5.0 DISCUSSION

5.1 Cultivation of *Ganoderma australe* mycelia in submerged culture

Mass production of *G. australe* mycelia was achieved through submerged culture cultivation. According to Lomberh and coworkers (2002), submerged cultivation of mushroom mycelia in liquid media is a promising method, which can be used in novel biotechnological processes for obtaining pharmaceutical substances of anticancer, antiviral, immunomodulating, and antisclerotic action from fungal biomass and cultural liquids and also for the production of liquid spawn. It provides fast growth and high productivity of the mycelia. When compared to solid culture cultivation for production of bioactive compound, submerged culture provide more advantages including short fermentation period, availability of convenient control, high product concentration and easy down stream processing (Wasser *et al*., 2000). Thus, submerged culture system was chosen for mass production of mycelia in this study.

5.2 Preparation of extract for antimicrobial activity test

The mycelia were extracted with dichloromethane in the first step to remove all the organic solvent dissolving compounds from the cultivated mycelia. Dichloromethane's volatility and ability to dissolve a wide range of organic compounds makes it a useful solvent for this process. Aqueous ethanol was used to extract proteinaceous material from the mycelia of *G. australe* strain KUM60813. Ethanol extraction was chosen because of it’s commonly used in protein extraction. Dulger *et al*. (2004) reported the antimicrobial

properties of four different extracts of macrofungus (*Cantharellus cibarius*) against fifty important human pathogens. He observed good antimicrobial activity with ethanol and acetone extracts against most pathogens. The presence of protein fractions in gel filtration and protein band in Tricine SDS PAGE verified efficient and appropriate extraction methods used in the study.

5.3 Isolation, purification and identification of protein

In the present study, isolation and purification of protein with antimicrobial activity was reported for the first time from the mycelia of *G. australe*. The protein was purified using chromatographic techniques similar with other mushroom protein reported earlier. However, the strategy involved in this study followed different step of extraction and isolation methods. Extraction of mycelia proteins was achieved through double extraction with dichloromethane and 50 percent ethanol, followed by precipitation of protein using 90 percent ammonium sulfate. The extraction protocol used is a modification method to the commonly used ammonium sulfate precipitation and ethanol extraction method in protein purification. Wang *et al*. (2000) reported purification of protein from mycelia of *Tricholoma lobayense* by cold saline extraction and ammonium sulfate precipitation while another study by Zheng *et al*. (2010) used homogenization prior to precipitation of protein by ammonium sulfate from *Clitocybe sinopica*.

The behaviour of the *G. australe* protein on various chromatographic media employed in the isolation procedure varied from other fungal proteins. The protein was absorbed neither on CM cellulose nor on DEAE cellulose, unlike some of the fungal proteins reported in literature. Zheng *et al*. (2010) purified an antibacterial protein from the

fruiting bodies of the wild *Clitocybe sinopica*, which was absorbed on DEAE cellulose. A number of reported mushroom proteins also absorbed on Affi gel blue and Mono S, such as antifungal protein from *Lyophyllum shimeiji* (Lam and Ng, 2001a) and antifungal protein from *Lentinula edodus* (Ngai and Ng, 2003). The protocol found to be satisfactory for purification of protein from *G. australe* KUM60813 was gel filtration chromatography. The protein was purified after chromatographic steps using Sephacryl S-100 and Superdex 75 HR. It is remarkable that only two chromatographic steps are required for the purification of this protein. To our knowledge, there are only few reports of a two-step chromatographic protocol to purify a fungal protein.

Molecular mass of the purified protein is within the range of reported fungal proteins. In Tricine SDS PAGE, the protein posses approximately 16.6 kDa, less than reported antibacterial protein from *Clitocybe sinopica* that composed of two subunits with the same molecular mass of 22 kDa (Zheng *et al*., 2010). Protein isolated from *Ganoderma lucidum* and *Cordyceps militaris* both possess molecular weight of 15 and 10.9 kDa respectively (Wang and Ng, 2006b; Wong *et al*., 2010).

The purified protein showed similarity to an antifungal protein from *Aspergillus giganteus*, with a very high probability and Z score (Table 4.5). Antifungal protein from *A. giganteus* exhibited high level of antifungal activity against species of the genera *Aspergillus* and *Fusarium*, but did not affect the growth of yeast or bacteria (Theis *et al*., 2003). Several classes of these proteins involve inhibition of the synthesis of the fungal cell wall or disrupt cell wall structure or function; or perturb membrane structure, resulting in fungal cell lyses (Selitrennikoff, 2001). The antifungal protein showed 32 percent coverage to the purified protein from *G. australe*. Experiences gathered from decades of structural and molecular biology which showed that those domain that possess similarities in

sequences (>30 % identity) also possess a common similarity in function (Ponting and Birney, 2005).

When the matched antifungal protein was subjected to protein to protein BLAST analysis, the result revealed the classification of the protein in a Conserved Domain Database (CDD) of Antifungal protein. This suggested that the purified protein are homologous and are products of genes that are member of the same family. Few other antifungal protein sequences producing significant alignments with antifungal protein from *Aspergillus giganteus* are given in Table 5 (Appendix D). Interestingly, this is the first report of antimicrobial protein from mushroom (other proteins are derived from microfungi and bacteria). The CDD is the protein classification component of NCBI's Entrez query and retrieval system. Proteins domains are distinct unit of molecular evolution, which usually associated with particular aspects of molecular function and generally represent discrete unit of three-dimensional (3D) structure. The identification of functionally characterized domains in protein sequences may give the first clues as to their molecular and cellular function (Bauer, *et al*., 2005).

5.4 Antimicrobial activity of PM and protein fractions

Many studies have reported the existence of antimicrobial activities in mushroom. Recent in vitro study of extracts from polypore mushroom species show 75 percent demonstrated antimicrobial activity and 45 percent of 204 mushroom species (polypores and gilled mushroom alike) inhibited variety of microorganisms (Suay *et al*., 2000).

The present study investigates the antimicrobial activity of crude proteins known as PM (powdery materials) of *Ganoderma tsugae* and selected *Ganoderma australe* strains in

primary screening prior to isolation of bioactive protein. The used of combination of human pathogenic microorganisms include Gram-positive bacteria, Gram-negative bacteria and yeast was to evaluate spectrum activities of the protein extracted.

As shown in Table 4.1, antibacterial activity was detected from all PM of *Ganoderma* strains tested with varied intensity. However, they are not effective against all pathogenic fungi tested. The PM were observed to be weakly antagonistic against *P. aeruginosa* and *B. subtilis.* Possible reason for the low activity could be insufficient dose of the PM used that effectively kill the pathogen. The PM of KUM60813 exhibited good inhibitory activity against *B. cereus*, *E. coli* O157:H7 and *S. typhi* with clear inhibition zone recorded in the range of 9 – 11 mm. Smania *et al*. (2007) observed MIC value of 2 mg/ml for *E. coli* and *P. aeruginosa* while 1 mg/ml in case of *S. aureus* and 0.25 mg/ml for *Bacillus* species with *G. australe* extract. Besides, extracts from *G. pfeifferi* (Monthana *et al*., 2000) and *G. applanatum* (Smania *et al*., 1999) were also reported to exhibit significant antibacterial activity against *E. coli.*

In the preliminary screening of antibacterial activity by PM from *Ganoderma* strains, the results obtained showed a broader spectrum of activity than that of the purified protein. The PM showed antibacterial activity against both Gram-positive and Gram-negative bacteria in term of clear inhibition zone. The results obtained against *S. typhi* in the initial test are also worth discussing. The fact that all the PM are active against this bacterium is very interesting. This pathogenic bacterium was shown to be most susceptible towards three out of five PM tested namely *G. australe* KUM60813 and KUM70069 and also *G. tsugae*. All the PM also showed activity against *E. coli* O157:H7, *E. coli* serogroups that cause significant human diseases including diarrhoea, haemorrhagic

colitis (HC) and occasionally complications such as hemolytic-uremic syndrome (HUS) and thrombocytopenic purpura (TTP) (Voravuthikunchai *et al*., 2004).

Higher activity in Gram-negative bacteria compared to Gram-positive bacteria contradict with the common reports revealed most antibiotic of fungal origin are rather selective in their inhibitory action and most of them being inhibitory to Gram-positive bacteria (Burn, 1988). Venturini *et al*., (2008) had also reported higher antibacterial activity against Gram-positive bacteria by extracts from edible wild and cultivated mushrooms. The studied bacteria were food-borne pathogens. Therefore, it is worthwhile to examine the mechanisms of activity of the proteins from *G. australe*. As indigenous community in Malaysia consumes the fungus, it can be considered as a safe additive in food to prevent bacterial contamination.

When PM from *G. australe* KUM60813 with the highest antibacterial activity in the primary screening proceed to purification with chromatography, reduction in activity was observed. Out of eight fractions eluted from Sephacryl S-100, only two showed activity against half of the tested bacteria. Both S6 and S7 showed almost similar degree of inhibition except that no inhibition activity was observed against *E. coli* O157:H7 by S7. No activity was detected from three fractions namely S2, S4 and S8.

The sixth fraction eluted from Sephacryl S-100, exhibited broadest spectrum of activity against six out of ten (60%) bacteria tested, followed by seventh fraction (against *S. aureus, B. cereus, B. subtilis, E. coli* and *Salmonella* sp.), first fraction with mild activity (against *S. aureus, B. cereus, B subtilis* and *E. coli* 0157:H7), fifth fraction (against *S. aureus* and *B. cereus*) and third fraction (against *B. subtilis*). *Escherichia coli* O157:H7 only sensitive to fraction one and sixth with higher activity demonstrated by the latter fraction. Higher activity existed in crude protein compared to separated fractions might be

due to synergism effect which is increased intensity caused by the combination of more than one substances present in the crude protein extracts.

In general, reference disc are more active than extracts in inhibiting test bacteria in vitro. Extract of *G. australe* KUM60813 and S6 showed comparable inhibitory activity to standard antibacterial proteins Cecropin and Dermaseptin, when tested against *B. cereus*, *E.coli* O157:H7 and *Salmonella* sp.. However, chloramphenicol showed higher level of inhibition against all tested bacteria. Though the activity of the PM were not equivalent to the standard antibiotic (chloramphenicol), microorganism get resistance to the antibiotic after sometime, therefore protein extract of *G. australe* maybe still a promising source of future antimicrobial agent.

In the present study, all the test plates with antibacterial activity were observed to demonstrate clear inhibitory zone. This indicated bactericidal effect of the crude protein extracts and protein fractions on the tested bacteria. According to MacMillan and Hibbitt (1969), protein produces considerable morphological changes in the fine structure involving the plasma membrane of bacteria. Study done on the region of the *Staphylococcal* cell showed direct affect of cationic peptides which suggests that the plasma membrane may be the target site of action for the proteins. The ability of enzymes and peptides to rapidly kill broad spectrum microorganisms including multidrug resistant bacteria, fungi and virus, are probably because of disruption of the plasma membrane which lead to the lysis of the cells (Reddy *et al*., 2004). Some antibacterial compound inhibit bacterial growth without killing the bacteria, denotes by hazy inhibition zone on test plates. Study conducted by Kho (2008) on bioactive compound from *Auricularia auricula-judae* showed most antibacterial activity in the study has bacteriostatic effects rather than bacteriocidal effects.

In conclusion, *G. australe* strain KUM60813 was selected for further isolation and characterization of bioactive protein/s because of its positive and strong inhibitory activity against all bacteria tested. Lack of antifungal or antiyeast activity demonstrated in the test suggested that the crude protein extracts of the mushrooms studied might not be effective antifungal agents. The best fraction with highest clear inhibitory zone recorded from all susceptible pathogenic bacteria was further fractionated using Superdex 75 HR to purify the protein.

5.5 Antimicrobial activity of purified protein

The purified protein from *G. australe* showed antibacterial activity against *E. coli* O157:H7 and *Salmonella typhi* but was not active against *Escherichia coli*, *Salmonella* sp., *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus*. Weak antibacterial activity with hazy inhibition zone was observed against *Plesiomonas Shigelloides.*

Another antibacterial compound reported from *G. australe* is methyl australate with inhibition against *E. coli, P. aeruginosa, S. aureus, Klebsiella pneumonia, B. subtilis* and *S. typhi* (Smania *et al*., 2007). Compared to our purified protein, methyl australate showed broad spectrum activity against both Gram-negative and Gram-positive bacteria. The purified protein lost its antibacterial activity against Gram-positive bacteria after purification. The reduction in activity of the purified protein might be due to lost of synergism effect present in S6 before purification. Another possibility could be cause by instability of protein after long storage as well as freezing and thawing process. Indeed it is well known that high temperatures can cause denaturation, but some proteins are even sensitive to low temperatures in that they are cold labile. Consequently when working with

new biological extracts with the aim of protein purification, loss of activity may result from the traditional practice of chilling the sample (Bonner, 2007).

In the present study, the mechanism of antibacterial action especially against Gram-negative bacteria was still unknown and should be further investigated. Analyses of bacteria treated with the purified protein for certain incubation time using scanning electron microscope (SEM) can be conducted to observe its mechanism of action. According to Nair *et al*. (2007), the mode of action of cationic proteins that affect Gram-positive bacteria is thought to be due to their interaction with the cytoplasmic membrane, its ultimate disruption and leakage of cytoplasm, resulting in cell death. Since the cell wall structure of the Gram-negative bacteria is more complex than Gram-positive bacteria, the protein mechanisms of action against them are more difficult to explain. Hancock and Chapple (1999) had proposed mechanisms of interaction of cationic antimicrobial peptide with the cell envelope of Gram-negative bacteria in which the peptides bind to the negative charges of the outer membrane or only to the divalent cation binding sites on the outer membrane in order to disturb the outer membrane and interact directly with the hydrophobic part of the membrane. The peptide later binds to the negatively charged surface of the cytoplasmic membrane and inserts further into the membrane interface. The peptides were then either aggregate into micelle-like complex (which spans the membrane) or flip-flop across the membrane and interact in the cytoplasm with negatively charged molecules such as DNA and RNA.

Protein, peptides and polysaccharopeptides from mushrooms have been reported to be capable of inhibiting human immunodeficiency virus type 1 (HIV-1) reverse transcriptase and protease. Both enzymes are very important in the life cycle of HIV-1. A number of studies have reported inhibitory effect of the anti viral compounds derived from

mushrooms at the very early stage of virus replication. A polysaccharopeptides from Turkey tail fungus *Trametes (coriolus) versicolor* has been reported to be capable of inhibiting HIV-1 reverse transcriptase and protease (Ng *et al*., 2006b). Anti HIV-1 reverse transcriptase activity also has been reported of peptide from *Russula paludosa* (Wang *et al*., 2007).

The purified protein from *G. australe* also exhibits HIV-1 reverse transcriptase activity similar with the protein isolated from the previous mention mushrooms. In the present study, promising result were obtained with the purified protein which inhibited the HIV-1 reverse transcriptase activity at a minimal inhibitory concentration of 1.08 mg/ml and 97 percent inhibition at 5 mg/ml.

The activity was similar to previously reported ribosomal-inactivating protein from winter mushroom, *Flammulina velutipes* with complete inhibition of HIV-1 reverse transcriptase at 5 mg/ml or 362.3 µM (Wang and Ng, 2001a). When compare to anti HIV-1 reverse transcriptase peptide from *Russula paludosa*, higher activity was observed. The peptide exhibited 99.2, 89.3 and 41.8 percent when tested at 1, 0.2 and 0.04 mg/ml respectively, giving 50 percent inhibition at 11 µM.

Though the anti HIV-1 reverse transcriptase activity of protein from *G. australe* KUM60813 was lower, modification of the protein can be done to enhance its activity. This supported by research done by Wang and Ng (2001a) on protein from *Flammulina velutipes*. Succinylation potentiated the HIV-1 reverse transcriptase activity of the protein from total inhibition at more than 5 mg/ml to minimal inhibitory concentration as low as 5 µg/ml. Wang and Ng (2000a) also demonstrated that ability of protein to inhibit HIV-1 reverse transcriptase could be increased after chemical modification with succinic

anhydride. Among the reported proteins including those purified from *Pleurotus ostreatus*, milk proteins, protein isolated from American ginseng and Chinese ginseng.

5.6 Other biological activities of purified protein

In the present study, hemolytic activity test was conducted to investigate the possibility of the purified protein to induce haemolysis in human erythrocytes. Despite many research reports on antimicrobial activity of proteins from mushrooms, only few include hemolysis test in their study. The hemolytic activity of the purified protein was compared with melittin, a peptide derived from the honey bee. This peptide is often use as a positive control peptide in the hemolytic assays. The purified protein was shown to demonstrated hemolytic activity, but at a concentration much higher than melittin. At 140 μg/ml, the protein showed 3% hemolytic activity whereas at the same concentration, total hemolysis (100%) was demonstrated by melittin. No activity was detected at concentration lower than 140 μg/ml. However, the purified protein caused toxicity to erythrocytes at concentration lower than those required for antimicrobial activity while melittin was toxic at concentration in the same order of magnitude as its antimicrobial activity (Javadpour *et al*., 1996). Thus, another aspect to be considered is inspection of the effect of the purified protein on the viability of mammalian cells, to see its mechanism of action. According to Zasloff (2002), the toxicity of melittin to human cells is due to hydrophobicity. The ability of antibacterial peptide to lyses cells is the result of complex interrelationship of factors involving conformation, charge, hydrophobicity and amphipathicity (Yeaman and Yount, 2003).

A hemolytic protein was isolated from cultured mycelia of mushroom, *Termitomyces clypeatus* by Khowala *et al*. (1993) and also from *Pleurotus ostreatus*, *Pleurotus eringii* and *Pleurotus nebrodensis* (Bernheimer and Avigad, 1979; Ngai and Ng,

2006; Lv *et al*., 2009). Some of the proteins with hemolytic activity also pose apoptosis-inducing and anti HIV-1 effects. The hemolysin from *Pleurotus nebrodensis* (nebrodeolysin) exhibited remarkable hemolytic activity towards rabbit erythrocytes and caused efflux of potassium ions from erythrocytes. The estimated concentration for nebrodeolysin to induced 50 % hemolysis was 0.1 µg/ml. The protein also showed strong cytotoxicity against Lu-04, Bre-04, HepG2, L929, and HeLa cells and possess anti-HIV-1 activity in CEM cell culture at approximately 40 µg/ml and 65 µg/ml, respectively (Lv *et al*., 2009).

In the case of hemolytic protein from *T. clypeatus* studied by Khowala *et al*. (1993), the protein was analyzed to be a lipoprotein and delipidation removed its hemolytic property. The mode of action was studied by observing protections of sugar and lipid components to the protein mediated lyses of red blood cells. It was observed that the hemolysin possibly interacted with the phospholipid components of the blood cells causing lysis. On the other hand, the hemolytic activity of bee venom toxin, melittin against red blood cells was observed to be abolished on permethylation of the ammonium groups into quartenary trimethylammonium groups. The loss of activity in permethylated melittin may result partly from the absence of the hydrogen bonding potential and partly from steric effects involving the bulky trimethylammonium groups. Displacing the trimethylammonium groups away from the backbone to relieve steric effects (by acylating melittin with glycine or 5-aminopentanoic acid followed by permethylation) restored moderate activity at 5-fold increase in concentration (Ramalinggam and Bello, 1992). Therefore, modification of the purified protein can possibly be done to remove its hemolytic property and induce its antimicrobial activity.

Inhibition of intracellular function in pathogen was suggested as one of antimicrobial peptide mechanism of action, contributed to cell death in pathogen as well membrane dysfunction and inhibition of extra cellular biopolymer synthesis (Yeaman and Yount, 2003). This was described by studies conducted by Xiong *et al*. using tPMPs (trombin-induced-platelet microbicidal protein) in which tPMP mediated inhibition of DNA and/or RNA synthesis corresponded temporarily with cell death in *Staphylococcus aureus* cells but was not observed until 30 or more minutes after membrane permeabilization, verified that non-membranolytic mechanism are responsible for cell death (Xiong *et al*., 2002).

In the present study, the protein from *G. australe* exhibited some deoxyribonuclease activity of 37.3 ± 6.1 units/mg toward salmon sperm DNA. A novel single-chained ribosome-inactivating protein (RIP) with a molecular weight of 29 kDa from fruiting bodies of the edible mushroom *Volvariella volvacea* also exerted deoxyribonuclease activity, on supercoiled SV-40 DNA (Yao *et al*, 1998). An anti tumor lectin from A*grocycbe agaerita* has also been identified to hold endonuclease activity (Zhou *et al*., 2003). DNase assay gave another possible mechanism of antitumor activity of the purified protein. According to Linardou *et al*. (1994) deoxyribonuclease I (DNase I), a compact, monomeric enzyme, serve as a very attractive candidate for targeting to tumor cells. Only a small amount of enzyme targeted to a cell needs to enter the nucleus in order to degrade the chromosomal DNA, making a cell incapable of further replication.

5.7 Comparison of antimicrobial protein from *G. australe* with other *Ganoderma* species

Antimicrobial protein with activity against pathogenic bacteria has not been reported from *Ganoderma australe*. Even among *Ganoderma* species, only few research done on isolation and purification of antimicrobial proteins and peptides from this mushroom. Among reported antibacterial activity was from crude protein extracts of *Ganoderma recinaseum* against *E. coli*, *B. cereus*, *Staphylococcus* sp. and *S. poona* with average activity against all susceptible bacteria (Hearst *et al.*, 2010). However, no further test reported on activity of purified protein from this mushroom.

In the present study, isolation and purification of antibacterial protein have been undertaken. When compared to other reported antimicrobial protein from *Ganoderma*, the purified protein from *G. australe* is different from protein with antifungal activity namely ganodermin and protein with laccase activity purified from *Ganoderma lucidum* (Wang and Ng 2006a; Wang and Ng, 2006b). This is obvious from comparison of their purification protocol and molecular mass. The isolation procedure for ganodermin involved four chromatographic steps on DEAE-cellulose, Affi-gel blue gel, CM-Sepharose and Superdex 75 wheares the laccase was purified by sequential chromatography on DEAE-cellulose and Affi-gel blue gel and adsorption on Con A-Sepharose. Meanwhile, our protein from *G. australe* was purified using only two steps, by gel filtration chromatography on Sephacryl S-100 and Superdex 75. When compared between molecular masses, protein from *G. australe* demonstrated a slightly higher molecular weight compared to ganodermin but lower molecular weight compared to the laccase. Ganodermin exhibits molecular mass of 15 kDa while laccase from *G. lucidum* posses 75 kDa in SDS PAGE.

The proteins from *G. australe* and *G. lucidum* also varied in biological activities. The purified protein from *G. australe* showed anti HIV-1 reverse transcriptase, antibacteria (against Gram-negative bacteria), and deoxyribonuclease activity, with no anti fungal activities against both yeast and plant pathogenic fungi whereas ganodermin showed promising result in inhibiting the mycelial growth of *Botrytis cinerea*, *Fusarium oxysporum* and *Physalospora piricola*. However, ganodermin lacks antibacterial, deoxyribonuclease and HIV-1 reverse transcriptase inhibitory activities. The laccase from *G. lucidum* demonstrated potent inhibitory activity towards human immunodeficiency virus (HIV)-1 reverse transcriptase. There was no study done on hemolytic activity of both proteins from *G. lucidum* towards red blood cells. All the proteins had no ribonuclease activity.