4.0 RESULTS

4.1 Fungal strain

The mycelia of all *Ganoderma australe* strains and *Ganoderma tsugae* grew well on GYMP agar media and full colonization were observed after 7 days incubation at 28^{o}C. The cultures also grew well in GYMP liquid medium and were harvested after 10 days. The culture of selected strain, *G. australe* KUM60813 is shown in Plate 4.1.

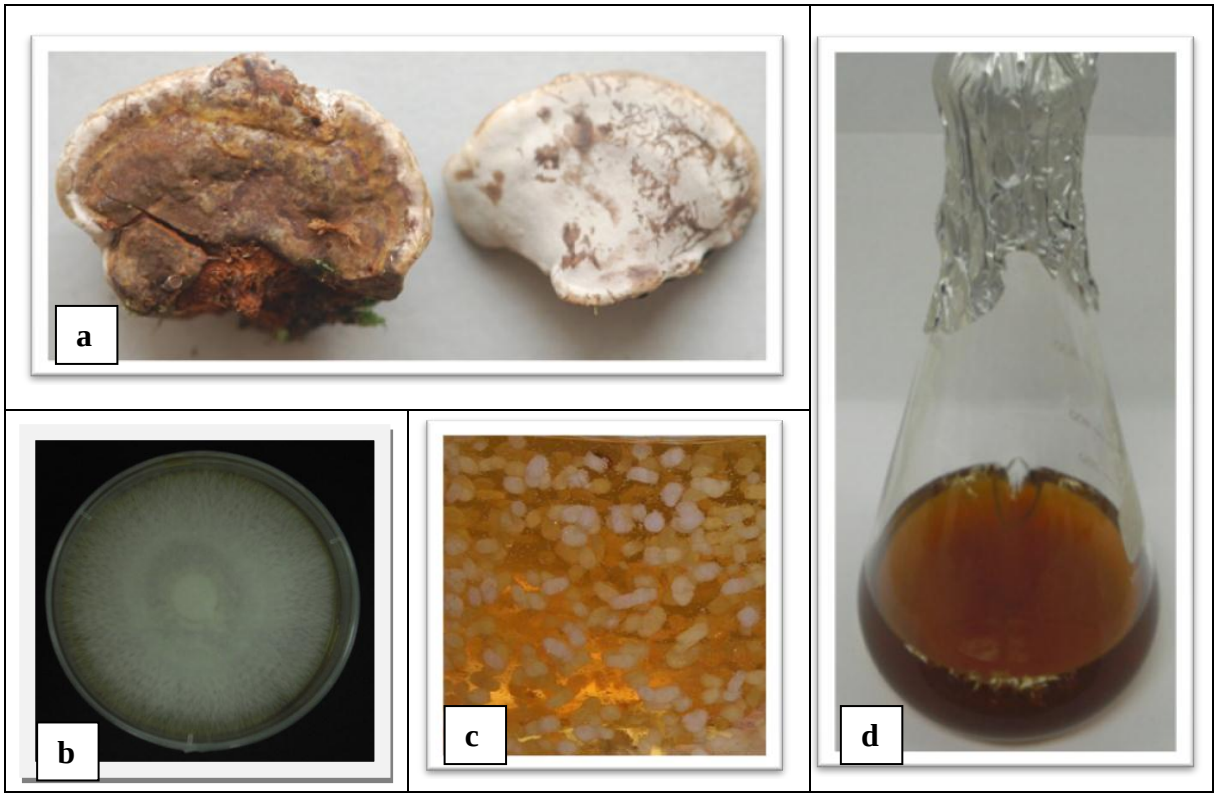

Plate 4.1 : KUM60813. (a) *Ganoderma australe* fruiting bodies; (b) 7 days old mycelia culture on GYMP agar plate; (c) *Ganoderma australe* in liquid culture on 10th day after inoculation; (d) *Ganoderma australe* in liquid culture before incubation for growth.

4.2 Antimicrobial activities of powdery materials (PM) from ammonium sulfate precipitation

4.2.1 Antibacterial activity in primary screening

In vitro antimicrobial activities of the powdery materials from ammonium sulfate precipitation from the *G. australe* strains and *G. tsugae* were tested with Gram-positive and Gram-negative human pathogenic bacteria. *Ganoderma tsugae*, a known mushroom with medicinal properties was used as control to compare its antimicrobial activity with *G. australe* strains. The antibacterial activities of the PM (Section 4.3) were verified by the presence or absence of inhibition zones (Plate 4.2).

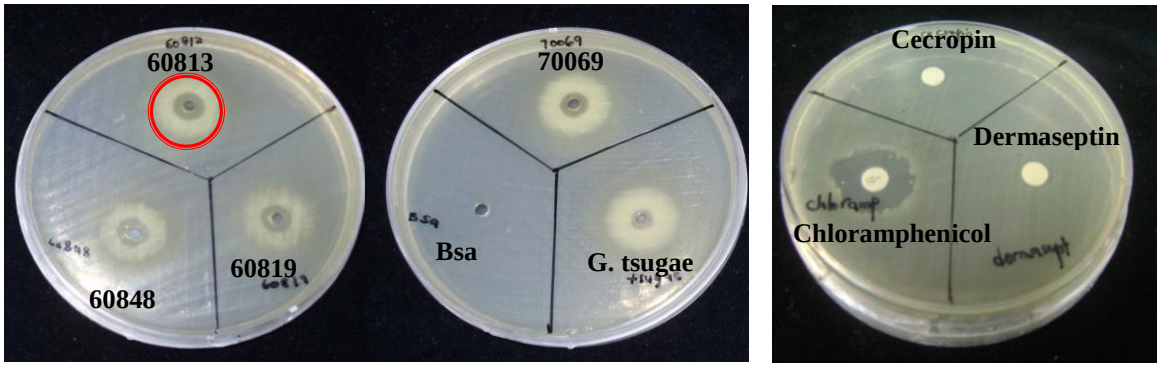

Plate 4.2 : Inhibition plates of *E.coli* O157:H7 on Mueller Hinton agar after 12 hours incubation at 37 ± 2ºC with inhibition zone produced by the PM compared to control.

Table 4.1 shows the antibacterial activity of PM from the mycelia of *Ganoderma* strains tested. Most of the PM of *Ganoderma* strains tested at a concentration of 50 µg / well showed low inhibition activity with clear inhibition zones of 5.5 to 8 mm, especially KUM60848 which indicated the lowest inhibition activity against all bacteria tested. Moderate inhibitory activity with clear inhibition zones of 8.5 to 9.5 mm in diameter was

demonstrated against *B. cereus*, *E. coli* O157:H7 and *S. typhi* by strain KUM60813, whereas *S. aureus* was inhibited moderately by KUM60819. There was also moderate antibacterial activity against *S. typhi* by strain KUM70069 and *G. tsugae* with clear inhibition zones of 9.5 and 11 mm respectively.

Table 4.1 : Antibacterial activity of PM (50µg/well) of *Ganoderma* strains evaluated by well diffusion method after 12 hours of incubation on MH agar medium at 37 ± 2ºC.

Ganoderma strains	Diameter of inhibition zone include 4mm of well (± 1mm)					
	Pathogenic bacteria					
	Gram-positive			Gram-negative		
	S. aureus	*B. cereus*	*B. subtilis*	*E. coli* O157:H7	*S. typhi*	*P. aeruginosa*
KUM60813	8.25	8.5	8	8.5	9.5	7.5
KUM60819	8.5	7.5	7.5	7.75	7.5	7
KUM60848	7.25	8	7.25	7.25	8	5.75
KUM70069	7	8	7.75	7.75	9.5	6.5
G. *tsugae*	6.5	6.5	6.25	6	11	5.5
BSA (30 µg) (Negative Control)	-	-	-	-	-	-
Cecropin (60µg) (Positive Control)	10.3	9	7.5	9	11	9.5
Dermaseptin (60 µg) (Positive Control)	10	9	8	10	10	9
Chloramphenicol (Positive Control)	15	22	13.5	16.3	17.2	21

Figure 4.1 shows antibacterial activity of all *Ganoderma* strains tested against selected pathogenic bacteria. All PM from strains of *G. australe* demonstrated higher percentage of inhibitory activity against tested bacteria which is approximately 30% when

compared to medicinal mushroom *G. tsugae* except for *S. typhi* which was more sensitive to this mushroom.

Negative control which was bovine serum albumin illustrated no inhibition activity while cecropin, dermaseptin and chloramphenicol as standard antibiotic showed moderate and high level of inhibition activity against all Gram-positive and Gram-negative bacteria. In the preliminary screening of antibacterial properties of PM prepared from *G. australe* strains and a *G. tsugae, G. australe* strain KUM60813 showed moderate activities against a broader spectrum of tested organisms. Thus, PM from this strain was selected for further purification of bioactive proteins.

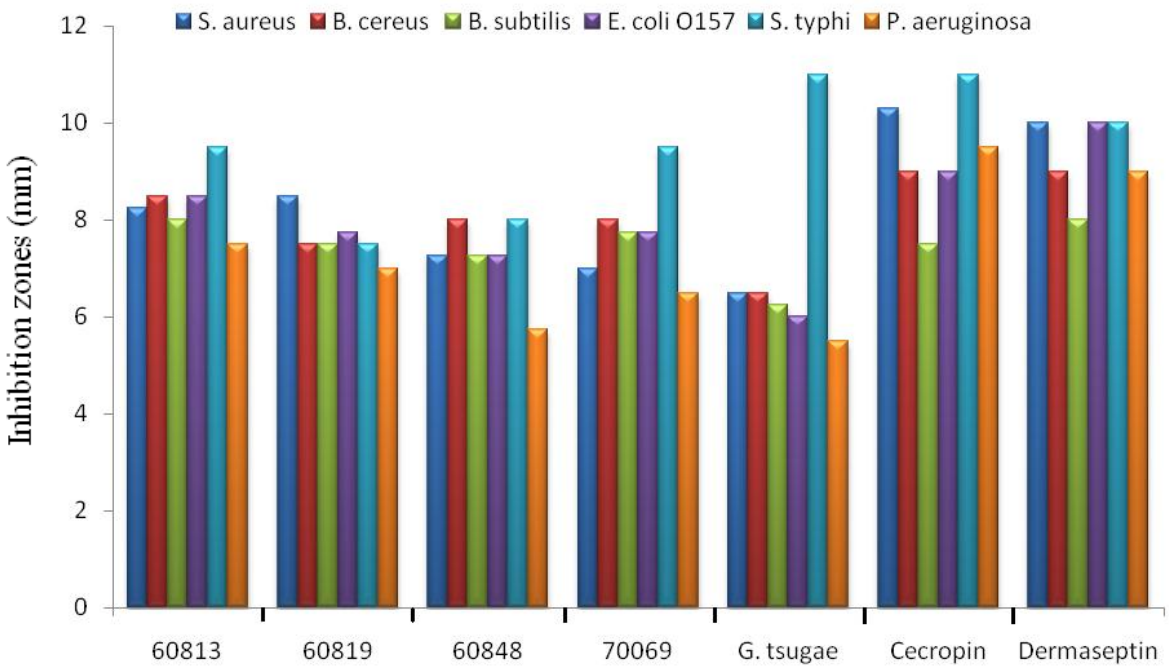

Figure 4.1 : Antibacterial activities of PM of *Ganoderma* strains against human pathogenic bacteria compared to dermaseptin (60 μg) and cecropin (60 μg).

4.2.2 Antifungal activity

The PM from strains of *G. australe* and *G. tsugae* did not exhibit antifungal activity against *Candida albicans*, *Candida parasilopsis* and *Schizosaccharomyces pombe*.

4.3 Purification of protein with antimicrobial activity from *Ganoderma australe* strain KUM60813

In this study, an antimicrobial protein was purified from the mycelia of *G. australe* strain KUM60813. Isolation of the protein involved steps started with extraction of the mycelia with dichloromethane to remove organic compounds. The mycelia were later extracted with 50 % ethanol in order to extract proteinaceous molecules. The ethanolic extract was precipitated by 90 % saturation of ammonium sulfate. This produced a dark brown PM when freeze dried for 24 hours at -50 ± 2ºC. The PM from KUM60813 was selected for purification due to good inhibitory activity demonstrated against both Gram-positive and Gram-negative bacteria compared to other *Ganoderma australe* strains tested. The PM was run on a column of Sephacryl S-100, resulting a separation of eight fractions labeled as S1 to S8 (Figure 4.2).

The dried pooled fractions were used in antibacterial activity test against few human pathogenic bacteria. Table 4.2 shows antibacterial activity of fractions from Sephacryl S-100. From eight protein fractions eluted, only S1, S3, S5, S6 and S7 demonstrated antibacterial activity against tested bacteria. No activity was recorded by S2, S4 and S8. Low antibacterial activity with clear inhibition zones of 6 to 7.3 mm in diameter were recorded by S1, S3 and S5 against susceptible pathogenic bacteria at a concentration of

1mg/well. Fraction S6 illustrated moderate inhibition activity with clear inhibition zones of 9 to 11 mm in diameter against all pathogenic bacteria except for *B. subtilis* which showed lower inhibitory activity. Moderate inhibition activity was also observed against *S. aureus*, *E. coli* and *Salmonella* sp. by S7. All Gram-positive and Gram-negative bacteria tested were sensitive to Cecropin and Dermaseptin, illustrated by moderate antibacterial activity with clear inhibition zones.

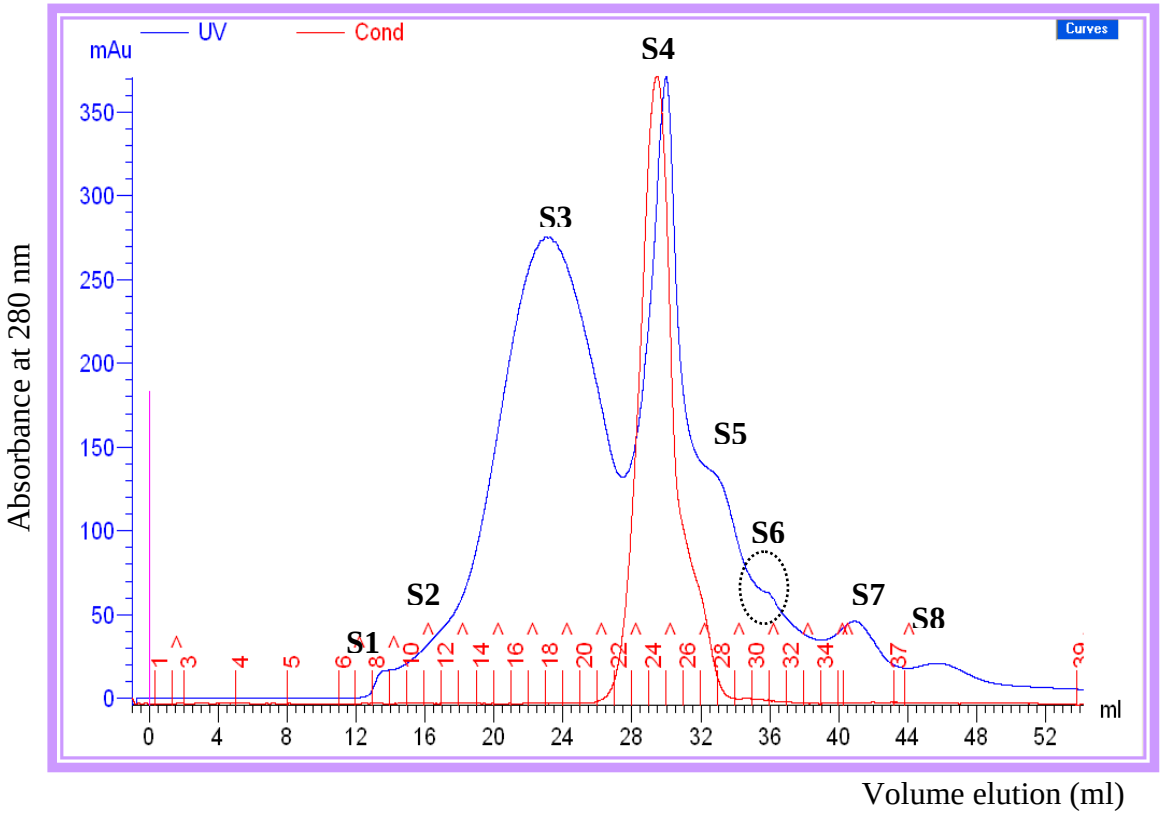

Figure 4.2 : Chromatogram of fractions eluted from S-100 column. Proteins eluted with 10mM Tris HCl buffer (pH 7.4) added with 0.15M Na_2Cl at a flow rate of 1 ml/min. The circle represents the pooled peak that demonstrated good inhibitory activity against few pathogenic bacteria (Table 4.2).

Table 4.2 : Antibacterial activity of protein fractions from Sephacryl S 100 (1mg/well) evaluated by well diffusion method after 12 hours of incubation on MH medium at 37 ± 2ºC. Inhibition zones were measured (in mm) and the diameter of inhibition zones reported included the diameter of the wells (4mm).

Protein Fractions		Gram-positive			Gram-negative		
		S. aureus	*B. cereus*	*B. subtilis*	*E. coli* O157:H7	*E. coli*	*Salmonella* sp.
Time / Elution volume (ml) ↓	S1	6.7 ± 0.3	6 ± 0	7 ± 0	7 ± 0	-	-
	S2	-	-	-	-	-	-
	S3	-	-	7 ± 0.5	-	-	-
	S4	-	-	-	-	-	-
	S5	7 ± 0	7.3 ± 0.6	-	-	-	6.2 ± 0.3
	S6	10.3 ± 0.6	9 ± 0.5	7 ± 0	11 ± 0.5	10 ± 0	9.5 ± 0.5
	S7	9 ± 0	6.0 ± 0	6.5 ± 0.5	-	9.5 ± 0.5	9.5 ± 0
	S8	-	-	-	-	-	-
BSA(30 µg) (Negative Control)		-	-	-	-	-	-
Cecropin (60µg) (Positive Control)		10.3 ± 0.6	9 ± 0	7.5 ± 0.3	9 ± 0.5	11 ± 0.5	9.5 ± 0
Dermaseptin(60 µg) (Positive Control)		10 ± 0	9.2 ± 0.3	7.8 ± 0.3	10 ± 0	10 ± 0	9.2 ± 0.3

* Antibacterial activity against *Salmonella typhi*, *Pseudomonas aeruginosa* and *Plesiomonas shigelloides* was not detected.

p = 0; **p < 0.05

Analysis carried out for antibacterial activity of positive protein fractions from Sephacryl S-100 showed significant difference ($p<0.05$) in width of inhibition zone between fractions and standard proteins against *B. subtilis* and *E. coli* (Table 1 and 2, Appendix E). Fraction S6 demonstrated significant difference ($p=0$) in width of inhibition zone against *S. aureus* and *B. cereus* when compared to other fractions with antibacterial

activity. Conversely, inhibition activity of S6 against *E. coli* O157:H7 demonstrated significant difference (p=0) when compared to standard proteins, denoted by higher inhibition zone. No significant difference in width of inhibition zone was exhibited by S1, S3, S6 and cecropin against *B. subtilis*. For inhibition activity against *E. coli* and *Salmonella* sp., significant difference (p=0) in inhibition zones was illustrated by cecropin to other positive fractions and fraction 5th (S5) to other positive fractions and standard proteins, respectively (Table 1 and 2, Appendix E).

Figure 4.3 illustrates antibacterial activity of protein fractions from Sephacryl S-100. Only S6, cecropin and dermaseptin exhibited antibacterial activity against all tested bacteria. Most of the tested bacteria were not susceptible to S2, S3, S4, S5 and S8. Fractions S6 and S7 demonstrated almost similar inhibitory activity except that no activity was recorded by S7 against *E. coli* O157:H7. The inhibition zone recorded by S6 against Gram-negative bacteria was almost comparable to standard proteins.

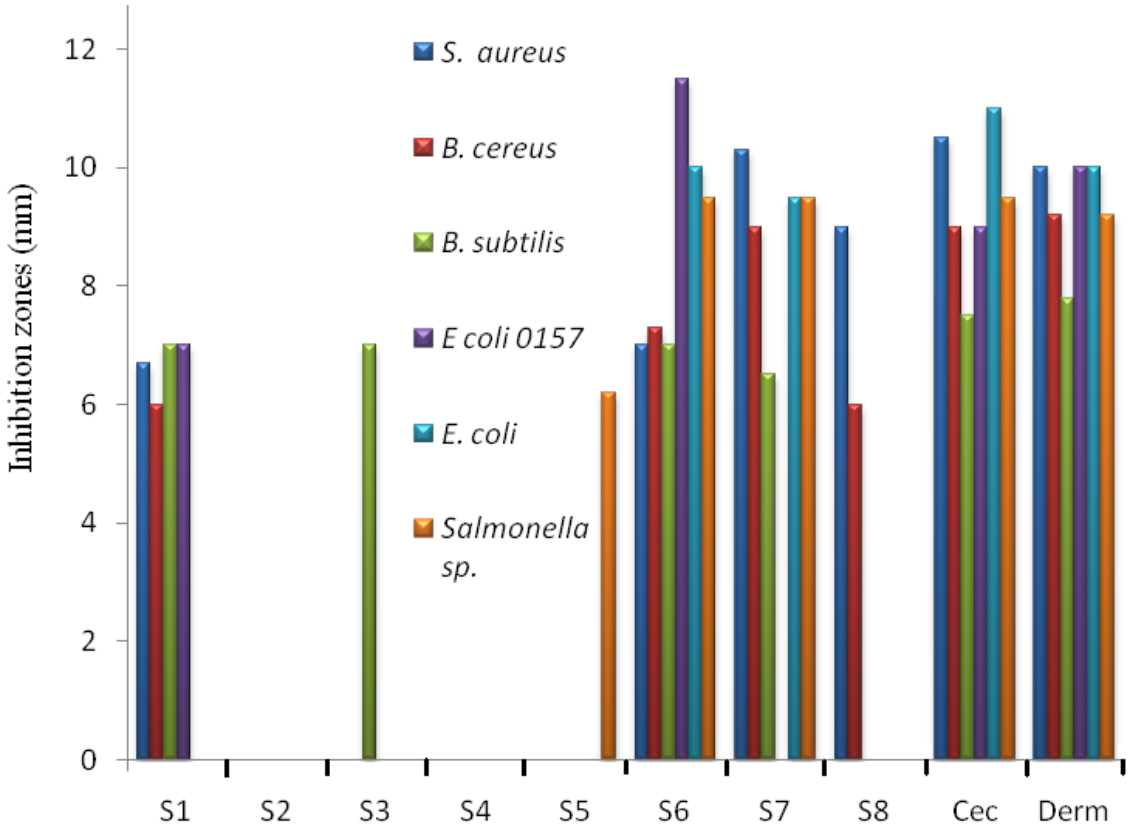

Figure 4.3 : Antibacterial activities of protein fractions from Sephacryl S-100 labeled as S1 to S8 against few pathogenic bacteria compared to dermaseptin (60 µg) and cecropin (60 µg).

Fraction S6 with good antibacterial activity amongst all fractions was subjected to gel filtration on Superdex 75 HR. This fraction demonstrated antibacterial activity against most of the tested bacteria with antibacterial activity against Gram-negative bacteria almost similar to cecropin and dermaseptin. The purification step on Superdex 75 HR yields two peaks, SP1 and SP2, of different size. The SP2 was the major peak (Fig. 4.4). Antibacterial activity was only existed in this peak. The protein was colorless in solution but appeared as whitish powder when freeze dried for 24 hours at -50 ± 2ºC.

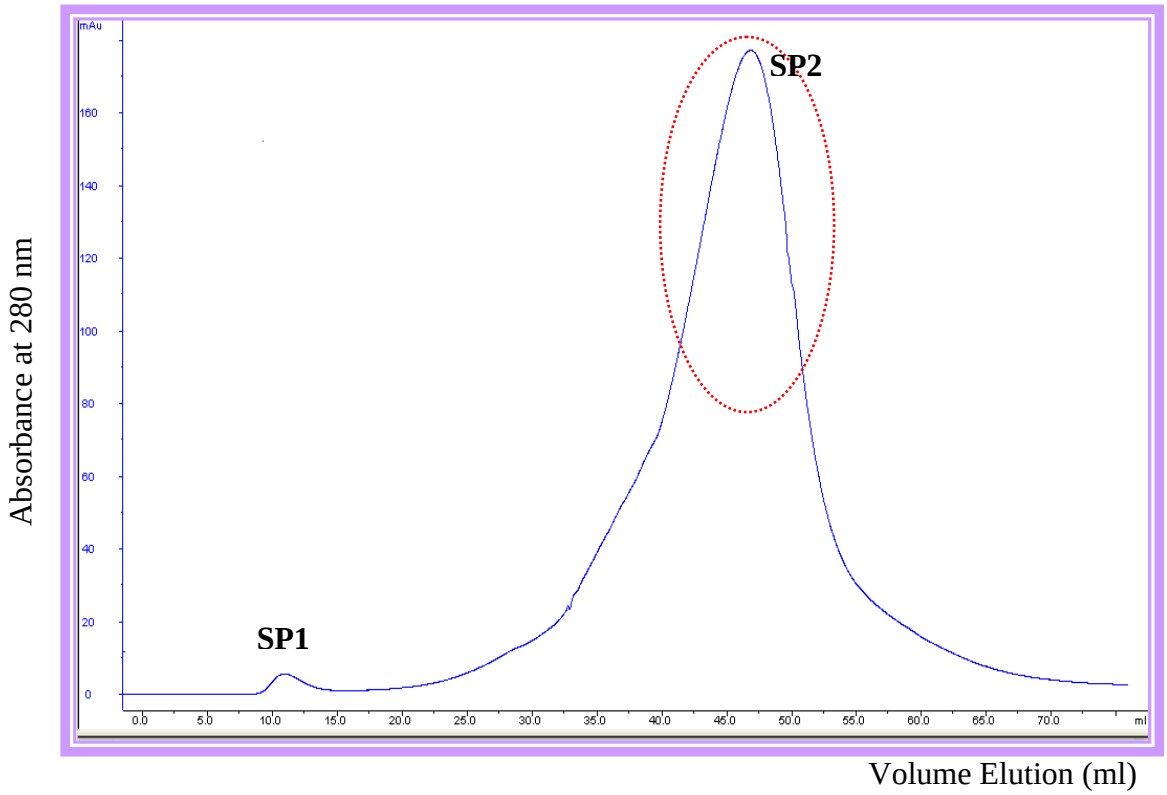

Figure 4.4 : Chromatogram of fractions eluted from Superdex 75 HR column. Proteins eluted with 10Mm Tris Hcl buffer (pH 7.4) added with 0.15M Na_2Cl at a flow rate of 1 ml/min. The circle represents the pooled peek demonstrated inhibitory activity against *E. coli* O157:H7 and *S. typhi*.

Table 4.3 shows the antibacterial activity of SP2 from Superdex 75 HR. The fraction exhibited clear inhibition zone of 11 mm in diameter against *E. coli* O157:H7 and 9 mm against *S. typhi*. The remaining seven pathogenic bacteria were not sensitive to the purified protein except for *P. shigelloides* which exhibited hazy inhibition zone of 8 mm in diameter. Positive antibacterial activity was only demonstrated against Gram-negative bacteria. Cecropin as positive control showed moderate inhibition activity against all pathogenic bacteria tested. Statistical analysis of antibacterial activity of SP2 and cecropin

demonstrated significant difference ($p<0.05$) in width of inhibition zone (Table 3, Appendix E).

Table 4.3 : Antibacterial activity of protein fraction, SP2 from Superdex 75 HR 10/30 (1mg/well) evaluated by well diffusion method after 12 hours of incubation on MH medium at 37 ± 2ºC. Inhibition zones were measured (in mm) and the diameter of inhibition zones reported included the diameter of the wells (4mm).

Pathogenic bacteria	Purified protein	Cecropin (50ug)	BSA
Escherichia coli	-	11 ± 0.5	-
Escherichia coli O157:H7	11 ± 0	9 ± 0	-
Salmonella sp.	-	10 ± 0.5	-
Salmonella typhi	9 ± 0.9	11 ± 0	-
Plesiomonas shigelloides	8* ± 0.5	-	-
Bacillus subtilis	-	7.7 ±0.6	-
Bacillus cereus	-	9 ± 0	-
Staphylococcus aureus	-	10.3 ± 0.6	-

*indicated hazy inhibition zone

The purity of the purified protein was further analyzed with Tricine SDS PAGE (Fig. 4.5). The eluted fraction from Superdex 75 HR, SP2 with antimicrobial activity appeared as single band with molecular weight estimated to be 16.6 kDa. The overall purification yield of the protein is summarized in Table 4.4.

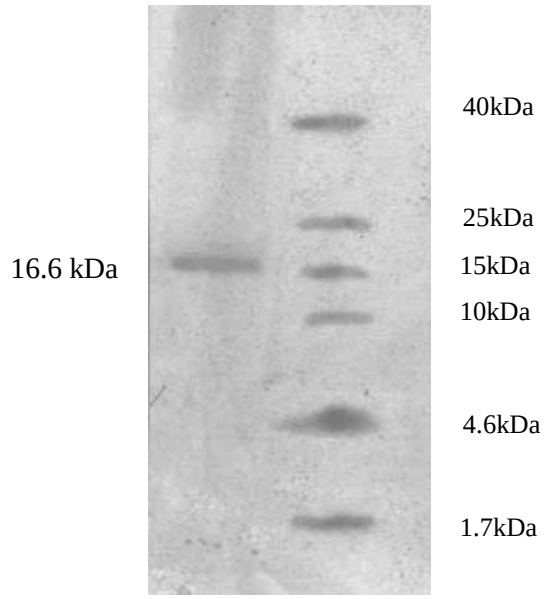

Figure 4.5 : Determination of molecular mass by Tricine-SDS PAGE. The molecular mass of isolated protein shown in lane B was estimated at 16.6 kDa. Lane B shows Fermentas Spectra Multicolor Low Range Protein Ladder (1.7 – 40 kDa).

Table 4.4 : Purification of bioactive protein from *G. australe* KUM60813 by using gel filtration on Sephacryl S-100 and Superdex 75 HR.

Sample	Total protein (mg)	Total yield (%)
Powdery material (PM)	3260	100
S6 (Sephacryl S 100)	156	4.79
SP2 (Superdex 75 HR)	27.98	0.86

Table 4.4 shows summary of protein yield at different stages of purification. From 111.4g of dried mycelia, 0.028g antimicrobial protein was successfully purified. The purified protein was later tested for its medicinal properties.

4.4 Identification of purified protein

The purified protein was analysed using ProFound program. Table 4.5 showed the result for homology searching of the *Ganoderma australe* purified protein from the ProFound program. The analysis showed a high similarity between purified protein and the antifungal protein from *Aspergillus giganteus*. The match protein showed the coverage of 32% (observed between full length amino acid sequences) in the highest case and no other homologous sequence has been found. Percent coverage demonstrated the ratio of the portion of protein sequence covered by matched peptides to the whole length of protein sequence.

Table 4.5 : Identification of purified protein from *Ganoderma australe* strain KUM60813 using ProFound.

Rank	Probability	Est'd Z	Protein Information & Sequence Analyses Tools (T)	%	pI	kDa
+1	1.0e+000	2.43	gi | 2306 | emb | CAA37523.1 | antifungal protein [*Aspergillus giganteus*]	32	9.5	8.58

The probability of the purified protein sequence to the antifungal protein is 1.0e+000. The probability value (1.0e+000) describes the probability that the respective protein identified in the ProFound database is the protein analysed based on data, experimental conditions and other background information. The Z score of the purified protein to the antifungal protein from *A. giganteus* is 2.43. A score of 2.43 means that the search is in the 99th percentile, which means there are only about 1% of random matches that could yield higher Z score than the given search.

Table 4.6 shows amino acid sequence of the antifungal protein from *Aspergillus giganteus*. The sequence was subsequently analyzed using protein to protein BLAST to find similarity with other sequences in the database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), producing significant alignments with antifungal proteins in the Conserved Domain Antifungal protein Super-family (Appendix D, Table 5).

Table 4.6 : Peptide sequence of antifungal protein from *Aspergillus giganteus* (CAA37523.1)

Average Mass	Monoisotopic Mass	Residues		Peptide Sequence
		Start	End	
8145.404	8139.889	1	75	VATPVEADSLTAGGLDARDESAVLATYNGK CYKKDNICKYKAQSGKTAICKCYVKKCPRD GAKCEFDSYKGKCYC

(http://www.ncbi.nlm.nih.gov/protein/2306?report=genbank&log$=protalign&blast_rank=2&RID=1JBJW9G7016).

4.5 Bioactive properties of purified protein

The purified protein was tested for other bioactivities including antimicrobial activity, ribonuclease, deoxyribonuclease, anti HIV-1 Reverse Transcriptase and hemolytic activities

4.5.1 Minimal Inhibitory Concentration against *Escherichia coli* O157:H7 and *Salmonella typhi*

Table 4.7 shows the result of antibacterial activity of the broth micro dilution assays against *E. coli* O157:H7 and *S. typhi*. The purified proteins reduced 3.9 % growth of *E. coli*

O157:H7 at concentration as low as 5 μg/ml and inhibited 9 % growth of *S. typhi* at 0.5 μg/ml. The inhibition of bacterial growth increased proportionate to the increased of concentrations of the purified protein.

Analyses carried out in antibacterial activity by micro dilution method showed significant difference (p = 0) in inhibition activity, demonstrated by different concentration of purified protein against *E. coli* O157:H7 and *S. typhi*. (Table 5, Appendix E). This denoted by the existing of six different homologous groups in Multiple Range Test table for activity against *E. coli* O157:H7 and five different homologous groups for activity against *S. typhi*.

Table 4.7 : Antibacterial activity of purified protein at different concentration evaluated by broth micro dilution method after 18 hours of incubation in MHB medium at 37 ± 2ºC.

Protein concentration (mg/ml)	Percent inhibition	
	Escherichia coli O157:H7	*Salmonella typhi*
5.0	60.46 ± 2.5 [a]	48.2 ± 2.4 [a]
0.5	13.07 ± 1.5 [b]	22.97 ± 3.4 [b]
0.05	9.8 ± 1.0 [c]	20.27 ± 2.0 [b]
0.005	3.92 ± 1.0 [d]	14.41 ± 1.4 [c]
0.0005	0	9.0 ± 3.0 [d]
Without sample (Control)	0	0

Figure 4.6 and 4.7 illustrates growth inhibition activity of purified protein against tested bacteria. The purified protein reduced the growth of *E. coli* O157:H7 by more than 50% at a concentration of 4 mg/ml whereas *S. typhi* was inhibited at 50% at 5.21 mg/ml.

The increase in protein concentration was significantly increasing the percent inhibitory activity (Tables 5 and 6, Appendix E). Comparison in percent inhibition of antibacterial activity was also illustrated against both pathogenic bacteria at different level of protein concentrations (Tables 7 and 8, appendix E).

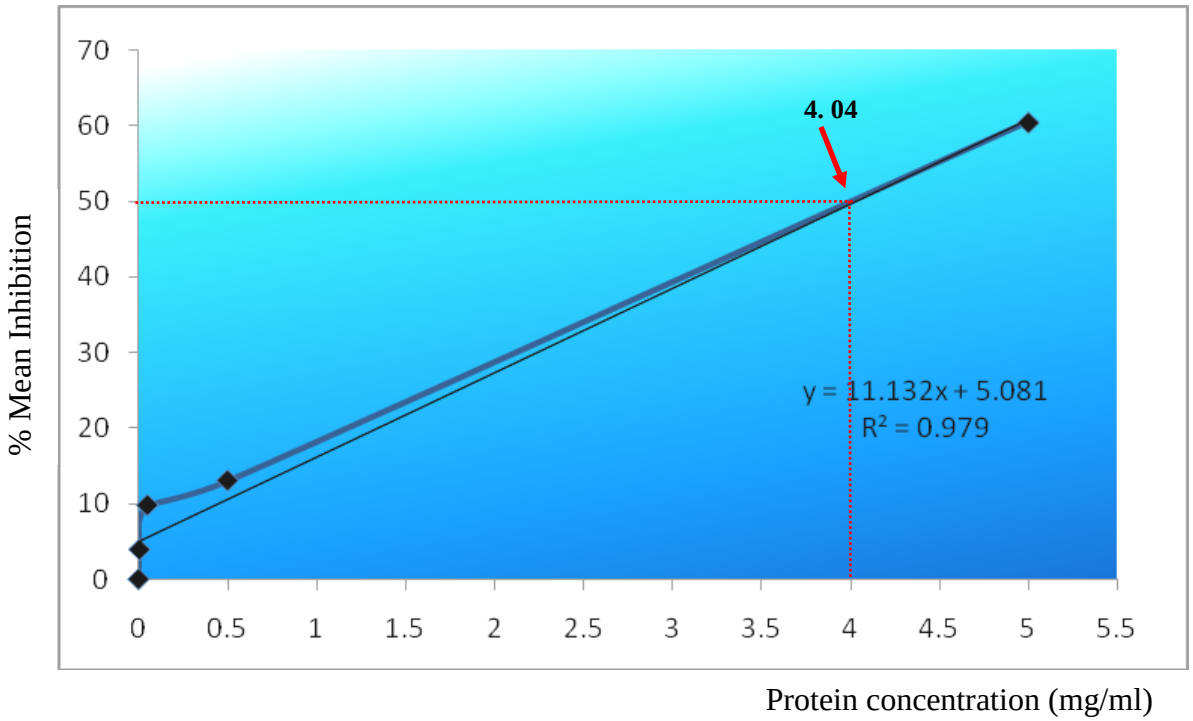

Figure 4.6 : Antibacterial activity of purified protein against *E. coli* O157:H7 by broth micro dilution method. Minimal inhibitory concentration of purified protein was indicated by arrow.

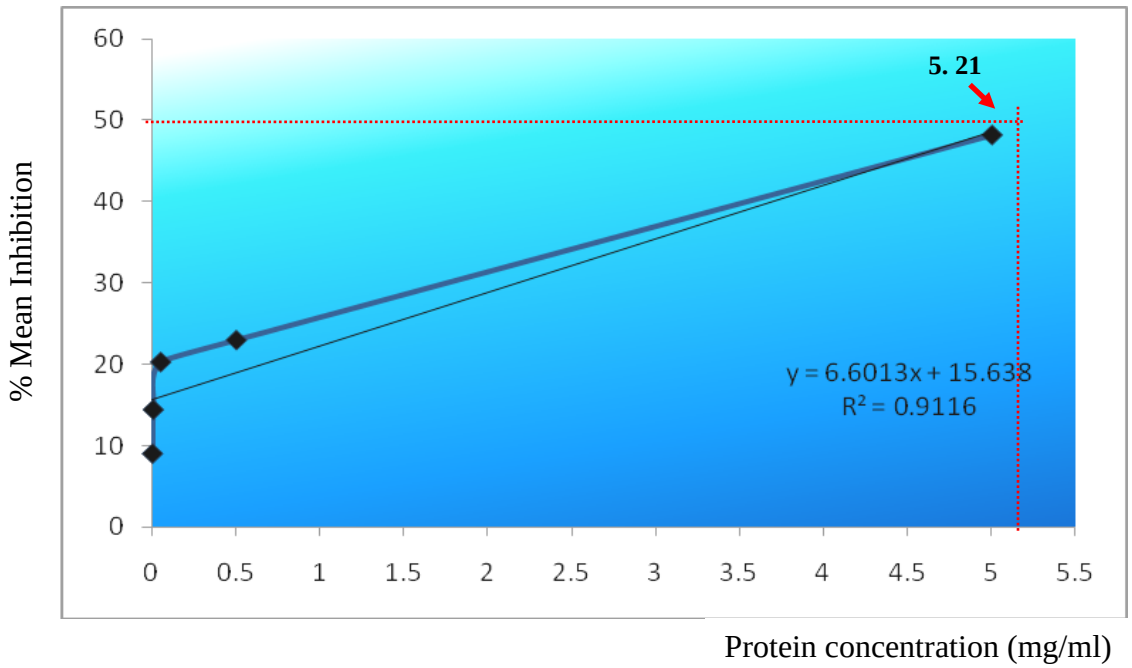

Figure 4.7 : Antibacterial activity of purified protein against *S. typhi* by broth micro dilution method. Minimal inhibitory concentration of purified protein was indicated by arrow.

4.5.2 Anti HIV-1 Reverse Transcriptase activity

The HIV-1 RT inhibition activity of purified protein was assayed using Reverse Transcriptase assay, calorimetric. The percent inhibition of HIV-1 RT increased with increasing concentrations of the purified protein. The activity ranged from 19.4% to 97% when tested at the concentration ranged from 39 µg/ml to 5.0 mg/ml (Table 4.8). It was inhibited 50% of HIV-1 RT activity at a concentration of 1.08 mg/ml (Fig. 4.8). The analysis carried out for anti HIV-1 RT activity of purified protein showed significant difference ($p = 0$) in percent inhibition at different concentrations (Table 9 and 10, Appendix E).

Table 4.8 : Anti HIV activity of purified protein from Superdex 75 HR 10/30 evaluated with the reverse transcriptase calorimetric assay. The inhibitory activity of the protein was calculated as percent inhibition as compared to a control without the protein.

Protein concentration (mg/ml)	Inhibitory activity (%)
5	97.0 ± 0.1[a]
2.5	95.7 ± 0.2 [a]
1.25	56.6 ± 2.0[b]
0.625	34.1 ± 1.6[c]
0.313	25.9 ± 0.4[d]
0.156	24.6 ± 2.0[d]
0.078	22.0 ± 0.6[e]
0.0039	19.4 ± 1.7[f]
Without sample	No activity
ABTS solution (Blank)	No activity

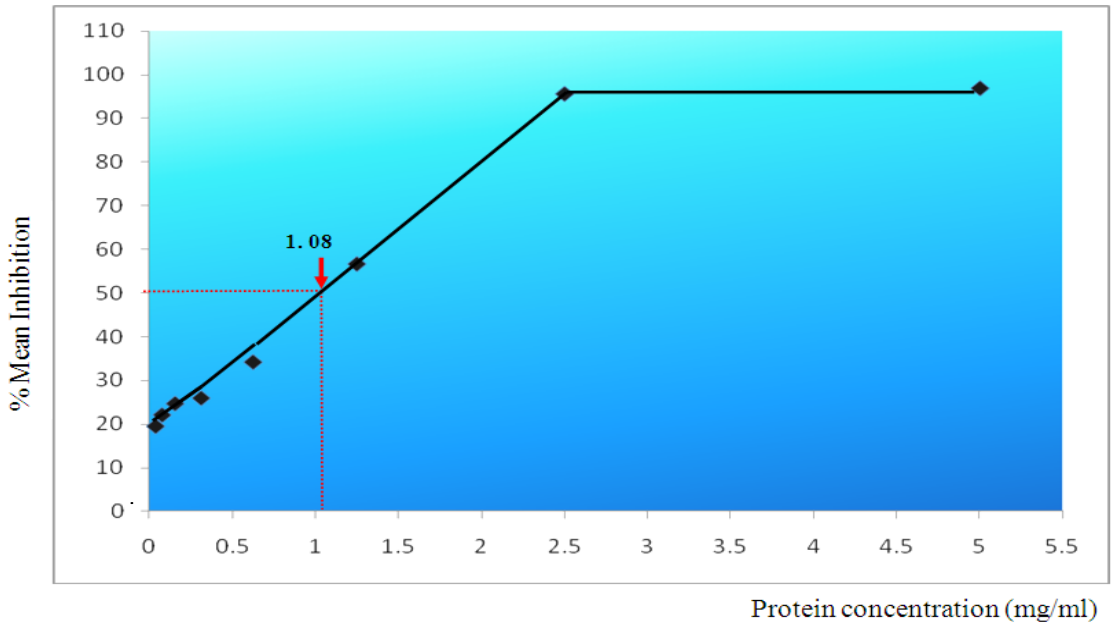

Figure 4.8 : Inhibitory effect of purified protein on activity of HIV-1 reverse transcriptase. (Data represent means ± SD, n = 3)

4.5.3 Deoxyribonuclease activity

Deoxyribonuclease activity of the purified protein was tested toward salmon sperm DNA. One unit of enzymatic activity was defined as the amount of enzyme which produces an absorbance increase at 260 nm of 0.001 min^{-1} ml^{-1} at pH 5.5 at 37^{o}C. The purified protein exhibited an activity of 37.3 ± 6.1 units/mg toward herring sperm DNA.

4.5.4 Hemolytic activity

The hemolytic activity of the purified protein was done to measure its cytotoxicity against human erythrocytes. The activity was compared to melittin, a toxin from honeybee venom. The purified protein was found to be hemolytic at concentration started from 140 μg/ml. When both proteins were tested against human erythrocytes at concentrations from 0.27 to 70 μg/ml, melittin as a control showed high hemolytic activity while purified protein exhibited no activity at the same concentrations (Table 4.9). Total hemolysis was demonstrated by melittin at concentration ranged from as low as 8.75 μg/ml to 140 μg/ml after incubation for one hour.

Table 4.9 : Hemolytic activity of purified protein compared to Melittin from honeybee.

Concentration (ug/ml)	% Inhibition compared to Control	
	Purified protein	**Melittin**
140	3.0 ± 1.5^{e}	102.8 ± 2.5^{a}
70	-	101.7 ± 1.6^{a}
35	-	101.9 ± 1.5^{a}
17.5	-	100.1 ± 3.3^{a}
8.75	-	100 ± 0.8^{a}
4.38	-	59.9 ± 4.6^{b}
2.19	-	12.4 ± 3.7^{c}
1.09	-	9.5 ± 2.0^{cd}
0.55	-	7.3 ± 4.3^{de}
0.27	-	0
0.1% tween 20 (control)	100	100
PBS	-	-

*Values represent means ± SD

4.5.5 Antifungal and ribonuclease activity

The purified protein did not have antifungal activity against *Fusarium oxysporium cubense I* (FOC1), *Fusarium oxysporium cubense II* (FOC2), *Fusarium oxysporium cubense IV* (FOC4), *Ganoderma boninense* and *Colletotrichum* sp. at concentrations of 40 and 200 μg. The purified protein also did not have ribonuclease activity at a concentration of 50 μg.