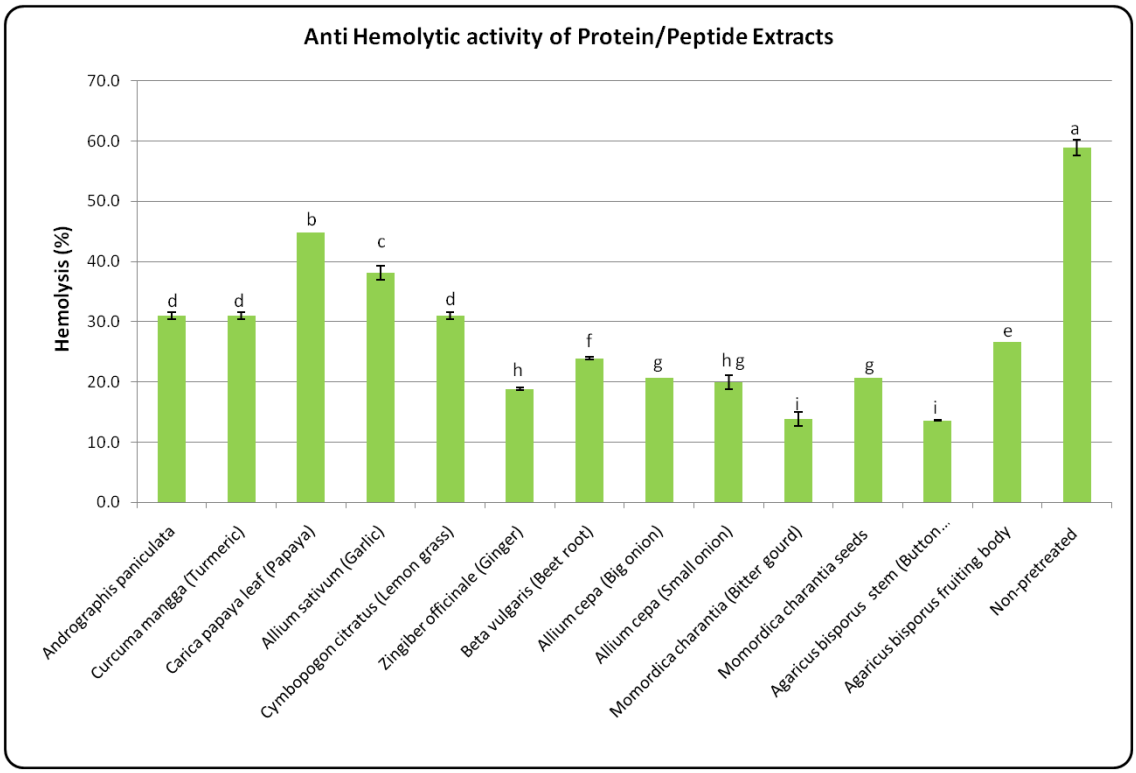

Figure 4.12: Percentage of anti hemolytic activity of various protein/peptide extracts
All tests were done in triplicates. Letters in lower case show significant difference ($p<0.05$) determined by Duncan's multiple range tests.

4.7 Toxicity Studies

The acute toxicity results of plant extracts from *Andrographis paniculata*, *Curcuma mangga*, *Cymbopogon citratus* and *Allium sativum* tested did not reveal any toxic symptoms or mortality at concentration up to 2500mg/kg doses. The result confirmed that all four plants do not cause any acute toxic effect. It was observed that the animals fed with the extracts were healthy with no unusual changes in behavior or in locomotor activity. No ataxia and no signs of intoxication were observed during the 14 days period. No differences were found in growth rate between the control group and the animals fed with different levels of the extracts. The food consumption of male and female rats of control and experimental groups were similar, indicating that the feed intake and utilization was not influenced by the gender.

Table 4.5: Mice deaths at various concentrations of ethanolic extracts for toxicity levels

Source of ethanolic extracts	Dose (mg/kg)											
	0		500		1000		1500		2000		2500	
	M	F	M	F	M	F	M	F	M	F	M	F
Andrographis paniculata	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Curcuma mangga	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Allium sativum	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Cymbopogan citratus	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

M= Male mice
F= Female mice
0/5= Total number of mice death/ total number of mice used

4.8 Chromatogram of selected protein/peptide extracts from HPLC

Figures 4.13, 4.14 and 4.15 displays the various chromatograms obtained from the HPLC. The chromatograms were obtained at a flow rate of 1ml/minute of 0.1% TFA in ultrapure water at ambient temperature. All the samples were run separately for a durations of 10 minutes. *Allium sativum* protein/peptide extracts resolved into two fractions, whole *Momordica charantia* protein/peptide extract resolved into a single fraction while *Momordica charantia* seeds protein/peptide extracts resolved into four fractions. Protein/peptide standard chromatogram is enclosed in appendix J.

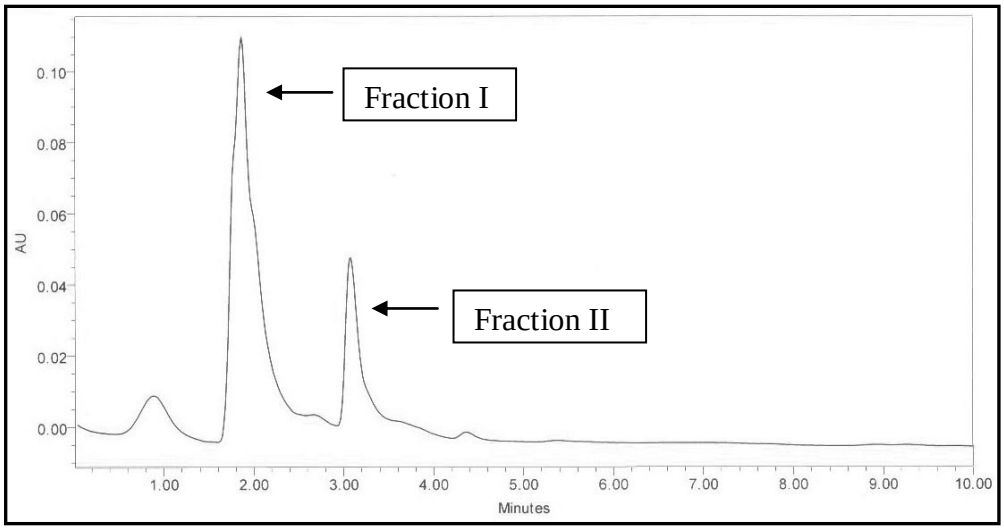

Figure 4.13: HPLC chromatograms of protein/peptide compound separated from *Allium sativum*

1mg/ml of *Allium sativum* protein/peptide compound is injected with the volume of 10µl every injection. The samples were run separately for duration of 10 minutes at a flow rate of 1ml/minute.

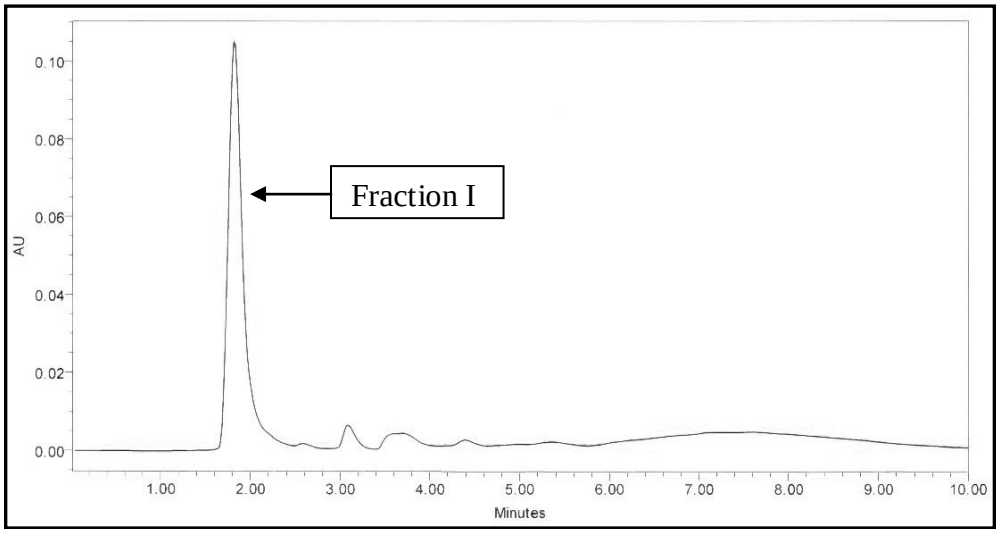

Figure 4.14: HPLC chromatograms of protein/peptide compounds separated from *Momordica charantia*

1mg/ml of *Momordica charantia* protein/peptide compound is injected with the volume of 10µl every injection. The samples were run separately for duration of 10 minutes at a flow rate of 1ml/minute.

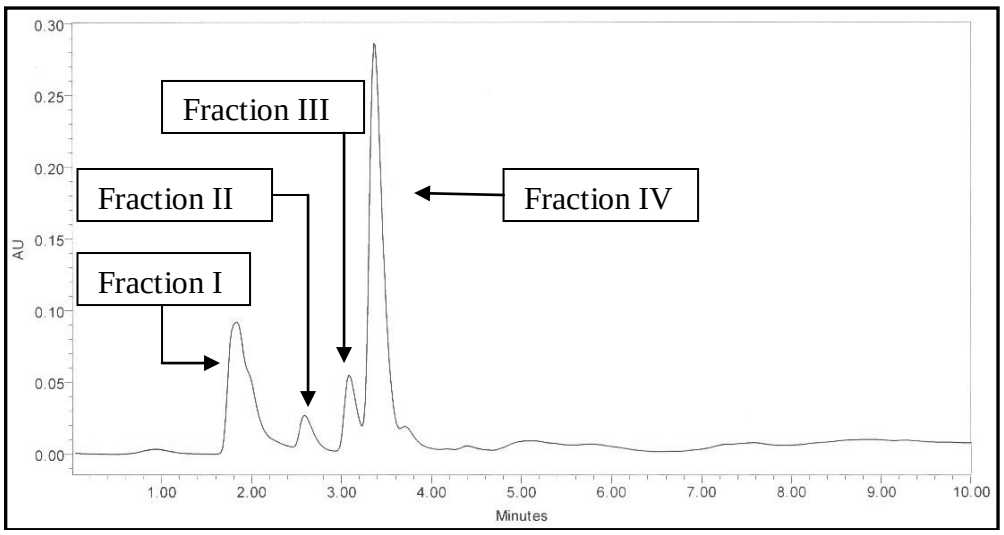

Figure 4.15: HPLC chromatograms of protein/peptide compounds separated from *Momordica charantia* seeds.

1mg/ml of *Momordica charantia seeds* protein/peptide compound is injected with the volume of 10µl every injection. The samples were run separately for duration of 10 minutes at a flow rate of 1ml/minute.

4.9 Matrix Assisted Laser Desorption/Ionisation (MALDI) Time of Flight (TOF)/ (TOF)

Table 4.6, 4.7 and 4.8 displays the protein/peptide identified from the MALDI TOF/TOF analysis. The tables show three protein/peptides identified from MALDI TOF/TOF analysis with the highest protein score for each fraction. The fractions were obtained from HPLC using a C-18 reverse phase column. The HPLC chromatogram of *Allium sativum*, *Momordica charantia* and *Momordica charantia* seeds protein/peptide extracts which are used for MALDI TOF/TOF analysis are shown in figure 4.13, 4.14 and 4.15 respectively.

Allium sativum protein/peptide extracts consist of two fractions. Fraction I of *Allium sativum* has been tentatively identified to content wound induced proteinase inhibitor 2 precursors, DEMETER-like protein 3 and an unknown protein from 2D-PAGE of needles. Probable glycine cleavage system H, thioredoxin F-type, and annexin D6 has been tentatively identified in fraction II of *Allium sativum*. *Momordica charantia* protein/peptide extracts has been tentatively identified to contain trypsin inhibitor 3, trypsin inhibitor 2 and trypsin inhibitor 1. Lastly *Momordica charantia* seeds protein/peptide extracts consist of four fractions. Tentatively fraction I of *Momordica charantia* seed protein/peptide extracts has been identified to contain fructose-bisphosphate aldolase 1, DNA ligase 4 and sufE-like protein. Fraction II of *Momordica charantia* seeds protein/peptide has been tentatively identified to contain chloroplast 30S ribosomal protein S7, ribulose bisphosphate carboxylase and ribosome recycling factor. Hypothetical mitochondrial protein, chloroplast 50S ribosomal protein L33 and 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase has been tentatively identified from fraction III of *Momordica charantia* seeds protein/peptide. Lastly fraction IV of

Momordica charantia seeds protein/peptide has been tentatively identified to contain histone H2B.1, tubulin beta-3 chain and uncharacterized protein At2g41620.

Table 4.6: Protein/peptide analysis of *Allium sativum* protein/peptide extract by MALDI TOF/TOF

Sample source	Protein name	Accession number	Protein Score
Allium sativum (Fraction I)	Wound-induced proteinase inhibitor 2 precursor	IP21_SOLLC	37
	DEMETER-like protein 3	DML3_ARATH	26
	Unknown protein from 2D-PAGE of needles (N140) (Fragments) - *Pinus pinaster* (Maritime pine)	UN03_PINPS	26
Allium sativum (Fraction II)	Probable glycine cleavage system H protein 2, mitochondrial precursor - *Arabidopsis thaliana*	GCSH2_ARATH	35
	Thioredoxin F-type, chloroplast precursor (TRX-F) - *Brassica napus*	TRXF_BRANA	29
	Annexin D6 (AnnAt6) - *Arabidopsis thaliana*	ANXD6_ARATH	24

Allium sativum protein/peptide two fractions obtained from HPLC with a C-18 reverse phase column. The table shows three protein-peptides identified by MALDI TOF/TOF analysis with the highest protein score for each fraction.

Table 4.7: Protein-peptide analysis of *Momordica charantia* protein/peptide extract by MALDI TOF/TOF

Sample source	Protein name	Accession number	Protein Score
Momordica charantia (Fraction I)	Trypsin inhibitor 3 (Trypsin inhibitor III) (MCoTI-III) - *Momordica cochinchinensis*	ITR3_MOMCO	22
	Trypsin inhibitor 2 (Trypsin inhibitor II) (BDTI-II) - *Bryonia dioica*	ITR2_BRYDI	19
	Trypsin inhibitor 1 (Trypsin inhibitor I) (CVTI-I) – *Citrullus lanatus*	ITR1_CITLA	18

Momordica charantia protein/peptide fraction obtained from HPLC with a C-18 reverse phase column. The table shows three protein/peptides identified by MALDI TOF/TOF analysis with the highest protein score for each fraction.

Table 4.8: Protein-peptide analysis of *Momordica charantia* seeds protein/peptide extract by MALDI TOF/TOF

Sample source	Protein name	Accession number	Protein Score
Momordica charantia seeds (Fraction I)	Fructose-bisphosphate aldolase 1, chloroplast precursor - *Chlamydomonas reinhardtii*	ALFC_CHLRE	38
	DNA ligase 4 (EC 6.5.1.1) (DNA ligase IV) (Polydeoxyribonucleotide synthase [ATP] 4) –Arabidopsis	DNL4_ARATH	24
	SufE-like protein, chloroplast precursor – *Arabidopsis thaliana*	SUFE_ARATH	23
Momordica charantia seeds (Fraction II)	Chloroplast 30S ribosomal protein S7 – *Zygnema circumcarinatum*	RR7_ZYGCR	40
	Ribulose bisphosphate carboxylase small chain, chloroplast precursor	RBS_SACHY	39
	Ribosome recycling factor, chloroplast precursor (Ribosome-releasing factor, chloroplast)	RRFC_SPIOL	33
Momordica charantia seeds (Fraction III)	Hypothetical mitochondrial protein AtMg00970 (ORF117) - *Arabidopsis thaliana*	M970_ARATH	33
	Chloroplast 50S ribosomal protein L33 - *Populus alba*	RK33_POPAL	30
	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	ARD4_ORYSA	28
Momordica charantia seeds (Fraction IV)	Histone H2B.1 (H2B-I) - *Chlamydomonas reinhardtii*	H2B1_CHLRE	43
	Tubulin beta-3 chain (Beta-3 tubulin) (Fragment) - *Anemia phyllitidis*	TBB3_ANEPH	42
	Uncharacterized protein At2g41620 – *Arabidopsis thaliana*	Y2162_ARATH	40

Momordica charantia seeds protein/peptide four fractions obtained from HPLC with a C-18 reverse phase column. The table shows three protein/peptides identified by MALDI TOF/TOF analysis with the highest protein score for each fraction.

CHAPTER 5

DISCUSSION

Discussion

Discussion

5.1 Yields of ethanolic extracts

The most commonly used solvents for investigations of antimicrobial activity in plants are methanol, ethanol, and water (Parekh *et al*., 2005; Bisignino *et al*., 1999; Lourens *et al*., 2004; Rojas *et al*., 2006). Most antimicrobially active components that have been identified are not water soluble and thus organic solvent extracts have been found to be more suitable (Nair *et al*., 2005; Parekh *et al*., 2006). Water-soluble compounds, such as polysaccharides and polypeptides, including fabatin and various lectins, are commonly more effective as inhibitors of pathogen adsorption and have no real impact as antimicrobial agents (Cowan, 1999). Therefore 95% ethanol solvent was used to extract the dried plant materials because it is commonly used for extraction and this experiment is mainly focusing on antimicrobial properties of the samples.

The extraction duration was shortened and made more effective by grinding the plant materials to fine powder. The samples were left for 3 days on a shaker and the solvent was later evaporated using rotary evaporator. According to a study done by Eloff (1998), 5 minutes extraction of very fine particles of diameter 10µm gave higher quantities than values obtained after 24 hours in a shaking machine with less finely ground material. Some researchers employ Soxhlet extraction of dried plant material using organic solvents (Kianbakht and Jahaniani, 2003). In the Soxhlet extraction, the sample is continually exposed to fresh solvent which improves the efficiency of the method. This method works well with compounds that can withstand the heat but is not suitable for thermo-labile compounds as prolonged heat could degrade the compound of interest (De Paira *et al*., 2004). Result in table 4.1 shows that fermented extract sample yielded the highest amount from the original sample at 44.65% compared to the rest of

the treatment samples. The list is followed by functional food paste at 29.81%, fermented vinegar with lactic acid bacteria 19.81% and fermented bean 14.62%.

From the results obtained, the yields from fermented samples are higher as compared to others. This is attributed to a significant increase in the soluble fraction of a food substrate during fermentation. Fermentation results in a lower proportion of dry matter in the food and the concentrations of vitamins, minerals and protein appear to increase when measured on a dry weight basis (Adams, 1990). By tradition, lactic acid bacteria (LAB) are the most commonly used microorganisms for preservation of foods. Their importance is associated mainly with their safe metabolic activity while growing in food media utilising available sugar as carbon source for the production of organic acids and other metabolites.

5.2 Yields of protein/peptide extracts

There are four spectroscopic methods that are commonly used to determine the concentration of protein in a solution (Stoscheck, 1990). These include measurement of the protein's intrinsic UV absorbance and three other methods which generate a protein-dependent color change namely the Lowry assay (Lowry *et al.*, 1951), Smith copper/bicinchoninic assay (Smith *et al*., 1985) and the Bradford dye assay (Bradford, 1976). The UV absorbance method is simple and fast but it requires a pure protein with known extinction coefficient in a solution free of other interfering UV absorbing substances and the measurements should be made using a quartz cuvette which is quite expensive.

The Lowry and copper/bicinchoninic assays are based on reduction of Cu^{2+} to Cu^{+} by amides. Although it makes them potentially quite accurate, they require the preparation

of several reagent solutions, which must be carefully measured and mixed during the assay. This is followed by lengthy, precisely timed incubations at closely controlled, elevated temperatures, and then immediate absorbance measurements of the unstable solutions. The lengthy procedures are susceptible to errors and produce inconsistency in the results. Both assays may be affected by other substances frequently present in biochemical solutions, including detergents, lipids, buffers and reducing agents (Stoscheck, 1990).

A rapid method for the estimation of protein concentration is essential in many fields of protein study. An assay originally described by Bradford (Bradford, 1976) has become the preferred method for quantifying protein in many laboratories. This technique is faster, involves fewer mixing steps, does not require heating, and gives a more stable colorimetric response than the other assays. Moreover, when compared with the Lowry method, it is subject to less interference by common reagents and non protein components of biological samples.

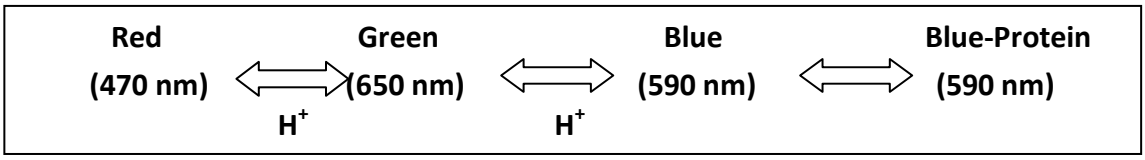

Figure 5.1: The colour changes that occur during Bradford assay

Therefore in this study the Bradford assay method was performed. The Bradford dye assay was based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acid conditions, the dye was most stable as a doubly-protonated red form. Upon binding to protein, however, it was most stable as an unprotonated, blue form. Figure 5.1 shows the colour changes that occur during Bradford assay. In this study the samples were extracted with the extraction buffer and centrifuged. Next the

supernatant was collected and freeze dried. The freeze dried samples were reconstituted in 5 ml of buffer and the protein concentration was measured.

The current study shows that *Allium sativum* yielded the highest amount of protein/peptide extract at 1.82mg/g followed by *Andrographis paniculata* protein/peptide extract at 1.77mg/g. The amounts of protein/peptide extracted from both samples are significantly the same. The result was followed by *Zingiber officinale* protein/peptide extract at 1.56mg/g. *Zingiber officinale* protein/peptide extract were significantly lower compared to *Andrographis paniculata* and *Allium sativum* protein/peptide extract. *Allium sativum* yielded the highest amount of protein/peptide because it contains high level of protein, according to USDA Nutrient Database for every 100g of raw *Allium sativum* it contain 6.39g of protein. Raw *Zingiber officinale* on the other hand contains only 1.82g of protein for every 100g (USDA Nutrient Database). Next the list was followed by both *Curcuma mangga* and *Cymbopogan citratus* at 1.36mg/g. Statistical analysis between the latter two quantities are significantly the same. The lowest total extractable protein/peptide was obtained from *Momordica charantia* seeds at 0.49mg/g. *Momordica charantia* yielded the lowest total extractable protein/peptide because most of the protein in *Momordica charantia* is available in its leafy tips. The leafy tips of raw *Momordica charantia* contains 5.30g of protein whereas the fruiting body contains only 1g of protein for every 100 g (USDA Nutrient Database).

5.3 Identification of test microorganisms

Prior to doing the antimicrobial susceptibility testing assay the bacterial species were confirmed by molecular methods. Bacterial identification was done using a rapid and reliable 16S rDNA sequence analysis method. The bacteria was identified based on the

highest score (bits) and lowest E value of the BLAST result. The bacteria identified are *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus* and *Staphylococcus aureus. Escherichia coli* and *Pseudomonas aeruginosa* are Gram negative bacteria whereas *Bacillus cereus* and *Staphylococcus aureus* are Gram positive bacteria.

5.4 Antimicrobial activity

All the antimicrobial susceptibility testing procedures was in accordance to the guidelines for performing susceptibility testing which was published by the Antimicrobial Susceptibility Testing Subcommittee of the National Committee for Clinical Laboratory Standards (NCCLS). The NCCLS subcommittee develops standard methods for susceptibility testing, provides quality control parameters, establishes interpretive criteria, provides suggestions for testing and reporting strategies that are clinically relevant and cost-effective, and develops methods to detect emergence of resistance in microorganisms (NCCLS, 1997 a, b; NCCLS 1998).

Muller Hinton medium was used for all antimicrobial susceptibility testing because this formula conforms to National Committee for Clinical Laboratory standards (NCCLS, 1997 a, b). Mueller Hinton medium contains heat stable ingredients (Gordon and Hine, 1916). Bauer *et al*. (1966) recommended Mueller Hinton agar for performing antibiotics susceptibility tests using single disk of high concentration (refer to chapter under Materials and Methods). This non supplemented medium was chosen by NCCLS as the antimicrobial susceptibility testing because this medium is low in sulphonamide, trimethoprim and tetracycline inhibitors which provides satisfactory growth of most non-fastidious pathogens and demonstrates batch to batch reproducibility.

The antimicrobial assay was performed against both Gram positive and Gram negative bacteria so that it can be determined whether the extracts tested are having broad, narrow or limited spectrum. Extracts effective against pathogen that kill or inhibit a wide range of Gram positive and Gram negative bacteria are referred as broad spectrum. If they are effective mainly against Gram positive or Gram negative bacteria, they are narrow spectrum. Lastly, if they are just effective against a single organism, they are referred to as limited spectrum.

Lipopolysaccharide (LPS) is the major component of the pathogenic Gram negative bacteria whereas Gram positive bacterium lacks LPS and instead they have lipoteichoic acid (LTA) on their cell wall. LTA was reported capable of stimulating the innate immunity and contributing to septic shock. Both Gram positive and Gram negative bacteria differ in the structural details and the signaling transduction (Ryu *et al*., 2009). Therefore both Gram positive and Gram negative bacteria should be included to determine the antimicrobial properties of the extracts.

5.4.1 Disk diffusion assay

In the current study *Andrographis paniculata* showed the most promising result for the ethanolic extracts with inhibition of 14mm against *B. cereus*, 11.5mm against *P. aeruginosa*, and 11mm against *S. aureus*. Whereas protein/peptide extract of *Andrographis paniculata* did not inhibit any bacteria. Results from the current sudy contradict the result obtained by Singha *et al*. (2003). A study done by Singha *et al*. (2003) showed that aqueous extract and arabinogalactan proteins fraction inhibits *E. coli* and *P. aeruginosa* but not *S. aureus*. Besides that Singha *et al*. (2003) also reported that 80% methanol and chloroform extraction of *Andrographis paniculata* did not show inhibition against *E. coli*, *P. aeruginosa* and *S. aureus*. This could be due to different

solvents and their variable concentrations that are used to extract the plant material. Moreover the strain of the bacteria used could also affect the results.

In the current study protein/peptide extracts of *Allium sativum* showed promising results with 15mm inhibition against *E. coli*, 28mm against *S. aureus*, 16.3mm against *B. cereus* and 9mm against *P. aeruginosa*. In the previous study, Jabar and Al-Mossawi (2007) used water extract and raw juice of *Allium sativum* to conduct disk diffusion and MIC antimicrobial test. The result obtained by Jabar and Al-Mossawi (2007) showed that *Allium sativum* showed promising result against *E. coli* and *S. aureus* for both water extract and fresh juice of *Allium Sativum*. Besides that in the previous study, Daljit and Jasleen (1999) conducted antimicrobial activity of *Allium sativum* aqueous extract and obtained inhibition zone of 20mm against *E. coli, S. aureus* and *P. aeruginosa.*

Allium sativum was shown to inhibit the growth of Gram positive, Gram negative and acid-fast bacteria, as well as toxin production by the bacteria (Delaha and Garagusi, 1985). *Allium sativum* also inhibited beneficial intestinal microflora, but it is more effective against potentially harmful enterobacteria. This is probably due to greater sensitivity of enterobacteria to allicin (Miron *et al*., 2000). *Allium sativum* has also been nicknamed Russian penicillin for its widespread use as a topical and systemic antimicrobial agent (Agarwal, 1996). Although much has been reported on the medicinal properties of *Allium sativum* (Ali *et al*., 2000) not much has been reported for its proteinaceous constituents (Smeets *et al*., 1997).The present study shows that the antibacterial protein activity of *Allium sativum* is similar to that of other regions and thereby an added information in scientific literature showing benefits in terms of health and well being to the consumer as a functional food.

5.4.2 MIC and MBC assay

The plant and fermented samples that showed inhibition were selected to test for minimum inhibitory concentration (MIC). This can be achieved by dilution of antimicrobials in either agar or broth media. In this study MIC was determined by doing broth dilution assay. Broth dilution assay was chosen because it has the added advantage that the same tubes can be taken for minimum bacterial concentration (MBC) test as well. The MIC of an antibacterial compound is defined as the lowest concentration that will still inhibit the growth of the test bacteria. Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents.

MBC is defined as the lowest concentration where no bacterial growth is observed (bactericidal concentration). This is determined from the broth dilution resulting from the MIC tubes by sub culturing to antimicrobial free agar as described by Vollekova *et al*. (2001). The lowest concentration of the extract which shows no bacterial growth is noted and recorded as the MBC.

Ethanolic extract of *Andrographis paniculata* shows MIC and MBC values below 10mg/ml for both *B. cereus* and *S. aureus*. This result proves that *Andrographis paniculata* extract at a concentration of 10mg/ml is both bacteriostatic and bacteriocidal. The other ethanolic extracts tested only show bacteriostatic properties by limiting the growth of the bacteria. Mishra *et al*. (2009) tested *Andrographis paniculata* ethanolic extract against 11 different bacteria by disk diffusion and MIC methods. In her study *Andrographis paniculata* ethanolic extract inhibited *E. coli* K-12, *S. aureus*-2737, *S. aureus*-ML-50 and various other bacteria. The results obtained by Mishra *et al*.

(2009) showed that *Andrographis paniculata* extract is capable of inhibiting *S. aureus* which is similar with this study but on the other hand *Andrographis paniculata* extract was not able to inhibit *E. coli* as stated by Mishra *et al*. (2009). This contradiction could be due to different strains of bacteria used and variable methods used to extract the sample. In my study, on the other hand, protein/peptide extracts of *Allium sativum* showed some promising results in the MIC values by inhibiting the growth of the bacteria tested. The MBC results showed that *Allium sativum* is only bacteriostatic and not bacteriocidal.

5.5 Antioxidant activity

The formation of free radicals reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated as the cause of food oxidative deterioration. Besides, free radicals have also been implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, diabetes mellitus, atherosclerosis, cataracts, chronic inflammation, and neurodegenerative diseases, such as Alzheimer's or Parkinson's disease (Gutteridge, 1993; Knight, 1995; Frankel and German, 2006; Valko *et al.*, 2007).

5.5.1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) which catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen is one of the most important antioxidative enzymes (Weisiger and Fridovish, 1973). Numerous direct and indirect methods for the assay of SOD have been developed since the discovery of SOD (McCord *et al.*, 1976). Indirect SOD assays are more popular compared to direct ones due to their convenience and ease of use. The most commonly used indirect method involves enzymatic generation of the superoxide anion in the assay medium and

competition between the superoxide scavenger and the SOD-catalyzed dismutation of superoxide. Nitroblue Tetrazolium (NBT) is a method that is commonly used in SOD assay. However, NBT will form mono- and di-formazan, which tends to precipitate in the water. Precipitation during the reaction can give variability in absorbance readings and does not allow adaptation to a reliable microtiter plate assay. Therefore this property is not suitable for a precise assay of SOD. In this assay a water-soluble tetrazolium salt, WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt] was used. WST-1 has the highest solubility and sensitivity compared to other tetrazolium salts with the same SOD preparation (Haraguchi *et al*., 1998). Therefore SOD assay using WST-1 tetrazolium salts was used for this study.

There is no significant difference between the inhibition rate of Vitamin C at 1mg/ml and 10mg/ml. *Allium sativum* showed high inhibition rate at 93.00% and 92.73% for ethanolic and protein/peptide extract respectively. A research study done by Metwally (2009) showed that fish fed on diets constituted with *Allium sativum* showed significant increase in SOD activity in blood serum and in liver tissue homogenates. Besides that there is also studies showing that *Allium sativum* powder increases the antioxidant capacity in hamster (Yaoling *et al*., 1998). *Allium sativum* oil and its component enhanced SOD activity in liver (Lee *et al*., 1999). Carmia (2001) reported that it also enhances the cellular antioxidant enzymes SOD in the cells.

Ethanolic extract of *Cymbopogon citratus* showed high inhibition rate at 91.50% for SOD assay which is 1.5% lower compared to *Allium sativum*. The rhizome of *Cymbopogan citratus* has long ago been used in oral healthcare. Besides that it's also used as a diuretic and an abortifacient (Morton, 1981). The essential oil also shows

significant antimalarial activity in the four day suppressive *in vitro* tests in mice (Tchoumbougnang, 2005). In the present study, *Momordica charantia* seeds showed the highest inhibition rate for protein/peptide extracts at 93.61%. This result is similar to that done by Sathishekar and Subramanian (2005) on antioxidant properties of *Momordica charantia* seeds. They reported that administration of *Momordica charantia* showed hypoglycemic effect reducing blood glucose level and thereby preventing the formation of free radicals or it may scavenge the reactive oxygen metabolites through various antioxidant compounds in them. Besides that, study done by Sathishsekar and Subramanian (2005) showed that rats treated with *Momordica charantia* seeds reduces reactive oxygen species (ROS) and improves the activities of antioxidant enzymes. A study done by Sekar (2005) showed that glucose level was significantly lower after oral administration of the aqueous extracts prepared from *Momordica charantia* seeds. Different parts of this plant have been used in the Indian system of medicine (ayurveda) for a number of ailments besides diabetes (Ganguly *et al*., 2000; Jayasooriya *et al*., 2000).

5.5.2 DPPH

DPPH is a stable free radical and has been widely used to evaluate the free radical scavenging ability of various botanical materials (Amarowicz *et al*., 2004). Antioxidants, on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character. A purple coloured DPPH is a stable free radical, which is reduced to α,α-diphenyl-β-picryl hydrazine (yellow colored) by reacting with an antioxidant. Antioxidants interrupt radical chain oxidation by donating hydrogen from hydroxyl groups to form a stable end product, which does not initiate or propagate further oxidation of lipids (Castro *et al*., 2006). *Curcuma mangga* shows the highest scavenging activity at the value of 86.46%. The scavenging activities of

Curcuma mangga and vitamin C showed the same value at a concentration of 10mg/ml. *Curcuma mangga* contains high amount of curcumin (Abas *et al*., 2005). Curcumin is the phytochemical that gives a yellow color to turmeric and is now recognized for most of the therapeutic effects (Sharma *et al*., 2005). Although ethanolic extract of *Cymbopogon citratus* shows high SOD activity and significantly lower erythrocyte hemolysis, it shows relatively low DPPH radical scavenging activity. Yoo *et al*. (2008) also reported that *Cymbopogon citratus* shows lower DPPH radical scavenging activity compared to the other extracts tested.

In the current study, the results from DPPH assay of protein/peptide extracts showed very low positive DPPH activity. This is because the protein/peptide precipitated in the alcohol reaction medium. The steric accessibility of DPPH radicals is a major determinant of the reaction because smaller molecules which are easily accessible to the radical site will eventually have higher antioxidant capacity (Huang *et al*., 2005). On the other hand, larger antioxidant compounds might react slower or even be inert in this assay. Therefore DPPH assay is not suitable for protein/peptide extracts.

5.6 Hemolysis

Oxidative stress plays an important role in many physiological and pathological disorders. Free radicals are known to play a pivotal role in tissue damage, as well as have an adverse effect on erythrocytes. Erythrocytes have been used as a model to investigate oxidative damage in biomembranes because of their high vulnerability to peroxidation. Although there is extensive antioxidant defense system to protect the erythrocyte, oxidative damage of membrane protein and lipids that contribute to the senescence of normal cells will result in a shorter life span for damaged cells. Autoxidation of oxyhemoglobin is the major source of intracellular ROS in the

erythrocyte which generates superoxide and through dismutation produces hydrogen peroxide (Nagababu and Rifkind, 2003). There is a correlation between exercise and oxidative stress. Oxidative stresses that occur during exercise in various tissues and blood can be prevented by antioxidant interventions in animals and humans (Sen, 1995; Ji, 1995). Besides that antioxidant vitamin supplementation could also prevent certain hemolytic situations due to non exertion from oxidative stress (Etlik *et al.*, 1995).

Ethanolic extract of *Cymbopogon citratus* exhibits the lowest hemolysis in erythrocyte at 17.09% compared to non pretreated erythrocyte at 58.90%. The result showed that *Cymbopogon citratus* has the strongest effect in lowering the hemolysis of erythrocyte compared to other samples tested. De Freitas *et al*. (2008) reported that aqueus extracts of *Cymbopogon citratus* protects human erythrocytes against hypotonic shock preventing it from hemolysis. Besides that *Cymbopogon citratus* was believed to have anxiolytic, hypnotic, anticonvulsant and antifungal properties (Shadab, 1992). Besides, it also acts as an antidepressant and as mood enhancer (Blanco, 2009).

The list is followed by ethanolic extract of *Andrographis paniculata* at 19.45%. *Andrographis paniculata* is a remedy for a number of ailments related to digestion, hepatoprotection, vermicidal, anti acne, analgesic, anti-inflammatory, antibacterial, antityphoid, antibiotic activities, hypoglycemic, and immune enhancement (Matsuda *et al*., 1994). The result shows that ethanolic extract of *Andrographis paniculata* is not significantly different compared to *Cymbopogon citratus* in lowering the hemolysis of erythrocytes. Akowuah *et al*. (2009) reported that methanolic extracts of *Andrographis paniculata* leaves were shown to exhibit significant lipid peroxidation inhibition and free radical scavenging activity using *in vitro* models. The results of this study support the results obtained in their research. On the other hand the result obtained by this study

contradicts the result obtained by Liu and Ng (2000). Liu and Ng (2000) reported that *Andrographis paniculata* extract inhibited generation of superoxide and hydroxyl radicals but not hemolysis or lipid peroxidation. This could be due to different extraction methods used. Liu and Ng (2000) soaked the dried *Andrographis paniculata* leaves in water at room temperature overnight and then boiled under reflux for 2 hours whereas in this research the *Andrographis paniculata* leaves was soaked in ethanol and the temperature of the extract was maintained below 40ºC throughout the research.

Protein/peptide extract from the fruiting body of *Momordica charantia* and the stem of *Agaricus bisporus* shows the lowest hemolysis activity at 13.87% and 13.60% respectively. Both of the protein/peptide extracts are not significantly different but they are significantly lower compared to the non-pretreated erythrocyte. The result obtained from this research is in line with the research done by Barros *et al*. (2008). Barros (2008) proved that *Agaricus bisporus* has antioxidant properties, namely free radical scavenging activity and lipid peroxidation inhibition capacity.

5.7 Toxicity studies

This study was conducted using the fixed-Dose Procedure method. The fixed-dose procedure method was first proposed by the British Toxicology Society in 1984 (British Toxicology Society, 1984). This procedure was incorporated into the OECD guidelines 420 (OECD, 1992). The result obtained could be interpreted in relation to animal survival and evidence of toxicity. It is possible to assign the chemical into OECD classification categories (Fielder, 1995). In a study done with 2000 animals it was found that animal models overall is a good predictability for human toxicity in 71% of the cases, with 63% for nonrodents alone and 43% for rodents alone (Olson *et al*., 2000)

The result obtained from the acute toxicity test was used to study the biological activity of the chemical and gain insight into its mechanism of action. Information that is acquired from the test is used in hazard identification and risk management in production, handling and use of chemicals. The LD_{50} value, when administered in an acute toxicity test with expected death rate of 50% of the treated animals in a given period of time, is currently the basis for toxicological classification of chemicals. The dosed animals are closely observed during the first 24 hr and then day by day for as long as 2 weeks (Rhodes *et al*., 1993).

The acute toxicity results of all four plant extracts namely *Andrographis paniculata*, *Curcuma mangga*, *Cymbopogon citratus* and *Allium sativum* tested did not show any toxic symptoms or mortality at 2500mg/kg doses. These results confirmed that all four plants do not cause any acute toxic effect. Under the system of classification that was set by OECD all three samples are classified as no label, therefore they are considered non toxic samples. Carlini *et al*. (1986) also reported that high doses of the essential oil from *Cymbopogon citratus* showed no adverse effects on either the intestinal or central nervous systems in animal studies. Therefore, the extracts in the study are suitable for development and use in functional foods and nutraceuticals.

5.8 High Performance Liquid Chromatography (HPLC)

Reverse-phase high performance liquid chromatography (RP-HPLC) has become the most widely used technique in the separation and analysis of protein and peptides. Its ability to resolve both small peptides and large proteins has made it an essential analytical technique (Larive *et al*., 1999).

The chromatograms were obtained with 1ml/min flow rate of 0.1% TFA in ultrapure water at ambient temperature. All the samples were run separately for durations of 10 minutes. The samples were run several times to make sure the retention time of the chromatogram are the same. *Allium sativum* protein/peptide extracts consist of two fractions with the retention times of 1.9 minutes and 3.1 minutes. *Momordica charantia* protein/peptide extracts consist of one fraction with the retention time of 1 minute and *Momordica charantia* seeds protein/peptide extracts consist of four fractions with the retention times of 1.8 minutes, 2.6 minutes, 3.1 minutes and 3.4 minutes. The fractions of the repeated runs were collected and pooled together for MALDI TOF/TOF analysis.

5.9 Matrix Assisted Laser Desorption/Ionization (MALDI) Time of Flight (TOF)/TOF

MALDI mass spectrometry has been shown to be a robust technique tolerating considerable salt loads and permitting the analysis of complex mixtures because of the soft ionization (Goheen *et al.*, 1997). The plant extracts were fractionated with HPLC on a C-18 reverse phase column. The fractions collected were used for MALDI TOF/TOF analysis. *Allium sativum* protein/peptide extract consists of two fractions. The proteins tentatively identified with the highest protein score for fraction I of *Allium sativum* to be wound-induced proteinase inhibitor 2 precursor with the protein score of 37%, and DEMETER-like protein 3 with protein score of 26%.

Wound-induced proteinase inhibitor 2 precursor is a potent inhibitor of both trypsin and chymotrypsin. They are induced due to mechanical damage to the plant tissue resulting in systemic release from the wound sites (Graham *et al.*, 1985). DEMETER-like protein 3 functions as a potential transcriptional activator that may act by nicking the target promoter. It catalyzes the release of 5-methylcytosine (5-meC) from DNA by a

glycosylase/lyase mechanism (Mayer *et al.,* 1999). The protein fraction that was identified with the highest protein score for fraction II of *Allium sativum* is glycine cleavage system H protein 2 with protein score of 35%. The glycine cleavage system catalyzes the degradation of glycine (Theologis *et al.,* 2000).

Momordica charantia protein/peptide extract consists of one fraction. The proteins identified with the highest protein score for *Momordica charantia* are trypsin inhibitor 3, trypsin inhibitor 2 and trypsin inhibitor 1 with protein score of 22%, 19% and 18% respectively. Trypsin inhibitor 3 functions to inhibit trypsin and probably participates in a plant defense mechanism (Hernandez *et al.,* 2000).

Momordica charantia seeds protein/peptide extract consists of four fractions. The proteins identified with the highest protein score for fraction I of *Momordica charantia* seeds is fructose-bisphosphate aldolase 1, chloroplast precursor with protein score of 38%. Fructose-bisphosphate aldolase plays an important role in the glycolytic pathway. The proteins identified with the highest protein score for fraction II as chloroplast 30S ribosomal protein S7 with protein score of 40%. It functions as a primary rRNA binding protein and it binds directly to 16S rRNA where it nucleates assembly of the head domain of the 30S subunit (Turmel *et al,* 2005). For fraction III the proteins identified with the highest protein score was the hypothetical mitochondrial protein with the protein score of 33%. Lastly the proteins identified with the highest protein score for fraction IV is histone H2B.1 with protein score of 43%. This protein plays a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability (Walther and Hall, 1995).

CHAPTER 6

CONCLUSION

Conclusion

Andrographis paniculata extract showed the most promising result for the ethanolic extracts samples with inhibition zones of 14mm against *B. cereus*, 11.5mm against *P. aeruginosa*, and 11mm against *S. aureus* while *E.coli* showed no inhibition. These results were comprehensive as it encompassed gram positive and gram negative test microorganisms for a plant that is now grown indigenously unlike earlier published studies.

The screening of the plants used in the study indicated the scope of Malaysian grown turmeric based on ethanolic extracts. This result attributes to useful functional food property of tumeric in terms of antimicrobial and antioxidant values. The same is also true of lemon grass that is used for aroma in Malaysian cuisine such as *nasi lemak.* Lemon grass is also justifiably used in aromatherapy based on the bioactivity shown in this study.

The results from this study prove that *Andrographis paniculata* extract at a concentration of 10mg/ml is not only inhibitory to bacterial growth but also have bacteriocidal properties. Protein/peptide extracts of *Allium sativum* showed some promising results in the MIC values by inhibiting the growth of the bacteria tested. The MBC results in this study also specifically showed that *Allium sativum* extract is only bacteriostatic but do not have bactericidal properties.

Ethanolic extract of *Cymbopogon citratus* exhibits the lowest hemolysis in erythrocyte at 17.09% compared to non pre treated erythrocyte at 58.90%. The list is followed by ethanolic extract of *Andrographis paniculata* at 19.45%. Protein / peptide extract from the fruiting body of *Momordica charantia* and the stem of *Agaricus bisporus* showed

the lowest hemolysis activity. Both of the protein/peptide extracts are not significantly different but are significantly lower compared to the non-pretreated erythrocyte. The erythrocyte hemolysis showed to be a reliable method in the study based on the replicability of the results.

The scavenging activity of *Curcuma mangga* extract and vitamin C showed to be significantly similar at the concentration of 10mg/ml. *Curcuma mangga* extract contains high amount of curcumin that contributes to therapeutic effects. Although ethanolic extract of *Cymbopogon citratus* showed high SOD activity and significantly lowered the erythrocyte hemolysis, it showed relatively low DPPH radical scavenging activity.

The acute toxicity results of all four plant extracts namely *Andrographis paniculata*, *Curcuma mangga*, *Cymbopogon citratus* and *Allium sativum* tested did not show any toxic symptoms or mortality at 2500mg/kg doses. These results confirmed that all four plants do not cause any acute toxicity. This is an important quality for plant samples that may be used as functional foods.

Suggestions and Future Work

The MALDI results obtained did not yield any conclusive results. Future work should be done to enable the crude extracts to be further resolved into pure fractions using HPLC methods coupled with preparative column to determine the characteristics of specific peptides based on their antimicrobial and antioxidant properties. This can provide more conclusive results with MALDI. The final results may be promising in terms of developing biopharmaceuticals and nutraceuticals.