2.0 LITERATURE REVIEW

2.1 Medicinal mushrooms

Since Greek and Roman antiquity, mushroom has been used as food and medicine (Anke, 1989). The ancient Roman believed mushrooms to be 'the food of the Gods', the Egyptians considered them to be 'a gift from Osiris' and the Chinese regarded them as 'the elixir of life' (Samorini, 2001). Many cultures worldwide, especially in the orient recognized that extracts from certain mushrooms could have profound promoting benefits, and consequently became essential components in many traditional Chinese medicines. In fact, mushrooms have seen a summation of more than 3000 years of use for the prevention and treatment of diseases in Chinese medicine (Chang, 2006).

Mushrooms produce a large number of metabolites that show antibacterial, antifungal, antiviral, antitumor, hypoglycemic, anti allergenic, immunodulating, anti-inflammatory, hypolipidemic, and hepatoprotective activity (Yamac and Bilgili, 2006). There are at least 270 species of mushrooms that known to posses various therapeutic properties and the term ‘medicinal mushroom’ is now increasingly gaining worldwide recognition (Ying *et al.*, 1987).

Medicinal mushrooms are macrofungi that contain pharmaceutical substances with potent and unique health enhancing properties. They posses enormous potential and should be included into the medical biotechnology industry for the benefits of humankind (Wasser and Weis, 1999).

Historically, most medicinal mushrooms species were relatively scarce and were collected from the forest where they grew on dead or living trees and forest litter. They are

predominantly lignocelluloses degraders. For medicinal purposes, they were almost always prepared either as hot water extracts, concentrates or in powdered. To date, almost all of the important medicinal mushrooms have been subjected to large-scale artificial cultivation by solid substrate or low moisture fermentation, thus removing the historical scarcity factor and allowing large commercial operation to develop (Stamets, 2000).

2.2 Pharmacological potential of medicinal mushrooms

There have been a number of reviews published on the bioactive substances found in mushrooms and their medicinal properties (Rai *et al*., 2005; Stamets, 2002; Lindequist *et al*., 2005). Mushroom has many significant medicinal values. Experiences from Asian and Eastern countries show that mushroom could play important role in cancer prevention and treatment. Besides, in some East Asian countries, Japan, China and Korea, mushrooms are used as 'immunomodulator' (biological response modifier, immune potentiators and immuno stimulants). They are the most important medicinal mushroom drugs used in these countries (Lindequest *et al*., 2005). The used of mushroom *I. obliquus* as a folk medicine for cancer and stomach have also been reported in Eastern Europe since the 16th and 17th century (Molitoris, 1994).

Besides the ability of many mushroom to stimulate the immune system, some medicinal mushroom were demonstrated to suppress immune responses. This is very attractive mechanism especially for the treatment of allergic diseases. Anti allergic affects of few ethanol extracts have been investigated in-vivo (Sano *et al*., 2002). Furthermore, eating of *Tricholoma populinum* J. E. Lange was proven to lead to the regression of severe

allergic symptoms in a patient with thrombongitis obliterans and in another patient with urticaria (Kreisel *et al*., 1990).

The mushrooms also have the ability to lower blood pressure and free cholesterol in plasma, as well as accelerate the accumulation of lipid in the liver by removing them from circulation (Rai *et al*., 2005). The control of blood lipids especially the cholesterol is important in reducing the risk of atherosclerosis development. A pronounced hypocholesteremic effect of *Pleurotus ostreatus* with inhibition of lipid peroxidation was demonstrated in rats and rabbits. Ten percent intake of dried fruiting bodies of this mushroom has significantly reduced the incidence and size of atherosclerotic plaques in rabbits (Bobek *et al*., 1998). Gunde-Cinerman (1999) has suggested that *Pleurotus* mushroom could be recommended as a neutral cholesterol lowering substances in the human diet. Biosynthesis of cholesterol was also inhibited by some of Triterpenes from *Ganoderma lucidum* (Komoda *et al*., 1989).

In addition to medicinal potentials mention above, mushrooms also exhibit anti-inflammatory effect. This is supported by studies conducted by Kim *et al.,* in 2003 and 2004. In these studies, ethanolic extracts and a proteoglycan from *P. linteus* showed anti-inflammatory effect in the collagen-induced arthritis and the croton oil-induced ear edema test in mice and antinociceptic effect in the writhing test (Kim *et al*., 2003; Kim *et al*., 2004).

2.3 Cultivation of medicinal mushrooms

Cultivation of mushroom is a global practice. To date, there are only 20 mushroom species currently cultivated on an industrial scale out of about 35 mushroom species

cultivated commercially. World production of mushrooms is increasingly being dominated by species that are both edible and have medicinal properties or only medicinal such as *Ganoderma* and *Trametes* (Smith *et al*., 2002). According to Chang (1999), the overall production of cultivated edible and/or medicinal mushrooms was recorded as 4909 x 10^3 tons in 1994, increasing to 6158 x 10^3 in 1997, with an estimated value in access of 14 billion US dollar.

Medicinal mushrooms can be cultivated through a variety of methods. Production of the fleshy mushroom fruit-bodies utilizes various forms of solid substrate or low moisture fermentations whereas for mycelial biomass production, liquid tank fermentations are now becoming increasingly important particularly for nutriceutical and pharmaceutical productions (Smith *et al*., 2002).

According to Stamets (2000), the rapid worldwide cultivation of medicinal mushroom is due largely to the use of specially designed polypropylene bags or containers with microfilter window for ion exchange. The bags contain the substrate of sawdust and selected nutrients, and sterilized prior to inoculation with the mushroom mycelia. The entire growing process is carried out under controlled environmental conditions over a reduced time scale (1-3 months). The composition of basic substrate and supplementary ingredients can vary considerably since the basic raw materials are derived from lignocellulosics from agriculture or forestry, thus there will be some degree of variation in size and age which will influence specific biochemical composition of mushrooms (Gunde-Cimerman, 1999; Wasser *et al*., 2000). This is not critical for the production of fruit bodies for fresh market but undoubtedly could create problems and preclude standardization of the extracted products for nutriceuticals and pharmaceuticals purposes.

Submerged cultivation of mushroom mycelia in liquid media is a promising method used due to the advance in biotechnology. As stated by Smith *et al*. (2002), submerged pure culture fermentation techniques have been widely developed for most of the main medicinal mushrooms and used in the propagation of mycelia for three main applications: (1) liquid spawn for solid substrate fruit-body production; (2) biomass that can be used for food and dietary supplements; and (3) biomass and/or extruded metabolites especially exo-polysaccharides as raw materials for pharmaceutical studies. In all cases, the underlying principle in each approach is to use mycelia in the active physiological state and of known purity. Submerged cultivation in liquid media provides fast growth and high productivity of desired specific compounds in optimum physical-chemical conditions thus enhance the nutritional and nutraceutical values of the mycelia (Wasser *et al*., 2000).

2.4 *Ganoderma*

Ganoderma species belong to the kingdom of Fungi, the division of Basidiomycota, the class of Homobasidiomycetes, the order of Aphllophorales, the family of polyporaceae (Ganodermataceae) and the genus of *Ganoderma* (Chang, 1995; Wasser and Weis, 1999). They are regarded as polypores because they possess tiny pores on the underside of their pileus (cap) and contained reproductive spores. The caps are spongy when fresh, hardening to a shiny, smooth woody structure when matured. The colour of the caps range from brown, to yellowish, with reddish-brown being typical. The pore surface is cream in colour and the spores are brown (Zoberi, 1972). *Ganoderma* species could not be eaten directly and are not listed among the list of edible mushrooms because the fruiting bodies are

always thick, corky, and tough. They also do not have the fleshy texture characteristics of true edible fungi (Jong and Birmingham, 1991; Jonathan *et al*., 2008).

2.4.1 *Ganoderma australe*

Ganoderma australe is non-laccate species and are often found in tropical and sub-tropical regions. In Malaysia, *G. australe* has been found on mango (*Mangifera indica*) stumps (Abdullah *et al*., 1997) and as wood degrading fungi in forest reserves and plantation forests (Zakaria *et al*., 2009). According to Zakaria *et al*. (2009), there was no proper record on the occurrence of *G. australe* on different types of forest trees in Malaysia. Existence of biological species of *G. australe* was reported in Taiwan (Yeh *et al*., 1995) and southern India (Kaliyaperumal and Kalaichelvan, 2008).

Most of the research conducted on medicinal properties of *Ganoderma* species have been performed on species isolated from other parts of the world and there are only few reports on Malaysian *Ganoderma*. Also, there was no research done on antimicrobial activity of Malaysian indigenous *G. australe*, particularly in isolation and purification of protein from the species. Even in literature, only few compounds have been successfully isolated and not many focused on antibacterial action of this mushroom (Smania *et al*., 1999). The majority of medical research within Ganodermataceae has been performed on *Ganoderma lucidum* because of its great potential as source of bioactive compounds, thus the study of local species may yield compounds with novel medicinal qualities.

2.5 Proteins from mushroom

Many molecules with interesting biological activities are produced by mushrooms. One of the important components of mushroom is protein. Mushrooms have a high protein content. In fact, qualitative analyses by Barbisan *et al*. (2003) demonstrated that mushrooms have eight essential amino acids in addition to non-essential amino acids. A variety of proteins with remarkable biological actions are elaborated by mushrooms and many of these proteins have potential bioactivities. They include antifungal and antibacterial proteins, ribosome inactivating proteins, proteins with nuclease activity, ubiquitin-like proteins and enzymes (Ng, 2004).

2.5.1 Antimicrobial proteins

Proteins isolated from mushrooms have broad spectrum of antimicrobial activities including antifungal, antibacterial and antiviral activity. Antifungal protein is an extensively isolated protein from mushroom. According to Chu *et al*. (2005), antifungal peptides and proteins have great economic implications because they are protective against the destructive effects of pathogenic fungi. They can be classified according to their structure or function into chitinases and chitinase-like proteins, glucanases, lipid transfer proteins, protease inhibitors, ribonucleases, deoxyribonucleases, peroxidases, miraculin-like proteins, cyclophilin-like proteins, allergen-like proteins, thaumatin-like proteins, ribosome-inactivating proteins, antifungal peptides, glutamine-rich proteins, calcyon-like protein, defensin-like protein, and pathogenesis-related protein.

Antifungal proteins have been isolated from only a few species of mushrooms, despite the existence of numerous mushrooms species. Among the reported antifungal proteins are from the fruiting bodies of *Tricholoma giganteum* (Guo *et al*., 2005), *Lyphyllum shimeji* (Lam and Ng, 2001a), *Lentinula edodes* (Ngai and Ng, 2003), *Polyporus alveolaris* (Wang *et al*., 2004), and *Ganoderma lucidum* (Wang and Ng, 2006b).

In contrast to antifungal proteins from mushroom, literature relating to antibacterial proteins is only a handful. Antibacterial proteins have been isolated from the mycelia of *Cordyceps sinensis* (Zheng *et al*., 2006) and the fruiting bodies of *Clitocybe sinopica* (Zheng, 2010). Antibacterial protein from *Clitocybe sinopica* possessed potent antibacterial activity against *Agrobacterium rhizogenes, A. tumefaciens, A. vitis, Xanthomonas oryzae* and *X. malvacearum* whereas antibacterial protein from *Cordyceps sinensis* inhibit the growth of both Gram negative and Gram positive bacteria.

Proteins, peptides and polysaccharopeptides from mushrooms have been reported to be capable of inhibiting human immunodeficiency virus type 1 (HIV-1) reverse transcriptase and protease, the two enzymes of paramount importance to the life cycle of the HIV. Inhibitory effects on hepatitis B and Herpes simplex virus type I have also been reported. The anti-viral effects of mushrooms do not seem to be related to viral adsorption or virucidal effects (i.e. they do not kill the virus). However, a number of studies have reported inhibitory effects at the initial stage of virus replication (Roupas *et al*., 2010).

Proteins with activity against HIV-1 reverse transcriptase have been isolated from mushrooms such as *Russula paludosa* (Wang *et al*., 2007) and *Pleurotus ostreatus* (Wang and Ng, 2000b). A novel anti-viral protein has been isolated from an extract of *Grifola frondosa* (Maitake) fruiting bodies. The protein inhibited Herpes simplex virus type 1 (HSV-1) replication *in vitro* with an IC_{50} value of 4.1 mg/ml. It was reported that the

protein directly inactivated HSV-1 while simultaneously inhibiting HSV-1 penetration into Vero cells (Gu *et al*., 2006).

2.5.2 Other proteins with biological activities from mushroom

Ribosome inactivating proteins (RIPs) is another protein isolated from mushroom. This protein boasts a variety of activities including immunosuppressive, anti-proliferative and antiviral activities (Ng *et al*., 1992).

Ribosome inactivating proteins have been isolated from few mushrooms species including *Volvariella volvacea* (Yao *et al*., 1998), *Flammulina velutipes* (Ng and Wang, 2004c), *Lyophyllum shemeiji* (Lam and Ng, 2001a), *Calvatia caelata* (Ng *et al*., 2003) and *Hypsizigus marmoreus* (Lam and Ng, 2001b). It is remarkable that, from the fruiting bodies of *Flammulina velutipes*, four RIPs have been isolated designated flammulin (Wang and Ng, 2000), velutin (Wang and Ng, 2001a), flammin and velin (Ng and Wang, 2004c). All RIPs from *Flammulina velutipes* inhibit cell-free translational in the rabbit reticulocyte lysate system. Velutin exhibited ability to inhibit β-glucosidase and β-glucoronidase which are implicated in viral infection and also human immunodeficiency virus-1 (HIV-1) reverse transcriptase (Wang and Ng, 2001a).

Ribonuclease is also another medicinally protein reported from several mushroom species including *Dictyophora indusiata*, *Ganoderma lucidum*, *Irpex lacteus*, *Lentinula edodes*, *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Pleurotus sajor-caju*, *Pleurotus tuber-regium*, *Russulus virescens*, and *Volvariella volvacea* (Wang and Ng, 2004a). Ribonuclease demonstrates an array of activities including antiviral, immunomodulatory and anti-neoplastic activities.

Besides the previous mention proteins, there are significant amount of literature regarding mushroom enzymes exist. These enzymes play an important role in the saprophytic mode of life in mushroom. Together with protease, these enzymes find important application in biotechnology and industry (Ng, 2004). Laccases which play an important role in lignin degradation demonstrated to be very potential applications in detoxification of polluted water, biosensor, pulping and textile dyes. These ligninolytic enzymes have been isolated and characterize from some mushroom species including *Coriolus versicolor*, *Panus tigrinus*, and *Phlebia tremellosa* (Ng, 2004). Besides, there are numerous researches done on isolation of fungal cellulases and xylanase from mushroom.

2.6 Isolation, purification and characterization of proteins

Protein purification is a multi-step process exploiting a wide range of biochemical and biophysical characteristics of the target protein, such as its source, relative concentration, solubility, charge and hydrophobicity. Rosenberg (1996) stated few targets to achieve in designing a purification protocol for those working with proteins: (1) high recovery, (2) high purified end product, (3) reproducibility, within the lab, in other lab and also when either scale up or down, (4) Economical use of reagent and convenience with regard of time. Most successful isolation procedures involve only a few steps, chosen to give the highest yields.

2.6.1 Extraction

The first step for purification of bioactive compounds is extraction process. There are few factors affecting the type of compounds that can be extracted from fungi or any living sources including the type of solvent used, the extraction process employed and the age, and part or type of cultivation of the living tissue (Roberts, 2004). Many compounds have been extracted from both the fruiting body and mycelia of *Ganoderma*. These compounds found to exhibit remarkable biological activities (Paterson, 2006).

2.6.2 Ammonium sulfate precipitation of proteins

Ammonium sulfate precipitation is a highly effective method for proteins separation and used as an early step in purification protocol (Rosenberg, 1996). In this method, protein precipitation is achieved by dehydration in the microenvironment of the protein molecule. Principally, a large number of water molecules are bound to the sulfate ion (SO_4^{-2}) in solution, thus reduce the amount of water available to interact with the protein molecules. At a particular concentration of ammonium sulfate, an insufficient quantity of unbound water will remain to keep a given protein species in solution, resulting in the precipitation of that protein (Rosenberg, 1996).

As stated by Bonner (2007), practice ammonium sulfate is used for the routine precipitation of proteins for the following reasons, (1) ammonium sulfate dissolves at high concentrations (about 4 M at 0°C) generating little heat; (2) the density of saturated ammonium sulfate solution (1.235 gml^{-1}) is less than the density of aggregated protein (1.29 gml^{-1}) which allows collection of the precipitate by centrifugation; (3) ammonium

sulfate precipitation is a mild method of protein concentration giving very good recoveries of activity; (4) proteins can be stored as an ammonium sulfate precipitate (covered in saturated ammonium sulfate at –25°C) for long periods with little loss of activity when the precipitate is redissolved.

2.6.3 Chromatographic techniques for protein purification

Chromatography refers to a group of separation techniques that involves a retardation of molecules with respect to the solvent front that progresses through the material. The name literally means "color drawing" and was originally used to describe the separation of natural pigments on filter papers by differential retardation. The same principle is now commonly used for protein separation. Column chromatography is the most common physical configuration, in which the stationary phase is packed into a tube, a column, through which the mobile phase, the eluent, is pumped. The degree to which the molecule adsorbs or interacts with the stationary phase will determine how fast it will be carried by the mobile phase. Chromatographic separation of protein mixtures has become one of the most effective and widely used means of purifying individual proteins (Bonner, 2007).

Chromatography technique allowed biomolecules to be separated according to differences in their specific properties such as size (gel filtration chromatography), charge (ion exchange chromatography), hydrophobicity (hydrophobic interaction chromatography) and biorecognition-ligand specificity (affinity chromatography).

In the present study, purification of protein was achieved through size exclusion chromatography, which is also known as gel permeation chromatography and gel filtration

chromatography. According to Booner (2007), the separation in gel filtration chromatography does not involve binding between the sample and the resin and it is independent of the eluent used. These properties have made gel filtration chromatography a technique that is useful for the separation of biological (proteins, nucleic acids and oligosaccharides) and organic polymers.

Gel filtration can be used to isolate one or more components, to determine molecular weight, or to analyze the molecular weight distribution in the sample. The best result for high resolution fractionation can be achieved with sample that originally contain few components or with samples that have been partially purified by other chromatography techniques. This is different from ion exchange or affinity chromatography. Thus, a significant advantage of gel filtration is that condition can be varied to suit the type of sample or the requirement for further purification, analysis or storage without altering the separation. Besides, it is also suitable for the final polishing step in the purification scheme (Amersham, 2002).

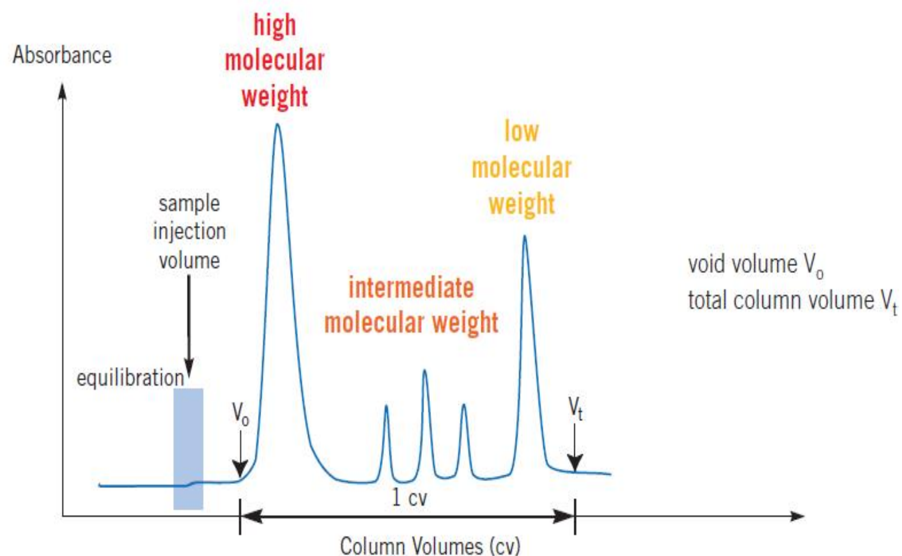

Figure 2.1 : Theoretical chromatogram of high resolution fractionation (UV absorbance).
Source : Amersham (2002)

Figure 2.1 shows the theoretical elution profile (chromatogram) of a high resolution fractionation. Molecules that do not enter the matrix are eluted in void volume, V_o as they pass directly through the column at the same speed as the flow of buffer. Small molecules such as salts move down the column but not separate from each other and usually elute just before one total column volume, V_t of buffer has passed through the column.

2.6.4 Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is a fundamental procedure in the protein characterization in the sense that analytical reactions probing the structure and composition of the target protein are carried out before and after the separation of complex mixture of macromolecules by PAGE. A lot of information can be obtained using PAGE including molecular weight determination, purity of proteins, post-translational modifications, subunit structure, enzyme activity, protein processing and amino acid sequences (Rosenberg, 1996).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) is a very important technique for separation of peptides and proteins. The Laemmli system has advantages for separation of large protein and the Tricine SDS-PAGE system is particularly useful for small proteins and peptides, in the mass range of 1-100kDa. In the present study, Tricine SDS-PAGE was used due to the capacity of this electrophoretic system to give better resolution for protein smaller than 30kDa (Schagger, 2006).

2.6.5 Protein sequencing and identification

Most analytical proteomics problem begin with a protein mixture. This mixture contains intact protein of varying molecular weight, modifications, and solubilities. The protein must be cleaved in order to obtain the peptides sequences. This is due to the inability of mass spectrometers (MS) to perform measurement of peptide masses or obtain peptide sequence directly on intact proteins. Although modern MS instruments can obtain a tremendous amount of data even from relatively complete protein mixtures, simplification of the mixtures allows data to be collected on the greatest number of components. Therefore, to analyze protein mixture by MS, the complete mixtures of many components must be separated into less complex mixtures containing fewer components. It is possible to separate the intact proteins then cleave them into peptides or cleave the proteins into peptides first then separate the peptides prior to analysis. The peptides are then analyzed by mass spectrometer. The data from the mass spectrometer is then used, with the aid of specialized software, to identify peptides and peptides sequences from database that match the data from the MS data (Fig. 2.2) (Liebler, 2002).

The use of database searches is very important in protein sequence analysis. When the partial or complete amino acids of the target protein is known, computer assisted searches should perform to compare the new sequences to a database of known sequences. This will reveal whether there is a protein of known structure or homologous sequence that is sufficiently similar to the sequence of the target protein to suggest a familiar relationship and a possible function. Two mathematical aspects to database searches are the algorithm which used to find sequence similarities, and the method used to determine which

similarities are statistically significant and therefore, potentially interesting (Rosenberg, 1996).

The two popular algorithm used for rapid searches of databases are FASTA and BLAST which look for intervals or segments of good matching between sequences. FASTA is a multistep algorithm for sequence alignment (Wilbur and Lipman, 1983). Basic Local Alignment Search Tool (BLAST) is the most popular computer program used to perform database similarity searches. The program allows rapid identification of sequences from large databases that are similar to the query and provides sound statistical evaluation of the significant of the finding (Dong and Brendel, 2005).

In the present study, Profound is used for identification of the purified protein. Profound is a tool for searching a protein sequence collections with peptide mass maps. A Bayesian algorithm is used to identify proteins from protein databases using mass spectrometric peptide mapping data. The algorithm ranks protein candidates by taking into account individual properties of each protein in the database as well as other information relevant to the peptide mapping experiment. The program consistently identifies the correct protein(s) even when the data quality is relatively low or when the sample consists of a simple mixture of proteins (Zang and Chait, 2000). This system of estimating the risk of a random match versus a true match is used in most conventional sequence homology matching systems, such as BLAST. It has the distinct advantage of being independent of the scoring system: the expectation value is calculated from a distribution of scored sequences, rather than on a particular result.

According to Rossenberg (1996), if database screening based on overall sequences similarity does not identify the target protein as a family member, a short sequence of the target protein may be recognized as a commonly occurring structural motif, which may be

helpful for assigning a function. The fundamental unit of protein structure, the domain, may possess functions independently of whether they are present in isolation or a part of a larger multidomain protein. The domain is defined as the region or regions of a polypeptide that fold independently and possesses a hydrophobic core with a hydrophilic exterior (Ponting and Birney, 2005).

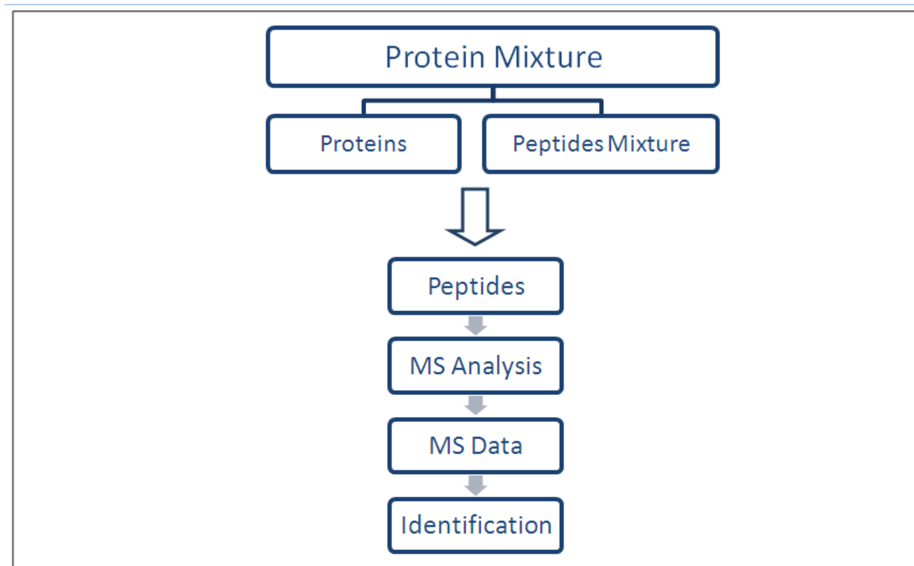

Figure 2.2 : General flow scheme for proteomic analysis started from protein mixture to identification of protein.

2.7 Antimicrobial activity

2.7.1 Pathogenic bacteria

Bacteria tested in this study are divided into 2 categories which are environmental bacteria and skin opportunistic bacteria. *E. coli* (ATCC 29552), *E. coli* (O157:H7), *Salmonella* spp (ATCC 13076), *Salmonella typhi*, *Shigella* spp., *Bacillus cereus*, and

Plesiomonas shigelloides can be found in environment and are common cause of food and water poisoning. On the other hand, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* are common opportunistic skin bacteria which can cause variety of infections including skin infection, bacteremia, eyes and ears infections. They are also common cause of hospital/healthcare associated infection (HAI/HCAI) and often turn out being multiple resistant organisms.

Health care-associated infection (HCAI), which also referred to as “nosocomial” or “hospital” infection, is defined as: “An infection occurring in a patient during the process of care in a health-care facility which was not present or incubating at the time of admission. This includes infections acquired in the hospital but appearing after discharge, and also occupational infections among staff. HCAI is acknowledged as the most frequent adverse event in health care. In Malaysia, the estimated incidence of HCAI from 1995 and 2008 was reported by World Health Organization (WHO) to be 13.9 percent (http://www.who.int/gpsc/country_work/summary_20100430_en.pdf).

2.7.2 Pathogenic yeast

In the present study, antimicrobial activity of the protein from *Ganoderma australe* strains and *Ganoderma tsugae* were tested against pathogenic yeast *Candida albicans*, *Candida parasilopsis* and *Schizosaccaromyces pombe*.

Candida is the fourth most common cause of hospital related bloodstream infections (Banerjee *et al.*, 1991). *Candida albicans* is a dimorphic fungus lacks a sexual cycle and is a diploid organism. It exhibits many different morphological forms under different environmental conditions, including budding yeast cells, pseudohyphae, true hyphae and

clamydospores (Odds and Baillihre, 1988). The fungus which is the most isolated yeast, exist as a commensal of mammalian including humans. According to Molero *et al*. (1998), *C. albicans* colonize mucosal surfaces of the oral and vaginal cavities and the digestive tract and is also able to cause various infections, depending on the nature of the underlying host defect. This yeast cause infections known as candidacies which can be divided into superficial and deep seated and represent a major clinical problem.

Candida parasilopsis is pathogenic yeast which infects hospitalized patients. According to Levy *et al*. (1998), this fungus particularly affects critically ill neonates and surgical intensive care unit (ICU) patients. The hands of the healthcare workers are suggested as the predominant environmental source responsible for the spreading of infections (Lupetti *et al*., 2002).

Schizosaccaromyces pombe which is called “fission yeast” is a unicellular eukaryote, used as a model organism in molecular and cell biology. The cells are rod-shaped and have approximately 14.1 million base pairs in its genome, which is estimated to contain 4,970 protein-coding genes and at least 450 non-coding RNA (Wilhelm *et al*. 2008). This fungus is an important organism in studying the cellular responses to DNA damage and process of DNA replication. Furthermore, the full genome sequence of this yeast has been published in 2002, with many genes homologous to human disease genes being identified (http://en.wikipedia.org/wiki/Schizosaccharomyces_pombe).

2.7.3 Pathogenic fungi

There are five phytopathogenic fungi tested in this study including *Fusarium oxysporium cubense I* (FOC1), *Fusarium oxysporium cubense II* (FOC2), *Fusarium oxysporium cubense IV* (FOC4), *Ganoderma boninense* and *Colletotrichum* sp.

Fusarium oxysporum f sp. *cubense* (FOC) is plant pathogenic fungus which causes Fusarium wilt or Panama disease, the most destructive disease of banana worldwide. The disease have been threat to banana industry since 1900s especially in the developing countries where banana is one the important staple foods. In 1940s, almost 40,000 ha of banana plantation were affected by the pathogen, which lead to the very severe loss to the industry (Ploetz and Pegg, 2000).

There are four races of *Fusarium* were found to be virulent towards Cavendish recognized as FOC 1, FOC 2, FOC 3 and FOC 4. According to Stover (1972), races 1 and 2 are virulent to Gros Michel and Bluggoe banana cultivar while race 3 is a pathogen of *Heliconia* sp. and only have only mild effect on *Musa* sp. The most virulent race is race 4 which affects Cavendish and other banana cultivar susceptible to races 1 and 2. In Malaysia, areas cultivated with banana have decreased in recent years, due to the effect of Fusarium wilt as one of the contributing factors (Leong *et al.*, 2010). The most susceptible is 'pisang berangan' and 'pisang rastali'. Currently, there is no effective chemical control measure for this disease other than resistant varieties (Getha and Vikineswary, 2002).

Colletotrichum is another most important plant pathogens, with worldwide distribution but found mainly in subtropical and tropical regions (Bailey and Jegger, 1992). This pathogen cause economically significant diseases of plants (anthracnose) that affect a large number of crops such as cereals and grasses, legumes, vegetables, and perennial

crops, including fruit trees (Bailey and Jegger, 1992). *Colletotrichum* species are commonly isolated as endophytes from healthy plants and have been identified as saprobes on dead plant materials. Most endophytic, saprobic and many pathogenic strains in the genus have been recently classified as *Colletotrichum gloeosprioides* or *Colletotrichum* sp. (Photita *et al*., 2005). According to Cano *et al*. (2004), there are few key morphological features to identify the genus of *Colletotrichum* including its acervular conidiomata, often with setae, producing elongated slimy conidia, and the presence of appresoria. The key criterion for identification of the species is based mainly on determining the plant host.

Ganoderma is the most important pathogen which caused serious oil palm disease in this region. The infection, known as basal stem rot (BSR) disease has resulted in very severe losses especially in parts of west Malaysia. The taxonomy of *Ganoderma* species involved in this disease was very confusing for many years until being introduced as *Ganoderma boninense* by Ho and Nawawi (1985). Progressive destruction of the trunk's basal tissues was the final effect of infection by *Ganoderma*. The infection can be observed by symptoms of wilting and malnutrition of the leaves. These external symptoms occur as a consequence of restricted water and nutrient supply to the aerial parts of the oil palm. These appear when almost one-half of the basal stem tissue had been killed by the fungus (Turner and Bull, 1967).

There were few available control measures for BSR disease such as cultural practices and mechanical and chemical treatment but have not proven satisfactory due to the fact that *Ganoderma* has various resting stages such as resistant mycelia, basidiospores, chlamydospores and pseudosclerotia (Susanto *et al*., 2005). Besides, studies of the chemical control of BSR with fungicides were limited to the laboratory and did not offer any solution to the field problems. Current control recommendation involving surgeries is unsatisfactory

since removals of all infected tissues frequently result in physical collapse of the oil palm (Turner, 1968).

2.7.4 Importance of antimicrobial agent

Antimicrobial chemotherapy has played a vital role in the treatment of human infectious diseases in the 20th century. Hundreds of antimicrobial agents have been developed or synthesized since 1920 and dozens of these are currently available for clinical use (Murray *et al*., 1999). According to Alanis (2005), there are few mechanisms of action of bacterial killing by these antimicrobial agents such as inhibition of cell wall synthesis by Beta lactams, inhibition of protein synthesis by Tetracyclines, Aminoglycosides, and Macrolides, inhibition of DNA or RNA synthesis by Fluoroquinolones and Rifampin, and membrane disorganizing agent by Polymyxins. However, it seems that bacteria have overcome most of the killing mechanisms of these antimicrobes.

2.7.5 Drug resistance of microbes

Antimicrobial resistance is the result of complex interactions between antimicrobial agents, microorganisms, and the environment. Environmentally mediated resistance is defined as resistances that result from physical or chemical characteristic that directly alter either antimicrobial agents or alter normal physiologic response of the microorganisms to particular antimicrobial agents such as pH, atmosphere and cation concentration. Microorganisms mediated resistance refer to antimicrobial resistances that occur due to genetically encoded traits of the microorganisms either intrinsically (inherent resistance) or

acquired resistance. Intrinsic resistance is resulting from normal genetic, structural or physiological state which is natural in particular bacterial group, genus or species for example resistance of Gram-negative bacteria toward Vancomycin whereas acquired resistance occur from altered cellular physiology and structure either by genetic mutations (Penicillin Binding Protein alteration), genetic sharing (conjugation, transformation, or transduction) or both (Murrey, 1999).

2.7.6 Antimicrobial compounds from medicinal mushrooms

Mushroom is one of the exploited sources of bioactive compounds, to date. This is due to the fact that whole mushroom (mainly fruiting bodies), extracts (from fruiting bodies or mycelia) or the culture fluid and isolated compounds can be explored for their biological activity (Lindequist *et al*., 2005). There are some advantages of using filamentous fungi over plants as sources of bioactive compounds including the production of fruiting body in much less time, the reliability of mycelia to be produced rapidly in liquid culture and the ability to manipulate culture medium for optimal production of bioactive products (Roberts, 2004).

Although the production of important antibiotic such as penicillin, cephalosporin and griseofulvin by fungi is well known, the occurrence of antibiotic in mushroom is less well documented for discovery of new antibiotic with different structural types (Yamac and Bilgili, 2006). In 1941, Achel, Harvey and Wilkins performed studies on potential of mushrooms as sources of antibiotics. Diverse antimicrobial activity was detected in analysis of either fruiting bodies or mycelial cultures of more than 2000 fungal species (Rosa *et al*., 2003). The study was succeeded by the isolation and identification of Pleuromutilin by

Kavanagh *et al*. (1950) which served for the first development of the first commercial antibiotic of Basidiomycetes origin. Since then, more and more studies were being performed to detect antimicrobial activity of mushrooms for discovery of novel antibiotic.

A recent study by Yamac and Bilgili (2006) presented antimicrobial activities of fruit bodies and mycelia cultures of some mushrooms isolates and detected 61 out of 80 extracts have antimicrobial against at least one of the microorganisms employed and most of the activities were antibacterial. Similar study conducted by Rosa *et al*. (2003) demonstrated significant activity of 14 mushroom isolates against one or more of the test microorganisms.

Most of antimicrobial activity reported was from extracts of the mycelia or fruiting bodies of mushroom. Nevertheless, bioactive compounds with potent antibiotic activity were also being purified and isolated from mushroom (Table 2.1). Their dose compensation and the mode of action is a subject for research. Undoubtedly, the antimicrobial potential of extracts of several medicinal mushroom type and other Basidiomycetes not yet exploited, must warrant further examination (Rai *et al*., 2005).

Table 2.1 : Compounds showing antimicrobial activities from mushrooms.

Mushrooms	Bioactive compounds	Bioactivity
Cheimonophylum candissimum	Cheimonophyllon A-E	Antibacterial Weak antifungal
Clitocybe cyathiformis	Cyathiformine A	Antibacterial and antifungal
Clitocybe diatreta	Diatretol	Antibacterial
Coprinus atramentarius	Illudin C2, Illudin C3	Antimicrobial
Crepidotus fulvotomentosus	Strobilurin E	Antifungal

Favolaschia pustulosa	9-methoxystrobilurin L	Antifungal and antibacterial
Favolaschia sp.	Favolon	Antifungal
Flagelloscypha pilatii	Pilatin	Antibiotic
Ganoderma lucidum	Ganoderan	Antiviral
Lentinula edodes	Lentinan	Antiviral
Mniopetalum sp.	Mniopetals	Antimicrobial
Mycena sp.	Strobilurin M, Tetrachloropyrocatechol	Antifungal, cytostatic Antifungal, antibacterial
Omphalotus illudens	Illudinic acid	Antibacterial
Oudemansiella radiate	Oudemansin x	Antifungal
Poria cocos	Lanostene	Phospolipase A2 inhibitor (group of anti-inflammatory agents)

Source : Rai *et al*. (2005)

2.8 Anti Human Immunodeficiency Virus -1 Reverse Transcriptase activity

2.8.1 Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) is a lentiviruses (a member of the retrovirus family), which are pathogens that causes an illness trajectory, characterized by long period of clinical latency and asymptomatic infection caused by weak humoral immune response and persistent viremia (Green, 1997). According to Barre-Sanoussi (1996), there are two different viral strains of HIV been identified since 1983 : HIV type 1 (HIV-1), which is distributed worldwide, and the HIV type 2 (HIV-2), which is seen predominantly in West Africa. Both viruses can cause primary immunodeficiency in human.

HIV-1 can infect cells that possess the CD4+ marker / receptor such as CD4+ T-lymphocytes (T-helper cells), macrophage / monocytes, some bone marrow progenitor cells and follicular dendritic cells, resulting in direct and indirect effects on human immune system. Direct interaction of HIV-1 and the affected host CD4+ cell is the direct effects of HIV-1 on the immune system (Staprans and Feinberg, 1997) while indirect affects and dysregulation of the immune system is caused by the substances released as part of the immune response to HIV-1 infection, resulting to the deterioration of CD4+ cell which leads to the dysfunction and cellular death (Brennan and Porche, 1997)

HIV-1 is transmitted by body fluids that contain HIV-1 or CD4+ HIV-1 infected lymphocytes. These include serum, seminal fluid, vaginal secretions, amniotic fluids and breast milk (Staprans and Feinberg, 1997). According to Brennan and Porche (1997), HIV transmission happens by few mechanisms or behaviors that allow the exposure of these body secretions to other persons. These include form of unprotected sexual activity, injection of blood or blood products, and perinatal infection (from mother to the fetus or infant). In Malaysia, the first reported cases of HIV/AIDS were in 1986, and since then there are now over 91, 000 reported cases of HIV infections in Malaysia. There are more than 16,000 people have died from AIDS as of December 2010. The majority of HIV infections are found in adult aged 30-39 (43% of cases) and those aged 20-29 (33% of cases) with 90% reported cases are among male (http://www.rumahjaireh.com/PDFs/AIDSinMalaysia2010.pdf).

2.8.2 Anti HIV compounds

Enormous efforts have been dedicated in searching for the cure for HIV/AIDS. Until now, there is no cure for this pandemic disease. Because HIV-1 is one of the main targets for inhibiting the reproduction of HIV, many inhibitors to the enzyme have been and still are extensively discovered in small and big scale screening. However, only a small number is used in therapy and these compounds are nucleoside and non-nucleoside inhibitors (De Clercq, 2001). Many natural products such as ribosome inactivating proteins, alkaloids, flavanoids, lignans, and so on, have been reported to inhibit unique enzymes and protein crucial to the life cycle of HIV, including the reverse transcription process, virus entry, the integrase or protease (Cos *et al*., 2004; De Clercq, 2000). As HIV demonstrates a high ability to developed resistance against therapeutic agents, new promising compounds with anti HIV inhibitory activity have to be discovered (Mlinaric *et al*., 2005).

2.8.3 Anti HIV-1 reverse transcriptase activity

Most of natural inhibitors of HIV-1 reverse transcriptase described are obtained from plants, but not from fungi (Mlinaric *et al*., 2005). According to Mlinaric *et al*. (2005), the kingdom of fungal which represent a vast and promising source of novel therapeutic agents are often overlooked. Still, there are many reports of proteins from mushrooms with inhibitory activity against human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, an enzyme of paramount importance to the life cycle of the HIV.

In the screening of new compounds with anti HIV-1 RT activities, many strategies and bioassays have been developed. According to Tan and Pezzuto (1991), the early

developed assays for screening of HIV-1RT inhibitory activity were relatively complicated, time consuming and included work with radioactive material. A simple, non-radioactive ELISA based assay become commercially available recently. This assay allows rapid, reliable and safe screenings with a minimum amount of sample (Mlinaric *et al*., 2005).

In this study, the purified protein was assessed for anti HIV-1 reverse transcriptase activity using the transcriptase colorimetric enzyme immunoassay. The assay determined the quantitative retroviral reverse transcriptase activity by incorporation of digoxigenin and biotin-labeled dUTP into DNA. Lyophilize recombinant HIV-1 RT was used in the screening. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/ primer hybrid poly(A) oligo(dT)15. The digoxigenin and biotin-labeled nucleotides in an optimized ratio are incorporated into one of the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows sandwich ELISA protocol. Biotin labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405nm can be determined using microtiter plate (ELISA) reader and is directly correlated to the level of RT activity.

2.9 Hemolytic activity

In the present study, the ability of the purified protein to induce hemolysis against erythrocytes was investigated. Hemolysis test is used to see potential toxicity issues early in the drug discovery process. Besides, the examination of the in vitro cytotoxicity of the purified protein is important before they can be considered in clinical use (Javadpour *et al*., 1996). According to Zafloff *et al*. (2002), erythrocyte is used in the test because its represent prokaryote cyte negatively charged cell containing lipid bilayer. This is due to the fact that erythrocyte plasma membrane is a natural membrane in the body which contains amnionic surface and a lipid monolayer. The activity was compared to melittin, a protein from honeybee venom which can cause total hemolysis at a very low concentration (Dempsey, 1990).