3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and Disposables

Sigma Aldrich Co. USA.

Acetonitrile

Ammonium bicarbonate

Bovine Serum Albumin (BSA)

Cecropin

Dermaseptin

Ethylene diamine tetra acetic acid (EDTA)

Gluteraldehyde solution

Glysine

Lanthanum nitrate

Magnesium sulfate

Perchloric acid

Sodium acetate

Sodium carbonate

Thiourea

Triton X-100

Trizma base

Yeast tRNA

MES buffer

Systerm, Classic Chemicals Sdn. Bhd, Selangor.

85% Phosphoric acid solution

Absolute ethanol

Dichloromethane (DCM)

Di-potassium hydrogen phosphate

Formaldehyde

Glucose

Glycerol

Magnesium sulfate

Potassium di-hydrogen phosphate

Silver nitrate

Sodium chloride

Sodium hydrogen phosphate (Na_2HPO_4)

Sodium thiosulphate

Oxoid Limited, United Kingdom

Bacto Agar

Chloramphenicol

Malt extract

Mueller Hinton Broth (MHB)

Nutrient Agar (NA)

Nutrient Broth (NB)

Sabouraud Dextrose Agar (SDA)

BBL Difco Microbiology Spectrum USP Chemicals USA Instruments, USA.

Bacto peptone

Bacto yeast extract

Mueller Hinton Agar (MHA)

Potato Dextrose Agar (PDA)

Merck and Co., Inc. USA.

2-Mercapto ethanol

Ammonium acetate

Sodium hydroxide

Biorad Laboratories, Inc. California, USA.

Ammonium persulfate (APS)

Coomassie Brilliant Blue G-250

Sodium Dedocyl Sulfate (SDS)

R & M chemicals Ltd. Edmonton, Alberta.

Acetic acid

Hydrochloric acid

Trichloro acetic acid

Urea

BDH, United Kingdom.

Ammonium chloride

Invitrogen, Carlsbad, California, USA.

Herring sperm DNA

Novex® Tricine Pre-Cast Gels

XCell Sure Lock Mini-Cell

Tricine SDS Running Buffer (10X)

Tricine SDS Sample Buffer (2X)

Fermentas, Vilnius, Lithuania.

Spectra Multicolor Low Range Protein Ladder

GE Healthcare, United Kingdom.

Sephacryl S 100 HR

Superdex 75 HR

3.1.2 Instrumentation

Electrophoresis Power supplies (Scie-PLAS Ltd, United Kingdom)

Image Scanner III (GE Healthcare, United Kingdom)

ACTA Prime plus (GE Healthcare, United Kingdom)

ELISA plate reader (Tecan Group Ltd, Austria)

Freeze Dryer –CHRIST (Martin Christ GmbH, Germany)

Rotary Evaporator – BUCHI (BUCHI, Flawil, Switzerland)

3.1.3 Samples

The mushrooms used in this study were *Ganoderma tsugae* and *Ganoderma australe* strains KUM60813, KUM60819, KUM60848, and KUM70069. Both strains KUM60813 and KUM60819 were collected from Fraser Hill, Pahang. Strains KUM60848 and KUM70069 were collected at Institute of Biological Studies, University Malaya and Taman Negara Endau Rompin, Selai, Johor, respectively. All *Ganoderma australe* strains and *Ganoderma tsugae* (a commercial strain from Ganofarm Sdn. Bhd. Malaysia) were maintained as the live culture collections and provided by Mushroom Research Centre, Institute of Biological Science, University of Malaya.

3.1.4 Test organisms

The bacteria used in the test were *Staphylococcus aureus, Bacillus cereus*, and *Bacillus subtilis* for Gram-positive and *Escherichia coli* O157:H7, *Salmonella typhi*, and *Pseudomonas aeruginosa* for Gram-negative bacteria while pathogenic yeast tested included *Candida albicans, Candida parasilopsis* and *Schizosaccharomyces pombe*. The phytopathogenic fungus used in antifungal test included *Fusarium oxysporium cubense I* (FOC1), *Fusarium oxysporium cubense II* (FOC2), *Fusarium oxysporium cubense IV* (FOC4), *Ganoderma boninense* and *Colletotrichum* sp.

The Gram-positive bacteria were obtained from culture collections of Prof Thong Kwai Lin while the Gram-negative bacteria were obtained from Dr. Noni Ajam of Institute of Biological Sciences, University of Malaya. All yeasts species were obtained from Prof Ng K.P. from University Malaya Medical Center, UMMC. The *Fusarium* sp. and

Colletotrichum sp. cultures were obtained from Dr. Vijaya S.K. from University Putra Malaysia (UPM) while *Ganoderma boninense* strain Per71 was obtained from Sime Darby, Banting.

3.2 METHODS

3.2.1 Media preparation

The mushrooms were maintained on GYMP agar while the bacteria were maintained on NA. In the antimicrobial test, MHA, SDA and yeast peptone glucose (YPG) agar were used. Preparation of these agar medium included steps of dissolution of ingredients (Appendix A) in distilled water according to standard volume, followed by sterilization at 121^{o}C at 15 psi for 15 minutes. It was then cooled and poured into several sterile Petri plates and left to solidify before use.

3.2.2 Submerged cultivation for mycelia production

In this study, submerged cultivation was chosen for mass production of mycelia because of its short fermentation period, fast growth and high productivity compared to solid substrate fermentation. *Ganoderma* strains were initially subcultured on GYMP agar media for 7 days. Ten milliliter of sterilized distilled water was poured on this plate and only mycelia were scraped with inoculation loop to obtain mycelia suspension. The ten milliliter mycelia suspension was transferred into 200ml of GYMP liquid medium in 500ml Erlenmeyer conical flask. Cultivation of *Ganoderma* mycelia was carried out under

agitation of 120rpm for 10 days. The ten days cultured mycelia were filtered and weighed. The experimental flow chart of the study is as indicated in Figure 3.1.

3.2.3 Preparation of extracts

The fresh mycelia of *Ganoderma* spp. were soaked in dichloromethane in a ratio of 1:2 at 28 ± 2 ^{o}C and agitated at 120 rpm for 24 hours. The mycelia were then separated and dried at room temperature . The mycelia were soaked in 50% ethanol in a ratio of 1:2. The ethanolic extract was collected by filtration through Whatman No. 4 filter paper.

3.2.4 Ammonium sulfate precipitation of proteins

The ethanolic extracts were concentrated using rotor-evaporatorprior to use for precipitation process. Precipitation of protein was performed at 90% of ammonium sulfate. Ammonium sulfate was added slowly to a final concentration of 90% and stirred at 4^{o}C until all the salt dissolved. For 90% precipitation, the quantity of ammonium sulfate needed was determined from the Table 1 (Appendix D). This step was followed by centrifugation of the solution at 12000 rpm at 4^{o}C for 30 minutes. The pellet was separated from the supernatant and resuspended in an appropriate volume of 10mM Tris HCl buffer (pH 7.4) prior to freeze drying. After freeze-dried, the dark brown powdery materials (PM) were stored at -20^{o}C for further analysis.

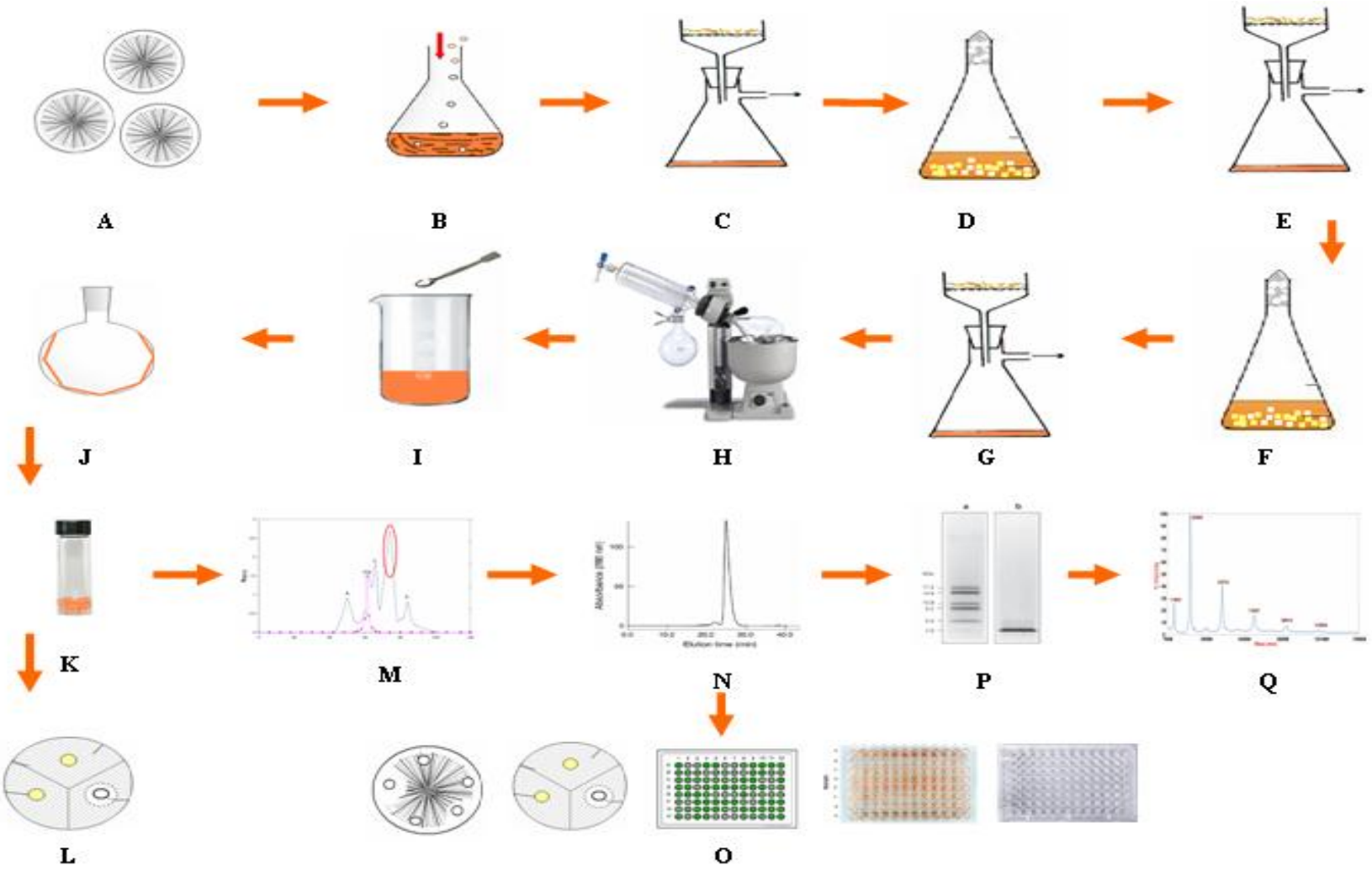

Figure 3.1 : Flow chart of whole experimental methods involved in the study.

Descriptions of flow chart of whole experimental methods involved in the study

code	Descriptions
A	*Ganoderma* strains were sub cultured in GYMP agar media and grew for 7 days.
B	10 ml mycelial suspension was transferred into 200ml of GYMP liquid medium and shook at 120rpm for 10 days.
C	The cultured mycelia were filtered to separate them from culture medium and weighed.
D	The weighed mycelia were extracted with 100% Dichloromethane (DCM) in a ratio of 1:2 at 120rpm for 24 hours.
E	The extracted mycelia were again filtered to separate from the solvent prior to self evaporation under fume hood overnight.
F	The weight of mycelia was measured again before extraction with 50% ethanol in a ratio of 1:1 at 120rpm for 24 hours.
G	The extracted mycelia were filtered again to remove them from the ethanol.
H	The ethanolic extracts were concentrated using rotor-evaporator.
I	The dissolved proteins were precipitated out using 90% ammonium sulfate and then separated from supernatant at 12000rpm for 30 minutes.
J	The pellet was separated from the supernatant and resuspended in an appropriate volume of buffer prior to freeze dried for 24 hours at -50 ± 2ºC to get crude protein powder.
K	The powdery materials (PM) were kept at -20^{o}C for next test and purification steps.
L	PM were used in antimicrobial test. Best strain was selected for future isolation and purification of protein using gel filtration.
M	Gel filtration of crude protein from selected strain on Sephacryl S-100. Fraction with highest antibacterial activity was selected for further purification step.
N	Gel filtration of selected fraction from Sephacryl S-100 on Superdex 75 HR 10/30.
O	Assays on purified protein.
P	Molecular weight determination of purified protein using Tricine SDS PAGE.
Q	Identification of protein using ProFound

3.2.5 Primary screening of antimicrobial activities

The screening for antimicrobial activities of the PM is divided into antibacterial and anti-yeast test using modified agar well diffusion method (Stroke and Ridgway, 1980).

The assay for antifungal activity against few human pathogenic yeasts and antibacterial activity towards selected pathogenic Gram-positive and Gram-negative bacteria were carried out in 100mm x 15mm petri plates containing 15 ml of SDA media for *Candida* sp., YPG media for S. *pombe* and MHA media for bacteria. Twenty four hours old cultures of bacteria and two days old cultures of fungi were lawned using sterile cotton swap onto the entire surface of the test media (adjusted to 10^5 cfu/ml), three wells with diameter of 4mm were on the surface test media using a sterile cork borer. Each well was loaded with 50 µg PM in 20 µl of sterile distilled water. The plates were incubated at 37 ± 2 ^{o}C except for *S. pombe* at 27± 2 ^{o}C (Fig. 3.2).

A control experiment was set up by loading 20µl of Nystatin for antifungal test. The 20µl of 60µg Cecropin, 60µg Dermaseptin and standard disc of 30µg Chloramphenicol for antibacterial test were used as positive control while BSA was used as negative control. Diameters of inhibition zones were recorded after 24 hours. The inhibitory zones were measured in millimeters and the diameter of inhibition zone reported included the diameter of the disc (6 mm) and the diameter of the wells (4mm). Negative results were regarded as those in which no inhibition zone was observed after 24 hours. All tests were carried out in duplicates and their means were recorded. The *Ganoderma* strain which had the strongest antimicrobial activity was then selected for isolation and purification of the bioactive material from the PM.

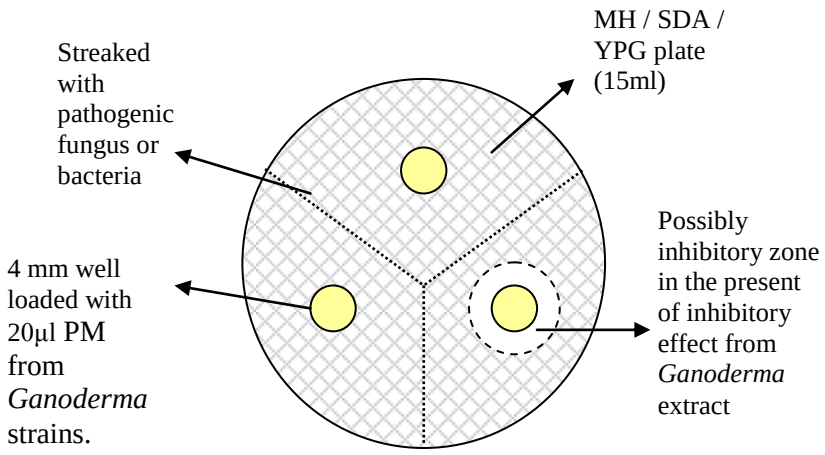

Figure 3.2 : Well diffusion method for antifungal and antibacterial test of PM from *Ganoderma* strains against pathogenic fungus and bacteria (SDA media for *Candida* sp., YPG media for S. *pombe* and MHA media for bacteria, all plates were incubated at 37 ± 2 ^{o}C except for *S. pombe* at 27± 2^{o}C).

Antibacterial activity of S1 to S8 (Sephacryl S-100) and SP1 and SP2 (Superdex 75 HR) were monitored using previous method used for primary screening except the use of protein fractions in place of PM (see section 3.2.6). The bacteria tested were *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus and Bacillus subtilis* for Gram-positive and *Escherichia coli* O157:H7, *Salmonella typhi*, *Shigella* sp., *Plesiomonas shigelloides* and *Pseudomonas aeruginosa* for Gram-negative bacteria. The test plates were prepared in triplicates.

3.2.6 Purification of protein

The PM of the *Ganoderma* strain with the strongest antimicrobial inhibitory activity was selected for bioactive protein purification. The selected strain was *Ganoderma australe* strain KUM60813. Protein purification was carried out using chromatography technique.

In the first purification step, Sephacryl S-100 packed in column (16 x 240mm) was used to fractionate PM obtained from *Ganoderma australe* strain KUM60813 as described in 3.2.4. The PM was dissolved in 10mM Tris HCl buffer (Appendix B) to obtained concentration of 100 mg/ml and filtered through 0.22 um filters to ensure the sample clear and free from particulate matter. A 50 ul sample (0.1g/ml) was loaded into the column for each run. The sample was loaded by injecting it onto the gel bed through the flow adaptor. The flow rate used was 2ml/min. The sample was layered carefully onto the upper bed surface, allowing it to drain into the bed. This method was followed with additional buffer to wash the sample into the bed. The column was attached to reservoir. Fractions were collected and monitored using Amersham Pharmacia AKTA Prime. The collected fractions were identified as S1 to S8 and used for antibacterial test after freeze-dried for 24 hours at -50 ± 2ºC.

The fraction with the strongest antibacterial activity was further fractionate using Superdex 75 HR, packed in column (10x30mm) followed similar method with fractionation using Sephacryl S-100. The peaks eluted were identified as SP1 and SP2.

3.2.7 Protein quantification

Quantitative estimation of protein amount in sample solution was performed using spectrophotometeric and turbidimetric methods by Layne (1957).

The spectrophotometer was zero at 280 nm with distilled water (blank). Stock standard protein, BSA (1mg/ml) was prepared and diluted to 20 – 160 μg. The absorbance of the proteins standard were recorded at 280 nm. A standard curve (Fig. 1, Appendix D) was plotted to determine the protein concentration in the crude extract and the fractions.

Similar procedure was used for the freeze-dried PM and fractions to estimate their protein contents.

3.2.8 Tricine Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

Tricine SDS-PAGE was conducted using Novex X Cell SureLock Electrophoresis Cell system (Invitrogen) with a Scie-PLAS power supply. The assembly and preparation of the electrophoresis apparatus was followed according to the instruction manual. The concentration of purified protein from Superdex 75 HR (SP2) was adjusted to a suitable amount of protein to be loaded onto the gel. About 0.1 mg/ml of the purifed protein was used for Coomassie staining. For silver staining, 100-fold less protein was applied.

The buffer used was Novex Tricine Sample Buffer (2X). The sample was mixed with 5µl buffer and 5µl of MiliQ water before heated at 85°C for 2 minutes prior to loading. Pre cast Tricine gel 10 – 20 % was mounted in the vertical electrophoresis apparatus. The upper and lower buffer chambers were filled with 200 and 600 ml of Tricine SDS running buffer (preparation in Appendix C).

Samples were subsequently loaded under the cathode buffer. Ten microliter sample volumes were applied to 0.7 × 5 mm sample wells. Two microliter low range protein standard from Fermentas was used as standard for molecular weight determination. Tricine SDS PAGE was performed at 125 V constant for 90 minutes. Molecular weight was determined from linear plot of log10 MW vs. mobility's of protein standards. Band obtained from the protein standard were used to construct calibration curve.

3.2.9 Staining of SDS PAGE gel

3.2.9.1 Colloidal Coomassie blue staining (MALDI-TOF compatible)

The staining was performed according to the protocol by Neuhoff *et al*. (1988). The gel was incubated in fixing solution prior to staining with Colloidal Coomassie dye (preparation in Appendix C). The length of incubation with fixing solution required was 30 minutes. After that, the gel was stained with Colloidal Coomassie stain overnight. The following step was destaining of the gel twice in 25% methanol overnight. The gel was stored at 4 - 10°C.

3.2.9.2 Silver staining

Silver staining was carried out using method of Shagger (2006). Coomassie-stained gels can be reused for silver staining after removing the protein-bound Coomassie dye by washing with 50% methanol, 50 mM ammonium hydrogen carbonate, followed by several washings with water. After the step, the gel was incubated in fixing solution for 30 minutes. The gel later was washed twice with water for 30 minutes. In the next step, the gel was sensitized by incubating with 0.005% sodium thiosulfate ($Na_2S_2O_3$) for the same amount of time as the gel was incubated in fixing solution.

The gel was subsequently incubated with 0.1% silver nitrate for 15–60 minutes and washed with water for seconds. Developing solution was added to the gel for 1–2 minutes. Development was stopped by incubating the gel in 50 mM EDTA for a total of 15–60

minutes. Finally, the gel was washed twice with water and maintained in preserving solution at 4 - 10°C.

3.2.9.3 Modified silver staining for Mass Spectrometry

Few modifications had been made in silver staining protocol for protein identification using mass spectrometry. Fixing solution was prepared using 50% methanol, 12% acetic acid and 0.0475% formaldehyde. The length of incubation with fixing solution required was 2 hours or overnight. The gel was then washed three times with 35% absolute ethanol in miliQ water for 20 minutes each. In the next step, the gel was sensitized for 2-3 min. The gel was then washed three times with miliQ water for 5 minutes.

For silver reaction, the gel was incubated with 0.2% silver nitrate and 0.072% formaldehyde for 20 minutes before subsequently washed with water twice for 5-10 seconds. Developer was added to the gel for 3–5 minutes. Development was stopped by incubating the gel in stopping solution (refer to appendix D for preparation) for 5 minutes. The gel was preserved in 1% acetic acid at 4-10ºC.

3.2.10 Protein visualization

The stained gel was scanned using Image Scanner III (GE Healthcare) which compatible with Image Master Software. The Image Master Software was used to visualize and analyze the gel. The gel was placed directly on the scanner and the gel image was later cropped and scanned at 600 DPI. Finally, the image was saved as tiff file at 600 DPI.

3.2.11 Identification of purified protein

Silver stained gel band was sent to Proteomics International Pty Ltd, Australia for Peptide Sequencing by Mass Spectrometry. Protein sample was destained, trypsin digested and peptides extracted according to standard techniques (Bringans *et al*., 2008). Peptides were analysed by electrospray ionisation mass spectrometry using the Ultimate 3000 nano HPLC system (Dionex) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems). Tryptic peptides were loaded onto a C18 PepMap100, 3 mm (LC Packings) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). The standard used was BSA.

Identification of the fingerprint was done using ProFound. Search was made against Fungi in NCBI NR database. Once the monoisotopic masses were loaded onto the program, the parameter such as one missed cleavage allowed, carbamidomethylation of cyctine, methione oxidation and a mass tolerance of 0.50 Da were assumed. The value of the Z score and the percentage of the sequences coverage was used as criteria for the identification of the protein. The following are Z score and its corresponding percentile in an estimated random match population: (Z = 1.282 Percentile = 90.0; Z = 1.645 Percentile = 95.0; Z = 2.326 Percentile = 99.0; Z = 3.090 Percentile = 99.9). The identity of the protein was selected as the protein that produced highest score and consequently the best match with its peptide sequence. Databases search was performed to see protein sequences alignments and comparison to other proteins (BLAST, National Center for Biotechnology Information, USA).

3.2.12 Bioactive properties of purified protein

3.2.12.1 Determination of minimal inhibitory concentration (MIC)

MIC determination was done according to modified microtitre broth dilution method recommended by the National Commitee of Laboratory Safety and Standards (NCLSS) (Amsterdam, 1996).

The media used were Mueller Hinton (MH) agar for maintaining bacteria cultures and Mueller Hinton Broth (MHB) for MIC determination test (Appendix A). The tested bacteria were grown overnight on MH agar plates. The bacteria tested were *Escherichia coli* O157:H7 and *Salmonella typhi*.

Serial dilutions of test protein (at 10 times the required test concentrations) were made in 0.01% acetic acid, 0.2% BSA in polypropylene or coated glass tubes. The test protein was then dissolved in a dH_2O at 20 times the required maximal concentration (enough final volume for all tests to be performed on a given day). It was then diluted into an equal volume of 0.02% acetic acid, 0.4% BSA to get 10 times the required maximal concentration. Serial doubling dilutions was prepared in 0.01% acetic acid, 0.2% BSA to get serial dilutions of proteins at 10 times required test concentrations. Final concentration of protein tested was 5.0 mg/ml to 0.5 µg/ml.

Each tube containing 5 ml MHB was inoculated with test strain from MH agar plates and grown overnight at 37^oC with a shaking rate of 180rpm. The assay was performed in 96 wells microtiter plates. Bacterial cultures were diluted in MHB to give 2 - $7x10^5$ colony forming units/ml and each well was loaded with 100 µl of the tested bacteria. To each well, 11 µl of 10x purified protein was added. Mueller Hinton broth was used as

sterile control blank and blank for the plate scanner while 100 µl bacteria without purified protein was a control for bacteria assay.

The plates were incubated at 37^{o}C for 18-24 hours to verify the MIC. MIC can be taken as the lowest concentration of protein that reduced bacterial growth by more than 50%. The plate was measured at 600 nm wavelength in an ELISA plate reader. The inhibitory activity was calculated as percent inhibition as compared to control without the protein.

3.2.12.2 HIV-1 Reverse Transcriptase inhibition activity assay

The anti HIV activity of the isolated protein was determined with the reverse transcriptase calorimetric assay. It was carried out according to instructions suplied with the assay kit from Boehringer mannheim (Germany).

The isolated protein was tested in triplicate at different concentration ranging from 5 mg/ml to 0.078 mg/ml. 6 ng recombinant HIV-1-RT, diluted in lysis buffer (20 µl/well) was added in a separate reaction tube with 20 µl of isolated protein diluted in lysis buffer and 20 µl reaction mixture. The negative control was made of 40 µl lysis buffer with no HIV-1-RT with 20 µl reaction mixture. The reaction tubes were incubated for 1 hour at 37ºC. The samples (60 µl) were transferred into the wells of the microplate modules and incubated at 37ºC for an hour after which it was washed five times with 250 µl washing buffer per well per washing cycle.

The 200 µl of anti-Digoxigenin-POD solution (200 mU/ml) was added to each well and the plate was incubated at 37ºC for an hour. The plate was washed again five times with 250 µl washing buffer per well per washing cycle. 200ul of ABTS (2,2'-Azinobis [3-

ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate solution was added to each well and incubated at 15 to 25ºC until color development (green color) was sufficient for photometric detection. The plate was measured at 405 nm wavelength in an ELISA plate reader. The inhibitory activity of the isolated protein was calculated as percent inhibition as compared to a control without the protein.

3.2.12.3 Deoxyribonuclease activity

The deoxyribonuclease activity of the isolated protein was carried out according to method by Wang and Ng (2001b). The reaction mixture was incubated at 25ºC for 15 minutes. The reaction mixture consists of 0.2 ml of 0.1M Ammonium acetate buffer (pH 5.5), 0.2 ml of a herring sperm DNA solution (5 mg/ml) and 10 µl of the solution of the purified protein (5 mg/ml). The reaction was terminated by addition of 0.3 ml of ice-cold 20mM lanthanum nitrate in 1.2% (v/v) perchloric acid for 20 minutes at 0ºC. The reaction mixture was subsequently centrifuged at 3000 x *g* for 5 min.

The pellet was discarded while the supernatant was diluted three-fold with water and the optical density was read at 260 nm against a blank reaction mixture without the purified protein. One unit of enzymatic activity is 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 and 37ºC using herring sperm DNA. It is slightly different from 1 Kunitz unit of DNase activity which is based on an assay conducted at a pH of 5, a temperature of 25ºC and DNA type 1 as substrate.

3.2.12.4 Hemolysis test

The experiment was conducted according to method described by Helmerhorst *et al*. (1999). Human erythrocytes were collected in vacuum tubes containing heparin (final concentration 20.4 U/ml) as anti-coagulant. The erythrocytes were harvested by centrifugation for 10 minutes at 2000 x g at 20ºC before being three times washed with phosphate buffer saline (9mM sodium phosphate, pH 7.0 in 150 mM NaCl). Phosphate buffer saline (PBS) was added to the pellet to yield a 20% (v/v) erythrocytes in the buffer suspension. The 20% suspension was later diluted 1:20 in PBS and from this suspension 100µl was added to 100 µl of a two-fold serial dilution series of protein in the same buffer in 96-well-V-bottomed microtitre plate. The activity was compared to melittin, a toxin from honeybee venom with known ability to hemolysed erythrocytes (Ramalinggam and Bello, 1992). Both proteins were tested against human erythrocytes at concentrations from 0.27 to 140 µg/ml. Hemolysis was measured at 450nm with an ELISA plate reader after incubation at 37ºC for one hour. Zero percentage and 100% hemolysis was determined in PBS (phosphate buffer saline) and 0.1% Tween 20, respectively.

3.2.12.5 Antifungal activity

Antifungal activity of purified protein was done according to method by Wang and Ng (2002). The test fungi included *Fusarium oxysporium cubense I* (FOC1), *Fusarium oxysporium cubense II* (FOC2), *Fusarium oxysporium cubense IV* (FOC4), *Ganoderma boninense* and *Colletotrichum sp.*

The assay was carried out in 100mm x 15mm Petri plates containing 10 ml of potato dextrose agar. After 72 hours of inoculation and the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony and 40 and 200 µg of purified protein in 20 µl Tris HCl buffer (pH 7.4) was added to the disks.

The plates were incubated at 23ºC for 72 hours until mycelial growth had enveloped disks in the controls and had formed crescents of inhibition around disks containing samples with antifungal activity. A positive control experiment was set up by loading 2µg of nystatin in 20 µl of sterile distilled water. Bovine serum albumin was used as negative control (30 µg). The inhibitory zones were measured in millimeters and negative results were regarded as those in which no zone of inhibition was observed after 72 hours.

3.2.12.6 Ribonuclease activity

The activity of the isolated protein toward yeast tRNA was performed according to method by Wang and Ng (2004c). Yeast tRNA (200µg) from Sigma was incubated with the 50µg isolated protein in 150µl 100mM MES buffer (pH 6.0) at 37ºC for 15 minutes. The reaction was terminated by introducing 350µl of ice-cold 3.4% perchloric acid. After standing on ice for 15 minutes, the mixture was centrifuged at 15,000 x *g* for 15 minutes at 4ºC.

The absorbance of the supernatant was measured at 260 nm after suitable dilution. One unit of ribonuclease activity is defined as the amount of ribonuclease that produces an absorbance increase of one per minute at 260 nm in the acid-soluble fraction per milliliter of reaction mixture under the specified conditions. The absorbance of the control

(i.e. buffer only) was approximately 0.1 while the absorbance of a sample with high RNase activity was close to 2. The control absorbance was deducted from the sample absorbance for the calculation of RNase activity.

3.2.13 Statistical analysis

The results of this study were subjected to the analysis of variance (ANOVA) and the significance of difference between means of triplicate values was determined by the Duncan's multiple range tests at 95% least significant difference ($p<0.05$). Results of analyses were provided as tables (Appendix E).