การแยก การทำให้บริสุทธิ์ การหาลักษณะเฉพาะและฤทธิ์ต้านจุลชีพของเปปไทด์ต้านจุลชีพที่แยกได้จากเห็ดขอนขาวสายพันธุ์ที่เพาะในประเทศไทย

นายศิวัช พุ่มพวง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2558

ISOLATION, PURIFICATION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF ANTIMICROBIAL PEPTIDES ISOLATED FROM *LENTINUS SQUARROSULUS* CULTIVATED IN THAILAND.

Mr. Siwat Poompouang

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biomedicinal Chemistry
Department of Biochemistry and Microbiology
Faculty of Pharmaceutical Sciences
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Thesis Title	ISOLATION, PURIFICATION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF ANTIMICROBIAL PEPTIDES ISOLATED FROM *LENTINUS SQUARROSULUS* CULTIVATED IN THAILAND.
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Field of Study	Biomedicinal Chemistry
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ศิวัช พุ่มพวง : การแยก การทำให้บริสุทธิ์ การหาลักษณะเฉพาะและฤทธิ์ต้านจุลชีพของเปปไทด์ต้านจุลชีพที่แยกได้จากเห็ดขอนขาวสายพันธุ์ที่เพาะในประเทศไทย (ISOLATION, PURIFICATION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF ANTIMICROBIAL PEPTIDES ISOLATED FROM *LENTINUS SQUARROSULUS* CULTIVATED IN THAILAND.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. ดร. มณีวรรณ สุขสมทิพย์, 114 หน้า.

จากสถานการณ์การเพิ่มขึ้นของจุลชีพก่อโรคที่ดื้อยาปฏิชีวนะในปัจจุบัน ทำให้เกิดความสนใจในการศึกษาและพัฒนายาปฏิชีวนะใหม่ๆจากแหล่งธรรมชาติเพื่อใช้ในการยับยั้งจุลชีพเหล่านั้น เปปไทด์ต้านจุลชีพซึ่งเป็นโปรตีนจากสิ่งมีชีวิตและมีคุณสมบัติในการยับยั้งจุลชีพ อาจจะถูกพัฒนาเป็นยาปฏิชีวนะตัวใหม่เพื่อแก้ปัญหาสถานการณ์ดื้อยาดังกล่าว การศึกษานี้ได้ทำการแยก, ทำบริสุทธิ์และศึกษาคุณลักษณะของเปปไทด์ต้านจุลชีพจาก *Lentinus squarrosulus* (Mont.) ซึ่งเป็นเห็ดใช้บริโภคที่เพาะในประเทศไทย ส่วนสกัดหยาบของโปรตีนจะถูกสกัดแยกจากส่วนดอกเห็ดด้วยการบดละเอียดในบัฟเฟอร์ Tris-HCl, pH 7.4 ความเข้มข้น 10 มิลลิโมลาร์ แล้วตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตโดยใช้ความเข้มข้นสุดท้ายเป็น 40-80 % ของน้ำหนักต่อปริมาตร ส่วนสกัดหยาบของโปรตีนที่ได้ถูกนำมาทำให้บริสุทธิ์ขึ้นด้วยคอลัมน์แบบแลกเปลี่ยนไอออนชนิด DEAE cellulose โปรตีนที่มีคุณสมบัติยังยั้งจุลชีพจะถูกชะออกจากคอลัมน์ด้วยโซเดียมคลอไรด์ความเข้มข้น 0.5 โมลาร์ ในบัฟเฟอร์ Tris-HCl ความเข้มข้น 10 มิลลิโมลาร์, pH 7.4 หลังจากนั้นนำมาทำให้บริสุทธิ์ด้วยคอลัมน์ Sephadex G-25 โดยโปรตีนที่มีคุณสมบัติต้านจุลชีพจะถูกชะออกจากคอลัมน์ในช่วงพีคที่สี่ การทดสอบคุณสมบัติยับยั้งจุลชีพโดยใช้วิธีอะการ์ดิสดิฟฟิวชั่น (agar disc diffusion) พบว่าโปรตีนที่ได้สามารถยับยั้งเชื้อราก่อโรคในมนุษย์หลายสายพันธุ์ ทั้งสายพันธุ์มาตรฐานได้แก่ *Candida albicans* ATCC 10231และสายพันธุ์ที่แยกได้จากผู้ป่วย 3 สายพันธุ์คือ *Candida tropicalis, Trichophyton mentagrophytes* และ *Trichophyton rubrum* อย่างไรก็ตามพบว่าโปรตีนดังกล่าวให้ผลน้อยต่อเชื้อรา *Aspergillus niger* และไม่มีผลยับยั้งแบคทีเรียแกรมบวกและแกรมลบ นอกจากนั้นยังไม่มีผลเสริมฤทธิ์กับยาปฏิชีวนะ เตตราไซคลีน, ไซโปรฟลอกซาซิน และคลอแรมเฟนิคอล การศึกษาอื่นๆพบว่าโปรตีนดังกล่าวจะสูญเสียคุณสมบัติยับยั้งเชื้อราเมื่ออยู่ในสภาวะที่มีเอนไซม์โปรติเอส, รวมทั้งสภาวะที่มีค่า pH ต่ำหรือสูงกว่าช่วง pH 3.0 -9.0 และอุณหภูมิสูงกว่า 40 องศาเซลเซียส โปรตีนดังกล่าวยังมีคุณสมบัติย่อยสลายดีเอ็นเอได้ทั้งจีโนมิกดีเอ็นเอและพลาสมิดดีเอ็นเอ โปรตีนบริสุทธิ์มีน้ำหนักโมเลกุลประมาณ 17 กิโลดาลตันเมื่อวิเคราะห์ด้วยวิธีโซเดียมโดเดซิลซัลเฟตพอลิอะคริลาไมด์เจลอีเลคโทรโฟริซิสภายใต้สภาวะรีดิวซิ่ง สำหรับการหาลำดับกรดอะมิโนของโปรตีนด้วยวิธี MALDI-TOF/MS พบความเหมือนของกรดอะมิโนของโปรตีนนี้ในช่วงของลำดับอนุรักษ์ของโปรตีนไซโคลฟิลินซึ่งเป็นโปรตีนที่มีคุณสมบัติยับยั้งเชื้อรา ผลที่ได้จากการศึกษานี้แสดงให้เห็นว่า โปรตีน/เปปไทด์ต้านเชื้อราจาก *L. squarrosulus* (Mont.) มีความน่าสนใจที่จะนำไปประยุกต์ใช้ทางคลินิกซึ่งควรต้องทำการศึกษาต่อไป

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเวชเคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558

5576227633 : MAJOR BIOMEDICINAL CHEMISTRY
KEYWORDS: LENTINUS SQUARROSULUS (MONT.) / ANTIFUNGAL PEPTIDE / PROTEIN PURIFICATION / CYCLOPHILIN PROTEIN / ANTIMICROBIAL PEPTIDES

SIWAT POOMPOUANG: ISOLATION, PURIFICATION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF ANTIMICROBIAL PEPTIDES ISOLATED FROM *LENTINUS SQUARROSULUS* CULTIVATED IN THAILAND.. ADVISOR: ASSOC. PROF. MANEEWAN SUKSOMTIP, Ph.D., 114 pp.

The emergence of microbial pathogenic resistance to current antibiotic drugs has increased the interest in developing alternative antibiotics from natural resources. Antimicrobial peptides (AMPs), a family of proteins from various organisms with antimicrobial activity, may be potential new antibiotic drugs which can be utilized to overcome the antibiotic resistance. The aim of this study is to isolate, purify and characterize the AMP from *Lentinus squarrosulus* (Mont.), the common edible mushroom cultivated in Thailand. Crude proteins were extracted from fresh fruiting body by homogenization with 10 mM Tris-HCl buffer, pH 7.4 and the proteins were precipitated by solid ammonium sulfate at the final concentration of 40-80% (w/v). The crude protein extracts were further purified by anion exchange chromatography using DEAE - cellulose column. The partial purified protein collecting from anion exchange column using the elution buffer (0.5 M NaCl in 10 mM Tris-HCl buffer, pH 7.4) showed the antifungal activity. After further purification by Sephadex G-25 gel filtration, the active protein was eluted in the fourth peak. The antimicrobial activity test using agar disc diffusion method exhibited the strong activity against various species of human fungal pathogen including *Candida albicans* ATCC 10231, *Candida tropicalis*, *Trichophyton mentagrophytes* and *Trichophyton Rubrum* which the last 3 species were clinical isolates. However, the activity against *Aspergillus niger* was low. Furthermore, it had no activity against both gram positive and gram negative bacteria as well as no synergistic effect with antibiotic drugs (ciprofloxacin, chloramphenicol and tetracycline). The antifungal activity of protein was completely lost in the presence of proteases, the pH above or below the range of 3.0 - 9.0 and the temperature above 40 $^{\circ}$C. The nuclease activity was also detected on both genomic DNA and plasmid DNA. The purified protein had the molecular mass of approximately 17 kilodalton determined by sodium dodecyl sulfate – polyacrylamide gel electrophoresis under reducing condition. Amino acid sequence determined by MALDI-TOF/MS showed high similarity to the conserved sequences of cyclophilin, the protein with the antifungal property. The findings from this study suggested that antifungal protein/peptide from *L. squarrosulus* (Mont.) may have potential clinical application which need further investigation.

Department:	Biochemistry and Microbiology	Student's Signature	.....
Field of Study:	Biomedicinal Chemistry	Advisor's Signature	.....
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LIST OF ABBREVIATIONS

%	percentage
A	absorbance
AMP	antimicrobial peptide/protein
ATCC	American Type Culture Collection
APS	Ammonium persulfate
bp	base pair
BSA	bovine serum albumin
cm	centimeter
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
gDNA	genomic deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
et al.	*et alii*, and others
F	fraction
g	units of times gravity
h	hour
HCl	hydrochloric acid
Kb	kilo bases
kDa	kilo Dalton
kg	kilogram
LB	Luria-Bertani

M	molar
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
mg	milligram
min	minute
mL	milliliter
mM	milli Molar
MS	mass spectrometry
N	Negative control
n	the number of subjects
NaCl	sodium chloride
P	Positive control
PAGE	polyacrylamide gel electrophoresis
pH	positive potential of hydrogen ion
rpm	revolutions per minute
SDA	sabouraud dextrose agar
SDS	sodium dodecyl sulphate
S.E.M	standard error of the mean
TAE	Tris-acetate-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
µM	micromolar
µL	microliter
UV	ultraviolet
V	volt

v/v	volume by volume
w/v	weight by volume
w/w	weight by weight

CHAPTER I
INTRODUCTION

All living organisms need protection from microbial infection and their ability to avoid from infection is believed to depend on their innate immune response. Antimicrobial peptides (AMPs) have been recognized in prokaryotic cells since 1939 when antimicrobial substances, named gramicidins, were isolated from *Bacillus brevis*, and were found to exhibit activity both *in vitro* and *in vivo* against a wide range of Gram-positive bacteria (Dubos, 1939a, b). AMPs are a group of immune-related peptides/proteins that protect the host from microbial infection (Boman, 1995). AMPs exhibit *in vitro* broad spectrum of activity against various targets, including Gram-positive and Gram-negative bacteria, fungi, parasites, enveloped viruses, and tumor cells (Baker *et al*., 1993 and Scott *et al*., 1999). Such peptides may have a direct effect on the microbe, such as by damaging or destabilizing the bacterial, viral, or fungal membrane (Martin *et al*., 1995).

AMPs are typically relatively short peptides which share common biophysical properties. They are small, from just 5-6 amino acid residues in some synthetic peptides to about 50 and even up to 100 in natural ones. Most of them (12 to 100 amino acids), are positively charged and have been isolated from insects and other invertebrates, vertebrates, plants, micro-organisms such as bacteria and fungi (Martin *et al*., 1995 and Wang and Ng, 2004). An updated database of AMPs is available online

at: http://aps.unmc. edu/AP/main.php. To date more than 1,500 AMPs of different origins have been reported (Guani-Guerra *et al*., 2010). The biological activities of AMPs from various organisms have been studied including AMPs from mushrooms.

Mushrooms, the macrofungi have long been used as a valuable food source and as traditional medicines around the world since ancient times (Akyuz *et al*., 2010). Asia is rich in various types of mushrooms and the asian people prefer to use a number of mushrooms for food, instead of treatment of infectious diseases and various ailments.

Fungi and macrofungi are good source of protein and other chemical compounds in health science. There are many reports of antimicrobial peptides (AMPs) from mushrooms with various biological activities such as eryngin, an antimicrobial peptide with antifungal property from *Pleurotus eryngii* (Wang and Ng, 2004), pleurostrin, an antimicrobial peptide with antifungal property from *Pleurotus ostreatus* (Chu *et al.*, 2005), cordymin, an antimicrobial peptide with antifungal property from *C. militaris* (Wong *et al.*, 2011).

There is also a report of AMPs with anticancer from *Cordyceps militaris* (Park *et al*., 2009). KREHG AccNo.55956 is the AMPs from *Russula paludosa* with HIV-1 reverse transcriptase inhibitory activity (Wang *et al*., 2007). The marmorin peptide with inhibitory activity on proliferation of hepatoma cells (HepG2) and breast cancer cells (MCF-7), as well as inhibitory HIV-1 reverse transcriptase activity is from *Hypsizigus marmoreus* (Lavanya and Subhashini, 2013). So proteins or peptides elaborated by

these fungi and macrofungi have shown several biological activities including antiproliferative, immunomodulatory, antiviral, antifungal and antibacterial effects (Lavanya and Subhashini, 2013 and Wang and Ng, 2004).

Lentinus is a small genus of wood-inhabiting macrofungi (mushroom). One of the most famous edible mushroom from this genus is *Lentinus edodes*, or Shiitake mushroom, the common Japanese name. Lentin, the antimicrobial peptide isolated from *L. edodes* has several biological activities. It can inhibit mycelial growth in a variety of fungal species including *Physalospora piricola*, *Botrytis cinerea* and *Mycosphaerella arachidicola*. Moreover, lentin from *L. edodes* also has activity against HIV-1 reverse transcriptase and is able to inhibit proliferation of leukemia cells (Ngai and Ng, 2003).

In Thailand, there are various species of mushroom including *Lentinus* spp. *Lentinus squarrosulus* (Mont) or "Hed khon khao" in local language, is one of edible indigenous mushroom species and abundantly grown in North East of Thailand. The local Thai people used *L. squarrosulus* mushroom for food for long time. They were also used as curative or tonic agents in Thai traditional medicine. However, there were no report, of AMPs isolated from this mushroom (กรมพัฒนาการแพทย์แผนไทยและการแพทย์ทางเลือก กระทรวงสาธารณสุข, 2554).

So, this study aim to isolate, purify, characterize and evaluate some biological activities such as antibacterial, antifungal and other activities of AMPs from *L. squarrosulus* (Mont.) cultivated in Thailand.

Thesis Objectives:

1. To isolate and purify AMPs from *L. squarrosulus* (Mont.) cultivated in Thailand.
2. To characterize the isolated AMPs.
3. To screen for antimicrobial activity and other potential activities.

Scope of study

This study focuses on the screening for antimicrobial peptide/protein from the Thai traditional edible mushroom, *L. squarrosulus* (Mont.). This specie has been reported to have antimicrobial activity especially, *L. edodes*. This study is to isolate, purify and characterize physicochemical and biological properties of peptide/protein from *L. squarrosulus* (Mont.) cultivated in Thailand.

Thesis outline

This thesis consists of the introductory chapter, Chapter 2, reviews the state of the research field of antimicrobial peptides. In particular, emphasis is put on reviewing the current knowledge about antimicrobial peptide. Chapters 3 and 4 contain research methodology and result of experiment, respectively. The final concluding chapter in Chapter 5, summarizes the findings of the thesis and briefly discusses future directions of the research.

CHAPTER II
LITERATURE REVIEW

Antimicrobial resistance (AMR) is the resistance of a microorganism to an antimicrobial medicine to which it was originally sensitive. Resistant organisms, including bacteria, fungi, viruses and some parasites are able to withstand attack by antimicrobial medicines, such as antibiotics, antifungals, antivirals, and antimalarial so that standard treatments become ineffective and infections persist increasing risk of spread to others. The evolution of resistant strains is a natural phenomenon that happens when microorganisms are exposed to antimicrobial drugs, and resistant traits can be exchanged between certain types of microbial organism such as bacteria. The misuse of antimicrobial medicines accelerates this natural phenomenon. Antimicrobial peptides (AMPs) have been proposed as a new class of antibiotics and are excellent candidates for the development of novel therapeutic agents acting against pathogens that are resistant to traditional antibiotics (Dubos, 1939a, b and Scott *et al*., 1999).

Antimicrobial peptides (AMPs)

The term “Antimicrobial peptides” (AMPs) is used to describe a large number of small proteins that can kill or inhibit growth of various microorganisms. Antimicrobial peptides are small molecular mass proteins with broad spectrum antimicrobial activity against bacteria, viruses, and fungi. These evolutionarily conserved peptides are generally defined as peptides of less than 100 amino acid residues with an overall positive charge imparted by the presence of multiple lysine and arginine residues and a substantial portion of amino acid residues type of both a hydrophobic and

hydrophilic side (Giuliani *et al*., 2007 and Wang and Ng, 2004). Antimicrobial peptides represented one of the most important groups of defensive polypeptides or peptides in prokaryotes and eukaryotes and they have an ancient mechanism of defense against pathogens (Odintsova *et al*., 2013). Antimicrobial peptides were produced by a wide variety of different organisms such as animals, plants, microorganisms including micro-algae (Silva-Stenico *et al*., 2011), bacteria (Hassan *et al*., 2012) and fungi (Chu *et al*., 2005 and Lavanya and Subhashini, 2013 and Park *et al*., 2009 and Wang and Ng, 2004 and Wang *et al*., 2007 and Wong *et al*., 2011) as shown in Table 1. However, many aspects of their natural composition are different based on organism species, mechanism of action, and specificity with respect to pathogens, as well as gene structure and regulation of gene expression (Odintsova *et al*., 2013).

Table 1 Various sources of AMPs (Pushpanathan *et al*., 2013)

Soure of AMPs	AMPs
Insect	Cecropin A, Sarotoxin IA, Ponericin G2, Ceratotoxin, Stomoxyn, Spinigerenin,Thanatin
Amphibians	Japonicin-1 and 2, nigrocin-1 and 2, Temporin-1Od, Brevinin-20a, Psedin-1
Echinoderms	Strongylocins, Centrocins, Betathymosins, Filamin A
Crustaceans	Callinectin,Aastacidin 2, Armadillidin, Homarin
Plants	Thionins, Plant defensin, Lipid transfer protein
Mammals	Defensin, Histamin, LL-37, Indolicidin
Bacteria	Iturin, Bacillomycin, Syringomycin
Fungi	Echinocandins, Aculeacins, Mulundocandins, FK463
Fishes	Paradaxins, Pleurocidins, Parasin, Oncorhyncin II and III

Antimicrobial and other activities

Because bacterial cell membranes are negatively charged with lipids such as phosphatidylglycerol (PG), cardiolipin (CL) or phosphatidylserine (PS), electrostatic interaction between these negatively charged lipids and the positively charged AMPs enable the cationic peptides to bind bacterial membranes. The outer membrane of a gram-negative bacteria is also negatively charged as it contains anionic lipopolysaccharides (LPS). LPS is normally stabilized by the divalent cations like Ca^{2+} and Mg^{2+} but AMPs displace them to interact with the outer membrane (Hoskin and Ramamoorthy, 2008). The exact mode of action of cationic antimicrobial peptides on gram-positive bacteria is unknown. However, it has been proposed and is widely believed that the peptides interact with and disrupt the cytoplasmic membrane, leading to the dissolution of the proton motive force and leakage of essential molecules, resulting in cell death. The damage of gram - negative and gram - positive bacteria cells after exposing antimicrobial peptide was shown in Figure 1-4 (Hartmann *et al*., 2010).

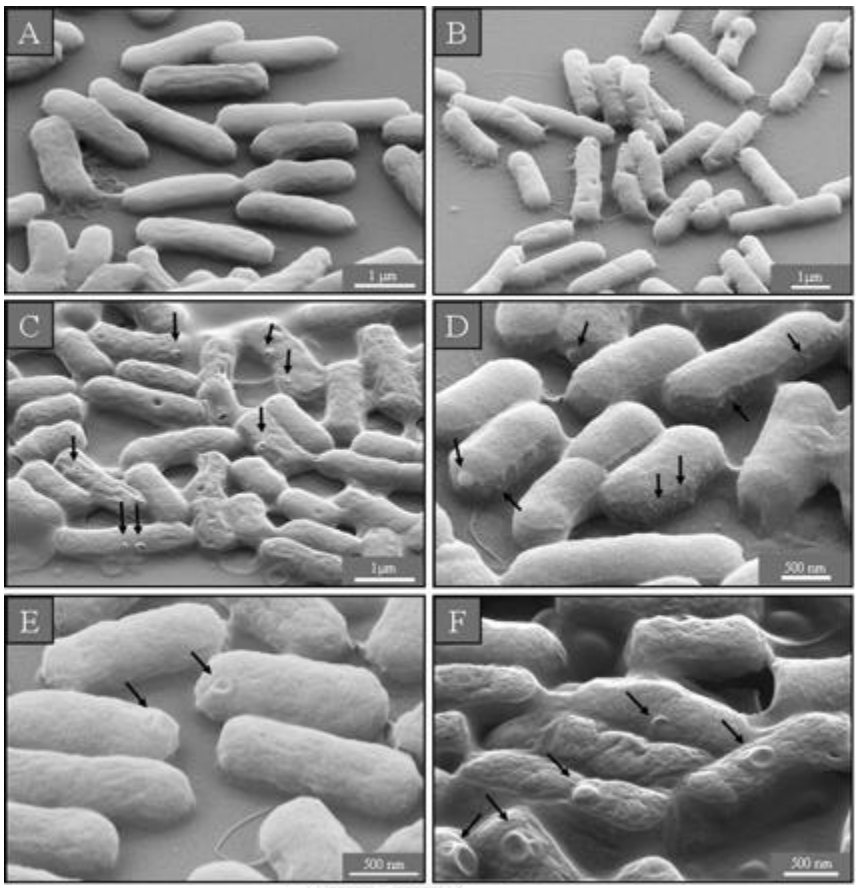

Figure 1 Scanning electron microscope (SEM) of untreated *E. coli* (A), B-F show bacterial cell damage after treating with AMPs (Hartmann *et al*., 2010).

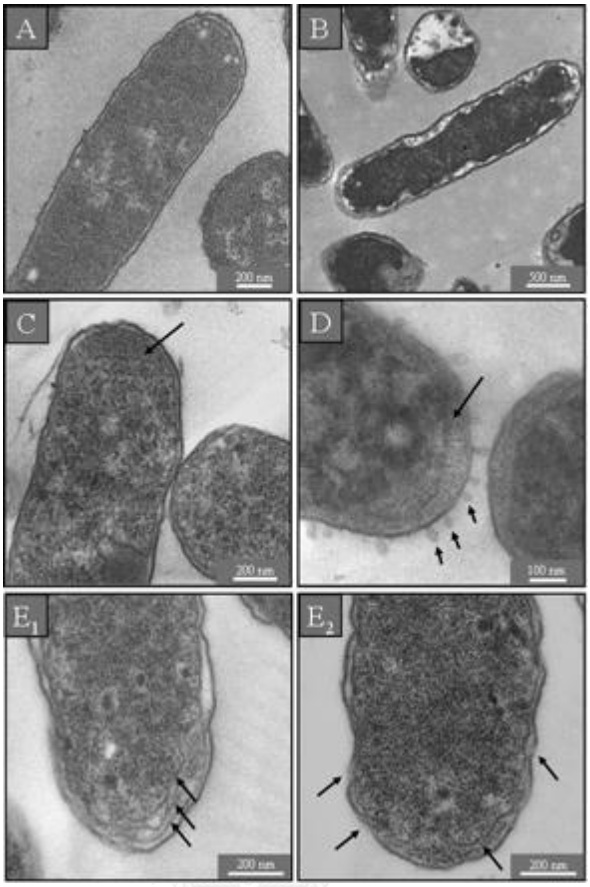

Figure 2 Transmission electron microscope (TEM) of untreated *E. coli* (A), B-E2 show bacterial cell damage after treating with AMPs (Hartmann *et al*., 2010).

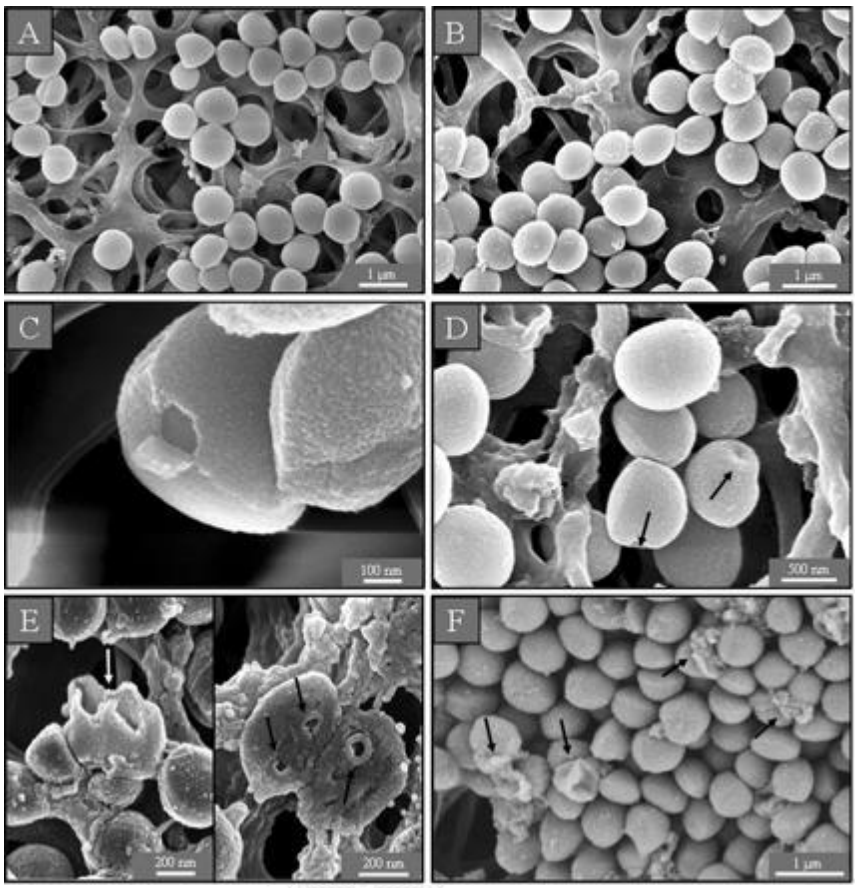

Figure 3 Scanning electron microscope (SEM) of untreated *S. aureus* (A), B-F show bacterial cell damage after treating with AMPs (Hartmann *et al*., 2010).

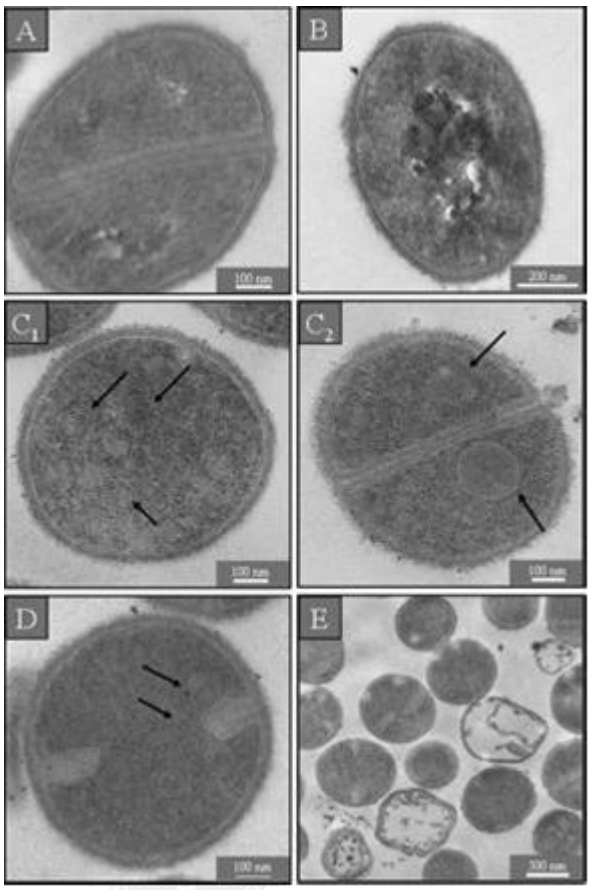

Figure 4 Transmission electron microscope (TEM) of untreated *S. aureus* (A), B-E show bacterial cell damage after treating with AMPs (Hartmann *et al*., 2010).

The antifungal activity and consequent therapeutic potential of AMPs have been recognized (Duncan and O'Neil, 2013). Activity against a range of fungal pathogens relevant to human health has been confirmed for a number of human, mammalian, amphibian, insect and plant derived AMPs (Muralidharan and Bobek, 2005). Bactericidal activity of AMPs are best characterized, whereas the antifungal properties and their mode of action are less studied. There are about 1,900 AMPs of different origins which are registered in online database of AMPs at http://aps.unmc.edu/AP/main.php. From these, more than 1,500 AMPs (79 %) have been assigned antibacterial activity compared to 648 antifungal AMPs (34 %) (Hegedüs and Marx, 2013). The effects of peptide on fungal cells are shown in Figure 5.

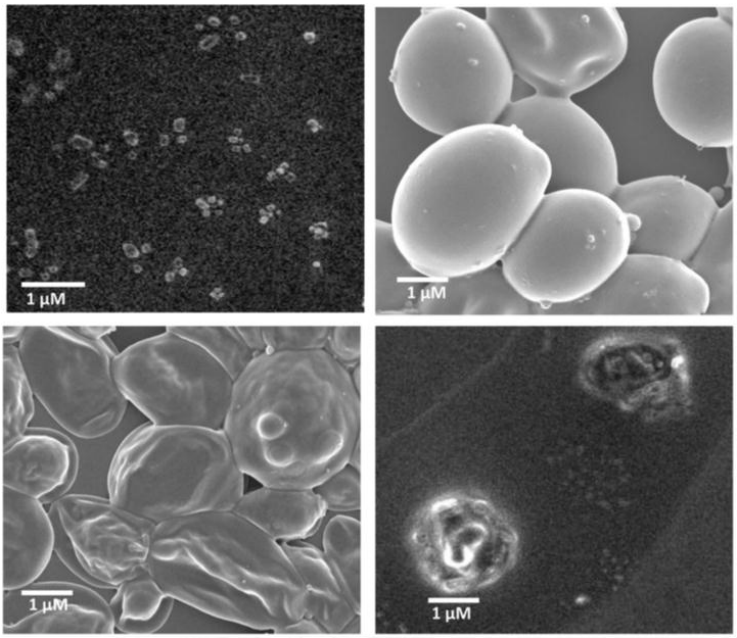

Figure 5 The scanning electron micrograph of fengycin, the antifungal peptide and its activity against *C. albicans* cells (Roy *et al*., 2013).

The most interesting property of antimicrobial peptides is their cell specificity by which they kill microbes without toxicity to normal mammalian cells (Lucca *et al*., 2012). The relative insensitivity of eukaryotic cells to antimicrobial peptides is generally ascribed to differences in lipid composition between eukaryotic and prokaryotic cell membrane (Ganz, 2003). It has been proposed that the net positive charge of the antimicrobial peptide accounts for their preferential binding to the negatively charged outer surface of bacteria, which is different from the predominantly zwitterionic surface of normal mammalian cells (Ganz, 2003). Also a less negative membrane potential in eukaryotes than in prokaryotes plays an important role in their selectivity (Ganz, 2003 and Hof *et al*., 2001). Tumor cells have lost part of their lipid asymmetry and therefore exhibit a more anionic character on the external leaflet of their plasma membrane, which thus preferentially binds cationic antimicrobial peptides (Giuliani *et al*., 2007 and Riedl *et al*., 2011). Recently, the studies on their anticancer activities are exciting and are most relevant. Although all AMPs are not able to kill cancer cells, those that do, can be divided into two broad categories: (a.) AMPs that are highly potent against bacteria and cancer cells but not against normal mammalian cells and (b.) AMPs that are cytotoxic for bacteria, cancer cells, and normal mammalian cells (Ausbacher *et al*., 2012 and Strömstedt *et al*., 2010). The effect of antimicrobial peptides on cancer cells is shown in Figure 6.

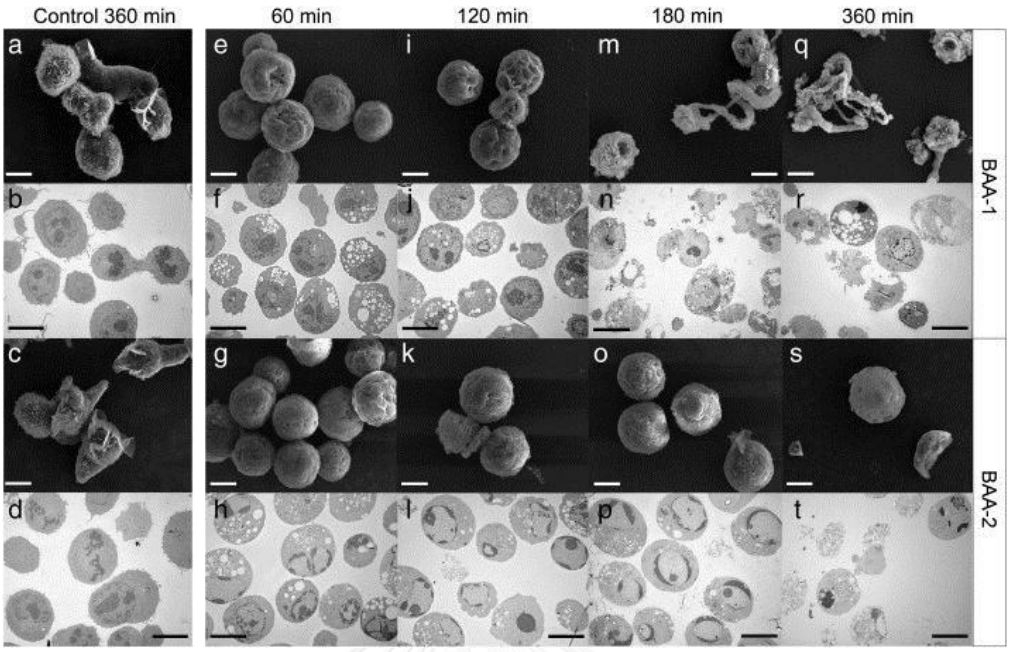

Figure 6 The anticancer activity of AMP. SEM and TEM images of Ramos cells treated with antimicrobial peptide, BAA-1 and BAA-2, and compared with control cells incubated for 360 min: A-D is control, E-T is cell damage when AMPs treated (Ausbacher *et al*., 2012).

Although the antibacterial, antifungal and anticancer activities of AMPs have been the main focus of the studies to date, some of these molecules have also been shown to be effective against viral pathogens (Gomes *et al*., 2007). Recent reports have drawn attention to the antiviral activity of antimicrobial peptides (AMPs) that were first defined by their antibacterial properties (Ganz, 2003a and Yasin *et al*., 2000). For example, alpha-defensins were shown to inhibit HIV-1 infection *in vitro* through binding to HIV-1 gp 120 and CD4 (Wang *et al*., 2004). LL-37 (cathelicidin in humans) binding to the formyl peptide receptor like-1 receptor induces down regulation of HIV-1 chemokine receptors and reduces susceptibility to HIV-1 infection *in vitro* (Bergman *et al*., 2007). The effects of antimicrobial peptides on virus are shown in Figure 7.

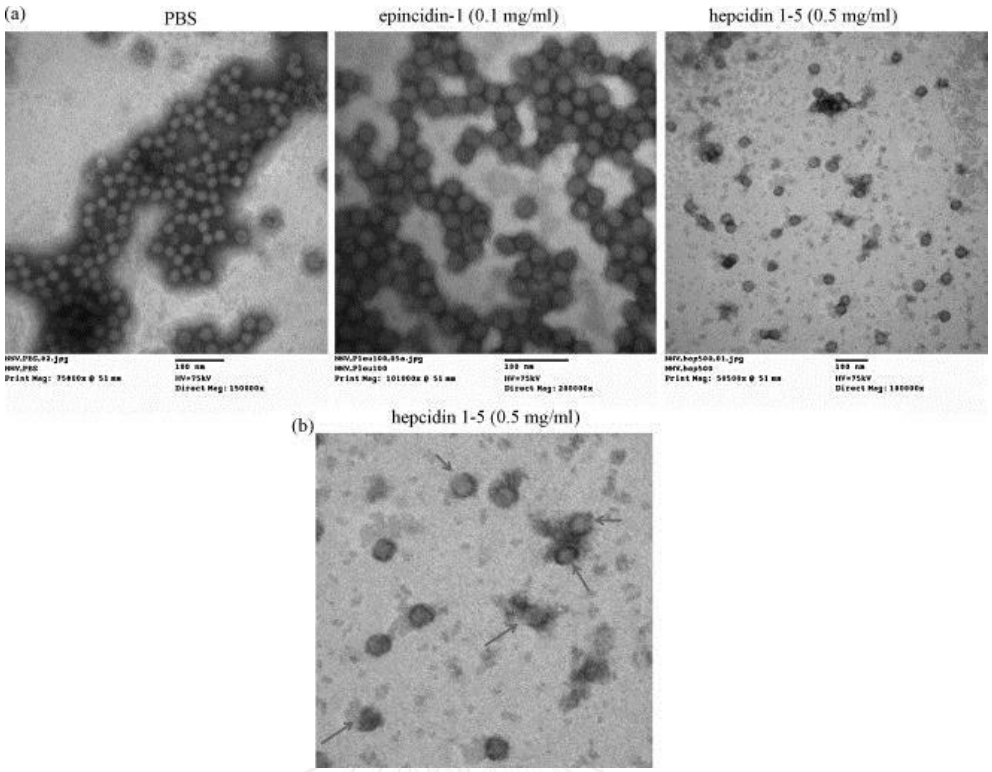

Figure 7 TEM analysis of nervous necrosis virus (NNV) exposed to epinecidin-1 and hepcidin 1–5 for 30 min at room temperature (Wang *et al*., 2010).

Category of antimicrobial peptides

More recently, Tomas Ganz proposed a structural classification of the AMPs based on their secondary structure (Duncan and O'Neil, 2013 and Friedrich *et al*., 2000). The classification proposed here contains 9 different peptide structure families. The last group consider hybrid structure of peptides possessing structural features of more than one AMP class. Types of antimicrobial peptides are 1. The linear amphipathic alpha-helix antimicrobial peptide, 2. Proline rich peptides, 3. Glycine/arginine rich peptides, 4. Brevinin (hook structure) peptides, 5. Defensins (cysteine knot structure), 6. Tachyplesin, 7. Arginine and Tryptophan rich antimicrobial peptides, 8. Histidine rich glycoprotein peptides and 9. Mixed structure peptides (Duncan and O'Neil, 2013 and Friedrich *et al.*, 2000). Various structures of AMPs based on database are shown in Figure 8 (http://aps.unmc.edu/AP/main.php and some reported articles).

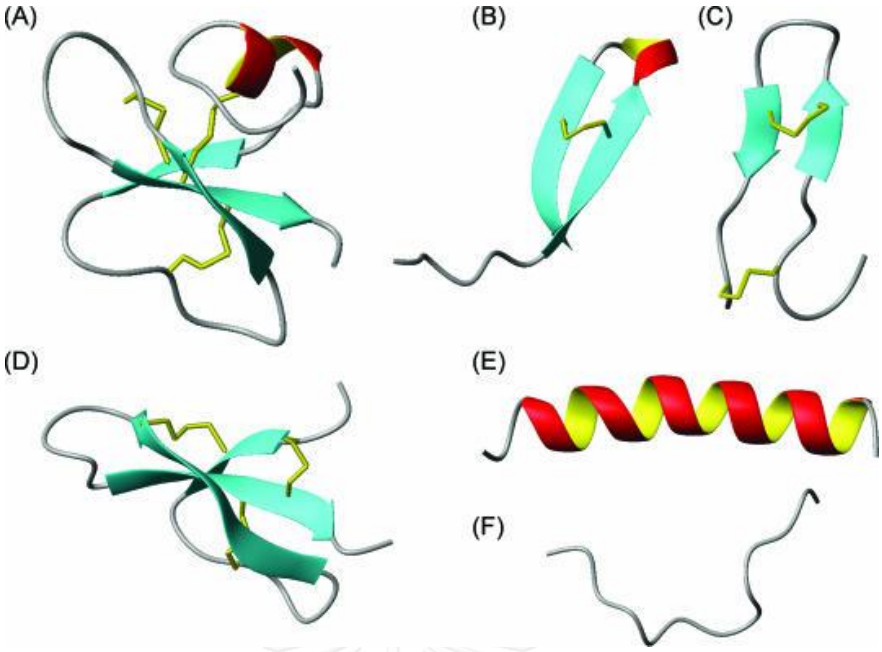

Figure 8 Various structures of antimicrobial peptide. A, Mixed structure of AMPs. B, looped. C, Beta-sheeted. D, Defensing. E, Alpha-helical and F, Extended (Jenssen *et al*., 2006).

Mechanisms of action of antimicrobial peptide (Laverty *et al*., 2011)

Antimicrobial peptide is a group of conservation of structure and charge. Antimicrobial peptides form amphipathic structures and are often cationic at physiological pH. As outlined above, amphipathicity and net charge are characteristics understandably conserved among many antimicrobial peptides. Furthermore, charge affinity is likely an important means conferring selectivity to antimicrobial peptides and mechanism of antimicrobial peptide is not clear. In the context of these paradigms, the following discussion highlights current concepts relating to the molecular basis of antimicrobial peptide mechanisms of action.

A; Aggregate model. Peptides reorient to span the membrane forming micelle-like complexes composed of peptides and lipid with no particular orientation and organization. Membrane permeabilization is characterized by the formation of informal channels with a variety of size and lifetimes, accompanied by the peptides translocation across the bilayer to inside the microorganism cell.

B; Toroidal model or wormhole model. In that case, the shorter peptides bind to the lipid head-groups causing the binding of the phospholipid layers from one leaflet to the other. Hydrophilic residues of the peptide that were laying on the membrane surface, insert in the core of the membrane by pulling together the lipid molecules, resulting in a pore structure in which the peptide chains and the lipid head groups line wall of the pore.

C; Barrel-stave model. In this model, a variable number of peptides insert into the bilayer in a perpendicular manner, either as monomers or a small aggregates in a “barrel-like” ring around an aqueous pore. In that case, hydrophobic interactions play the main role in the formation of the pore. The hydrophobic residue, of the peptide

are facing the non-polar lipid acyl chains whereas it's hydrophilic surface form the lining of a water-filled pore and the lipid head groups remain located at the membrane/water interface. The pore formation requires peptides sufficiently long to traverse the hydrophobic core of the membrane, without inserting the polar groups of the lipids.

D; Carpet-like model. This model includes peptides that act against microorganism through a relatively diffuse manner. Peptides accumulate on the membrane surface forming a localized carpet. Peptides chains orient themselves parallel with respect to the membrane surface and become trapped in the head groups region. As they accumulate, they create tension between the two leaflets of the bilayer which leads to disintegration or rupture of the membrane. The formation of transient hole in the bilayer may be an intermediate step before the collapse of the membrane. The mechanisms of antimicrobial peptide action are shown in Figure 9.

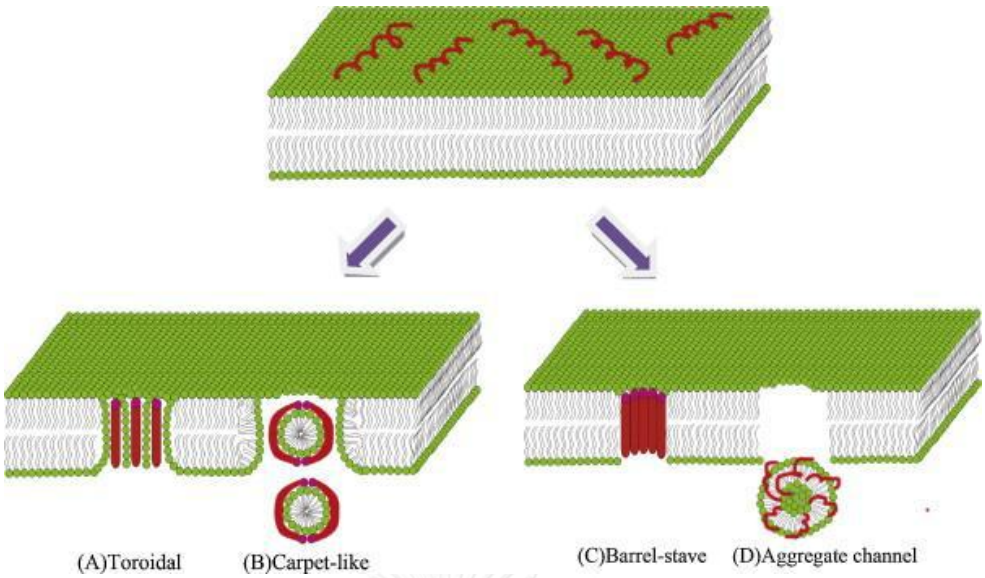

Figure 9 Mechanisms of antimicrobial peptide action (Laverty *et al*., 2011).

Potential as therapeutics

AMPs have many of the desirable features of a novel antibiotic class and can complement conventional antibiotic therapy. Moreover, they show synergy with classical antibiotics, neutralize endotoxin and are active in animal models. Minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) often coincide (less than a two-fold difference), indicating that killing is generally the highly desirable bactericidal mode of action (Seo *et al*., 2012). The clinical development of antimicrobial peptides are shown in Table 2 (Fox, 2013).

Table 2 Current status for clinical development of antimicrobial peptides (Fox, 2013).

Products	Description	Indication	Phase	Company (location)
Magainin peptide	22 amino acid linear AMPs Isolate from *Xenopus laevis*	Diabetic foot ulcers	3	Dipexium Pharma (white Planis, NY)
Novaxatin	Cyclic cationic peptide, 1093 dalton	Fungal infection	1/2	NavaBiotics (Aberdeen, UK)
MU1140	Lantibiotic	MRSA; *C.difficile*	preclinical	Organics (Teampa, Florida)

Fungi source of bioactive peptide or protein

Mushrooms, a macrofungi, have been playing an important role in several aspects of healthy food for long time. Edible mushrooms, for example, are used extensively in cooking. Wild and cultivated mushrooms contain a huge diversity of biomolecules with nutritional and some medicinal properties. Due to these properties, they have been recognized as functional foods, and as a source for the development of medicines and food supplements (Park *et al*., 2009). Fruiting bodies, mycelia and spores (as shown in Figure 10) accumulate a variety of some bioactive molecules with immunomodulatory, cardiovascular, liver protective, anti-fibrotic, anti-tumor, anti-inflammatory, anti-diabetic, , anti-oxidant, and antimicrobial properties (Lavanya and Subhashini, 2013).

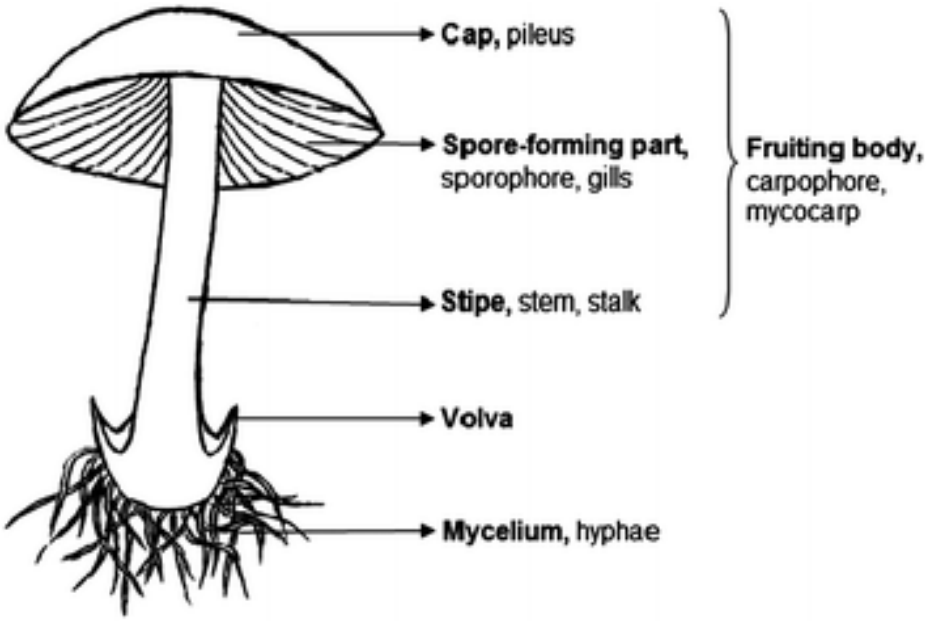

Figure 10 Basic Mushroom Anatomy (Ren, L *et al*., 2012)

In particular, mushrooms could be a source of natural antibiotics, which can be low or high molecular mass (LMW and HMW, respectively) compounds. LMW compounds are mainly secondary metabolites such as sesquiterpenes and other terpenes, steroids, antraquinone, benzoic acid derivatives and quinolones, they also include primary metabolites such as oxalic acid. HMW compounds include mainly peptides and proteins defensed against pathogenic and predatory organisms in their environment. It is estimated that there are 140,000 fungal species. Of these, around 2,000 species are edible, and about 200 have traditionally been gathered for food or for medicinal and other preparations (Erjavec *et al*., 2012 and Xu *et al*., 2011). Only 10% of mushroom-forming species are known, making them an enormous untapped pool of potentially useful substances (Xu *et al*., 2011). Antimicrobial peptides from mushrooms with antifungal activity such as Eryngin, an antimicrobial peptide with antifungal property from *Pleurotus eryngii* (Wang and Ng, 2004). Pleurostrin, an antimicrobial peptide with antifungal property from *Pleurotus ostreatus* (Chu *et al*., 2005). Cordymin, an antimicrobial peptide with antifungal property from *Cordyceps militaris* (Wong *et al*., 2011). There is also a report of AMPs with anticancer from *C. militaris* (Park *et al*., 2009). KREHG AccNo.55956 is the AMPs from *Russula paludosa* with HIV-1 reverse transcriptase inhibitory activity (Wang *et al*., 2007). The marmorin peptide with inhibitory activity on proliferation of hepatoma cells (HepG2) and breast cancer cells (MCF-7), as well as inhibitory HIV-1 reverse transcriptase activity is from *Hypsizigus marmoreus* (Lavanya *et al*., 2013). So proteins or peptides elaborated by these fungi and macrofungi have shown several biological activities including antiproliferative, immunomodulatory, antiviral, antifungal and antibacterial effects (Wang *et al*., 2004 and Lavanya *et al*., 2013).

Peptides with antimicrobial and other biological activities from Mushroom *Lentinus* spp.

The genus *Lentinus* is a white rot fungus, which has attracted the attention of many mycologists for many years (Kumar and Kaviyarasan, 2012). Some of them are known to be edible with well-known medicinal properties (Kumar and Kaviyarasan, 2012 and Sharma and Atri, 2015). This mushroom genus belongs to the *Basidiomycotina* sub-division and to the *Agaricaceae* family (Pegler, 1983). *Basidiomycotina* is known as mushrooms and their taxonomy has been found out by its macro and micro morphology. The species from *Agaricaceae* family are saprophyte, causing the degradation of wood and lignin/cellulosic matter. Some of them are important in food industry such as *Agaricus cretáceus, Agaricus silvicole, Clitocybe phyllphilla, Collybia longipes, Collybia radicata, Coprinus comatus, Lepiota gracilenta, Omphalia campanella, Panus torulos, Pleurotus columinus, Pleurotus pometi, Tricholoma georgii, Tricholoma jonides, Tricholoma nudum, Tricholoma personatum* and *Lentinus edodes* (Muralidharan and Bobek, 2005 and Thatoi and Singdevsachan, 2014).

Antibiotic, anti-carcinogenic and antiviral compounds have been isolated intracellularly (fruiting body and mycelia) and extracellularly (culture media) from *Lentinus* spp., and other mushroom (Cho *et al*., 2003 and Lindequist *et al*., 2005). Some of these substances from *Lentinus spp* were lentinan, lentin, lectin and eritadenin (Yildiz *et al*., 1998). Lentinan is a water soluble polysaccharide formed by β-1, 3 -glucan with ramification β-1, 6. Lectin is the denomination that receives a group of proteins chemically associated with carbohydrates. They are widely distributed in plants and cause blood coagulation (Karnchanatat, 2012). Hirasawa *et al*., (2005)

detected 3 different substances with anti-bacterial activity. Eritadenine is a compound that presents activity against cholesterol. Its activity was correlated to hepatic metabolism of lipids (Villas-Bôas *et al*., 2003). Lentin, the antimicrobial peptide isolated from *L. edodes* or Shitake mushroom which is one of the most famous edible mushroom from this genus, has several biological activities. It can inhibit mycelial growth in a variety of fungal species including *Physalospora piricola*, *Botrytis cinerea* and *Mycosphaerella arachidicola*. Moreover, lentin from *L. edodes* also has activity against HIV-1 reverse transcriptase and is able to inhibit proliferation of leukemia cells (Ngai and Ng, 2003).

In Thailand, there are various species of mushroom including *Lentinus* spp. *Lentinus squarrosulus* (Mont.) or "Hed khon khao" in local language, is one of edible indigenous mushroom species and abundantly grown in North East of Thailand. The local Thai people used *L. squarrosulus* mushroom as food for long time. Mycological data of *L. squarrosulus* was shown in Table 3 and the appearances of cultivated *L. squarrosulus* in Thailand was shown in Figure 11.

Table 3 Mycological data of *L. squarrosulus* (Mont.) (Pegler, 1983).

Taxonomy

Kingdom: Fungi

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Polyporales

Family: Polyporaceae

Genus: *Lentinus*

Species: *squarrosulus*

Scientific name: *Lentinus squarrosulus*

author: Mont. 1842

Figure 11 *L. squarrosulus* (Mont.) mushroom cultivated in Thailand
Retrieved from http://biodiversity.forest.go.th

Chapter III

MATERIALS AND METHODS

3.1 Equipments

- Analog Water Bath (LWB-206A, LabTech, USA)
- Balance (ML204, Mettler Toledo, Italy)
- Centrifuge (Allegra X-12 R, Zentrifugen, Germany)
- Electrophoresis apparatus
- Mini-PROTEAN® Tetra Cell (Bio-Rad, Hercules, Calif, USA)
- PowerPac™ Basic Power Supply (Bio-Rad, Hercules, Calif, USA)
- Sub-Cell® GT Agarose Gel (Bio-Rad, Hercules, Calif, USA)
- Freeze Dryer (TFD-5503, ilShin, China)
- Gel Doc (Gel Doc ™ XR+ System, Hercules, Calif, USA)
- General Incubator (Daeyang, China)
- Incubators (TFD-0713, ilShin, China)
- pH meter (FE20 – FiveEasy™ pH, Mettler Toledo, Italy)
- Microplate reader (Victor 3, Perkin-elmer, Massachusetts, USA)
- Spectrophotometer (Evolution 600 UV-Vis Spectrophotometer, Thermo Scientific, USA)

3.2 Materials

3.2.1 Microorganism

All bacterial and fungal strains were used for determination of biological activity of AMPs from *L. squarrosulus* (Mont.) mushroom. The strains used are as follows: gram-positive bacteria; *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633, gram-negative bacteria; *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 15442 and yeast; *Candida albicans* ATCC 10231 from Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The filamentous fungi and yeast of clinical isolates: *Trichophyton mentagrophytes*, *T. rubrum*, *C. tropicalis*, *Epidermophyton floccosum*, *Microsporum gypseum* and *Aspergillus niger* are from Department of Microbiology, Faculty of Medicine, Chulalongkorn University.

3.2.2 Plasmid DNA

pET28b (Cat. No. 15363-013, Invitrogen, USA) was obtained from Department of Microbiology, Faculty of Medicine, Chulalongkorn University and used as a substrate for determination of nuclease activity of AMPs.

3.2.3 Growth medium culture

All growth medium used in this study were as follows:

- **Sabouraud Dextrose Agar (SDA); 1 Liter**

Suspend 65 g of the medium in one liter of purified water. Heat with frequent agitation and boil for one minute to completely dissolve the medium. Autoclave at 121°C for 15 minutes. Cool to room temperature.

- **Mueller Hinton Agar (MHA); 1 Liter**

Suspend 38 g of the medium in one liter of purified water. Heat with frequent agitation and boil for one minute to completely dissolve the medium. Autoclave at 121°C for 15 minutes. Cool to room temperature.

- **LB (Luria-Bertani) liquid medium; 1 Liter**

Dissolve components (Tryptone 10 g, Yeast Extract 5 g and NaCl 10 g) in one liter of purified water. Heat the mixture to boiling to dissolve agar and sterilize by autoclaving at 121°C for 15 minutes. Cool to room temperature.

3.2.4 Enzymes

Two restriction endonuclease enzymes from Invitrogen (USA); *Eco*RI (Cat.No. 15202-013) and *Xba*I (Cat.No. 15172-011) were used according to the manufacturer's instructions. Proteinase K (Cat. No. RP2308) and Trypsin (Cat. No. T3924) from Sigma-Aldrich, Missouri, USA.

3.2.5 Chemicals and other materials

Chemicals

- ACRYL/BIS™ 29:1 (Cat. No. 0311-500ML, Amresco, Ohio, USA)
- Agar (Cat. No. 05038, Fluka, St. Gallen, Switzerland)
- Agarose (Cat. No. 05214, Fluka, St. Gallen, Switzerland)
- Amikacin (Cat. No. 231597, Sensi-Disc™, Becton Dickinson and company, USA)
- Ammonium persulfate (Cat. No. A3678, Sigma-Aldrich, Missouri, USA)
- Ammonium sulfate (Cat. No. A4418, Sigma-Aldrich, Missouri, USA)
- Barium chloride (Cat. No. B0750, Sigma-Aldrich, Missouri, USA)
- Bovine Serum Albumin (BSA) (Cat. No. A7906, Sigma-Aldrich, Missouri, USA)
- Bromophenol blue (Cat. No. 135869, Merck, Darmstadt, Germany)
- Chloramphenicol (Cat. No. C1919, Sigma-Aldrich, Missouri, USA)
- Ciprofloxacin (Cat. No. C17850, Sigma-Aldrich, Missouri, USA)
- Citric acid (Cat. No. 251275, Sigma-Aldrich, Missouri, USA)
- Coomassie blue R250 (Cat. No. BD100-25, Fisher Biotech, New Jersey, USA)

- Disodium hydrogen phosphate (Cat. No. 7558794, Sigma-Aldrich, Missouri, USA)
- DTT (dithiothreitol) (Cat. No. D0632, Sigma-Aldrich, Missouri, USA)
- 1 Kb DNA ladder (Cat. No. GA-100, GeneAll, Taiwan)
- 10 Kb DNA ladder (Cat. No. GA-1000, GeneAll, Taiwan)
- Ethidium bromide (Cat. No. E1510, Sigma-Aldrich, Missouri, USA)
- Ethyl alcohol (Cat. No. 64175, Sigma-Aldrich, Missouri, USA)
- Ethylenedinitrilotetraacetic acid, (EDTA) (Cat. No. 60004, Sigma-Aldrich, Missouri, USA)
- Glacial acetic acid (Cat. No. A537020, Sigma-Aldrich, Missouri, USA)
- Glycerol (Cat. No. G9012, Sigma-Aldrich, Missouri, USA)
- Glycine (Cat. No. 161-0718, Bio-Rad, Hercules, California, USA)
- Hydrochloric acid (Cat. No. 258148, Sigma-Aldrich, Missouri, USA)
- Ketoconazole (Cat. No. K1003, Sigma-Aldrich, Missouri, USA)
  Magnesium chloride (Cat. No. 208337, Sigma-Aldrich, Missouri, USA)
- β-Mercaptoethanol (Cat. No. M131, Amresco, Ohio, USA)
- Methanol (Cat. No. 106009, Merck, Darmstadt, Germany)
- Mueller Hinton ll Agar (Cat. No. 211438, Difco™, Becton Dickinson and company, France)
- Potassium dihydrogen phosphate (Cat. No. 7778770, Sigma-Aldrich, Missouri, USA)
- Protein marker (Mark12™) (Cat. No. LC5677, Invitrogen Life Sciences, USA)
- Sabouraud Dextrose Agar (Cat. No. 210950, Difco™, Becton Dickinson and company, France)
- Sodium Acetate (Cat. No. S2889, Sigma-Aldrich, Missouri, USA)
- Sodium Chloride (Cat. No. S7653, Sigma-Aldrich, Missouri, USA)
- Sodium Dodecyl Sulfate, (SDS) (Cat. No. 1610302, Bio-Rad, USA)
- Sodium hydroxide (Cat. No. 221465, Bio-Rad, USA)
- Tetracycline (Cat. No. C87128, Sigma-Aldrich, Missouri, USA)
- N,N,N',N'-Tetramethylethylenediamine (TEMED) (Cat. No. M146, Amresco, Ohio, USA)

- Tris-(hydroxymethyl) aminomethane (Cat. No. 93349, Sigma-Aldrich, Missouri, USA)
- Tryptone (Cat. No. 200150, Difco™, Becton Dickinson and company, France)
- Tween 80 (Cat. No. 93420, Fluka, St. Gallen, Switzerland)
- Yeast Extract (Cat. No. 211471, Difco™, Becton Dickinson and company, France)

Other material

- DEAE-Cellulose (Cat. No. DO909, Sigma-Aldrich, Missouri, USA)
- Dialysis tubing cellulose membrane (Cat. No. D9777, Sigma-Aldrich, Missouri, USA)
- Sephadex G-25 Medium (Cat. No. 17-0033-01, GE Healthcare, Sweden)
- Paper discs 6 mm (Whatman, UK)
- Petri Dish Glass, 150 mm x 15 mm (PYREX®, USA)
- Petri Dish, 90 mm × 15 mm (Cat. No. BI93D-01, Gosselin™, France)
- Microcentrifuge tube (Cat. No. 2500, US scientific, USA)
- 96-well plate (Cat. No. 655, Bioscience, USA)
- 50 mL Conical Centrifuge Tubes (Cat. No. 430828, Corning Inc., USA)
- 15 mL Conical Centrifuge Tubes (Cat. No. 430052, Corning Inc., USA)

3.3 Method

3.3.1 Mushroom sample collection.

L. squarrosulus (Mont.) mushroom samples were purchased from Khlong Toei market, Bangkok Thailand. They will be kept in sterile plastic bags and stored at - 20 ℃ until isolation. They were sent to the Department of Agriculture, Ministry of Agriculture and Cooperatives for phenotypic identification.

3.3.2 Antimicrobial protein (AMPs) Isolation, Purification and Characterization

3.3.2.1 Isolation of total protein using ammonium sulphate precipitation.

Fresh mushroom (1,500 g) of *L. squarrosulus* (Mont.) were cleaned with sterile distilled water and homogenized in 4,500 mL distilled water (3 mL/g) using a blender. The homogenate was centrifuged (12,000 ×g, 30 min, 4 ℃), and the resulting supernatant was collected. Tris–HCl buffer, pH7.4 was added to the supernatant until the concentration of 10 mM Tris-HCl buffer, pH 7.4 was reached. The crude extract was further used for precipitation of protein. The inorganic salt will be utilized for protein precipitation, with ammonium sulfate being the most common. Ammonium sulphate precipitation is the most commonly used because it is highly soluble in water. It stabilizes most proteins in solution and it reduces up to 50% of contaminating protein and therefore reduces the load for subsequent chromatography. Most precipitated proteins retain their activity and native conformation and can be redissolved easily and hence ideal for crude and dilute samples. Generally, most proteins precipitate between 20 - 80%. To minimize the possibility of protein denaturation, the saturated solution of ammonium sulfate should be used (Burgess, 2009). An experiment was performed to find out the optimal concentration required for protein precipitation by varying concentrations of ammonium sulphate ranging from 40 - 80% saturation. Proteins were precipitated from the supernatant by addition of ammonium sulfate to a final concentration of 40% (w/v), followed by incubation at 4℃ for 1 h and then centrifuged at 12,000 ×g for 30 min. The crude protein pellet from 40% NH_4SO_4 fraction was dissolved in 50 mL of 10 mM Tris–HCl buffer, pH 7.4. The supernatant from the first ammonium sulfate precipitation was further added with ammonium sulfate 40% (w/v) to a final concentration of 80% (w/v). The sample was incubated at 4 ℃ for 1 h and then centrifuged at 12,000 ×g for 30 min. The pellet from 80% NH_4SO_4 fraction was dissolved in 50 mL of 10 mM Tris–HCl buffer, pH 7.4. Both protein fraction samples were dialyzed at 4 ^{0}C against the same buffer using dialysis membrane (12,000 MWCO) for 24 h by changing buffer every 6 h (4 times). After dialysis, protein samples were concentrated with freeze-dried method. The antimicrobial

activity of each fraction was screened, and fractions possessing antimicrobial activity were further purified.

3.3.2.2 Ion Exchange Chromatography with DEAE – cellulose chromatography.

The most popular method for the isolation and purification of proteins and other charged molecules is ion exchange chromatography. In cation exchange chromatography, positively charged molecules are attracted to a negatively charged stationary phase. Conversely, in anion exchange chromatography, negatively charged molecules are attracted to a positively charged of stationary phase. Two milliliters of crude protein (dried protein pellet dissolved in 10 mM Tris–HCl buffer, pH 7.4) obtained in the above step was applied to DEAE cellulose column (Sigma-Aldrich) (2.6 x 25 cm) pre-equilibrated with 10 mM Tris–HCl buffer, pH 7.4. Elution was done with step-wise salt concentration gradient from 0.1 M-1 M NaCl in 10 mM Tris–HCl buffer, pH 7.4. After unadsorbed proteins were eluted with 10 mM Tris-HCl buffer, pH 7.4 at flow rate 0.2 mL/min, the adsorbed proteins were further eluted with increasing concentration of NaCl from 0.1 M to 1 M in 10 mM Tris-HCl buffer, pH 7.4. Each fraction, eluted with NaCl solution, was then dialyzed against 10 mM Tris-HCl buffer (pH 7.4) using dialysis bags (Sigma-Aldrich) MWCO. 12 kDa at 4 °C for 24 h by changing buffer every 6 h (4 times). After dialysis, protein samples were completely dried under high vacuum with the lyophilizer overnight and further determined for antimicrobial activity.

3.3.2.3 Size-exclusion chromatography with a G-25 Sephedex Column

Size Exclusion Chromatography (SEC) is the separation technique based on the molecular size of the components. Separation is achieved by the differential exclusion of the sample molecules from the pores of the packing material as they pass through a bed of porous particles. The principle feature of SEC is its gentle non-adsorptive interaction with the sample, enabling high retention of biological activity. The fractions with antimicrobial activity from DEAE cellulose column, were pooled and freeze-dried. Dissolve 50 mg of freeze-dried pellet into 1 mL of 10 mM Tris-HCl buffer, pH 7.4 were applied on a Sephadex G-25 column (GE Healthcare) (2 x 40 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.4. The column was eluted with the same buffer at a flow rate

of 0.2 mL/min. Filtrate fractions showing antimicrobial activity were pooled, lyophilized, stored at – 20 ^{0}C and used for further studies.

3.3.2.4 Determination of Protein content

The amount of total protein was estimated according to the protocol developed by Bradford (1976) against a bovine serum albumin (BSA) standard curve. In this method, Coomassie brilliant blue G-250 dye binds to proteins and its colour is changed from green to blue. That colour change is monitored at 595 nm with spectrophotometer. While the concentration of protein increases, the colour is getting darker and darker. Coomassie brilliant blue G-250 binds to arginine, lysine, and histidine residues in protein samples.

The 96 Well Plate Assay Protocol was used for determination of protein content, The standard calibration curve was generated using different concentration of BSA (50, 25, 12.5, 6.25 and 3.125 mg/mL) diluted in protein extraction buffer. The unknown samples were also diluted in the same buffer. Gently mix the Bradford Reagent in the bottle and bring to room temperature. Add 5 µl of each concentration of protein standard to separate wells in the 96 well plate. To the blank wells, add 5 µL of the extraction buffer. Prepare the unknown sample(s) in extraction buffer to final volume of 5 µL. To each well being used, add 195 µL of the Bradford Reagent and mix on a shaker for approximately 30 seconds. Let the samples incubate in the dark and keep in room temperature for 5 to 45 minutes. Then, measure the absorbance at 595 nm. The protein-dye complex is stable up to 60 minutes. Plot the net absorbance vs. the protein concentration of each standard. Determine the protein concentration of the unknown sample(s) by comparing the absorbance values against the standard curve. Protein content of unknown sample was estimated by expressed in mg of protein/mL of extract.3.3.3 *In vitro* Antimicrobial activity assay

The antimicrobial activity of each protein fraction will be screened by using paper disc diffusion method as described by Kirby-Bauer (1956) or Collins and Lyne (1987), Details are as follows:

3.3.3.1 Preparation of McFarland standard

McFarland standard is used as a reference to adjust the turbidity of microbial suspension so that microorganisms will be within a given range. Exactly 0.5 McFarland equivalent turbidity standards was prepared by adding 0.6 mL of 1% barium chloride solution ($BaCl_2$. $2H_20$) to 99.4 mL of 1% sulphuric acid (H_2SO_4) and mixed thoroughly. Exactly 0.5 McFarland gives an equivalent approximate density in Colony Forming Units per mL (CFU/ mL). A small volume of the turbid solution was transferred to cap tube of the same type that was used to prepare the test and control inoculate. It was then stored in the dark at 25°C (Stein *et al*., 2005).

3.3.3.2 Preparation of crude protein and purified protein

Lyophilized sample (crude and purified protein) was dissolved in 10 mL of 10 mM Tris–HCl, buffer (pH7.4) and determined for protein concentration with Bradford method (Bradford, 1976). For determination of antimicrobial activity, different amount of protein were used; 30 µg/disc for crude protein and various amount including; 3.5, 7.5, 15, 30, 40, 50 µg/disc for purified protein. Amikacin 30 µg/disc and Ketoconazole 20 µg/disc were used as positive control for bacteria and fungi, respectively. Moreover, 10 mM Tris–HCl buffer (pH 7.4) was used as negative control.

3.3.3.3 In vitro Antimicrobial Susceptibility Assays

The fungi and bacteria used for antimicrobial susceptibility test are as follows: *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, *E. coli* ATCC 35218, *K. pneumoniae* ATCC 4352, *P. aeruginosa* ATCC 15442, *T. mentagrophytes*, *T. rubrum*, *C. tropicalis*, *C. albicans* ATCC 10231, *E. floccosum, M. gypseum* and *A. niger*. The fungal and bacterial cultures were propagated on Sabouraud Dextrose Agar (Difco, France) and Mueller Hinton Agar (Difco, France), respectively and activated by periodic sub-culturing at monthly intervals.

The use of agar disc diffusion method to screen for antimicrobial activities of the crude protein extracts and purified protein was done according to the National

committee of clinical and laboratory standards (NCCLS, 2003 and 2007). The tested microorganisms (fungi and bacteria) will be suspended in 0.85% normal saline solution and standardized the turbidity of cell suspension with 0.5 McFarland standard, (approximately 1.5 x 10^8 colony-forming units (CFU)/mL for bacteria or.1.5 x 10^6 CFU/mL of yeasts and 1.0 x 10^5 CFU/mL of filamentous fungi) (Nweze *et al*., 2010). The tested filamentous fungi will be steriled swab dipped into steriled Tween 80 solution added into normal saline to final concentration of 0.05% w/v and used to pick the pure fungi (mixture of conidia and hyphal fragments). This was then suspended with sterile normal saline and vortexed. The turbidity of the homogenous suspension was adjusted to 0.5 McFarland standard. Sabouraud Dextrose Agar and Mueller-Hinton Agar plates were prepared for fungi and bacterial, respectively. The fungal and bacterial suspensions will be spread using steriled cotton swab on the surface of agar plates. After the surface of agar was dried, the paper disc impregnated with positive, negative control or protein samples with various amount of protein/peptide were placed on the dried surface of agar. The plates were incubated at 30°C for 24 h, 48 h for fungi and yeast as well as mold, respectively. The incubation for bacterial plates is 37°C for 24 h. After, completing the incubation, plates were then examined for the presence of inhibition zones and diameters were measured. The experiment was done in triplicate.

3.3.3.4 Effect of enzymes, temperature and pH on antifungal activity

The effect of temperature, protease enzyme and pH on antifungal activity of AMP from *L. squarrosulus* (Mont.) mushroom was checked according to the method described by Chihara *et al*., (2005), Teixeira *et al*., (2013) and Wang *et al* (2014) with minor modification. To determine the effect of temperature on antifungal activity, the purified protein pellet were dissolved in 10 mM Tris-HCl buffer, pH 7.4 to yield the final concentration of 1 mg/mL. Aliquot 500 µL purified protein suspension were incubated at 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C for 1 h. After incubation, the protein solution were then cooled to 4 °C and centrifuged (15,000 ×g, 10 min) to remove any precipitation before determining the antifungal activity assay. The assay

was performed by using agar disc diffusion method as described in Antimicrobial susceptibility assay. Each experiment was done in triplicate.

To determine the effect of pH on antifungal activity of purified protein, the purified protein solution (1 mg/mL) were incubated in the following buffers: 10 mM Na_2HPO_4 – Citrate Buffer; (pH 2.0 –7.0) and 10 mM Glycine – NaOH Buffer, (pH 7.0–10.0) at 4°C for 24 h. After 24 h of incubation in the appropriate buffers, the samples were dialyzed for 24 h against the 10 mM Tris-HCl buffer (pH 7.4) to remove the extreme pH buffers in order to prevent the inhibition of fungal growth in antifungal assay. After completing dialysis, the protein solution was further used to determine for antifungal activity according to the method described in antimicrobial susceptibility assays. Each experiment was done in triplicate.

The proteolytic stability of peptides toward trypsin (from bovine pancreas, EC 3.4.21.4, Merck, Germany) was determined by a typical trypsinization experiment. The 30 µg purified protein and 0.1 µg trypsin in 100 µL of PBS buffer (pH 7.4) were added into microcentrifuge tube and incubated at 37 °C for 6 h. After completing the incubation, the activity was terminated by heating at 80 °C for 5 mins and then the mixture was cooled to 4 °C before the protein was used for antifungal assay. The assay was determined by agar disc diffusion method as described in antimicrobial susceptibility assays. Each experiment was done in triplicate.

3.3.4 Other biological activity of AMPs from *L. squarrosulus* (Mont.)

3.3.4.1 Synergistic effects of purified protein and antibiotics against bacteria

Synergistic effect of purified protein and antibiotic against bacteria was determined using 30 µg of purified protein/disc. Tetracycline, Ciprofloxacin and Chloramphenicol were used as tested antibiotic (Naghmouchi *et al*., 2012). The stock reference antibiotics were prepared at concentration of 1 mg/mL in autoclaved Mill-Q water and stored at 4°C. Four different discs for each antibiotic were prepared by impregnated with different amount of standard antibiotic: 10, 20, 25 and 30 µg of

Tetracycline, Chloramphenicol and 5, 4, 3, 2 μg of Ciprofloxacin per discs. After the discs were dried, the 30 μg purified protein in suspension were dipped onto each dried antibiotic disc except the discs with 30 μg of Tetracycline and Chloramphenicol as well as the disc with 5 μg of Ciprofloxacin which have been used as reference. After the paper discs were dried, they were placed on the dried surface of agar plate spread with bacteria suspension. The discs impregnated with antibiotic alone were used as reference to compared diameter of inhibition zone. The disc impregnated with 10 mM Tris-HCl, buffer (pH 7.4) was used as negative control. The plates were incubated at 37°C for 24 h. After completing the incubation, plates were then examined for the inhibition zone and diameters were measured. The zone of inhibition occurred with the disc impregnated with antibiotic alone were compared with the discs impregnated with purified protein and antibiotic.

3.3.4.2 Nuclease activity of an antifungal protein

The method for nuclease activity assay was performed according to Jutur and Reddy, (2007) and Belkebir and Azeddoug, (2012) with minor modification. Different DNA substrates including genomic DNA from *E. coli* and circular pET28b plasmid (Invitrogen, USA) were used.

Genomic DNA extraction

5 mL bacterial cultures was spun down at 6,000 xg for 10 min. The pellet was washed two times with PBS buffer (120 mM NaCl, 16 mM Na_2HPO_4, 4 mM KH_2PO_4, pH 7.4) and resuspended in 3 mL lysis buffer (1 M Tris-HCl, pH 7.5, 1 M EDTA, pH 8, 1 M NaCl), 50 μL of 10% SDS and 100 μL of 10 mg/mL Proteinase K. The cell suspension was incubated at 60 °C for 3 h and subjected to phenol chloroform extraction. The genomic DNA was precipitated by adding 2 volumes of 3 M NaOAc and 2.5 volume of

absolute ethanol and incubated overnight at - 20 °C. The genomic DNA was washed with 70% ethanol and resuspended in 50 µL of purified water.

The digestion reaction was carried out at 37 °C for 1 h in 25 µL reaction mixture containing the following composition: 2.5 µL of 10x restriction buffer contain 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM dithiotheritol (DTT), 0.1 mg/mL of BSA, 0.1 M NaCl), 1 µg of DNA substrate, (gDNA or pET28b), 1 µL (15 µg) of purified protein and purified distill water to final volume of 25 µL. After completing 1 h incubation, the reactions were stopped by addition of 5 µL of 6X gel loading buffer contains 5% glycerol, 0.125% bromophenol blue, 25 mM EDTA (Invitrogen, USA) and placed on ice before electrophoresis. Seven microliters of sample was loaded on 1% agarose gel in TAE buffer. The gel was run for 60 min at 80 V and stained with Ethidium bromide. Banding pattern of DNA digested by purified protein was compared with the one digested by commercial endonuclease enzyme (*Eco*RI and *Xba*I).

3.3.5 Physicochemical properties of purified AMPs

3.3.5.1 Estimation of the molecular mass of AMPs using SDS-PAGE

SDS-PAGE was applied for the determination of MW of purified AMPs. Proteins were resolved on SDS-PAGE gels made up of a resolving gel (10, 12, 15 or 18 % etc. acrylamide depending on protein size), that was overlaid with a stacking gel (5 % acrylamide). All SDS-PAGE gels were made up with 30 % acrylamide with an acrylamide: bisacrylamide ratio of 37:5:1 (Bio-Rad Laboratories, USA). Analytical electrophoresis of proteins was carried out in polyacrylamide gels (Laemmli, 1970) in the Mini Protean II apparatus (Bio-Rad) according to the Laemmli discontinuous buffer system.

The 20 µg of protein sample was mixed with SDS-PAGE sample buffer (glycerol, 1 M Tris-HCl buffer pH 6.8, SDS, bromophenol blue, and dH2O) and 5% β-mercaptoethanol to avoid S-S bridge formation. The mixed and denatured samples were heated in a boiling water-bath for 10 min. SDS-PAGE gels consisting of 14% separating gel and 5% stacking gel (Appendix A) were cast as described. A clean 10 wells comb was inserted in between the plates sandwich. After the gels polymerized,

the electrophoretic apparatus was assembled and filled into with buffers. SDS running buffer was used for both chambers. Each well of the gel was washed (by pipetting up and down) 6-8 times with anode buffer using a micropipette before loading the samples. Samples were applied into the gel 10 µL in each well. Then, the electrophoresis assembly was connected to a power supply and constant current of 110V was applied. The samples were allowed to run until they migrated to the bottom of the resolving gel. The gel was stained using Coomassie brilliant blue staining buffer (0.1% Coomassie blue R250 in methanol: acetic acid: dH2O 40:10:50) for 20 mins and then destained using destaining buffer solution (25% methanol: 7% acetic acid and dH2O). The molecular mass of purified protein was determined by comparing with molecular mass of protein marker (Mark12TM, Invitrogen).

3.3.5.2 Amino acid sequence analysis

To determine the N-terminal amino acid sequence of the purified peptides, the eluted purified fractions with antimicrobial activity were further subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). The samples were reduced, alkylated, digested with trypsin, and analyzed in a 4800 Proteomics Analyzer MALDI-TOF mass spectrometer (Thermo Scientific™, USA) at Newcastle University. Briefly, the purified protein sample digested with trypsin enzyme was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 µL of a 3 mg/mL of **α**-cyano-4-hydroxy-transcinnamic acid matrix (Sigma-Aldrich, USA) in 50% acetonitrile were added to the dried peptide to digest spots and allowed again to air-dry at room temperature. MALDI-TOF MS analysis was performed in Proteomics Analyzer MALDI-TOF mass spectrometer. The acquisition method for MS analysis was 1 KV reflector positive mode. The parameters used to analyze the data were signal to noise = 20, resolution >6000. All mass spectra were calibrated externally using a standard peptide mixture. For protein identification, the amino acid sequence from the fragmentation spectra of selected peptides was performed using bioinformatics tool. Homology search of the deduced amino acid sequences were performed through the Mascot Server.

3.3.5.3 Bioinformatics analysis

The amino acid sequence obtained from N-terminal sequencing method were searched against protein databases using a BLAST Similarity Search Engine. The combination of bioinformatics tools and database such as NCBI as well as Peptide Search program from the EMBL Bioanalytical Research Group will be utilized (Mascot Server).

3.3.5.4 Statistical analysis

Results of zone diameters of growth inhibition were expressed as the mean ± standard error of the mean (S.E.M).

CHAPTER IV
RESULTS AND DISCUSSIONS

In order to identify the antifungal activity from the purified protein of *L. squarrosulus* (Mont.). The experiments were performed by using the simple test of paper disc diffusion assay on SDA media (a fungal growth-supporting medium). The antifungal activity of protein was detected by the formation of inhibition zone around the protein-containing paper disc which showed the antifungal activity against the tested fungus. The larger inhibition zone indicates the greater antifungal activity of proteins. To eliminate the possible release of antifungal compounds from endogenous microorganisms, mushroom and all materials were sterilized thoroughly before performing the experiment.

4.1 Collection and Morphological characteristics of L. squarrosulus (Mont.) mushroom.

The fresh fruiting bodies of mushroom specimens cultivated in Thailand were purchased from Klong Toey market in Bangkok, Thailand. Samples of mushroom fruiting bodies was sent to Department of Agriculture, Ministry of Agriculture and Cooperatives in order to confirm their identification relied on the morphological characteristics.

At the mature stage, the whole fruiting body was divided into three regions, namely a cap or pileus, a stem and a volva (Figure 12 A). The volva was a thin sheet of interwoven hyphae around the bulbous stem base. It was fleshy, light brown color (Ivory) and flat shaped (plane). The stem was attached to the center of lower surface

of cap and connected to the volva. The cap was fully expanded and has a circular shape, with an entire margin and a smooth surface having a Ivory color. The diameter of cap was about 3 - 5 cm. The lower surface of the cap contains many gills. They were straight and had an entire margin. The basidiospore morphology of the *L. squarrosulus* (Mont.) mushrooms was further studied using the light microscope. It was found that four basidiospores normally attached to each basidium. The shape of mature basidiospores was most asymmetric with a slight tendency to be egg-shaped. The average length of the egg-shaped spores was approximately 4 - 7 µm; the width of the widest end was approximately 2 - 3 µm and the narrow end was approximately 2 - 4 µm. The spores comprise a smooth and thick cover and there is a short, triangular spine sticking out at the narrow end of spores when observing by the transmitted light microscope, Figure 12 B (The Mycota, 2001).

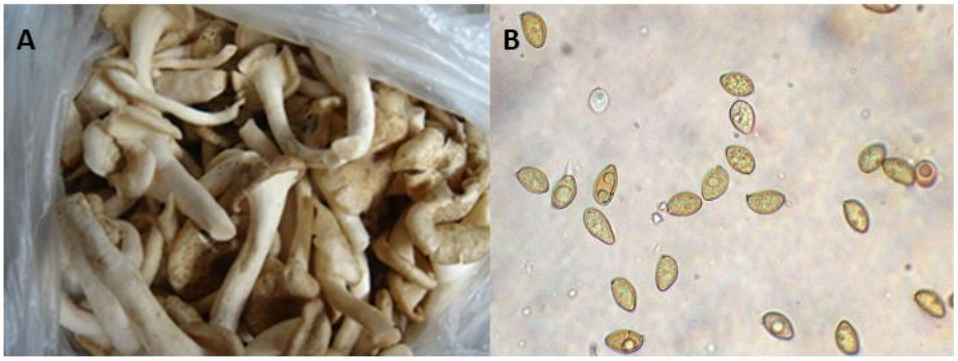

Figure 12 Morphological and physiological identification of *L. squarrosulus* (Mont.) mushroom, A) *L. squarrosulus* (Mont.) mushroom at mature stage and B) The basidiospore of the *L. squarrosulus* (Mont.) mushroom.

4.2 Purification of antifungal protein from *L. squarrosulus* (Mont.) using ammonium sulfate precipitation

The accumulation of antimicrobial proteins in crude extracts obtained by 40% w/v and 80% w/v ammonium sulfate precipitation were extracted from the fruiting bodies of *L. suarrosulus* (Mont.) mushroom. The antifungal activity of these extracts was detected by the disc diffusion assay using the clinical isolates of standard microorganisms. The crude protein extracts from the whole fruiting body displayed different antimicrobial activities.

The antimicrobial activity tests of crude protein extracts obtained from the 40% w/v ammonium sulfate precipitation did not show the inhibition of bacterial and fungal growth as illustrated in Table 4, 5 and Figure 13, 14. When comparing the activity of crude protein extracts to that of the standard antibiotics, amikacin and ketoconazole, the results confirmed that there is no inhibitory effect of this crude protein extracts on bacteria and fungi because the standard antibiotics kill both bacteria and fungi on the plate. The amount of standard antibiotics used in this study is the working amount to observe the zone of inhibition by this assay. In contrast to the previous crude extracts, the crude protein extracts derived from 80% w/v ammonium sulfate precipitation at the amount of 30 µg/disc displayed the inhibitory activity against four fungi and yeast, however, the antifungal protein has low activity against *A. niger* and no inhibitory effect on two filamentous fungi, *M. gypseum* and *E. floccosum*. The activity of crude extracts was then compared to the standard antibiotic, ketoconazole 20 µg/disc as shown in Table 5 and Figure 14. The findings indicated that the zones of inhibition formed by the standard antibiotic were larger than those generated from the crude extracts. This led to the fact that there was an

antifungal protein in the extract but the activity of proteins was not as good as the standard antibiotic. Although the fungi were susceptible to the crude protein extracts isolated by 80% w/v ammonium sulfate, gram-positive and gram negative bacteria were not. The antibacterial activity tests were achieved by using the crude protein extracts precipitated by 80% w/v ammonium sulfate at the amount of protein 30 µg/disc. The antibacterial activity of crude extracts was compared to the standard antibiotic drug, amikacin at the amount of 30 µg/disc (Table 4 and Figure 13). The amount of amikacin is the working amount used for this assay. Only 64.05% of the crude protein extracts having the antimicrobial activity was recovered by salting out method using 80% w/v ammonium sulfate (Table 6).

In the present study, the isolation and purification of protein with antimicrobial activity was reported. The protein was purified using the same chromatographic techniques as other mushroom proteins previously reported. However, the strategies involved in this study followed the steps of extraction and isolation. The extraction of proteins was achieved by the precipitation of protein using 40 - 80 % (w/v) ammonium sulfate. The extraction protocol used in this experiment is a modification of method commonly used for the protein extraction such as ammonium sulfate precipitation and ethanol extraction method. Wang *et al*., 2000 reported the purification of proteins from the mycelia of *Tricholoma lobayense* by the cold saline extraction and ammonium sulfate precipitation while another study by Zheng *et al*., (2010) used the homogenization prior to the precipitation of proteins by ammonium sulfate from *Clitocybe sinopica*.

Table 4 Diameter (mm) of inhibitory zone showing the antimicrobial activity of crude protein extracts from *L. squarrosulus* (Mont.). The test of antibacterial activity was achieved by the agar disc diffusion assay, on the soft agar plates for 24 h at 37 ºC. Results were expressed as mean ± S.E.M (n = 3), each performed in triplicate.

Bacterial	Samples		
	Amikacin 30 µg*	0-40 % w/v (30 µg)	40-80 % w/v (30 µg)
B. subtilis	15.71 ± 0.87	0	0
S. aureus	16.2 ± 0.43	0	0
K. pneumoniae	15.31 ± 1.02	0	0
P. aeruginosa	16.84 ± 0.53	0	0
E. coli	15.49± 0.82	0	0

*Amikacin are positive control (30 µg/disc)
10 mM Tris-HCl buffer, pH 7.4 and 0.85% Normal saline solution are negative control

Table 5 Diameter (mm) of antimicrobial activity zone obtained from the purified proteins from *L. squarrosulus* (Mont.), on the soft agar plates with filamentous fungi and yeast. The zone of inhibition was observed after incubating the plates for 48 h at 30°C. Results were expressed as mean ± S.E.M (n = 3), each performed in triplicate.

Fungi	Samples		
	Ketoconazole 20 µg*	0-40 % w/v (30 µg)	40-80 % w/v (30 µg)
T. mentagrophytes	24.81 ± 0.36	0	16.02 ± 0.38
T. rubrum	24.30 ± 0.16	0	17.13 ± 0.12
C. tropicalis	22.34 ± 0.81	0	16.51 ± 0.43
C. albicans	23.15 ± 0.28	0	15.22 ± 0.7
A. niger	14.83 ± 0.29	0	10.83 ± 0.6
M. gypseum	21.62 ± 0.51	0	0
E. floccosum	62.77 ± 0.64	0	0

*Ketoconazole are positive control (20 µg/disc)

10 mM Tris-HCl buffer, pH 7.4 and 0.85% Normal saline solution are negative control.

Table 6 Yield of proteins expressing the antifungal activity were obtained from the different stages of isolation and purification from *L. squarrosulus* (Mont.) fruiting bodies

Stages of purification	Volume (mL)	amount of proteins (mg) [a,b]	Recovery of protein (%)
Crude extract	480	331	100
Solubilized 40-80% of $NH_4(SO_4)_2$ precipitates	197	212	64.05
F3 (0.5 M NaCl eluted)	90	107	32.32
F4 (G-25 Sephadex column)	21	21	6.34

[a]; 1.5 kg fresh mushroom was used for purification and [b]; Protein concentration was determined by Bradford assay using BSA as a standard protein.

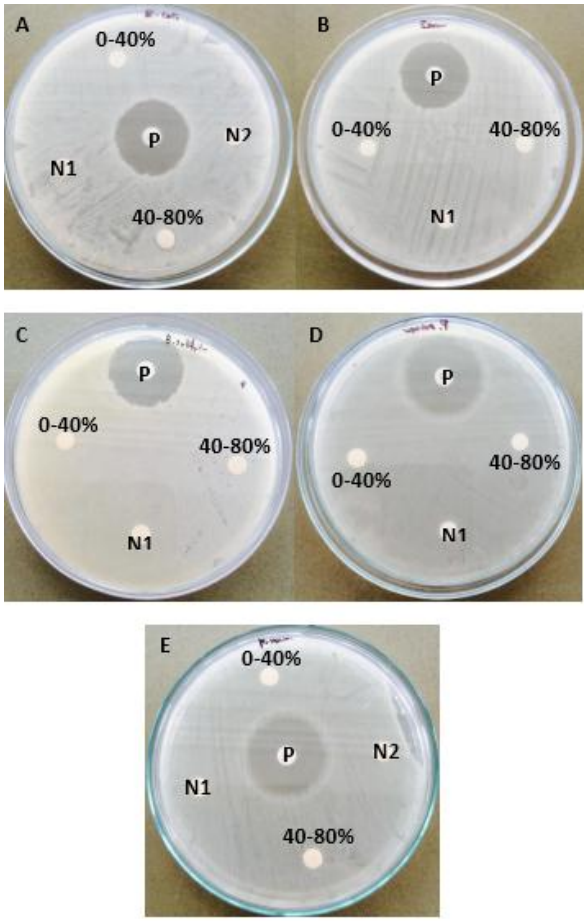

Figure 13 Inhibitory zone of crude proteins generated from *L. squarrosulus* (Mont.), against bacteria. Aliquots (30 µg, 2 mg/mL) of the crude protein samples were added to the 6 mm paper discs and plates were incubated at 30 °C for 24 h. (A) *E. coli* ATCC 35218, (B) *S. aureus* ATCC 6538, (C) *B. subtilis* ATCC 6633, (D) *P. aeruginosa* ATCC 15442, (E) *K. pneumoniae* ATTC 4352, Crude supernatants (40 % and 80 % w/v); Negative controls, (N1) 10 mM Tris-HCl buffer, pH 7.4, (N2) 0.85% Normal saline solution and Positive control (P) are Amikacin 30 µg/disc.

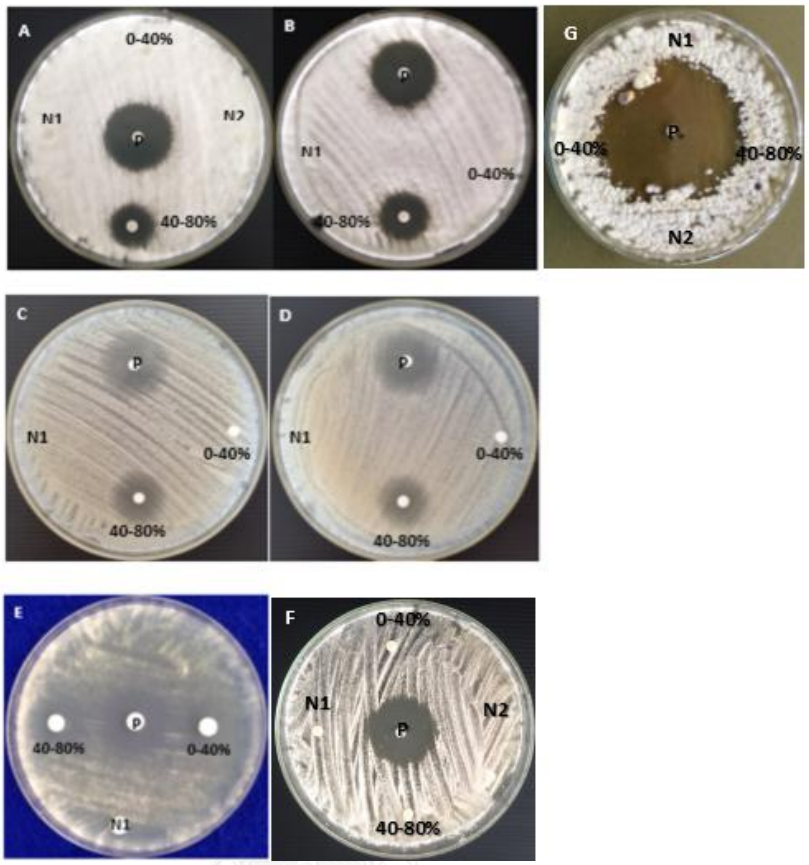

Figure 14 Inhibitory zone of crude proteins generated from *L. squarrosulus* (Mont.), against yeast and fungi. Aliquots (30 µg/discs) of the crude protein samples were added to the 6 mm paper discs and plates were incubated at 30°C for 24 h and 48 h for yeast and filamentous fungi, respectively. (A) *T. mentagrophytes*, (B) *T. rubrum*, (C) *C. albicans* ATCC 10231, (D), *C. tropicalis*, (E) *A. niger*, (F) *M. gypseum* and (G) *E. floccosum*. Crude supernatants (40% w/v and 80 % w/v); Negative controls, (N1) 10 mM Tris-HCl buffer, pH 7.4, (N2) 0.85% normal saline solution and Positive control (P) are Ketoconazole 20 µg/disc.

4.3 Purification of the selected antifungal protein from *L. squarrosulus* (Mont.) by ion-exchange chromatography

In order to separate the antifungal proteins from other contaminants in the 80% w/v ammonium sulfate extracts, the anion exchange chromatography was carried out. The analysis of molecules by the analytical anion exchange chromatography aims to differentiate between molecules having different net charges in certain pH. In the case of proteins, they can be separated using the anion exchange chromatography based on their pI. The pI presents the pH value at which a protein has no net electric charge. The anion exchange column used in this study was previously described in the experimental section. The protein mixture was eluted by a linear salt gradient, where the ionic strength of elution buffer was changed.

The 80% w/v ammonium sulfate precipitates after dialysis were loaded onto a DEAE cellulose column (5x30 cm) and then washed with 10 mM Tris-HCl buffer (pH 7.4). The crude proteins were eluted using the same buffer containing salts. A 0 – 1.0 M NaCl gradient was applied in order to disrupt the interaction between proteins and the matrix of anion exchange column. The protein-containing fractions were collected. The fractions collected from the column was monitored spectrophotometrically at 280 nm. Figure 15 displayed the elution profile of crude extract was obtained from the anion exchange chromatography. There were 4 peaks corresponding to one unabsorbed and three adsorbed proteins. The unabsorbed one was eluted by the buffer without salts while the absorbed ones came through the column after the elution with the salt-containing buffer. All fractions consisting of peptides or proteins were pooled according to the peaks of chromatogram. Each pool was then tested for the antimicrobial activity. The inhibitory activity corresponding to the third peak eluted

by 10 mM Tris-HCl buffer (pH 7.4) with 0.5 M NaCl on the chromatogram was shown in Figure 16 and Table 7. The data showed that, The partial purified protein at concentrations of 30 µg/disc inhibited the growth of *T. mentagrophytes*, *T. rubrum*, *C. albicans* and *C. tropicalis*, but the antifungal proteins had low activity against *A. niger*. When comparing to the standard antibiotic, ketoconazole at 20 µg/disc, ketoconazole has a greater antifungal activity than the purified proteins. Even though the less amount of ketoconazole was used, it formed a larger zone of inhibition on the plates. However, the antifungal activity of proteins improved after performing anion exchange chromatography because the impurities in the protein sample were removed. According to Table 6, the percent recovery of protein after the purification by anion exchange chromatography was equal to 32.32%. The recovery of proteins at this stage was calculated from the amount of proteins in the fractions collected from the third peak.

The behavior of the partial purified protein on various chromatographic media employed in the isolation procedure varied from other fungal proteins. Like some fungal proteins reported in literature, the protein was adsorbed on the matrix of anion exchange column such as DEAE cellulose. Zheng *et al*., (2010) purified an antibacterial protein from the fruiting bodies of the wild *C. sinopica*, which was adsorbed on DEAE cellulose. A number of reported mushroom proteins also absorbed on anion exchange chromatography, such as antifungal protein from *L. shimeiji* (Lam and Ng, 2001) and antifungal protein from *L. edodes* (Ngai and Ng, 2003).

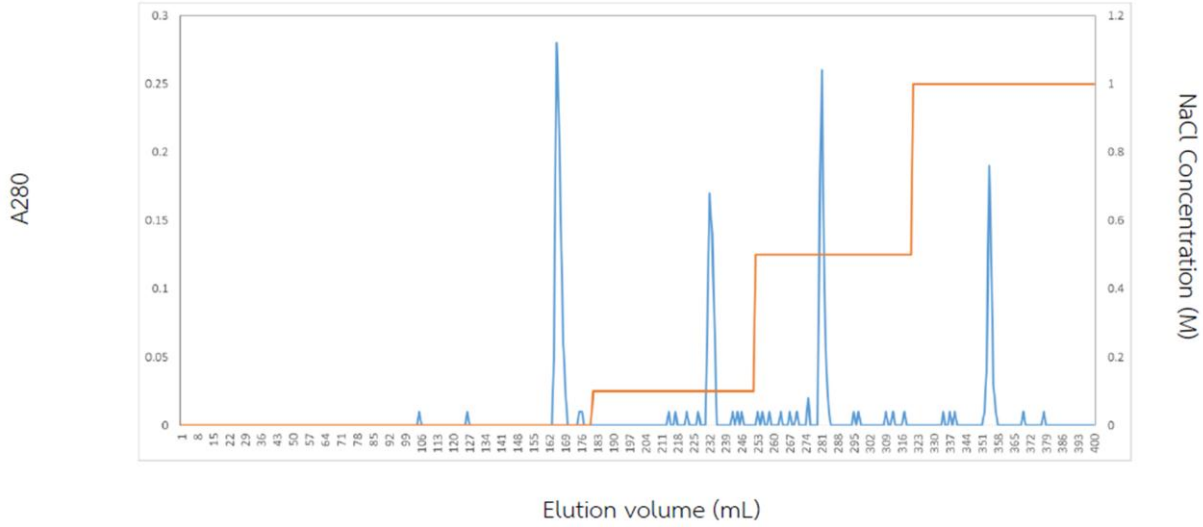

Figure 15 Elution profile of dialyzed 80% w/v ammonium sulfate precipitates using anion exchange chromatography on a DEAE cellulose column. Eluent 1, 10 mM Tris-HCl buffer (pH 7.4); Eluent 2, 0.1 M NaCl in 10 mM Tris-HCl buffer (pH 7.4); Eluent 3, 0.5 M NaCl in 10 mM Tris-HCl buffer (pH 7.4); Eluent 4, 1.0 M NaCl in 10 mM Tris-HCl buffer (pH 7.4); flow rate 0.5 mL/min; detection at 280 nm. The peak of antifungal activity eluted at a salt concentration of 0.5 M NaCl in 10 mM Tris-HCl buffer, pH 7.4. Salt concentration in fractions was shown as a solid line on the graph.

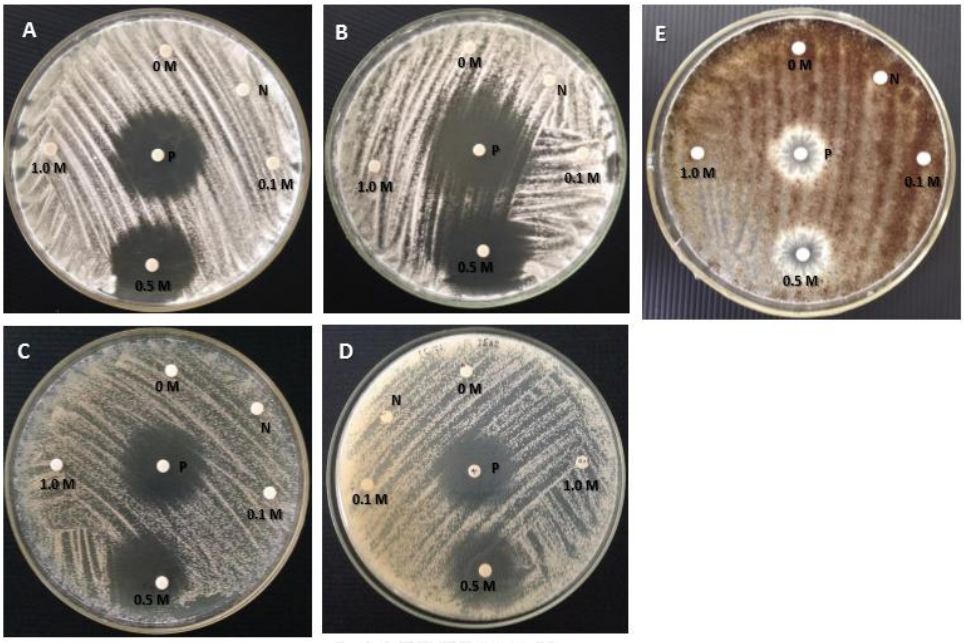

Figure 16 Inhibitory zone of partially purified proteins from DEAE chromatography of *L. squarrosulus* (Mont.), against Yeast and mold. Aliquots (30 µg, 2 mg/mL) of the test partial purified samples were added to the 6 mm paper discs and plates were incubated at 30°C for 24 h (yeast) and 48 h for filamentous fungi. (A) *T. mentagrophytes*, (B) *T. rubrum*, (C) *C. albicans* ATCC 10231, (D) *C. tropicalis*, (E) *A. niger*,. Samples (partially purified protein) are eluted with differences increase NaCl concentration (0 - 1.0 M); Negative control, (N) are 10 mM Tris-HCl buffer, pH 7.4 and Positive control (P) are Ketoconazole 20 µg/disc.

Table 7 Diameter (mm) of antimicrobial activity zone of eluted fraction 0.5 M NaCl (DEAE column) from *L. squarrosulus* (Mont.). The zones were observed on soft agar plate after incubation of yeasts and filamentous fungi at 30°C for 24 h and 48 h, respectively. Results were expressed as mean ± S.E.M (n = 3), each performed in triplicate.

	Samples				
Microbes	Ketoconazole*; 20 µg	0 M NaCl; 30µg	0.1 M NaCl; 30µg	0.5 M NaCl; 30µg	1.0 M NaCl; 30 µg
T. mentagrophytes	23.9±0.24	0	0	18.11±0.65	0
T. rubrum	24.42±0.1	0	0	17.28±0.47	0
C. tropicalis	21.71±0.12	0	0	18.62±0.5	0
C. albicans	22.48±0.21	0	0	18.39±0.63	0
A. niger	10.13±0.34	0	0	10.81±0.92	0

*Ketoconazole is a positive control (20 µg/disc)

10 mM Tris-HCl buffer, pH 7.4 are Negative control

4.4 Purification of the partial purified antifungal protein by gel filtration chromatography

To further purify the antifungal proteins, size-exclusion chromatography was used for this purpose. This technique is normally utilized to examine the apparent molecular mass distribution of peptide or protein mixture. The protein-containing fractions will be collected in order to perform bioactivity assays.

Sephadex G-25 gel filtration column was selected for the purification of the active antimicrobial activity protein. The samples were loaded onto the top of the column. Sephadex G-25 column was previously equilibrated with 10 mM Tris-HCl, pH 7.4 and then the proteins were eluted with the same buffer. The protein-containing fractions were collected and measured the absorbance at 280 nm. According to the elution profile of the proteins by the gel filtration, seven peaks were obtained by gel filtration. The proteins showing antifungal activity were derived from the peak at a volume of 50 to 58 mL or peak F4 (Figure 17 and Table 8). This peak was collected and represented purified protein. The recovery of purified protein after this purification step is equal to 6.34 % of total proteins. This amount of purified protein could be obtained from 1,500 g of fresh mushroom (Table 6). Furthermore, the antifungal activity test of the purified protein obtained from Sephadex G-25 column showed inhibitory activity against *T. mentagrophytes*, *T. rubrum*, *C. albicans* and *C. tropicalis*, but this protein has low activity against *A. niger* at concentrations of 30 µg/disc. The inhibition zones of proteins were compared to those of standard antibiotic, ketoconazole at the amount of 20 µg/disc (Figure 18).

The protocol found to be satisfactory for the purification of antifungal protein from *L. squarrosulus* (Mont.) was gel filtration chromatography. The protein was purified after the purification by ion exchange chromatography using Sephadex G-25 column. It is remarkable that only two chromatographic types are required for the purification of this protein (Wang and Ng, 2006 and Wang *et al*., 2004 and Zheng *et al*., 2010). To our knowledge, there are reports of a two-step chromatographic protocol to purify a fungal protein.

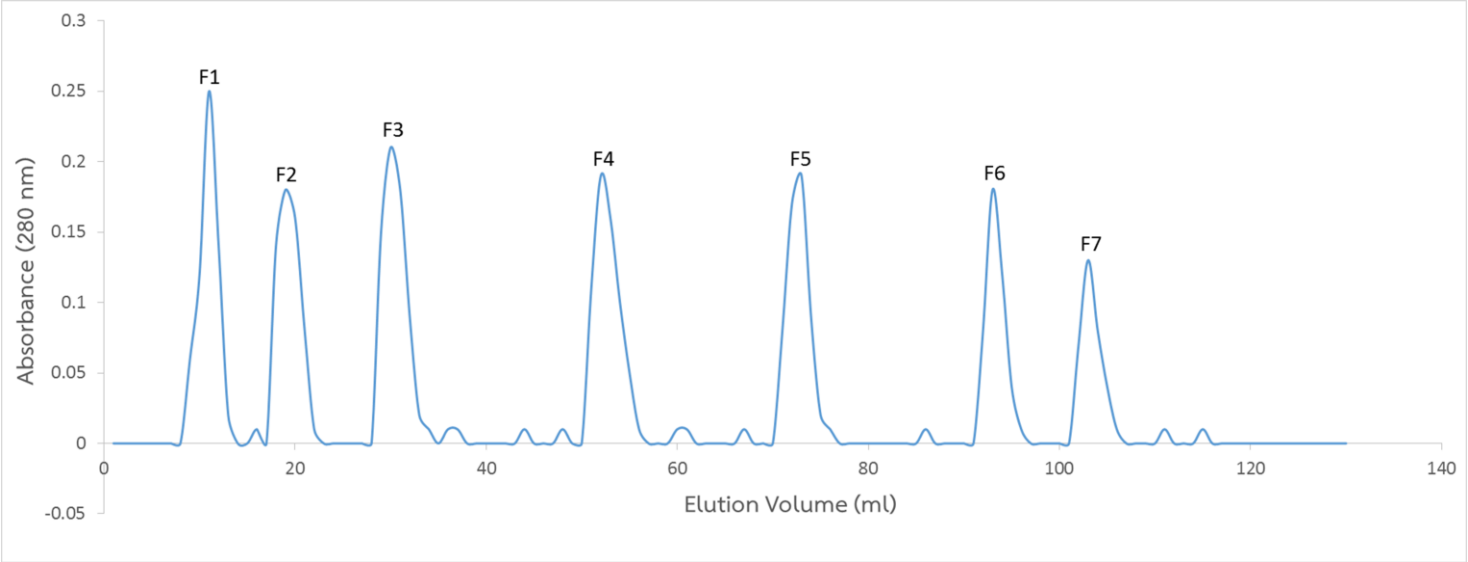

Figure 17 Chromatogram of purified protein from the fraction eluted with 0.5 M NaCl (DEAE cellulose column). The proteins were separated by gel filtration chromatography using a Sephadex G-25 column. The peak of protein having antifungal activity was eluted at 50 to 58 mL (Peak F4) after the sample was loaded to the column. The amount of protein in the collecting fractions was monitored spectrophotometrically at 280 nm. The fractions of defined volumes were collected at flow rate 0.5 mL/min using 10 mM Tris-HCl buffer, pH 7.4 as a running buffer.

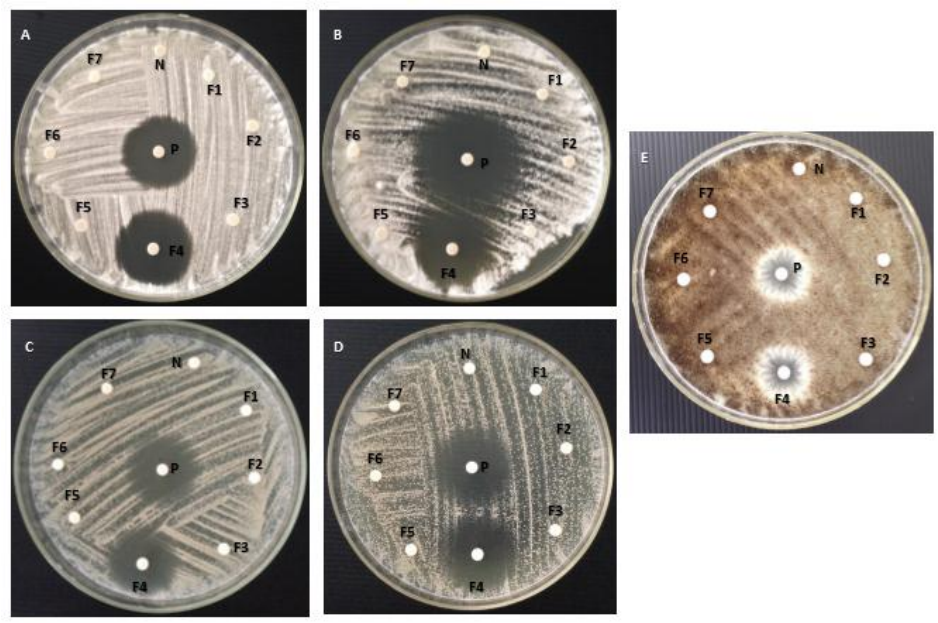

Figure 18 Zones of inhibition showing the antifungal activity of purified proteins from *L. squarrosulus* (Mont.) by Sephadex G-25 column. The antifungal activity of protein in peak No.4 (F4) was shown against Yeast and mold. Aliquots (30 µg, 2 mg/mL) of purified samples were added to the 6 mm paper discs and then the disc were placed onto plates. The plates were incubated at 30°C for 24 h and 48 h for yeast and filamentous fungi, respectively. (A) *T. mentagrophytes*, (B) *T. rubrum*, (C) *C. albicans* ATCC 10231, (D), *C. tropicalis*, (E) *A. niger*,. The purified protein sample was eluted with 10 mM Tris-HCl buffer, pH 7.4; Negative control, (N) is 10 mM Tris-HCl buffer, pH 7.4 and Positive control (P) is a standard antibiotic Ketoconazole at the concentration of 20 µg/disc.

Table 8 Diameter (mm) of antimicrobial activity zone of purified protein (Sephadex G-25 column) from *L. squarrosulus* (Mont.). The inhibition zones were measured on the soft agar plate after incubation at 30ºC for 24 h and 48 h for yeasts and filamentous fungi, respectively. Results were expressed as mean ± S.E.M (n = 3), each performed in triplicate.

Microbes	Samples							
	F1	F2	F3	F4	F5	F6	F7	Ketoconazole*; 20 µg
T. mentagrophytes	0	0	0	25.7±0.36	0	0	0	25.84±0.27
T. rubrum	0	0	0	22.81±0.43	0	0	0	25.88±0.51
C. tropicalis	0	0	0	20.54±0.35	0	0	0	22.8±0.21
C. albicans	0	0	0	20.62±0.82	0	0	0	22.37±0.35
A. niger	0	0	0	12.64±0.5	0	0	0	14.17±0.8

*Ketoconazole is a positive control (20 µg/disc)

10 mM Tris-HCl buffer, pH 7.4 are Negative control

4.5 Characterization of the purified antifungal protein from *L. squarrosulus* (Mont.)

The purified protein with known antifungal activity from mushroom was further characterized as follows:

4.5.1 Biological properties of the purified antifungal protein from mushroom

- **Stability of biological activity after exposure to proteolysis enzyme**

Purified protein with antifungal property from mushroom were further investigated for their stability in antimicrobial activity against *T. mentagrophytes*, *T. rubrum*, *C. albicans*, *C. tropicalis* and *A. niger* after exposure to protease digestion. After treating the purified protein with proteolytic enzymes i.e., trypsin, the protein became inactive providing the evidence that proteolytic enzymes have degraded the peptides leading to the loss of antimicrobial activity as shown in Table 9 and Figure 19.

The antifungal activity of purified protein from *L. squarrosulus* (Mont.) was lost in the presence of protease, whereas other antifungal proteins were stable and active in this condition (Leung and Alizadeh, 2011, Tan *et al*., 2013). However, the procedure used in this experiment including inactivation of enzyme at the end of reaction by heating at 80^0C for __ minutes. So, the lost of activity of purified protein may be due to the heat. Careful design of the experiment by including the prospered control will be needed before conclusion can be done.

Table 9 Antimicrobial activity of purified antifungal protein detected after protease enzyme treatment, expressed as diameter (mm) of antimicrobial activity zone and result were expressed as mean ± S.E.M (n = 3), each performed in triplicate.

Microbials	Samples	
	Ketoconazole 20 µg*	Antifungal protein 30 µg
T. mentagrophytes	24.96 ± 0.82	0
T. rubrum	23.63 ± 0.67	0
C. tropicalis	20.52 ± 0.94	0
C. albicans	22.63 ± 0.71	0
A. niger	12.41 ± 0.48	0

*Ketoconazole is a positive control (20 µg /disc)

10 mM Tris-HCl buffer, pH 7.4 and 0.85% Normal saline solution are negative control.

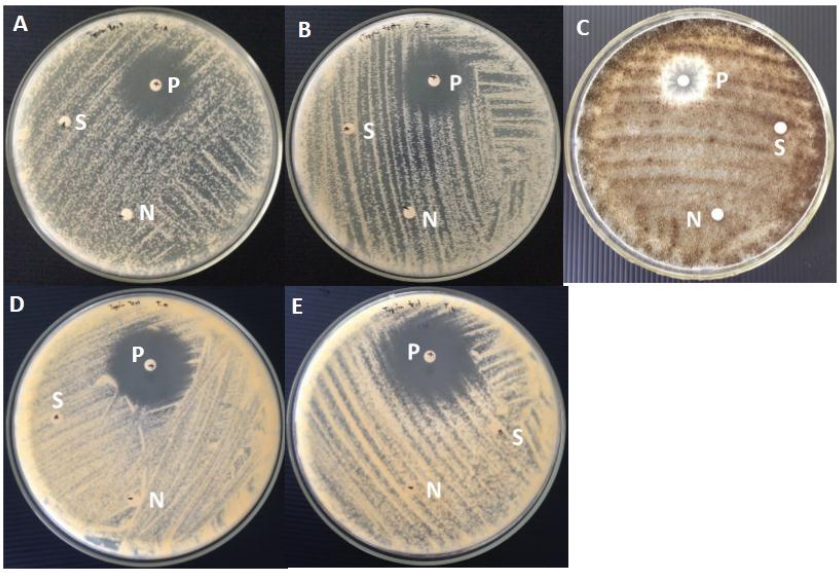

Figure 19 The figure shown antifungal activity of purified antifungal protein detected after protease enzyme treatment. Aliquots (30 µg) of purified protein were added to the 6 mm paper discs and incubated at 30°C for 24 h (yeasts) and 48 h for filamentous fungi. (A) *C. albicans*, (B) *C. tropicalis*, (C) *A. niger*, (D) *T. mentagrophytes* and (E) *T. rubrum*. Samples purified protein preparation with 10 mM Tris-HCl buffer, pH 7.4; Negative control, (N) are 10 mM Tris-HCl buffer, pH 7.4 and Positive control (P) are Ketoconazole 20 µg/disc.

- **pH stability**

The effect of various pH treatment on the antifungal activity of purified protein was shown in Table 10, Figure 20 and 21. After incubation in buffer between pH 2-10 for 24 hr, the highest activity against all microbes tested was observed after exposure to pH 7. When the pH is lesser than or more than 7, the antifungal activity of protein decrease dramatically. The protein still have activity until pH 9 and at pH 10, the protein completely lost activity. At pH 3, the activity of protein against *T. mentagrophytes* and *T. rubrum* was absolutely lost, whereas the present of activity against *C. albicans, C. tropicalis* was still detected. The purified protein completely lost activity against yeast at pH 2. In most cases, the activity of purified protein can be maintained between pH 6.0 to 8.0 and becomes inactive at below pH 5.0 and above pH 8.0 i.e. at more acidic and more basic ranges (Figure 20 and 21). With a change in pH, the net charge of the purified protein may be changed, depending on the pK_a of the functional groups involved, thus affecting binding to the outer cell wall, the initial step for the efficacy of the protein.

Table 10 Antimicrobial activity of purified antifungal protein detected after treatment with difference pH conditions. The activity was measured as diameter (mm) of antimicrobial activity zone and the results were expressed as mean ± S.E.M (n = 3), each performed in triplicate.

Fungi	Ketoconazole: 20 µg*	Condition (pH); Zone of inhibition (mm)								
		2: 30 µg	3: 30 µg	4: 30 µg	5: 30 µg	6: 30 µg	7: 30 µg	8: 30 µg	9: 30 µg	10: 30 µg
T. mentagrophyte	23.59±0.73	0	0	12.42±0.83	14.79±0.45	17.29±0.84	21.37±0.51	18.62±0.93	10.48±0.64	0
T. rubrum	22.27±0.92	0	0	11.97±0.61	14.17±0.53	18.38±0.96	20.83±0.89	17.57±0.82	10.33±03	0
C. albicans	21.14±0.35	0	10.2±0.14	11.53±0.47	12.21±0.21	17.1±0.7	20.2±0.62	15.83±0.26	9.2±0.17	0
C. tropicalis	21.32±0.63	0	8.52±0.81	12.11±0.36	13.51±0.6	16.24±0.51	20.15±0.3	16.22±0.11	10.41±0.29	0
A. niger	13.42±0.64	0	0	7.58±0.29	8.86±0.72	10.13±0.48	12.26±0.97	10.07±0.24	7.17±0.58	0

*Ketoconazole is a positive control (20 µg/disc) and 10 mM Tris-HCl buffer, pH 7.4 is negative control

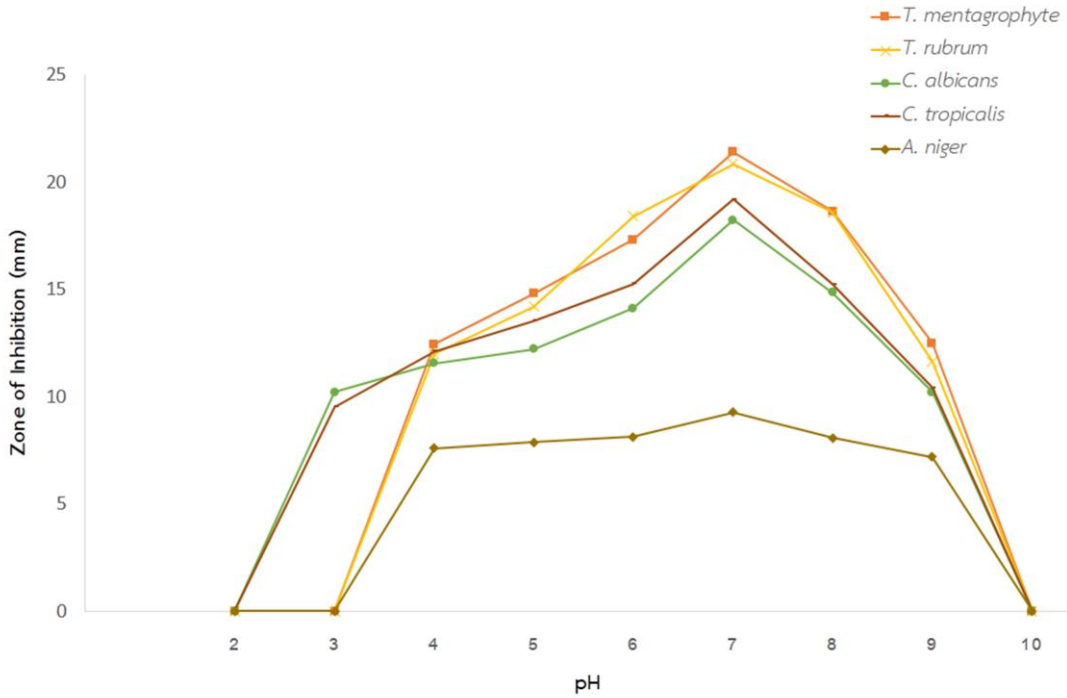

Figure 20 The figure shows antifungal activity of purified antifungal protein detected after different pH treatment (graph). Aliquots (30 µg) of purified protein after various pH treatment were added to the 6 mm paper discs and the discs were put onto the agar. The plates were incubated at 30°C for 24 h (yeasts) and 48 h for filamentous fungi.

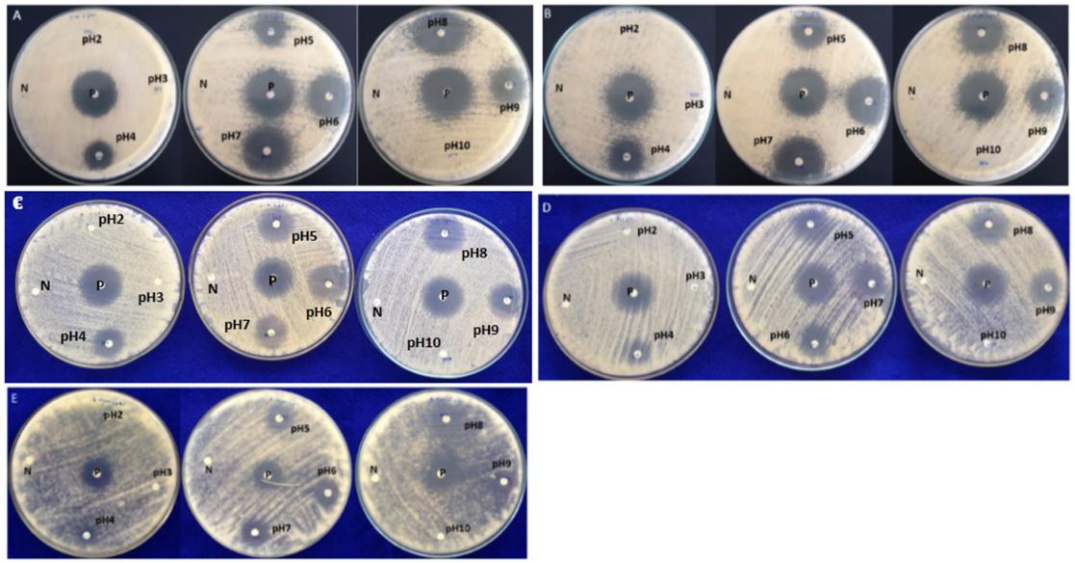

Figure 21 The figure shows antifungal activity of purified antifungal protein detected after differences pH treatment. Aliquots (30 µg) of purified protein after various pH treatment were added to the 6 mm paper discs and the discs were placed onto the agar. The plates were incubated at 30°C for 24 h (yeasts) and 48 h for filamentous fungi. (A) *T. mentagrophytes*, (B) *T. rubrum*, (C) *C. albicans*, (D) *C. tropicalis* and (E) *A. niger*. Negative control, (N) is 10 mM Tris-HCl buffer, pH 7.4 and Positive control (P) is Ketoconazole 20 µg/disc.

- **Temperature stability**

The effect of various temperatures on the antimicrobial activity of purified protein were checked against *T. mentagrophytes, T. rubrum, C. albicans, C. tropicalis* and *A. niger.* The peptides behaved differently at different temperatures against various fungi and yeasts. The purified protein with antifungal property was incubated at different temperatures from 20 to 100 ºC for 1 h. The results are compiled and presented in Table 11. Proteins showed the highest activity against .*T mentagrophytes, T. rubrum, C. albicans* and *C. tropicalis* at 20ºC, whereas the proteins incubated at 30°C and 40ºC showed lower activity against all microbes tested and completely lost the activity at 50°C (Figure 22 and 23).

The effect of temperature on the stability of the antifungal protein is presented in Figure 22. The optimum antifungal activities was found at 20°C. However, the results of thermostability showed that antifungal protein was more stable in narrow range of temperatures between 20°C and 40°C (Figure 23). The purified antifungal protein from *L. squarrosulus* (Mont.) did not have similar thermostability comparing with other antifungal peptides or proteins. Baciamin, the AMP from *Bacillus amyloliquefaciens* was preserved its antifungal activity after exposure to temperature range between 0 and 100 °C (Wong *et al*., 2008). PPEBL21, an AMP from *Escherichia coli* was also preserved its antifungal activity after exposure to the temperature range 0 – 80 °C (Yadav *et al*., 2010).

Table 11 Antimicrobial activity of purified antifungal protein detected after treatment with difference temperature conditions The results were expressed as diameter (mm) of antimicrobial activity zone and mean ± S.E.M (n = 3). Each performed in triplicate. *Ketoconazole is a positive control (20 µg/disc), 10 mM Tris-HCl buffer, pH 7.4 and 0.85% Normal saline solution are negative control

Fungi	Ketoconazole: 20 µg*	Condition (Temp,°C); (Clear zone in mm)								
		20	30	40	50	60	70	80	90	100
T. mentagrophyte	24.83±0.74	20.83±0.39	15.23±0.96	12.03±0.35	0	0	0	0	0	0
T. rubrum	23.96±0.88	20.11±0.74	14.369±0.68	11.72±0.73	0	0	0	0	0	0
C. albicans	22.25±0.31	19.87±0.45	15.5±0.2	11.87±0.1	0	0	0	0	0	0
C. tropicalis	21.81±0.29	20.02±0.1	15.14±0.63	10.76±0.43	0	0	0	0	0	0

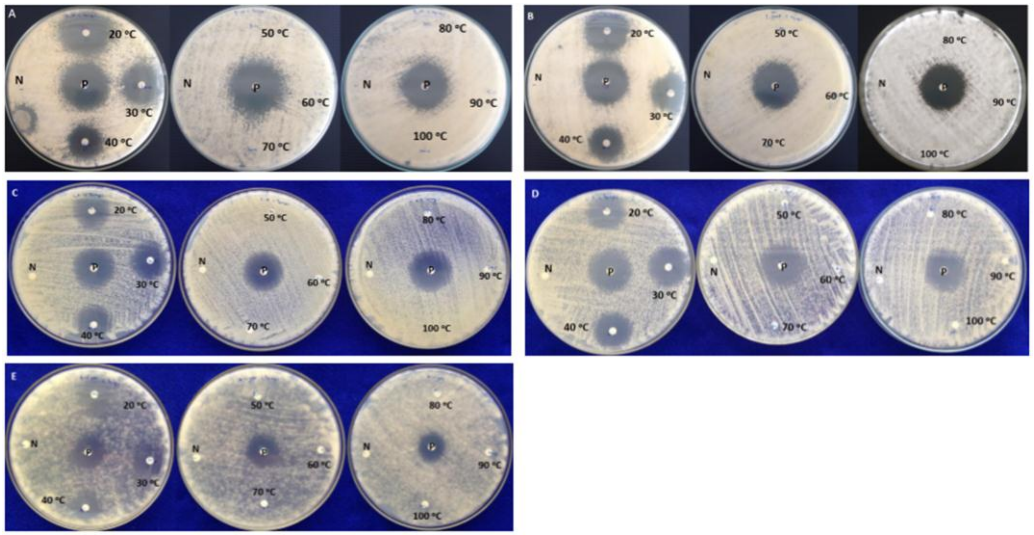

Figure 22 The figure shows antifungal activity of purified antifungal protein detected after various temperature treatment. Aliquots (30 µg) of purified samples after exposure to various temperature for 1 h were added to the 6 mm paper discs and then the disc were placed onto the plates. The plates were incubated at 30°C for 24 h (yeasts) and 48 h for filamentous fungi. (A) *T. mentagrophytes*, (B) *T. rubrum*, (C) *C. albicans*, (D) *C. tropicalis* and (E) *A. niger*. Negative control, (N) is 10 mM Tris-HCl buffer, pH 7.4 and Positive control (P) is Ketoconazole 20 µg/disc.

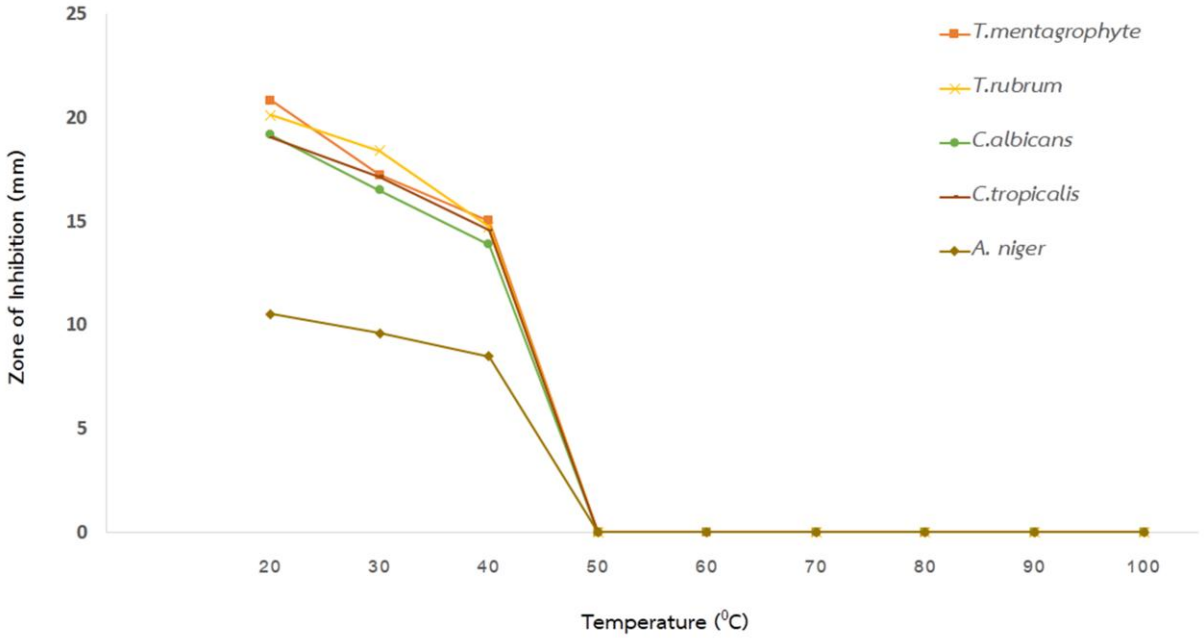

Figure 23 The figure shows antifungal activity of purified antifungal protein observed after temperature treatment (graph). Aliquots (30 µg) of purified protein after treatment with various temperature for 1 h were added to the 6 mm paper discs and then the discs were placed onto the agar. The plates were incubated at 30℃ for 24 h (yeasts) and 48 h for filamentous fungi.

- **DNase activity of purified antifungal protein**

Among antimicrobial peptides, they can be classified as several types, some proteins have antitumor and nuclease activities. To confirm whether antifungal protein from *L. squarrosulus* (Mont.) mushroom was a DNase, a DNase activity assay was performed. The DNase activity of purified antifungal protein was tested by using different types of DNA, genomic DNA and plasmid DNA as the substrates. The DNase activity determination of antifungal proteins on genomic DNA was done in 25 µL reaction containing the following: 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM dithiotheritol, 0.1 mg/mL of BSA, 0.1 M NaCl, 1 µg of DNA samples or 5, 10, 15 and 20 µg of purified protein. The results indicated that the purified antimicrobial protein acted on both DNA substrates.

Our results (Figure 24 and Figure 25) show that the catalytic property of antifungal protein is essentially the described DNase activity. This is in a good agreement with the previous published reports, antifungal protein (AFP) from mold (*Aspergillus giganteus* MDH 18894) (Liu *et al*., 2002) and VpPR-10.1 from *Vitis. Pseudoreticulata* (Xu *et al*., 2014)).

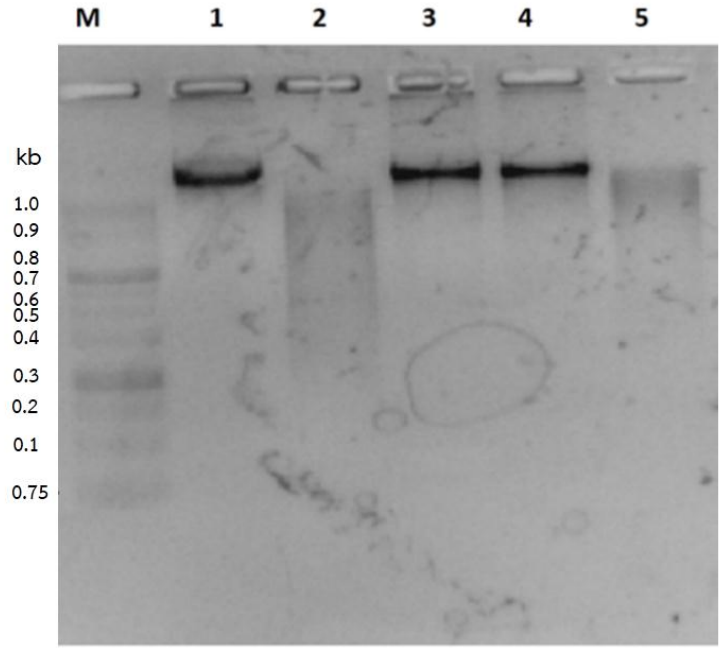

Figure 24 DNase activities of purified antifungal protein from *L. squarrosulus* (Mont.) mushroom on genomic DNA. Lanes 1 to 5 represent 1 μg genomic DNA of *E.coli* (DH5**α**) treated in difference conditions. Lane 1; untreated genomic DNA (negative control;), Lane 2; genomic DNA treated with the restriction enzyme (*Eco*RI) are positive control, lane 3; genomic DNA treated with commercial restriction enzyme buffer of *Eco*RI, lane 4; genomic DNA treated with modify buffer and lane 5; genomic DNA treated with 15 μg of purified antifungal protein. Lane M; 1 kb DNA ladder.

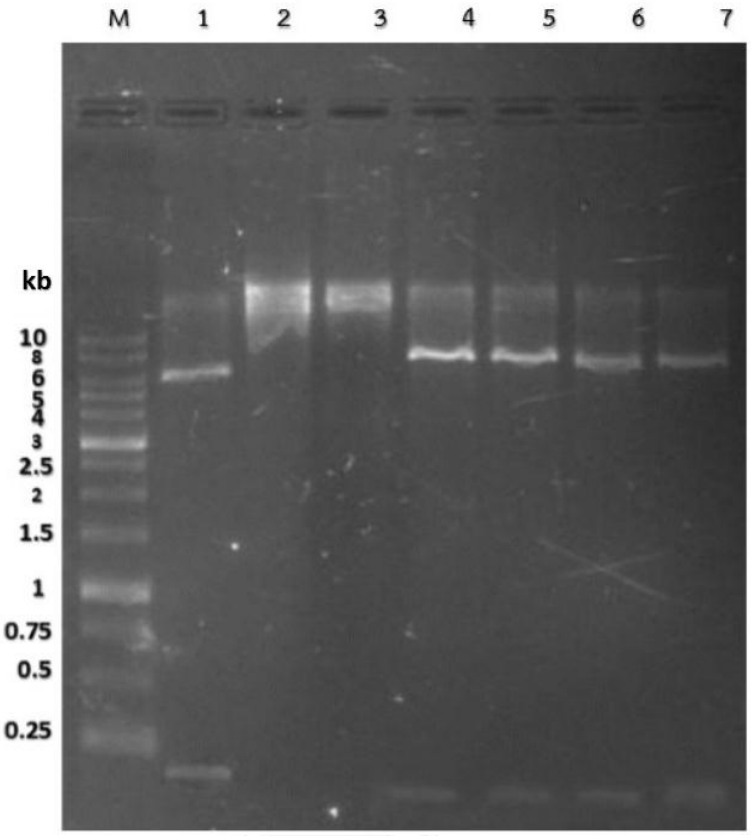

Figure 25 DNase activities of purified antifungal protein from *L. squarrosulus* (Mont.) mushroom on plasmid DNA. Lane M; 1 kb DNA ladder plus. Lane 1; pET28b treated with restriction enzyme *(EcoR*I + *Xbal),* a positive control. Lane 2 and 3; pET28b treated with reaction buffers, negative control. Lane 4 to 7; pET28b treated with 5, 10, 15 and 20 µg of purified protein from mushroom.

- **Synergistic activity of antibiotic drug and purified AMPs**

Synergistic activity between antibiotic drug and AMPs has been reported to have higher activity than using only antibiotic drugs or AMPs (Zhou and Peng, 2013). Some reports showed the inhibitory activity against pathogenic microorganism. It has been reported the synergistic effect on antifungal activity of Hep-20 together with amphotericin B (Tavanti *et al*., 2011). The combination of antibiotics with antimicrobial peptides may represent a promising novel approach to the treatment of *C. difficile* infections (Nuding *et al*., 2014). In the present study, in order to observe the synergistic activity of antifungal protein with antibiotics, five strains of bacteria including *E. coli* ATCC 35218, *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, *P. aeruginosa* ATCC 15442 and *K. pneumoniae* ATTC 4352 were tested. The results of synergistic activity were shown in Table 12 and Figure 26 in case of Chloramphenicol, Table 13 and Figure 27 in case of Ciprofloxacin, Table 14 and Figure 28 in case of Tetracycline. The findings did not exhibit the synergism of combined Chloramphenicol and purified antifungal protein; combined Ciprofloxacin and purified antifungal protein, combined Tetracycline and purified antifungal protein against tested bacteria. It is concluded that synergistic activity of all three test antibiotics with antifungal protein was not found against bacterial cells. The diameter of inhibitory zone from this test was compared to the standard following Current Concepts in Laboratory Testing to Guide Antimicrobial Therapy (Jenkins and Schuetz, 2012 and National committee for Clinical Laboratory Standards, 2007).

Table 12 Antimicrobial activity of 30 µg of purified antifungal protein in combination with various amount of antibiotic drug (Chloramphenicol: CP). Results were expressed as diameter (mm) of antimicrobial activity zone and mean ± S.E.M (n = 5). Each performed in triplicate.

Microbes	Zone of inhibition: mm							
	CP;30 µg * (alone)	CP;25 µg (alone)	CP; 25 µg+30 µg of protein	CP; 20 µg (alone)	CP; 20 µg+30 µg of protein	CP; 10 µg (alone)	CP; 10 µg+30 µg of protein	Negative
E. coli	18.25+0.53	16.11+0.75	15.58±0.31	12.62+0.81	11.36±0.54	9.83+0.70	10.51±0.76	0
S. aureus	18.67+0.53	17.54+0.83	17.31±0.52	16.36+0.78	16.52±0.48	13.54+0.85	13.77±0.64	0
K. pneumoniae	24.83+0.62	22.91+0.54	22.86±0.72	19.44+0.63	20.07±0.61	18.67+0.58	18.28±0.71	0
P. aeruginosa	21.41+0.58	18.93+0.66	18.76±0.43	18.75+0.88	18.17±0.35	15.03+0.69	14.35±0.59	0
B. subtilis	19.85+0.47	18.82+0.91	19.85±0.39	18.05+0.67	17.41±0.72	16.17+0.32	15.64±0.83	0

*CP 30 µg /disc (as Reference) and Zone of inhibition (Mean ± S.E.M)

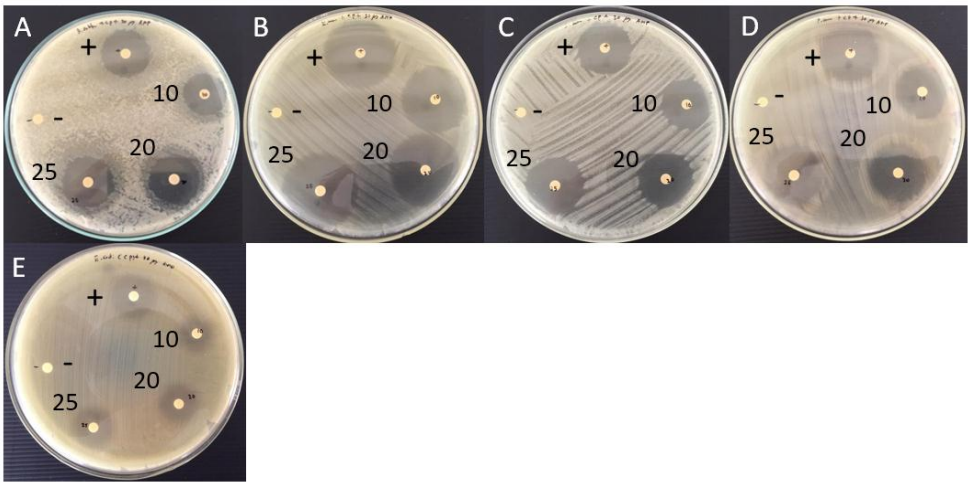

Figure 26 Antimicrobial activity of 30 µg purified antifungal protein in combination with various amount of antibiotic drug (Chloramphenicol: CP). (A) *B. subtilis*, (B) *K. pneumoniae*, (C) *S. aureus*, (D) *P. aeruginosa* and (E) *E. coli*. CP 30 µg /disc is used as reference (+), 10 mM Tris-HCl buffer, pH 7.4 is negative control (-) 10; CP 10 µg /disc + 30 µg of AMPs, 20; CP 20 µg/disc + 30 µg of AMPs and 25; CP 25 µg /disc + 30 µg of AMPs.

Table 13 Antimicrobial activity of 30 µg purified antifungal protein in combination with various amount of antibiotic drug (Ciprofloxacin: Cip), Results were expressed as diameter (mm) of antimicrobial activity zone and mean ± S.E.M (n = 5). Each performed in triplicate.

Microbes	Zone of inhibition: mm							
	Cip; 5 µg * (alone)	Cip; 4 µg (alone)	Cip; 4 µg+30 µg of protein	Cip; 3 µg (alone)	Cip; 3 µg+30 µg of protein	Cip; 2 µg (alone)	Cip; 2 µg+30 µg of protein	Negative
E. coli	24.14±0.41	21.53±0.56	21.75±0.61	13.38±0.91	12.36±0.79	10.74±0.52	10.63±0.74	0
S. aureus	17.75±0.73	15.82±0.78	15.44±0.53	13.61±0.44	14.37±0.31	11.02±0.78	11.67±0.56	0
K. pneumoniae	21.68±0.32	19.47±0.95	19.83±0.75	19.52±0.37	19.48±0.62	18.89±0.61	19.42±0.85	0
P. aeruginosa	18.12±0.95	16.31±0.46	15.38±0.69	13.09±0.62	12.27±0.82	10.92±0.57	11.03±0.71	0
B. subtilis	19.01±0.36	16.55±0.53	16.42±0.53	13.88±0.71	13.61±0.46	10.36±0.94	10.62±0.53	0

*Cip 5 µg /disc (as Reference) and Zone of inhibition (Mean ± S.E.M)

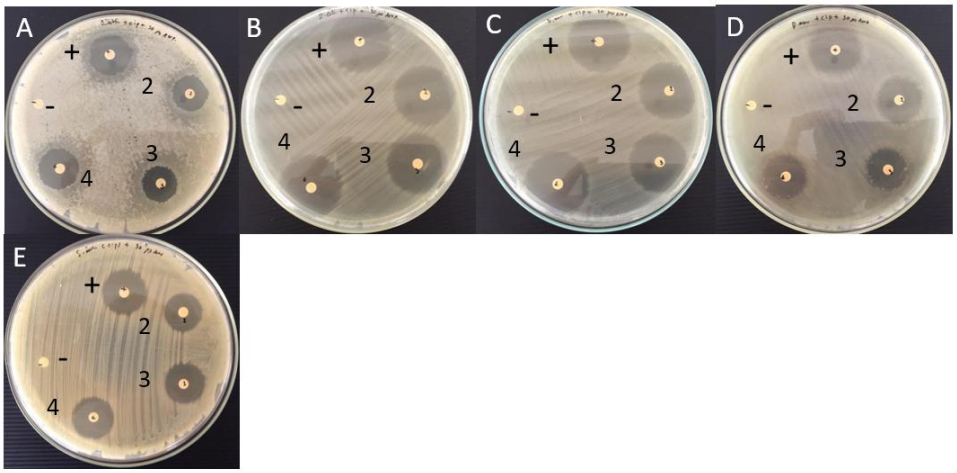

Figure 27 Antimicrobial activity of 30 µg purified antifungal protein in combination with various amount of antibiotic drug (Ciprofloxacin: Cip). (A) *B. subtilis*, (B) *K. pneumoniae*, (C) *S. aureus*, (D) *P. aeruginosa* and (E) *E. coli*. Cip 5 µg /disc is used as reference (+), 10 mM Tris-HCl buffer, pH 7.4 is negative control (-) ,2; Cip 2 µg /disc + 30 µg of AMPs, 3; Cip 3 µg /disc + 30 µg of AMPs and 4; Cip 4 µg/disc + 30 µg of AMPs.

Table 14 Antimicrobial activity of 30 µg purified antifungal protein in combination with various amount of antibiotic drug (Tetracycline: TE). Results were expressed as diameter (mm) of antimicrobial activity zone and mean ± S.E.M (n = 5). Each performed in triplicate.

Microbes	Zone of inhibition: mm							
	TE; 30 µg * (alone)	TE; 25 µg (alone)	TE; 25 µg+30 µg of protein	TE; 20 µg (alone)	TE; 20 µg+30 µg of protein	TE; 10 µg (alone)	TE; 10 µg+30 µg of protein	Negative
E. coli	16.43±0.76	16.95±0.75	15.78±0.61	14.82±0.63	15.48±0.37	11.72±0.39	12.09±0.55	0
S. aureus	22.16±0.84	22.43±0.69	21.63±0.52	20.59±0.42	19.52±0.61	16.31±0.74	15.37+0.46	0
K. pneumoniae	17.53±0.59	15.58±0.81	16.72±0.47	15.09±0.51	14.80±0.42	12.35±0.31	11.86±0.75	0
P. aeruginosa	15.81±0.37	14.22±0.52	13.46±0.65	12.05±0.38	11.41±0.88	11.31±0.87	10.91±0.73	0
B. subtilis	18.72±0.63	16.59±0.66	17.16±0.81	15.7±0.76	14.26±0.43	12.08±0.92	11.35±0.87	0

* TE 30 µg/disc (as Reference) and Zone of inhibition (Mean ± S.E.M)

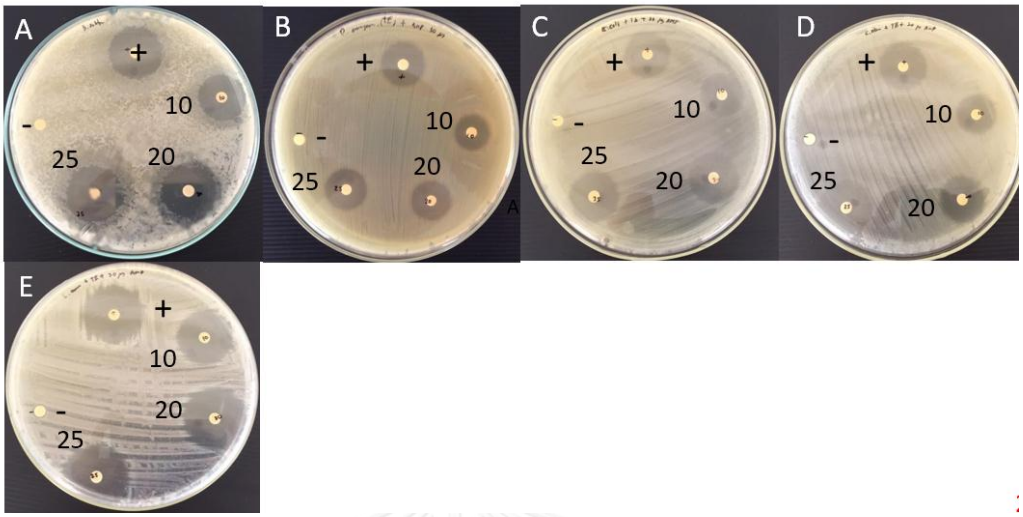

Figure 28 Antimicrobial activity of 30 µg purified antifungal protein in combination with various amount of antibiotic drug (Tetracycline: TE). (A) *B. subtilis*, (B) *K. pneumoniae*, (C) *S. aureus*, (D) *P. aeruginosa* and (E) *E. coli*. TE 30 µg /disc is used as reference (+), 10 mM Tris-HCl buffer, pH 7.4 is negative control (-), 10; TE 10 µg /disc + 30 µg of AMPs, 20; TE 20 µg /disc + 30 µg of AMPs and 25; TE 25 µg /disc + 30 µg of AMPs.

4.5.2. Physical properties of the purified antifungal protein from *L. squarrosulus* (Mont.)

- **Molecular mass**

Polyacrylamide gel electrophoresis in the presence of SDS was used to investigate the molecular mass of purified antifungal protein samples from each step of the purification processes. The purified antifungal protein samples, approximately 30 µg/well was loaded on to the SDS-PAGE (14% Gel). The antifungal protein was purified by anion exchange chromatography on DEAE-cellulose column followed by gel filtration chromatography on a Sephadex G-25 column. After completing purification, the protein bands on the gel were detected and appeared as a single band with a molecular mass of approximately 17 kDa (Figure 29). The molecular mass was estimated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition.

Molecular mass of the purified protein is within the range of other reported antifungal proteins. The purified protein with antibacterial activity from *C. sinopica* is composed of two subunits with the same molecular mass of 22 kDa (Zheng *et al*., 2010). The proteins isolated from *G. lucidum* and *C. militaris* possess molecular mass of 15 and 10.9 kDa, respectively (Wang and Ng, 2006[b]; Wong *et al*., 2010). There are other examples of proteins such as bacisubin (41.9 kDa) (Liu *et al*., 2007), YxjF (fragment) (12.23 kDa), endo-1-4-β-glucanase (46.60 kDa), YnfF (45.39 kDa), BamD (44.94 kDa), putative sensor kinase (53.38 kDa), bacillomycin D synthetase C (309.04 kDa) (NCBI search), and X98III (59.0 kDa) (Xie *et al*., 1998).

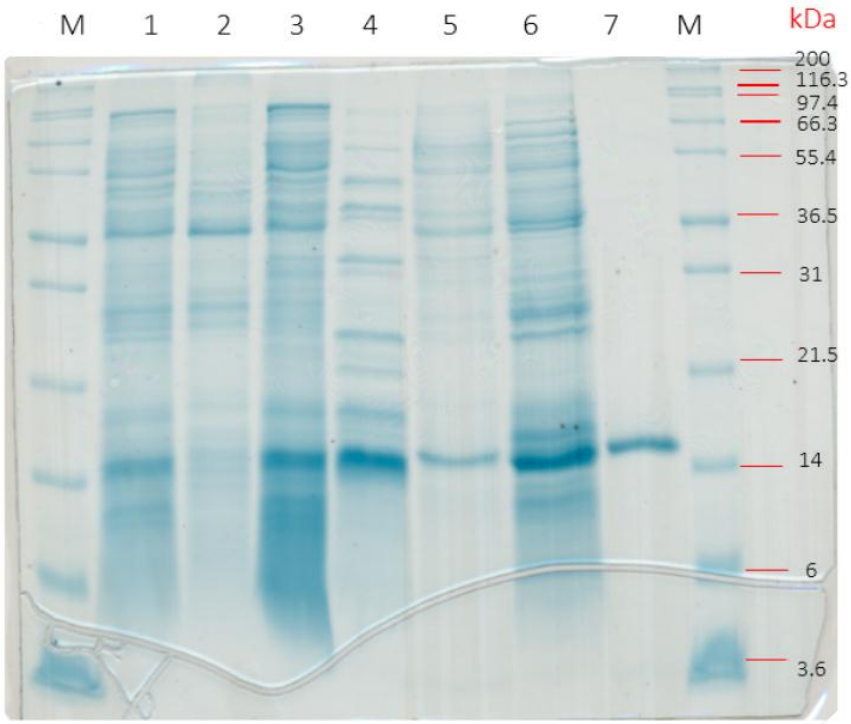

Figure 29 SDS-PAGE analysis (reducing condition) of antifungal protein purified from *L. squarrosulus* (Mont.). Lanes: M) protein markers (Mark12™, invitrogen), 1) Crude protein from fruiting bodies of mushroom before ammonium sulfate precipitatation, 2) Fractions (40% w/v) of partially purified by ammonium sulfate precipitation, 3) Fractions (80% w/v) of partially purified by ammonium sulfate precipitation, 4) EZ-Run™ Protein Marker BP, 5): 0.5M NaCl fraction from DEAE column (10 µg), 6). 0.5M NaCl fraction from DEAE column (30 µg), and 7). Fraction of purified antifungal protein by Sephadex G-25 column (10 µg).

- **Amino acid sequence**

The purified antifungal protein band (17 kDa) from SDS-PAGE (Figure 29) was excised and in-gel digested. The tryptic protein was analyzed by MALDI-TOF Mass Spectrometry and subjected to MS ion search in MASCOT search engine. The tryptic peptide sequences of purified antifungal protein were shown in Table 15. The partial peptide sequences of antifungal protein were then submitted to the protein database (the NCBI Blast database) for similarity searching. The result shown in Figure 30 that the sequences have high similarity with cyclophilin protein from other fungi. The results from the NCBI Blast database indicated that antifungal protein from *L. squarrosulus* (Mont.) has high similarity with conserved sequence of cyclophilin protein of *Dichomitus squalens*, *Ganoderma lucidum* and *Trametes versicolor* (Figure 31).

Table 15 Identification of tryptic peptides of antifungal proteins from *L. squarrosulus* (Mont.) mushroom by MALDI-TOF/MS.

Tryptic peptide	Observed mass	Calculated mass	Amino acid sequence
T1	474.7557	947.4964	KLYDDVVPKT
T2	566.7977	1131.5772	KVESLGSASGTPKA
T3	804.9133	1607.813	RIIPNFMLQGGDFTRG
T4	585.2783	1168.54	KFEDENFQLKH
T5	654.3208	1306.6306	RELATGQHGFGYKG
T6	616.3013	1845.8785	KSIYGEKFEDENFQLKH

1. gi|597981609 **Mass:** 17449 **Score:** 193 **Matches:** 12(5) **Sequences:** 4(2) **emPAI:** 0.81
cyclophilin [Dichomitus squalens LYAD-421 SS1]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
177	474.7557	947.4969	947.4964	0.49	0	(14)	51	2		K.LYDDVVPK.T
☑ 178	474.7559	947.4972	947.4964	0.87	0	(19)	13	1		K.LYDDVVPK.T
☑ 179	474.7560	947.4974	947.4964	1.01	0	38	0.15	1		K.LYDDVVPK.T
☑ 438	566.7977	1131.5809	1131.5772	3.31	0	37	0.35	1		K.VESLGSASGTPK.A
☑ 737	630.8445	1259.6744	1259.6721	1.82	1	45	0.037	1	U	K.KVESLGSASGTPK.A
☑ 1006	804.9130	1607.8115	1607.8130	-0.94	0	73	7.1e-005	1		R.IIPNFMLQGGDFTR.G
☑ 1007	804.9133	1607.8121	1607.8130	-0.56	0	(58)	0.0024	1		R.IIPNFMLQGGDFTR.G
☑ 1008	804.9142	1607.8138	1607.8130	0.50	0	(36)	0.31	1		R.IIPNFMLQGGDFTR.G
☑ 1015	812.9099	1623.8052	1623.8079	-1.70	0	(45)	0.034	1		R.IIPNFMLQGGDFTR.G + Oxidation (M)
☑ 1016	812.9103	1623.8061	1623.8079	-1.11	0	(30)	1.3	1		R.IIPNFMLQGGDFTR.G + Oxidation (M)
1017	812.9111	1623.8077	1623.8079	-0.13	0	(13)	66	2		R.IIPNFMLQGGDFTR.G + Oxidation (M)
☑ 1018	812.9117	1623.8088	1623.8079	0.55	0	(43)	0.057	1		R.IIPNFMLQGGDFTR.G + Oxidation (M)

2. gi|564733385 **Mass:** 17596 **Score:** 162 **Matches:** 12(4) **Sequences:** 5(1) **emPAI:** 0.48
cyclophilin [Ganoderma lucidum]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
438	566.7977	1131.5809	1131.5772	3.31	0	37	0.35	1		K.VESLGSASGTPK.A
☑ 504	585.2783	1168.5420	1168.5400	1.65	0	21	7.8	1		K.FEDENFQLK.H
795	654.3208	1306.6270	1306.6306	-2.72	0	16	28	3		R.ELATGQHGFGYK.G
1006	804.9130	1607.8115	1607.8130	-0.94	0	73	7.1e-005	1		R.IIPNFMLQGGDFTR.G
1007	804.9133	1607.8121	1607.8130	-0.56	0	(58)	0.0024	1		R.IIPNFMLQGGDFTR.G
1008	804.9142	1607.8138	1607.8130	0.50	0	(36)	0.31	1		R.IIPNFMLQGGDFTR.G
1015	812.9099	1623.8052	1623.8079	-1.70	0	(45)	0.034	1		R.IIPNFMLQGGDFTR.G + Oxidation (M)
1016	812.9103	1623.8061	1623.8079	-1.11	0	(30)	1.3	1		R.IIPNFMLQGGDFTR.G + Oxidation (M)
1017	812.9111	1623.8077	1623.8079	-0.13	0	(13)	66	2		R.IIPNFMLQGGDFTR.G + Oxidation (M)
1018	812.9117	1623.8088	1623.8079	0.55	0	(43)	0.057	1		R.IIPNFMLQGGDFTR.G + Oxidation (M)
☑ 1074	616.3007	1845.8801	1845.8785	0.91	1	(11)	1.2e+002	1		K.SIYGEKFEDENFQLK.H
☑ 1075	616.3013	1845.8820	1845.8785	1.91	1	17	29	1		K.SIYGEKFEDENFQLK.H

3. gi|636619459 **Mass:** 17501 **Score:** 148 **Matches:** 13(4) **Sequences:** 4(1) **emPAI:** 0.48
cyclophilin [Trametes versicolor FP-101664 SS1]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
177	474.7557	947.4969	947.4964	0.49	0	(14)	51	2		K.LYDDVVPK.T
178	474.7559	947.4972	947.4964	0.87	0	(19)	13	1		K.LYDDVVPK.T
179	474.7560	947.4974	947.4964	1.01	0	38	0.15	1		K.LYDDVVPK.T
504	585.2783	1168.5420	1168.5400	1.65	0	21	7.8	1		K.FEDENFQLK.H
1006	804.9130	1607.8115	1607.8130	-0.94	0	73	7.1e-005	1		R.IIPNFMLQGGDFTR.G
1007	804.9133	1607.8121	1607.8130	-0.56	0	(58)	0.0024	1		R.IIPNFMLQGGDFTR.G
1008	804.9142	1607.8138	1607.8130	0.50	0	(36)	0.31	1		R.IIPNFMLQGGDFTR.G

Figure 30 NCBI Blast database result. The result shows the peptide sequences of antifungal protein from *L. squarrosulus* (Mont.) matching with cyclophilin protein.

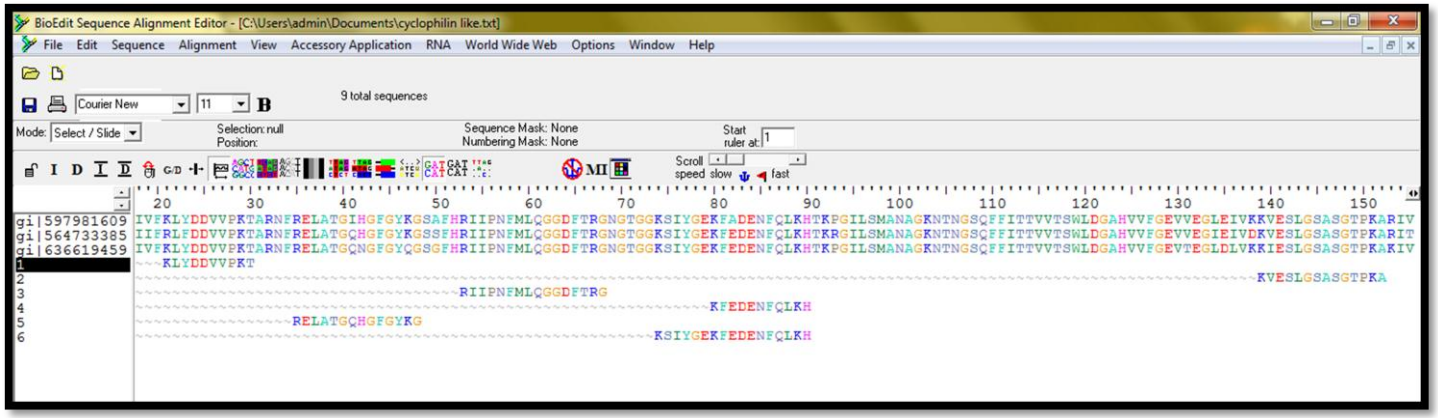

Figure 31 Amino acid sequences comparison between antifungal protein from *L. squarrosulus* (Mont.); peptide fragments number 1-6 and cyclophilin protein of *D. squalens* (gi597981609), *G. lucidum* (gi564733385) and *T. versicolor* (gi636619459) from NCBI database.

Antifungal protein has been isolated and purified from fruiting bodies of *L. squarrosulus* (Mont.), the edible mushrooms. The result of amino acid sequence of this antifungal protein showed high homology to cyclophilins protein from *D. squalens, G. lucidum* and *T. versicolor*. Molecular characterization of cyclophilin-like proteins from Mungbean (Ye and Ng, 2000), black-eyed pea (Ye and Ng, 2001), and Chickpea (Ye and Ng, 2002) were reported to have inhibitory effect on the growth of fungi including *Rhizoctonia solani*, *Mycosphaerella arachidicola* and *Botrytis cinerea*. The cyclophilin-like antifungal proteins of previous reports have a molecular mass of approximately 14 - 18 kDa which is comparable to antifungal protein from this study.

CHAPTER V
CONCLUSION

In the present study, isolation, purification and characterization of protein having antimicrobial activity were reported for the first time from the *L squarrosulus* (Mont.) mushroom in Thailand. The protein was purified using chromatographic techniques similar to other mushroom proteins reported earlier. However, the strategies used in this study followed the different extraction and isolation methods. The mushroom specimens (fruiting bodies): were purchased from the local market in Bangkok, Thailand. The specimens were then identified using their morphological characteristics. The collected fruiting bodies were separated into parts of fruiting body: cap and stalk, as well as whole fruiting. The extraction protocol was a modified method of the commonly used ammonium sulfate precipitation.

Crude antifungal proteins were extracted from fresh fruiting body by homogenization with Tris-HCl buffer (10 mM Tris-HCl, pH 7.4) and ammonium sulfate precipitation are 40 and 80 % w/v. The crude protein extracts were then tested for their antimicrobial activities in order to select protein samples for further purification and characterization. Regarding the antimicrobial activity test using agar disc diffusion method, the whole fruiting body extracts of *L squarrosulus* (Mont.) mushroom by ammonium sulfate precipitation (80% w/v) inhibited the growth of fungal pathogen of human (*T. mentagrophytes, T. rubrum, C. albicans* ATCC 10231, *C. tropicalis*), whereas the activity against *A. niger* is low. Furthermore the synergistic study between antifungal proteins and some antibiotics indicated that antifungal proteins did not potentially enhance the protein has no activity against gram positive and gram negative bacterial the antibacterial activity of antibiotic.

The purification of mushroom antimicrobial peptide/protein was achieved in three steps: 80% ammonium sulfate precipitation, anion exchange chromatography using DEAE-cellulose column and gel filtration using Sephadex G-25 fine column. Each antifungal protein has different behavior on various chromatographic media employed

in the isolation procedure. The protein was adsorbed onto anion exchange chromatography, DEAE-cellulose, like some of purified antimicrobial proteins previously reported in literature.

The partial purified antifungal peptide/protein collecting from anion exchange column using the elution buffer (0.5 M NaCl in 10 mM Tris-HCl buffer, pH 7.4) showed the antifungal activity. After further purification by Sephadex G-25 gel filtration, the active protein was eluted in the fourth peak. The activity of this purified protein against the tested fungal pathogen of human was more pronounced. Moreover, the potency was found to be comparable with Ketoconazole, the commercial antifungal drug. The purified antifungal protein had the molecular mass of approximately 17 kDa characterized by SDS-PAGE under reducing condition. Amino acid sequence of the purified antifungal protein showed the similarity to the conserved sequences of cyclophilin proteins from other fungi; *D. squalens* (gi597981609), *G. lucidum* (gi564733385) and *T. versicolor* (gi636619459). However, peptide mass fingerprint analysis of the peptide in this study yielded different fragments and the bioinformatics analysis of one of the largest peptide fragment sequence. The results of SDS–PAGE, MALDI-TOF mass spectrometry, and amino acid sequencing confirmed that the acquired protein was highly purified.

The nucleases activity of purified antifungal protein was tested by using different DNA substrates. The purified antifungal protein digested all genomic DNA and plasmid DNA in comparison with the standard restriction enzymes. The DNase activity of purified antifungal protein was similar to some peptide/protein having nuclease activity from other microorganisms.

The combination studies on synergies between antibiotic drugs (ciprofloxacin, chloramphenicol and tetracycline) and purified antifungal protein indicated that antifungal protein did not potentially enhance the antimicrobial activity of antibiotic.

Regarding the biological activity of purified antifungal protein, the heat, pH and protease stability was investigated. The protein was not stable in the proteolytic enzyme condition. Significant decrease in antimicrobial activity was observed when the peptide/protein was treated with trypsin. The results from the heat stability assay of the antimicrobial peptide showed that the antimicrobial activity was retained as high

as 40℃. There was sharp decrease in activity when temperature increased. The peptides showed biological activity profile between pH 4.0 - 9.0 however the activity sharply reduced to almost undetectable above pH 9.0 and below pH 4.0. Clearly, there was the complete loss of antimicrobial activity observed in the presence of proteases, the pH above or below the range of 4.0 - 9.0 and the temperature above 40 °C.

In conclusion, the results from this study was successfully proved that antifungal proteins can be isolated from *L squarrosulus* (Mont.) mushroom cultivated in Thailand. The antifungal activity of this protein provides us with additional reasons to continue investigating this molecules due to its ability to protect us from pathogenic fungi. Furthermore, it also shows promised DNase activity which will have medical application. Others biological activities will also be useful in future investigations.

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APPENDIX

APPENDIX A

PREPARATION of CHEMICAL SOLUTION AND REAGENTS

Antibiotic drug preparations

Ciprofloxacin (10mg/mL)

Stock Concentration: 10 mg/mL was dissolved in sterile water (10 mg powder in 1 mL sterile water), stored in the dark at -20℃ in aliquots.

Stock solutions in water must be filter sterilized through a 0.22 μm filter. Antibiotics dissolved in ethanol or other solvent need not be sterilized.

Chloramphenicol (10mg/mL)

Stock Concentration: 10 mg/mL was dissolved in 95% Ethanol (10 mg powder in 1 mL Ethanol), stored in the dark at -20℃ in aliquots.

Stock solutions in water must be filter sterilized through a 0.22 μm filter. Antibiotics dissolved in ethanol or other solvent need not be sterilized.

Ketoconazole (10 mg/mL)

Stock concentration: 10 mg/mL was dissolved in 100% dimethyl sulfoxide (DMSO) (10 mg powder in 1 mL DMSO), stored in the dark at -20℃ in aliquots. Ketoconazole must always be wrapped in foil and stored in the dark whether it is in stock solution,

Stock solutions in water must be filter sterilized through a 0.22 μm filter. Antibiotics dissolved in ethanol or other solvent need not be sterilized.

Tetracycline (10 mg/mL)

Stock Concentration: 10 mg/mL was dissolved in 50% Ethanol (e.g. 10 mg powder in 1 mL of 50%Ethanol), stored in the dark at -20℃ in aliquots. Tetracycline must always be wrapped in foil and stored in the dark whether it is in stock solution.

Stock solutions in water must be filter sterilized through a 0.22 μm filter. Antibiotics dissolved in ethanol or other solvent need not be sterilized.

Common reagents and solutions

1 M Tris-HCl buffer, pH 7.4 (1 L)

Solution is composed of

1) Tris-Base 121.1 g
2) Sterilizer distilled water 1,000 mL

Adjust pH 7.4 using concentrated HCl Sterilize by autoclaving for 20 minutes on liquid setting. Store this solution at room temperature. Dilute with sterilizer distilled water before use.

5 M NaCl (500 mL)

Solution is composed of

1) NaCl 146.1 g
2) Distilled water 500 mL

Dissolve 146.1 g NaCl (5 mol*58.44 g/mol) in exactly 880 ml ultrapure water. Bring the volume to 500 mL with distilled water. Sterilize by autoclaving for 20 minutes.

Electrophoresis buffers and solutions

APS solution (10%)

To prepare a 10% (w/v) solution: Dissolve 1 g of ammonium persulfate in 10 mL of ddH_2O and store at 4°C. Ammonium persulfate decays slowly in solution, so replace the stock solution every 2-3 weeks.

Coomassie destaining solution

Coomassie blue destaining solution is composed of:

1) 40% Methanol
2) 10% Glacial acetic acid

To make 1 L destaining solution:

1) Mix 400 mL MeOH with 500 mL of deionized water with stirring.
2) Slowly add 100 mL of glacial acetic acid with stirring.

3) Store destaining solution at room temperature. Destaining solution should not be reused.

Coomassie staining solution

Coomassie blue staining solution is composed of:

1) Coomassie R250	1 g
2) Glacial acetic acid	100 mL
3) Methanol	400 mL
4) Deionized H_2O	500 mL

To make 1 L staining solution:

1) Add 100 mL of glacial acetic acid to 500 mL of Deionized H_2O.

2) Add 400 mL of methanol and mix.

3) Add 1 g of Coomassie R250 dye and mix.

4) Filter to remove particulates (a coffee filter works great for this and is cheap), Store at room temperature in a sealable container.

SDS-PAGE Electrophoresis Running Buffer (10x) (250 mM Tris, 1.92 M glycine, 1% SDS)

Solution is composed of

1) Tris base	30.2 g
2) Glycine	144 g
3) SDS	10 g

To make 1 L solution:

All chemicals were added into Deionized H_2O, No need to adjust pH and adjust total volume to 1L.

SDS (10%)

Solution is composed of

1) SDS 1 g
2) Deionized H_2O 10 mL

To make 10 mL solution

1) Dissolve the SDS in 7 mL of deionized water with gentle stirring and make total volume up to 10 mL with deionized water.
2) Store the solution at room temperature.

Protocol online of preparation of SDS-PAGE:

http://josephroland.com/lab/acryl2.html

TAE, 50X

Solution is composed of

1) Tris Base 242 g
2) Glacial Acetic Acid 57.1 mL
3) 0.5 M EDTA 100 mL

To make 1 L solution

1) Mixed Tris base with stir bar to dissolve in about 600 mL of deionized water.
2) Added the EDTA and Acetic acid, bring final volume to 1 L with deionized water. Store at room temperature

Tris-HCl buffer, 0.5 M pH 6.8

Solution is composed of

1) Tris-Base 6.1 g
2) Deionized water 100 mL

To make 100 mL solution

1) Mix together and then adjust the solution to pH 6.8 with 5N HCl.
2) Make total volume up to 100 mL with deionized water and store at 4°C.

Tris-HCl buffer, 1.5 M pH 8.8

Solution is composed of

1) Tris-Base 18.15 g
2) Deionized water 100 mL

To make 100 mL solution

1) Mix together and then adjust the solution to pH 8.8 with 5N HCl.
2) Make total volume up to 100 mL with deionized water and store at 4°C.

Microbial Culture Medias

LB Agar (Luria Bertani Agar)

Culture media composed of

Tryptone	10.g
Yeast extract	5 g
Sodium chloride	5 g
Agar	10 g

Direction

1. Dissolve 30 g in 1 L of distilled water and adjust the pH to 7.2.
2. Sterilize by autoclaving at 121°C for 15 minutes.

LB Broth (Luria Bertani Broth)

Culture media composed of

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g

Direction

1. Dissolve 20 g of all ingredients in 1 L of distilled water and adjust the pH to 7.2.
2. Sterilize by autoclaving at 121°C for 15 minutes.

Sabouraud Dextrose Agar

Culture media composed of

Enzymatic Digest of Casein	5 g
Enzymatic Digest of Animal Tissue.	5 g
Dextrose	40 g
Agar	15 g

Directions

1. Suspend 65 g of the medium in 1 L of distilled water.

2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121°C for 15 minutes.

APPENDIX B

Mass-spectrometry data analysis

Identified peptides obtained from MALDI-TOF/MS and MASCOT database profile

MATRIX SCIENCE Mascot Search Results

```
User            : JoeG
Email           : joe.gray@ncl.ac.uk
Search title    : HW_AMP.RAW, all scans
Database        : NCBInr 20150617 (67897003 sequences; 24351009107 residues)
Taxonomy        : Other Fungi (4170847 sequences)
Timestamp       : 1 Dec 2015 at 11:15:58 GMT
Protein hits    : gi|597981609 cyclophilin [Dichomitus squalens LYAD-421 SS1]
                  gi|564733385 cyclophilin [Ganoderma lucidum]
                  gi|636619459 cyclophilin [Trametes versicolor FP-101664 SS1]
                  gi|646393807 hypothetical protein JAAARDRAFT_69498 [Jaapia argillacea MUCL 33604]
                  gi|449543842 hypothetical protein CERSUDRAFT_67221 [Ceriporiopsis subvermispora B]
                  gi|691796654 hypothetical protein BN946_scf184989.g46 [Trametes cinnabarina]
                  gi|50422985  DEHA2E17710p [Debaryomyces hansenii CBS767]
                  gi|126131986 hypothetical protein PICST_87511 [Scheffersomyces stipitis CBS 6054]
                  gi|67903236  hypothetical protein AN8605.2 [Aspergillus nidulans FGSC A4]
                  gi|730185157 hypothetical protein EV44_g3158 [Erysiphe necator]
                  gi|320587844 tor pathway phosphatidylinositol 3-kinase [Grosmannia clavigera kw1407]
                  gi|171684547 hypothetical protein [Podospora anserina S mat+]
                  gi|225681018 hypothetical protein PABG_01621 [Paracoccidioides brasiliensis Pb03]
                  gi|24636240  RecName: Full=Aegerolysin Aa-Pri1; Flags: Precursor [Agrocybe aegerita]
                  gi|636610503 glycoside hydrolase [Trametes versicolor FP-101664 SS1]
                  gi|759228345 hypothetical protein PV09_03846 [Verruconis gallopava]
                  gi|628275197 hypothetical protein A1O7_00211 [Cladophialophora yegresii CBS 114405]
                  gi|759319891 hypothetical protein Z519_03304 [Cladophialophora bantiana CBS 173.52]
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Comparison of the amino acid sequences of between Cyclophilin like protein from others source and Cyclophilin protein from *L. squarrosulus* (Mont.).

VITA

Mr. Siwat Poompouang was born on March 26, 1987 in Phichit Province, Thailand. In 2009, he received Bachelor of Science in Biochemistry from Burapha University. After graduation, he continued his Master degree program in Biomedicinal Chemistry at the Faculty of Pharmaceutical Science, Chulalongkorn University.