ฤทธิ์ขจัดอนุมูลอิสระและยับยั้งการเพิ่มจำนวนเซลล์มะเร็งของเพปไทด์จากเห็ดเผาะ *Astraeus hygrometricus*

นางสาวณัฐภัสสร ธัญญาธารินันท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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FREE RADICAL SCAVENGING AND ANTIPROLIFERATIVE ACTIVITIES OF PEPTIDE FROM HYGROSCOPIC EARTHSTAR *Astraeus hygrometricus*

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Thesis Title	FREE RADICAL SCAVENGING AND ANTIPROLIFERATIVE ACTIVITIES OF PEPTIDE FROM HYGROSCOPIC EARTHSTAR *Astraeus hygrometricus*
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ณัฐภัสสร ธัญญาธารินันท์ : ฤทธิ์ขจัดอนุมูลอิสระและยับยั้งการเพิ่มจำนวนเซลล์มะเร็งของเพปไทด์จากเห็ดเผาะ *Astraeus hygrometricus* (FREE RADICAL SCAVENGING AND ANTIPROLIFERATIVE ACTIVITIES OF PEPTIDE FROM HYGROSCOPIC EARTHSTAR *Astraeus hygrometricus*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.อภิชาติ กาญจนทัต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.อรภัค เรี่ยมทอง, 100 หน้า.

เห็ดได้ถูกนำมาใช้เป็นอาหาร และรักษาโรคมานานหลายทศวรรษ สารประกอบต่าง ๆ ที่ได้มาจากเห็ดเหล่านี้มีฤทธิ์ทางชีวภาพที่มีศักยภาพ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ขจัดอนุมูลอิสระของโปรตีนไฮโดรไลเสตที่ได้จากเห็ดเผาะ ในงานวิจัยนี้เตรียมโปรตีนไฮโดรไลเสตจากเห็ดเผาะโดยปฏิกิริยาการย่อยสลายด้วยโปรติเอสจาก จุลินทรีย์ คือ อัลคาเลส ฟลาโวไซม์ และนิวเทรส ปรากฏว่าที่ปฏิกิริยาการย่อยสลายด้วย 1 เปอร์เซ็นต์ (น้ำหนัก ต่อปริมาตร) ของ อัลคาเลส ให้โปรตีนไฮโดรไลเสตที่มีค่า IC_{50} ต่อ DPPH และ ABTS เท่ากับ 13.40±0.48 และ 5.53±0.59 ไมโครกรัมต่อมิลลิลิตรตามลำดับ จากการทำบริสุทธิ์โปรตีนไฮโดรไลเสตด้วยเทคนิคอัลตราฟิลเตรชันผ่านเยื่อกรองขนาด 10, 5 , 3, และ 0.65 กิโลดาลตัน พบว่าโปรตีนไฮโดรไลเสตเมื่อผ่านเยื่อกรองขนาด 0.65 กิโลดาลตัน (F_5) มีค่า IC_{50} ต่อ DPPH และ ABTS เท่ากับ 4.84±0.26 และ 1.49±0.39 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ เมื่อผ่านขั้นตอนการทำให้บริสุทธิ์ด้วยเทคนิคโครมาโตรกราฟีแบบเจลฟิลเตรชันด้วย Superdex 75 เป็นคอลัมน์ พบว่าแฟรกชัน F_{51} มีค่า IC_{50} ต่อ DPPH และ ABTS เท่ากับ 32.47±1.16 และ 15.58±0.73 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ พีค F_{51} ถูกนำไปทดสอบฤทธิ์ป้องกันความเสียหายของดีเอ็นเอโดยใช้ดีเอ็นเอพลาสมิด pKS, pUC19 และ pBR322 ผลการทดลองแสดงให้เห็นว่าดีเอ็นเอที่มีรูปร่างเป็นวงแหวนที่พันเกลียวซ้อนสามารถแปลงเป็นรูปแบบอื่นเมื่อดีเอ็นเอได้รับการป้องกันความเสียหายด้วยพีค F_{51} นอกจากนี้ งานวิจัยมีการศึกษาฤทธิ์การยับยั้งการเพิ่มจำนวนเซลล์มะเร็งจากเพปไทด์ F51 โดยทำการศึกษาในเซลล์มะเร็ง 5 ชนิด คือ เซลล์มะเร็งเต้านม (BT474) เซลล์มะเร็งปอด (Chago-k1) เซลล์มะเร็งกระเพาะอาหาร (KATO-III) เซลล์มะเร็งลำไส้ใหญ่ (SW620) และ เซลล์มะเร็งตับ (Hep-G2) พบว่า เพปไทด์ F51 มีฤทธิ์ยับยั้งการเพิ่มจำนวนเซลล์มะเร็งปอด (Chago-k1) ได้ดีที่สุด นอกจากนี้เทคนิค RP-HPLC ถูกนำมาใช้ในการทำบริสุทธิ์เพปไทด์ F51 พบว่า สามารถแยกย่อยเพปไทด์ F51 ได้ทั้งหมด 5 พีค คือ F51-1, F51-2, F51-3, F51-4 และ F51-5 นำเพปไทด์ที่ได้ไปพิสูจน์เอกลักษณ์ด้วยเทคนิค MS/MS (ESI-Q-TOF) ผลการวิจัยพบสายเพปไทด์ทั้งหมด 5 สาย ได้แก่ F51-1 ประกอบไปด้วยกรดอะมิโน Val-Thr-Thr-His-Lys-Thr-Val-Thr-Lys-His (VTTHKTVTKH) น้ำหนัก 1152 ดาลตัน, F51-2 ประกอบด้วยกรดอะมิโน Met-Pro-His-His-Tyr-Thr-Ile-Phe-Ala-Leu-Gly-Thr-Gln-Ser-Arg-Pro-Ser (MPHHYTIFALGTQSRPS) น้ำหนัก 1942 ดาลตัน, F51-3 ประกอบด้วยกรดอะมิโน Pro-Ser-Arg-Gly-Ser-Glu-Arg-Ala-Arg-Ala (PSRGSERARA) น้ำหนัก 1086 ดาลตัน, F51-4 ประกอบด้วยกรดอะมิโน Thr-Leu-Ser-Asn-Leu-Gly-Leu-Val-Leu-Val-His (TLSNLGLVLVH) น้ำหนัก1165 ดาลตัน และ F51-5 ประกอบด้วย Leu-Thr-Asn-Ser-Pro-Trp-Ala-His-Ala (LTNSPWAHA) น้ำหนัก 996 ดาลตัน ซึ่งเพปไทด์ทั้ง 5 สายมีกรดอะมิโนที่มีฤทธิ์ทางชีวภาพ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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NATPATSORN TUNYATARINUN: FREE RADICAL SCAVENGING AND ANTIPROLIFERATIVE ACTIVITIES OF PEPTIDE FROM HYGROSCOPIC EARTHSTAR *Astraeus hygrometricus*. ADVISOR: ASSOC. PROF. APHICHART KARNCHANATAT, Ph.D., CO-ADVISOR: ASST. PROF. ONRAPAK REAMTONG, Ph.D., 100 pp.

Mushrooms have been used as food and for therapeutic purpose for decades, and various compounds derived from them have potential biological activities. The present study aims to explore the free radical scavenging properties of peptides derived from the edible mushroom *Astraeus hygrometricus*. In this work, peptides from *A. hygrometricus* protein hydrolysates attainable with microbial proteases (Alcalase®, Flavourzyme® and Neutrase®) were prepared and their antioxidant activities were determined. Peptide fractions derived from *A. hygrometricus* hydrolyzed by 1% Alcalase®, showed the highest DPPH and ABTS radical scavenging activities with IC_{50} values of 13.40±0.48 and 5.53±0.59 µg/mL, respectively. Peptide fractions were obtained using molecular weight cut-offs of 10, 5, 3 and 0.65 kDa membranes and their antioxidant properties were further analyzed. Among the fractions, MW < 0.65 kDa (fraction F5) exhibited high levels of free radical scavenging activities towards DPPH and ABTS with IC_{50} values of 4.84±0.26 and 1.49±0.39 µg/mL, respectively. The fraction F51 from Superdex® 75 column presented the highest scavenging activities on DPPH and ABTS radicals with IC_{50} values of 32.47±1.16 and 15.58±0.73 µg/mL, respectively. Furthermore, the fraction F51 could protect hydroxyl radical-induced DNA damage as shown in pKS, pUC19, and pBR322. In addition, fraction F51 was used for determine the antiproliferative activity. The results showed that purified peptide fraction F51 was not cytotoxic on normal and cancer cell lines and the lung cancer cell line (Chago-k1 cell) had the highest antiproliferative activity. Moreover, fraction F51 was used for determine the induction of apoptosis that used flow cytometry and the caspase 3, 8 and 9 activities analysis method. The results indicated that purified peptide fraction F51 could induce the highest apoptotic cells and caspase 3, 8 and 9 activities on Chago-k1 cell at 72 hours. Furthermore, the peptide fraction F51 was purified using RP-HPLC and collected sub-fraction F51-1 to F51-5. The result was found to possess the highest antioxidant activity on fraction F51-5. Last, sub-fraction F51-1 to F51-5 was identify amino acid sequence using MS/MS (ESI-Q-TOF). The results showed that amino acid compositions on peptide sequences. The sequences of purified peptide fractions F51-1 to F51-5 had amino acid sequence Val-Thr-Thr-His-Lys-Thr-Val-Thr-Lys-His (VTTHKTVTKH, 1152 Da), Met-Pro-His-His-Tyr-Thr-Ile-Phe-Ala-Leu-Gly-Thr-Gln-Ser-Arg-Pro-Ser (MPHHYTIFALGTQSRPS, 1942 Da), Pro-Ser-Arg-Gly-Ser-Glu-Arg-Ala-Arg-Ala (PSRGSERARA, 1086 Da), Thr-Leu-Ser-Asn-Leu-Gly-Leu-Val-Leu-Val-His (TLSNLGLVLVH, 1165 Da) and Leu-Thr-Asn-Ser-Pro-Trp-Ala-His-Ala (LTNSPWAHA, 996 Da), respectively. Hence, the purified peptide fraction F51 could be used as natural antioxidant for drug development.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
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LIST OF ABBREVIATION

A, Abs	Absorbance
Amax	Maximum absorbance
ABTS	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
ACN	Acetonitrile
AIF	Apoptosis-inducing factor
APAF-1	Apoptotic protease activating factor 1
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
bp	Base pair
BSA	Bovine serum albumin
CAT	Catalase
$Cl^{\bullet}$	Atomic chlorine radical
CO	Carbon monoxide
CO_2	Carbon dioxide
$CO_3^{-\bullet}$	Carbonate radical
CAD	Caspase activated DNase
CARD	Caspase recruitment domain
Da	Dalton
DD	Death domain
DED	Death effector domain
DFF	DNA fracment factor
DIABLO	The direct inhibitor of apoptosis protein (IAP)-binding protein with low pI
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DRs	Cell surface death receptors
DR4	TRAIL-R1

DR5	TRAIL-R2
ESI-Q-TOF MS	Electrospray ionization quadrupole time of flight mass spectrometry
EQ	Ethoxyquin
et al.	and others
etc.	et cetera
FADD	Fas-associated death domain
FCS	Fetal calf serum
Fe^{2+}	Ferrous
FITC	Fluorescein isothiocyanate
g	Gram
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione-S-transferase
h	Hour
H	Hydrogen
HCl	Hydrochloric acid
HNO_2	Nitrous acid
H_2O	Water
H_2O_2	Hydrogen peroxide
$HOO^{\bullet}$	Hydroperoxyl radical
HPLC	High performance liquid chromatography
IAP	Inhibitor of apoptosis protein
IC_{50}	Median inhibitory concentration, 50% maximum inhibition
i.e.	in other words (it is or that is)
kDa	Kilodalton
kg	Kilogram
L	Liter
$L^{\bullet}$	Lipid radical
LC	Linear DNA

LDL	Low Density Lipoproteins
$LO^{\bullet}$	Lipid alkoxyl radical
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid peroxide
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
MS/MS	Tandem mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MW	Molecular weight
MWCO	Molecular weight cut off
m/z	Mass to charge ratio
N	Normal
NaCl	Sodium chloride
$NAD^{+\bullet}$	Pyridinyl radical
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometer
NO	Nitrogen oxide
$NO^{\bullet}$	Nitric oxide radical
$NO_2^{\bullet}$ and $NO_2^{-\bullet}$	Nitrogen dioxide radical
N_2O_3	Dinitrogen trioxide
NSS	Normal saline
O_2	Oxygen
$O_2^{-\bullet}$	Superoxide radical
$^1O_2$	Singlet oxygen
O_3	Ozone
OC	Open-circular DNA

$OH^{\bullet}$	Hydroxyl radical
$ONOO^{-}$	Peroxynitrite
PARP	Poly-(ADP-ribose)-polymerase
PBS	Phosphate buffered saline solution
PG	Propyl gallate
PI	Propidium iodide
pNA	p-nitroaniline
RCS	Reactive chlorine species
RNS	Reactive nitrogen species
$RO^{\bullet}$	Alkoxyl radical
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high performance liquid chromatography
rpm	Round per minute
SC	Supercoiled DNA
SD	Standard deviation
sec	Second
SMAC	Second mitochondria-derived activator of apoptosis
SOD	Superoxide dismutase
SO_2	Sulfur dioxide
TBHQ	Tertiary butylhydroquinone
TFA	Trifluoroacetic acid
TNF	Tumor necrosis factor
TNF-R1	TNF receptor type I
$TO^{\bullet}$	Alpha-tocopherol radical
TOH	Alpha-tocopherol
TRADD	TRAIL receptor-associated death domain
TRAILs	TNF-related apoptosis-inducing ligands
U	Unit activity
UV	Ultraviolet
V	Volt
w/v	Weight by volume

w/w	Weight by weight
°C	Degree Celsius
µg	Microgram
µL	Microliter
µm	Micrometer or micron
µM	Micromolar
/	Per
%	Percentage
:	Rati

CHAPTER I
INTRODUCTION

The oxidation reaction of biomolecules has been identified as a free radical-mediated process that occurs in all species. Free radicals in the human body can react with low-density lipoproteins (LDL) and also cause tissue damage to transform a normal cell into a cancer cell. In addition, free radicals can be generated from viral or bacteria infection, immunodeficiency diseases, smoke, grilled food and some drugs which cause oxidative stress in our body that can then lead to many diseases such as cancer, stroke, myocardial infarction and diabetes (Chen et al., 2012).

In recent times, the number of studies on antioxidant substances that can reduce strong free radicals has increased. There are many types of antioxidant substance mechanisms such as radical scavenging, singlet oxygen quenching, and metal chelation for inhibiting free radicals and enzymatic reactions which generate free radicals. The sources of antioxidant substances are synthetic antioxidants such as butylated hydroxyl toluene (BHT) and natural antioxidants such as vitamin C, vitamin E, flavonoid and xanthone. Synthetic antioxidants can inhibit free radicals but are unsafe for consumer health (Najafian and Babji, 2015). So, there has been renewed interest in natural antioxidants with few or no side effects. The sources of natural antioxidant are microbial, plant or animal. In addition, much research has reported that natural antioxidants contain phenolic compounds, especially polyphenol and flavonoid, that can inhibit free radicals.

Protein hydrolysate is a product derived from the hydrolysis reaction. It can be prepared by a treatment sample with chemical or enzyme and the product further

derived in smaller peptides and free amino acids. Preparation of protein hydrolysates by chemical means makes it difficult to control product quality because of its unspecific peptide bonds cleaving and severe reaction. Therefore, the enzymatic hydrolysis of protein attracts more attention as a process that produces high quality protein hydrolysates and that may have multifunctional bioactive compounds, especially bioactive peptide (Wisuthiphaet et al., 2015).

Edible mushrooms have been considered healthy and nutritious because they are high in vegetable proteins, vitamin, minerals, carbohydrates, dietary fiber and low in calories. Moreover, people have used mushrooms as potential medicinal products. For instance, lingzhi (*Ganoderma lucidum*), shiitake (*Lentinula edodes*), straw mushroom (*Volvariella volvacea*), oyster mushroom (*Pleurotus ostreatus*), Jew's ear (*Auricularia auricular*) and white jelly mushroom (Tremella fuciformis) for treatment of many diseases such as hypercholesterolemia, hypertension, platelet aggregation, and cancer (Valverde et al., 2015). Furthermore, much research indicates that extracts from some edible mushrooms can inhibit free radicals. For example, the induction of apoptosis in human and mice carcinoma cell lines by lectin from the yanagi mutsutake, anti-proliferative effect on liver cancer hepatoma hepG2 cells and breast cancer MCF7 cells of polysaccharide peptide LB-1b from Pleurotus ostreatus and antitumor of the polysaccharide from the oyster mushroom (J.-J. Zhang et al., 2016).

Astraeus hygrometricus is an edible mushroom. *A. hygrometricus* is commonly known as the hygroscopic earthstar because of its hygroscopic property. It helps plants in extracting powerful nutrients such as phosphorus, potassium, vitamin D and more, from very slightly soluble soil minerals and organic substances. On the other hand, *A. hygrometricus* obtains carbohydrate from photosynthesis. Much research has

demonstrated the potential advantages of mushroom extract from *A. hygrometricus*. For example, glucan extract enhances strong splenocyte activations, dry spores of *A. hygrometricus* are applied to heal a newborn's navel and used as poultices for the treatment of burns, anti-inflammatory, cardio- and hepatoprotective activities from damage (Biswas et al., 2010). The objective of this study is to produce peptide hydrolysate from *A. hygrometricus* with antioxidant and anti-proliferative activities which may have new bioactive peptides for drug development.

CHAPTER II
LITERATURE REVIEWS

2.1 Free radical

Free radicals are atom, molecules, or ions with unpaired valence electrons that have electrons available to react with various organic substrates such as lipids, proteins, and DNA. These free radicals lead to cell injury and death. Examples of free radical or reactive oxygen species (ROS) are the superoxide anion radical ($O_2^{\bullet-}$), alkoxyl radical ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), lipid peroxyl ($LOO^{\bullet}$) and hydroxyl radical ($OH^{\bullet}$). In particular, hydroxyl radicals are major active oxygen species generated from the Fe_2^{+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction) which attack the deoxyribose and can cause damage to DNA. Examples of reactive nitrogen species (RNS) and reactive chlorine species (RCS) are the nitric oxide radical ($NO^{\bullet}$), nitrogen dioxide radical ($NO_2^{\bullet}$ and $NO_2^{-\bullet}$), and atomic chlorine radical (Cl•). Moreover, non-radical species or oxidants are more stable than free radicals including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), ozone (O_3), peroxynitrite ($ONOO^-$), nitrous acid (HNO_2), dinitrogen trioxide (N_2O3) and lipid peroxide (LOOH) (Genestra, 2007).

The formation of free radicals can arise in the cells by enzymatic and non-enzymatic reactions. Enzymatic reactions generating free radicals contain those involved in the respiratory chain, the phagocytosis and the cytochrome P450 system. For instance, the superoxide anion radical is generated via several cellular oxidase systems such as peroxidases, NADPH oxidase and xanthine oxidase. It is deployed by the immune system to kill invading microorganisms and is also deleterious when produced as a byproduct of mitochondrial respiration. It is an important product of the

one-electron reduction of dioxygen (oxygen gas), which occurs widely in nature. Hypochlorous acid is generated in activated neutrophil-derived enzyme, myeloperoxidase, which oxidizes chloride ions in the presence of H_2O_2. It is a major inorganic bactericidal compound of innate immunity. Furthermore, free radicals can be produced from non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiations. The nonenzymatic process can also emerge during oxidative phosphorylation in the mitochondria (Pacher et al., 2007).

For example, peroxynitrite exhibits unique chemical reactivities such as DNA-strand breakage and protein nitration. It not only has a cytotoxic effect but also mutagenic. Free radicals are generated from either endogenous or exogenous sources. Endogenous free radicals as intermediates in cells are generated from inflammation, immune cell activation, excessive exercise, infection, cancer and aging. Exogenous free radicals result from air pollution, cigarette smoke, alcohol, heavy or transition metals (e.g., cadmium, mercury, lead and iron), industrial solvents, cooking (e.g., grilled food, used oil and fat), and radiation which causes oxidative stress in our body that can lead to many diseases such as cancer, stroke, myocardial infarction and diabetes (Chen et al., 2012). After invading the body by various routes, these exogenous compounds decompose or metabolize into free radicals. Free radicals in the human body can react with low-density lipoproteins (LDL) and also cause tissue damage to transform a normal cell into a cancer cell. Lipid peroxidation is respected as the main molecular mechanism that occurs when a hydroxyl radical abstracts an electron from an unsaturated fatty acid to cause damage to the cell or can respond directly to other biomolecules. Polyunsaturated fatty acids occur as a major part of the LDLs in the blood and oxidation of these lipid components play a vital role in atherosclerosis

(Esterbauer et al., 1991). The three most important cell types in the vessel wall are endothelial cells; smooth muscle cells and macrophages can release free radicals, which affect lipid peroxidation (Neužil et al., 1997).

2.2 Antioxidant

The human body requires some metabolites and enzymes called “antioxidants” that work together to prevent oxidative stress. Antioxidants are necessary to scavenge radicals and maintain balance by being electron donors, hydrogen donors, peroxide decomposers, enzyme inhibitors and metal-chelating agents (Tinggi, 2008). To improve the prevention and treatment of many diseases, antioxidants have two principle mechanisms of action. First, the chain-breaking mechanism donates an electron to the free radical. Second, radical initiators are removed by quenching the chain-initiating catalyst. Radical initiators are substances that can produce radical species under mild conditions and promote radical reactions (Rice-Evans and Diplock, 1993). Furthermore, cells are protected against oxidative stress by endogenous antioxidant systems including enzymatic, non-enzymatic or transition metals. In addition, exogenous antioxidants such as dietary antioxidants and synthetic antioxidants can also defend against free radicals and maintain balance in cells. Therefore, antioxidants are very important substances in protecting cells from damage (Bouayed and Bohn, 2010).

2.2.1 Endogenous antioxidants

Endogenous antioxidants are more powerful free radical fighters produced by the body of a living cell. Endogenous antioxidants, which are the primary antioxidant enzymes in the body, include those such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). Moreover, endogenous antioxidants, which are secondary antioxidant enzymes, can inactivate free radicals into intermediates. In

addition, endogenous antioxidants are divided into two categories: enzymatic and non-enzymatic antioxidants (Birben et al., 2012).

2.2.1.1 Enzymatic antioxidants

Antioxidant enzymes can protect cells from free radicals. Enzymatic antioxidants consist of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). SOD is a closely related enzyme that catalyzes the breakdown of the superoxide anion into hydrogen peroxide and oxygen (Zelko et al., 2002). It is present in almost all aerobic cells and in extracellular fluids. In humans, there are three major types of superoxide dismutase depending on the metal cofactor. SOD_1 (Cu, Zn-SOD) plays an important role in the catalyzing of O_2^- radicals to form hydrogen peroxide and oxygen located in the cytoplasm; SOD_2 (Mn-SOD) is of primary importance in removing O_2- located in the mitochondria and peroxisomes, and SOD_3 (Fe-SOD) is mainly in chloroplasts but has also been detected in peroxisomes (Wuerges et al., 2004). CAT is known to be a common enzyme found in nearly all living organisms especially in the liver which catalyze the decomposition of hydrogen peroxide into less reactive H_2O and O_2 (Chelikani et al., 2004; Eisner and Aneshansley, 1999). GPX is an enzyme that can catalyze the breakdown of hydrogen peroxide. There are particularly high levels of enzymes in the liver (Hayes et al., 2005).

2.2.1.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants are scavengers of free radicals such as glutathione (GSH), uric acid, alpha-lipoic acid (ALA), metallothioneine, melatonin, NADPH, coenzyme Q, albumin, ferritin, bilirubin and L-carnitine (Pisoschi et al., 2016). GSH is one of the most important cellular antioxidants that maintains balance by the enzyme glutathione reductase reacting directly with the radical (Meister, 1988). Melatonin (N-

acetyl-5-methoxytryptamine) is a natural hormone found in animals and algae. Melatonin is mainly produced by the pineal gland in the brain (Malhotra et al., 2004). Uric acid is an antioxidant in the human plasma that has been found in the central nervous system. Moreover, it can prevent lipid peroxidation only as long as ascorbic acid is present (Sautin and Johnson, 2008).

2.2.2 Exogenous antioxidants

Some phytochemicals derived from natural sources are exogenous antioxidants such as flavonoids, flavones, isoflavones, anthocyanins, catechins, isocatechins, coumarins, lignans, esculatin and gallic acid which are phenolic and polyphenol compounds. Also, exogenous antioxidants are obtained from out of the body by a diet rich in fruits, vegetables, herbs, vitamins and through the taking of supplements. There are three types of exogenous antioxidants: dietary antioxidants, synthetic antioxidants and the antioxidant properties of protein hydrolysate (Waqas et al., 2013).

2.2.2.1 Dietary antioxidants

Dietary antioxidants include vitamin C and vitamin E. Ascorbic acid, or vitamin C, is a monosaccharide antioxidant found in both plants and animals but it cannot be synthesized in humans. There are eight forms of vitamin E; namely, the alpha, beta, gamma and delta classes of tocopherol and tocotrienol which are synthesized by plants from homogentisic acid. It has been claimed that the α-tocopherol form is the most important to protect membranes from radicals by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Rizvi et al., 2014).

2.2.2.2 Synthetic antioxidants

Synthetic antioxidants can prevent oxidative stress caused by imbalance and cause a variety of chronic disorders. The structure of synthetic antioxidants are

developed from natural antioxidants. Synthetic antioxidants consist of butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), propyl gallate (PG) and ethoxyquin (EQ) and are widely used as ingredients in dietary supplements to protect lipid peroxidation. However, there are concerns regarding its side effects on human health (Wojcik et al., 2010). Therefore, many researchers have focused on natural antioxidants.

2.2.2.3 Antioxidant properties of protein hydrolysate

Protein hydrolysate is a protein derived from natural sources; for example, Zizyphus jujuba fruits, tuna backbone protein, defatted skipjack (*Katsuwonous pelamis*) and *Grifola frondosa* (DonG et al., 2015; Intarasirisawat et al., 2012; Je et al., 2007). Protein hydrolysate can be hydrolyzed by chemicals or enzymes. The advantages of enzymatic hydrolysis are specific to the cleavage site and maintain its activity. Enzymatic hydrolysis of any protein provides shorter peptides which may have bioactive and functional properties. Even though almost all species of life can repair oxidative damage in the systems, they are produced in limited quantities in nature. Researchers have tried to synthesize bioactive peptides. Bioactive peptides are protein fragments which have important function and great value for human health and their pharmaceutical potential including antimicrobial, antifungal, antioxidant and anticancer activities. Bioactive peptides usually contain 3-20 amino acid residues in their structure. However, the antioxidant activity of bioactive peptides depends on the amino acid composition in their structure (Shi et al., 2014). As for the types of antioxidant peptide, there are hydrophobic amino acids such as isoleucine, leucine and proline. In addition, the most reactive amino acids tend to be those containing an aromatic ring in the peptide structure such as phenyl, tyrosine and tryptophan including

the imidazole group in the histidine and thiol group in cysteine (Elias and Steinhoff, 2008). Besides, peptides with low molecular weight exhibit high antioxidant activity because they could interact more effectively with radicals interfering in the oxidizing process (Sharma et al., 2012). As previously reported, *G. frondosa* protein hydrolysates are obtained by trypsin with antioxidant activity. They separate peptides with different molecular weight distributions. The results show that the smallest peptide which research exhibited the highest antioxidant activity (DonG et al., 2015). Likewise, for protein hydrolysates from patin with antioxidant activity, the results show that the patin hydrolyzed fraction had higher amounts of hydrophobic and aromatic amino acids than the patin unhydrolyzed fraction. As a result, patin hydrolyzed fraction is believed to have high antioxidant activity (Najafian and Babji, 2015).

2.3 Protein extraction

Concerning extraction, organic solvents such as methanol and ethanol are mostly used. However, many researchers choose aqueous solvents because many papers have reported such usage as being more ecofriendly than organic solvents. Additionally, sample-solubility needs to be considered before solvent selection. As previously reported, the aqueous extract of the edible mushroom Pleurotus sajor-caju contains vitamins B1, B2, and C, and it can reduce cholesterol levels in the blood (Chakraborty et al., 2004; Pramanik et al., 2005).

2.4 Protein hydrolysate

Protein hydrolysate is a product derived from the hydrolysis reaction. It can be prepared by treatment sample with chemicals or enzymes and produced into smaller peptides and free amino acids. Protein hydrolysate from natural product generally contains 3-20 amino acid units. Preparing protein hydrolysates by chemicals is difficult

in terms of controlling product quality because of its unspecific peptide bonds cleaving and severe reaction. Therefore, enzymatic hydrolysis of protein attracts more attention as a process that produces high quality protein hydrolysates and may have multifunctional bioactive compounds, especially bioactive peptides (Wisuthiphaet et al., 2015). Several commercially available proteases have been used to hydrolyze protein such as papain, alcalase, neutrase and flavourzyme. As previously reported, the sequence of low molecular weight peptides from croceine croaker muscle hydrolyzed by papain and alcalase had good antioxidant properties on scavenging free radicals; moreover, the peptide from bluefin leatherjacket (*Navodon septentrionalis*) heads was hydrolyzed using papain. The results showed that the smaller molecular size of the bluefin leatherjacket (*Navodon septentrionalis*) head peptides with hydrophobic and aromatic amino acids in their sequences could have encouraged antioxidant activities (Chi, Hu, et al., 2015). Similarly, the peptide from the oyster *Crassostrea madrasensis* (Preston) prepared by using two proteases, papain and pepsin, showed better yield, total phenolic content and antioxidant activity (Asha et al., 2016).

Table 2.1 Protease, characteristics and optimal conditions

Protease	Hydrolysis reaction	Source of origin	Optimal conditions	
			pH	Temperature (ºC)
Pepsin	Digestive, acid protease and endopeptidase	Porcine gastric-mucosa	2	37
Trypsin	Digestive, Serine protease and endopeptidase	Bovine, porcine or human- pancreas	5	37
α-Chymotrypsin	Digestive, Serine protease and endopeptidase	Bovine pancreas	8	37
Papain	Cysteine protease, endopeptidase	Papaya latex (*Carica papaya*)	6	37
Alcalase®	Endoprotease	*Bacillus licheniformis*	8	50
Flavourzyme®	Endo- and exo-peptidase	*Aspergillus oryzae*	7	50
Neutrase®	Endoprotease	*Bacillus amyloliquefaciens*	7	50

2.5 Antioxidant activities

2.5.1 DPPH radical scavenging activity assay

The DPPH assay is based on the reduction of DPPH (2, 2-diphenyl-1-picrylhydrazyl) as a stable free radical in purple color crystalline powder. The DPPH radical is hydrophobic and dissolves in organic solvents such as methanol and ethanol solutions. When the DPPH radical receives an electron from an antioxidant substance that can donate a hydrogen atom, then this gives rise to the reduced form. The radical to the DPPH-H form turns a yellow color. More decolorization shows reduced ability. Then the absorbance reduced by using spectrophotometer at 517 nm is analyzed. The result of these methods is usually reported as inhibition activity calculated in terms of IC_{50}. The IC_{50} value is the concentration of test samples that inhibit the anti-oxidation

reaction by fifty percent. The DPPH assay is the most accepted model, rapid, simple and inexpensive for measuring the free radical scavenging activity. It has two major applications: first as a monitor of chemical reactions involving radicals and, second, as a standard of the position and intensity of electron paramagnetic resonance signals (Tailor and Goyal, 2014). In addition, DPPH is allowed to react with the whole sample and sufficient time given in the method allows DPPH to react slowly even with weak antioxidants (Kedare and Singh, 2011).

2.5.2 ABTS radical scavenging activity assay

An ABTS assay is a chemical compound used to screen the extracts for antioxidant activity. It is reported as a decolorized assay applicable to both lipophilic and hydrophilic antioxidants (Re et al., 1999). ABTS is most commonly used in simulated serum ionic potential solution (PBS; phosphate buffer containing 150 mM NaCl). $ABTS^{+\bullet}$ (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) is formed by the loss of an electron by the addition of potassium persulfate as a green radical cat ion, when its reactive toward antioxidant substances. Then, $ABTS^{+\bullet}$ is converted back to a colorless neutral form. Then, the absorbance reduced by using spectrophotometer at 743 nm is analyzed. The advantages of ABTS radical scavenging activity assay are that it is highly accurate, quick and easy to analyze. So, to sum up, comparison of ABTS and DPPH methods reveals that the former method is formed by adding potassium persulfate as a $ABTS^{+\bullet}$, while DPPH is a stable free radical that already exists (Shalaby and Shanab, 2013).

Table 2.2 Antioxidant activity of some mushrooms

Source of mushroom	Bioactive Compounds	Antioxidant Activity	References
Ramaria flava	Phenolic compounds	Scavenging of DPPH and OH radicals	(Liu et al., 2013)
Phellinus baumii Pilat	Polysaccharide	Scavenging of hydroxyl, superoxide and DPPH radicals	(Ge et al., 2013)
Agaricus bisporus	Polysaccharides, phenolics	Scavenging of superoxide, hydroxyl and DPPH radicals and hydrogen peroxide, enhancement of the activities of antioxidant enzymes in sera, liver, and heart of mice	(Tian et al., 2012)
Cordyceps taii	Polysaccharides	Scavenging of DPPH, hydroxyl, and superoxide anion radicals, and enhancement of antioxidant enzyme activities	(Xiao et al., 2012)

2.6 Antiproliferative activity

Cancer comprises a group of more than 100 diseases that develop across time and involve the uncontrolled division of the body's cells. Cancer begins when a cell can divide, from the normal restraints on cell division and begins to follow its own for proliferation. Mutation is one of the nodes in this network that can stimulate cancer and is influenced by certain environmental factors such as air pollution, uv light and tobacco smoke. Although cancer can develop in the body's cells and each type of cancer has its own unique properties (van Meerloo et al., 2011). Chemotherapy is cytotoxic therapy but it has side effects on your health. Therefore, much researchers interested in the

antiproliferative or cytotoxic activity from protein hydrolysates and use an MTT assay to assess the cellular cytotoxicity caused by any protein hydrolysates to decrease the growth rate of cancer cell lines and screen for anticancer activity. The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay is commonly used to assess the cellular cytotoxicity caused by anticancer substances. The MTT assay system is based on the reduction of the tetrazolium derivatives in living cells into formazan crystals by mitochondrial dehydrogenases, and this allows the estimation of the mitochondrial activity (Jo et al., 2015). The MTT assay is a colorimetric assay. The MTT molecule of yellow color is converted to an insoluble purple formazan product which is largely impermeable to cell membranes. Then, its accumulation within viable cells can detect the absorbance of this colored solution by using a spectrophotometer at 595 nm (Maioli et al., 2009). An MTT assay is usually used to measure the in vitro cytotoxic effects of anticancer substances on cell lines or primary patient cells. To crown it all, cytotoxicity refers to causing harm to cells and thereby killing them, while antiproliferative activity is the ability of a compound to stop the growth of cells that do not allow the cells to multiply rapidly (Riss et al., 2016).

2.7 DNA damage

DNA is the genetic information in all living cells. DNA which undergoes several types of spontaneous modifications, and it can also react with reactive oxygen species that are endogenous products of the cellular metabolism. In addition, exogenous products such as ionizing radiation and UV light are threats from the external environment. Damage to DNA can cause genetic alterations, and if genes that control cell growth are involved, these mutations can lead to the development of cancer. DNA damage may also lead to cell death which can have serious consequences for the

organism. DNA is able to be broken into three forms: supercoiled DNA (SC), open-circular DNA (OC) and linear DNA (LC) (Levy et al., 2000). Because ROS including hydroxyl radicals that are induced or generated by the Fenton reaction ($Fe^{2+}+H_2O_2$) leads to damage to DNA, accumulation of damaged DNA has also been considered to contribute to the onset of many diseases. Some of these DNA repair systems are very important (Wiesmüller et al., 2002). For this reason, many researchers are interested in antioxidant substances that can prevent cellular damage caused by hydroxyl radicals. Protein hydrolysate is one effective antioxidant substance and this exhibits a protective effect against DNA damage induced by hydroxyl radicals (Emonet-Piccardi et al., 1998). Therefore, protein hydrolysates are very important and necessary for use in the primary prevention of many diseases.

2.8 Apoptosis

Apoptosis is the process of programmed cell death. Apoptosis occurs when normal cells develop to small blebs form. Then the nucleus begins to break apart, through chromatin condensation, the DNA breaks into small pieces and organelle fragmentation. Afterward, the organelles are also located in the blebs and the cell breaks into several apoptotic bodies. Meanwhile, the organelles are still functional. On the other hand, inappropriate apoptosis is a factor in many human conditions including autoimmune disorders, Alzheimer's and cancer (Plati et al., 2011). Cancer can only develop from cells any place in the body when the body receives anti-apoptotic signals or loses pro-apoptotic signals. However, apoptotic cells are not always recognized by phagocytes and then they may undergo so-called necrosis. Necrosis is non programmed that small blebs fuse form as the structure of the nucleus change after that organelles are located in the blebs render the immediate loss of cell membrane (McCall, 2010).

Subsequently, the cell membrane releases the cell content and the organelles are not functional. The pattern of cell death of apoptosis and necrosis is shown in Figure 2.1. Generally, apoptosis is determined by characteristic morphological and energy-dependent biochemical features depending on the stimuli and the cell type. The ability of protein hydrolysates to induce apoptotic cells can be detected with an Annexin V-FITC/propidium iodide (PI) double-staining assay by using flow cytometer. Annexin-V binding is performed with phosphatidyl serine that is expressed on the outside of the plasma membrane. Annexin-V binding with phosphatidyl serine is used to indicate the amount of apoptotic cells while PI is used to indicate the amount of necrotic cells. The advantages of flow cytometry including single cell analysis, rapid analysis time and multi-parameter measurements that correlate to different cellular events at a time. As a result of much research reporting that the antioxidant and antiproliferative activities of protein hydrolysate derived from natural sources can induce apoptosis which is important for anticancer drug development (R. C. F. Cheung et al., 2015; Cummings et al., 2012; G. Zhang et al., 1997).

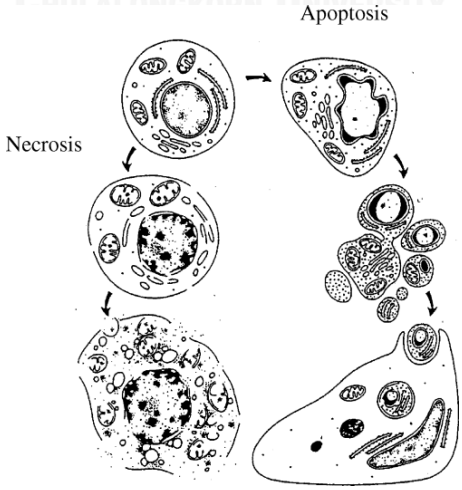


Figure 2.1 The different morphologies between necrosis (left) and apoptosis (right) (Goland, 2014)

2.8.1 The mechanisms of apoptosis

The major pathways for caspase activation in mammalian cells are the extrinsic death receptor pathway and the intrinsic death receptor pathway. The cell-intrinsic pathway can be induced by various stimuli consisting of Bcl-2 family proteins and initiated by internal sensors for severe cell distress. The cell-extrinsic pathway can be triggered by extracellular ligands through cognate death receptors at the surface of target cells (Reed, 2000).

2.8.1.1 Intrinsic mitochondrial apoptotic pathway

In the case of the intrinsic pathway, this can be induced by releasing cytochrome c from mitochondria and induced by various stimuli including high levels of pore-forming pro-apoptotic Bcl-2 family proteins. In the cytosol, the apoptosis protein activating factor 1 (Apaf-1) protein normally resides in an inactive conformation. However, Apaf-1 binding with cytochrome c renders caspase activation (Indran et al., 2011). Moreover, Apaf-1 binding with cytochrome c requires ATP or 2'-deoxy ATP (dATP) because both types of nucleotide (adenosine or deoxy-adenosine based) can be used for the hydrolysis of dATP to dADP that induces the formation of the heptameric apoptosome complex, which in turn activates pro-caspase 9. Then, active caspase 9 will convert caspases 3, 6 and 7 to active forms as shown in Figure 2.2. Furthermore, the mitochondria are able to release the apoptosis-inducing factor (AIF) which can translocate to the nucleus and induce apoptosis in a caspase-independent manner (Reubold et al., 2009). In addition, the intrinsic pathway can be induced when the body receives various stimuli including DNA damage, UV, chemical, and oxidative stress reflects an imbalance in an electrochemical gradient. Afterward, the cytochrome c being released from mitochondria into the cytosol together with the second

mitochondria-derived activator of caspase (SMAC; also known as the inhibitor of apoptosis protein (IAP)) binding with the direct inhibitor of apoptosis-binding protein with low pI (DIABLO) allowing the activation of caspases 3, 6 and 7 to active forms (J. Cai et al., 1998).

2.8.1.2 Extrinsic death receptor apoptotic pathway

Extrinsic apoptotic pathway is initiated by the association of cell surface death receptors (DRs) including tumor necrosis factor receptors (TNFR), TNF-related apoptosis-inducing ligands (TRAILs) and Fas binding with their respective activating cytokine ligands (also known as death ligands) on the cell surface containing TNF receptor type I (TNF-R1), TRAIL-R1 or TRAIL-R2 (DR4 or DR5) and FasR (also known as Apo-1 or CD95), respectively. Signal transduction through death receptors donate receptor oligomerization involving the aggregation of an intracellular motif common to the death receptor family members, known as the death domain (DD) (Khosravi-Far, 2004). Trimerization of death receptors is initiated by ligand binding, but later studies have provided basis to the formation of preassembled receptor oligomers in a ligand-independent manner. Ligand binding leads to the recruitment of DD-containing adaptor proteins such as FAS-associated death domain proteins (FADD) and TNFR1-associated death domain proteins (Elmore, 2007). These adaptor proteins interact with the death effector domain (DED) that contains pro-caspases, namely pro caspase 8 and pro caspase 10, to form the death-inducing signaling complex (DISC). DISC formation can activate caspases 8 and 10. As a result, downstream effector caspases, including caspases 3, 6 and 7 are activated and target cellular structures leading to cell death as shown in Figure 2.2.

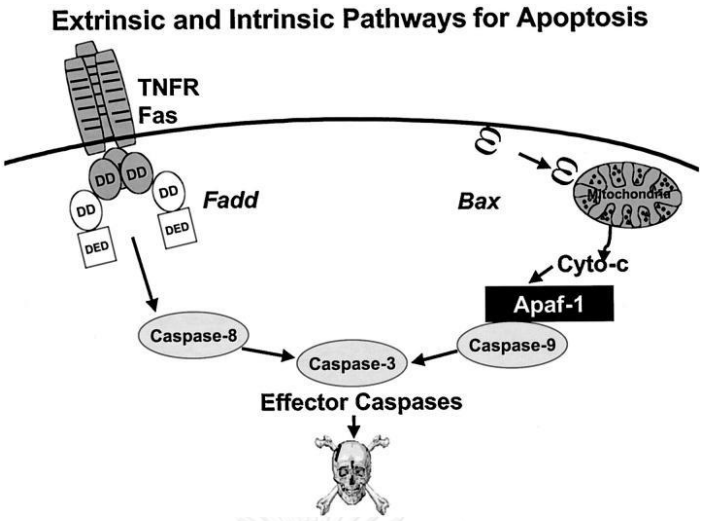


Figure 2.2 The intrinsic and extrinsic pathways of apoptosis (Reed, 2000)

2.8.2 The caspase activity

Caspases (also known as cysteine-aspartic proteases, cysteine aspartases or cysteine-dependent aspartate-directed proteases) are a family of protease enzymes playing essential roles in programmed cell death containing apoptosis, necroptosis and inflammation (Earnshaw et al., 1999). Caspases are divided into two types according to their functions in apoptosis: initiator caspases and effector caspases. Initiator caspases contain pro-domains of over 100 amino acids including caspases 1, 2, 4, 5, 8, 9, 10 and 12. In particular, caspases 8 and 10 have death effector domains (DED), caspases 4, 5 and 9 have caspase recruitment domains (CARD). DED and CARD are also used to hole with adaptor molecule FADD or Apaf-1 for activating effector caspase (Man and Kanneganti, 2016). Effector caspase consists of pro-domains less than 30 amino acids including caspases 3, 6, 7 and 14. Effector caspase can degrade death substrates by cleaving protein substrates such as actin, lamin and fodrin. Moreover, caspase activated DNase (CAD) and DNA fragment factor (DFF) effectively work

together to cleave poly-(ADP-ribose)-polymerase (PARP) causing increased DNA fragmentation and inactivating the cell cycle. PARP is also known as proteins involved in the cellular processes such as DNA repair, genomic stability and programmed cell death (Nowsheen and Yang, 2012). Therefore, caspase-dependent cell death is involved in the apoptosis process. As a result, it is able to expect that protein hydrolysate can induce apoptosis associated with caspases 3 and 8 activation.

2.9 *Astraeus hygrometricus*

Astraeus hygrometricus is a species of fungus in the Diplocystaceae family. *A. hygrometricus* is commonly known as the hygroscopic earthstar because of its hygroscopic property. Several Asian populations formerly thought to be *A. hygrometricus* were renamed in the 2000s once phylogenetic analyses exposed they were unique Astraeus species, including *A. asiaticus* and *A. odoratus*. *A. hygrometricus* can be found in tropical and temperate areas, flourishing during the period of July to October. *A. hygrometricus* is an ectomycorrhizal fungus, which grows in association with various trees below the surface of the soil. The pH ranges from 5.5 to 6.0. It helps plants in extracting powerful nutrients such as phosphorus, potassium, vitamin D and more, from very slightly soluble soil minerals and organic substances. On the other hand, *A. hygrometricus* obtains carbohydrate from photosynthesis. If you cut though a young fruit body the interior (gleba) is white. The external membrane of sporocarp can open its ray to increase humidity and close them up again in drier conditions, when it matures the hole gleba is disorganized and there remains only spores within the thick-walled endo and exo peridia. Much research demonstrates the potential advantages of mushroom extract from *A. hygrometricus*. For example, the glucan extract enhances strong splenocyte activations, the dry spores of *A. hygrometricus* are applied to heal a

newborn’s navel and used as poultices for treatment of burns, anti-inflammatory, cardio and hepato protective activities from damage.

CHAPTER III

EXPERIMENTAL

3.1 Preparation of A. hygrometricus protein hydrolysates

A. hygrometricus was collected from Banvalley School, Tak province, Thailand. 300 g of A. hygrometricus were washed and cut into small pieces and then mixed with 5 L of phosphate buffer saline (PBS; 20mM phosphate buffer with 0.15 M NaCl pH 7.2) using a mixer and subsequently stirred overnight at 4 ºC. A 100 ml sample was hydrolysed with Alcalase® (pH8.0), Flavourzyme® (pH7.0) and Neutrase® (pH7.0) in various concentrations (1, 2.5 and 5%) at 50 ºC, 150 rpm for 4 hours. The pH of each solution was adjusted using 2 M HCl or 2 M NaOH. Then, the enzyme was inactivated in a water bath at 80 ºC for 10 min. The hydrolysate was centrifuged at 11,000 rpm for 40 minutes. The supernatants were collected and kept in -20 ºC until time for use.

3.2 Amino acid composition

The amino acid composition of *A. hygrometricus* were estimated by ALS Laboratory group (Thailand CO., LTD) according to the method modified base on AOAC (2012). First, Hydrolyze 5mg of the purified protein to constituent amino acids by added 5 mL of 6N HCl, then flushed sample with N2 (Removing air) and incubated at 110 °C for 22 hours. Second, added 10 mL of 2.5 mM L-α-amino-n-butyric acid in 0.1 M HCl (Internal standard) and diluted with H_2O to 250 mL. After that, the solution was filtered and derivatized with 6 aminoquinolyl-N-hydroxy succinimidyl carbamate. Finally, it was heated at 55 °C for 10 minutes, then total amino acid content was determined by HPLC.

3.3 Determination of protein contents

The protein contents were determined with the Bradford method using bovine serum albumin (BSA) as a standard protein. Bradford assay was used to determine the protein contents of peptides derived from *A. hygrometricus*. The Bradford assay or colorimetric protein assay measured the concentration of total protein in the sample based on an absorbance shift of the dye Coomassie Brilliant Blue G-250. The principle of the Bradford assay involves the binding of the dye Coomassie Brilliant Blue G-250 with proteins to form the formation (Bradford, 1976). The dye exists in three forms: cationic (red), neutral (green), and anionic (blue). Under acidic conditions, the dye was predominant in the doubly protonated red-brown cationic form that was detected at 470 nm. Afterward, when the dye bound to the protein, it was converted to a stable unprotonated blue form that was detected at 595 nm (Carlsson et al., 2011). The advantages of this assay are that it is easy, sensitive and rapid. The Bradford assay was prepared using bovine serum albumin as a standard protein at 0, 0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 mg/mL. Then, 60 µL of each concentration of BSA was pipetted into each Eppendorf tube. Next, 600 µL of Bradford working solution was added and these solutions mixed by vortex. Then, 200 µL of these solutions was pipetted into 96-well plates and absorbance at 595 nm measured using a microplate reader spectrophotometer. Likewise, 60 µL of peptide samples derived from *A. hygrometricus* was pipetted into the Eppendorf tube and then 600 µL of Bradford working solution added. 200 µL of these solutions was mixed and pipetted into 96-well plates and absorbance at 595 nm measured using a microplate reader spectrophotometer.

3.4 Determination of antioxidant activities

3.4.1 DPPH radical scavenging activity

The DPPH radical scavenging assay was determined using the method of (Tanzadehpanah et al., 2012) with slight modifications. First, 100 µM of DPPH solution was prepared with 0.002 g of DPPH dissolved in 50 mL of 95% methanol. Then, this was added to each sample at the ratio of 1:4 in the dark at room temperature for 10 minutes. The mixture was centrifuged at 1,300 rpm for 5 minutes and the absorbance was measured at 517 nm with a spectrophotometer. 100 µg/mL of ascorbic acid was used as a positive control.

3.4.2 ABTS radical scavenging activity

ABTS radical scavenging assay was determined using the method of (Y. Cai et al., 2004) with slight modification. The ABTS solution containing 7 mM ABTS in 2.45 mM potassium persulfate at the ratio of 1:1 in the dark at room temperature for 12 hours. An aliquot of the ABTS solution was diluted with deionized water until it indicated an absorbance of 0.70 ± 0.02 at 734 nm. Then, this was mixed with peptide samples at the ratio of 1:30 (25 µL of sample and 750 µL of ABTS radical solution). The mixture was allowed to stand for 15 min in the dark and absorbance was measured at 734 nm with a spectrophotometer. 1 mg/mL of ascorbic acid was used as a positive control.

3.4.3 Percentage inhibition

The percentage of radical scavenging was calculated as follows:

$$\left[\frac{(\text{absorbance of the control} - \text{absorbance of the sample})}{\text{absorbance of the control}}\right] \times 100$$

Where absorbance of the control which no peptide sample, absorbance of sample was an absorbance of *A. hygrometricus* hydrolysates (peptide samples), absorbance of blank was an absorbance of the deionized water and absorbance of background was the color absorbance of the samples. All experiments, expect for radical scavenging activity, were performed in triplicate, and the results were reported as mean ± standard deviation (n=3). The IC_{50} values (i.e., the concentration of *A. hygrometricus* protein hydrolysates required to scavenge radical by 50%) was calculated using GraphPad Prism software version 6.

3.5 Isolation of peptides from *A. hygrometricus* protein hydrolysates

3.5.1 Ultrafiltration

Ultrafiltration was used for the separation through a semipermeable membrane that can classify molecules at different molecular weights. In this research, *A. hygrometricus* protein hydrolysates with the highest antioxidant activity was fractionated according to molecular weights using four different molecular weight cut-off (MWCO) membranes with 10, 5, 3 and 0.65 kDa. Then, five fractionates (≥10 (F1), 10-5 (F2), 5-3 (F3), 3-0.65 (F4) and ≤ 0.65 (F5) kDa) were collected and the radical scavenging activities calculated.

3.5.2 Gel filtration chromatography

Gel filtration chromatography is an effective method for separating bioactive substances with different molecular dimensions by columns of beads or gel matrix. Gel

filtration chromatography has been widely used for enhancing the antioxidant activity of protein hydrolysates. The larger molecules enter in the bead less or not at all and thus move through the bed more quickly as smaller molecules diffuse further into the pores of the beads and move through the bead more slowly. The advantages of this method include good separation with a minimal volume of elute. Moreover, various solutions can be applied without interfering with the filtration process, especially removing salts from protein solutions. The fraction F5 was shown to have the highest radical scavenging activities from molecular weight cut-off by ultrafiltration, and was further isolated using a Superdex 75 column (0.8 cm diameter × 60 cm length), pre-equilibrated using deionized water. The fraction was eluted with deionized water at a flow rate of 0.5 mL/min. Then, 5 mL of each eluted peptide fractions ($F5_1$ and $F5_2$) was collected and detected at 280 nm. Their radical scavenging activities were calculated.

3.6 Protective effect of the purified peptide against hydroxyl radical –induced DNA damage

3.6.1 Plasmid preparation from *E. coli*

Plasmid preparation was the method for DNA extraction. First, a streaked plate E.coli containing plasmid pBR322, pUC19 and pKS in LB agar (2% agar: peptone, yeast extract, NaCl and agar powder) was prepared. Next, about 100 mg/mL of ampicillin was added in agar to prevent plasmid. Then, an incubated E.coli plate was left over night at 37 °C. Afterward, a single colony from a streaked plate containing pBR322, pUC19 and pKS plasmid was selected. A single colony was inoculated with 5 ml of LB broth containing 5 µl of ampicillin. After that, it was incubated in a shaker at 37 °C, 250 rpm overnight. 3.0 mL of *E. coli* culture was isolated by centrifugation at 14,000 rpm for 30 seconds. The precipitants were collected. Finally, 1 mL of

precipitants of E.coli culture was pipetted and mixed with 500 µL of 100% glycerol, and kept at -70ºC until use.

3.6.2 Plasmid DNA purification check protocol

The precipitants were fractionated according to purified plasmid DNA using a Spinclean plasmid miniprep kit. Miniprep involves the rapid, small-scale isolation of plasmid DNA from bacteria, and is based on alkaline lysis. Miniprep was used in the process of molecular cloning to analyze bacterial clones. First, in lysate preparation, 1.5 mL of E.coli culture was transferred containing pBR322, pUC19 and pKs plasmid into a micro centrifuge tube and centrifuged at 14,000 rpm for 30 seconds. Then, only cell pellets were kept and 200 µL of resuspension buffer added including RNAse. After that, mixed by inverting 40 times and cell suspension incubated at room temperature for 5 minutes. Next, 250 µL of lysis solution was added to the cell suspension and mixed by inverting 40 times. In this step the cells began to lyse. It was observed that suspension become clear and viscous. Then, 350 µL of binding solution was added and mixed by inverting 40 times. Afterwards, this was centrifuged at 14,000 rpm for 10 minutes and then the lysate transferred into the spin column, and centrifuged again at 14,000 rpm for 1 minute. After that, the filtrate was discarded and the mini spin column put back into the collection tube. For washing bound DNA, 600 µL of wash solution was added and centrifuged at 14,000 rpm for 1 minute, the filtrate discarded and the filter dried by centrifuging again at 14,000 rpm for 2 minutes. The column was placed in a 1.5 mL receiver tube. Then, 50 µL of elution buffer was added to the column and incubated at room temperature for 1 minute. Later on, the column was centrifuged at 14,000 rpm for 2 minutes. Finally, the pure plasmid DNA was kept at -20 ºC until use.

3.6.3 Determination of DNA concentration and protein content of pure plasmid DNA

DNA concentration and protein content of pure plasmid DNA were measured by Nano drop technique using spectrophotometer from Denovix.

3.6.4 Protective effect of the purified peptide on hydroxyl radical-induced DNA damage

The protective effect of the purified peptide fraction against hydroxyl radical-induced DNA damage was based on the Fenton reaction using the method described by (Sheih et al., 2009). First, 3 µL of plasmid DNA (pBR322, pUC19 and pKS) was mixed with 4 µL of various concentrations of the purified peptide fraction and incubated for 20 minutes. Then, 3 µL of 2 mM FeSO4 and 3 µL of 18 % H_2O_2 was added. After that, the mixture was incubated at 37 °C for 30 minutes. Later, DNA bands were checked by 1% agarose gel electrophoresis.

3.6.5 Separating plasmid DNA on agarose gel electrophoresis

Agarose gel electrophoresis is another way to determine the yield, to size fractionate DNA molecules and purify DNA. Separating plasmid DNA on agarose gel electrophoresis by mixing 5 µL of peptide sample with 2µL loading dye. 3 µL of plasmid DNA mixed with 2 µL loading dye was used as a positive control. 3 µL of plasmid DNA and 4 µL of deionized water was mixed with 2 µL loading dye andused as a negative control and 5 µL of generuler 1 kb dna ladder was used as DNA maker. All of the samples were loaded into a well of 1% w/v agarose gel. The gel was run in 1xTAE buffer at 100 V for 30 minutes. The gel was stained in ethidium bromide for 10 minutes. Then, the gel was washed with water and plasmid DNA bands measured by UV light at 260 nm.

3.7 Antiproliferative activity

3.7.1 Cancer cell culture

In this part, five types of human cancer cell lines consisting of CHAGO-K1 (human undifferentiated lung carcinoma ATCC no. HTB 168), BT474 (human ductal breast carcinoma ATCC no. HTB 20), HEP-G2 (human hepatoblastoma liver ATCC no. HB 8065), KATO-III (human gastric carcinoma ATCC no. HTB 103) and SW620 (human colorectal adenocarcinoma (colon primary) ATCC no. CCL 227) were used to determine antiproliferative activity. In addition, WI-38 (human diploid lung fibroblast ATCC no. CCL 75) was a diploid human normal cell line used for antiproliferative activity to compare with the human cancer cell lines. To begin with, cancer cell lines cultured in a 25 cm^2 culture flask using a RPMI 1640 medium containing 10% fetal calf serum (FCS) while normal cell lines were cultured in a 25 cm^2 culture flask using MEM medium containing 10% FCS. Then, cancer cell line and normal cell line cultures were incubated at 37 ºC with 5% CO_2 in a CO_2 incubator.

3.7.2 Cytotoxicity assay

Antiproliferative activity was determined by cytotoxicity which can be monitored using MTT assay. The purified peptide fraction F51 was shown to have the highest radical scavenging activities from gel filtration chromatography and the cytotoxicity further determined on human cancer cell lines and human normal cell lines including CHAGO-K1 (lung cancer), BT474 (breast cancer), HEP-G2 (liver cancer), KATO-III (gastric cancer), SW620 (colon cancer) and WI-38 (lung fibroblast normal cell) by MTT assay. The method of MTT assay was modified from the method of (Gerlier and Thomasset, 1986). First, 200 µL of HEP-G2 and SW620 cells were plated at 0.5 x 104 cell/well in 96-well plates. Next, 200 µL of BT474, CHAGO-K1, KATO-

III and WI-38 cells were plated at 1 x 104 cell/well in 96-well plates. Then, cells were incubated at 37ºC with 5% CO_2 in a CO_2 incubator for 24 h. After that, the cell culture medium was removed and 200 µL of purified peptide fraction F51 added in the concentrations of 42.58, 21.29, 10.65, 5.32, 2.66, 1.33, 0.67, 0.33, 0.17 and 0.08 µg/mL. Afterwards, the mixture was incubated at 37ºC with 5% CO_2 in a CO_2 incubator for 72 h. Then, 10 µL of 5 mg/mL MTT in NSS solution was added and incubated at 37ºC with 5% CO_2 in a CO_2 incubator for 4 h. Later, 150 µL of 100% DMSO solution was added into 96-well plates to dissolve the insoluble purple formazan crystals. Finally, absorbance at 540 nm was measured using a microplate reader spectrophotometer, of which absorbance was directly proportional to the number of viable cells. The percentage of cell viability was calculated using the following equation:

$$\text{Cell survival (\%)} = \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

Where absorbance of sample was an absorbance of purified peptide fraction F51 from gel filtration chromatography and absorbance of control was 100% cell survival (no sample). All data were performed in triplicate, and the results were reported as mean ± standard deviation (n=3). The IC_{50} values (i.e., the concentration of *A. hygrometricus* protein hydrolysates required to inhibit the cancer cell growth by 50%) was calculated using GraphPad Prism software version 6.

3.8 Apoptosis analysis

3.8.1 Apoptosis by flow cytometer

The pattern of program cell death in apoptosis form was determined with membrane alteration. The membrane alteration was detected by dual staining packing with FITC conjugated Annexin V and propidium iodide (PI; the DNA-binding dye

molecule). After that, cells were separated with a flow cytometer. The flow cytometer was a biophysical technology in cell counting, cell sorting, protein engineering and biomarker detection by suspending cells in a stream of fluid and passing them by an electronic detection to a single cell for measuring fluorescence intensity and calculating the quantity of apoptotic cells. Then, the percentage of apoptotic cell was determined using a BD FACSCalibur Flow Cytometer. The results from the flow cytometer exhibited a pattern of program cell death in 4 quadrants consisting of living cells, early apoptosis, late apoptosis and necrosis (Figure 3.1).

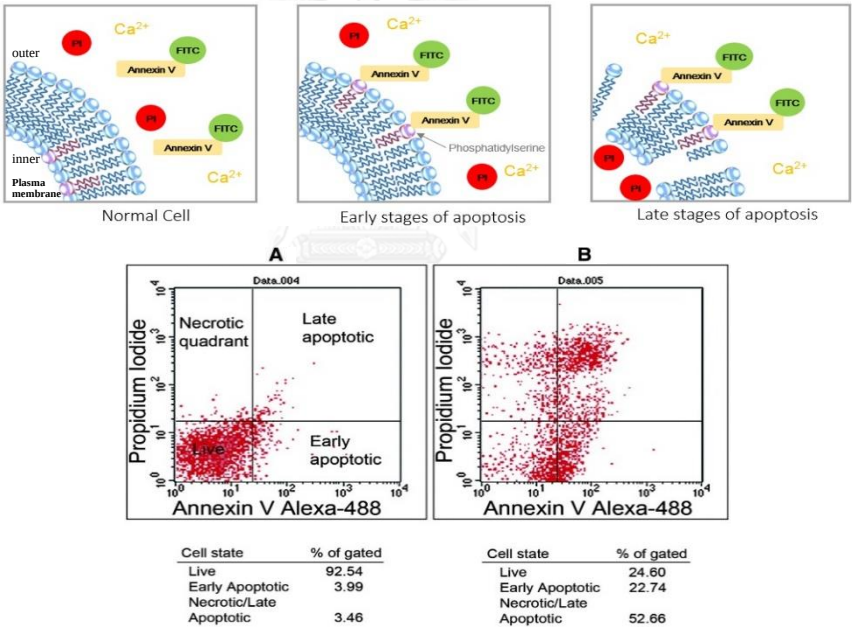


Figure 3.1 Annexin V and propidium iodide (PI) staining (Hospital, 2016)

In this study, Chago-k1 was the human cancer cell line treated by the purified peptide fraction F51 (protein content was 42.58 µg/mL) from gel filtration chromatography had the highest antiproliferative activity. First, the number of living cells in suspension was counted using 5 µL of living cells mixed with 5 µL of trypan blue added to the hemocytometer and the amount of living cells counted (trypan blue

was not absorbed). Then, cultured Chago-k1 in RPMI 1640 medium containing 10% FCS was added to a 35 x 10 mm sterile round cell culture dish at 5×105 cell/mL/dish. After that, Chago-k1 cultures were incubated at 37 ºC with 5% CO_2 in a CO_2 incubator. After 24 h, the Chago-k1 culture was treated with 1 mL of purified peptide fraction F51 (protein content was 42.58 µg/mL). In the same way, the Chago-k1 cultures were treated with 1 mL of 0.5 µg/mL doxorubicin used as a positive control and 1 mL of RPMI 1640 medium containing 10% FCS used as a negative control. Then, this was incubated at 37 ºC with 5% CO_2 in a CO_2 incubator for 24, 48 and 72 hours. Afterward, each treatment of Chago-k1 cells was collected and centrifuged at 2,500 rpm for 5 minutes and the precipitate collected. Next, the cells were washed by 5 mL of a 20 mM PBS buffer containing 1% FCS and centrifuged again at 2,500 rpm for 5 minutes. Then, the precipitate was collected and 0.5 mL of 20 mM PBS buffer added containing 1% FCS. The cell suspension was kept on ice. After that, the cells were analyzed using a FITC Annexin V Apoptosis Detection Kit with PI. First, the cell suspension was centrifuged at 3,000 rpm for 5 minutes and the precipitate collected. Second, 300 µL of Annexin V binding buffer was re-suspended in untreated cells and kept on ice, with 100 µL of Annexin V binding buffer added to the suspended cells and then 5 µL of FITC conjugated Annexin V solution added. Third, cells were incubated on ice in the dark room for 10 min. Next, 100 µL of Annexin V binding buffer and 10 µL of PI solution were added. Then, 300 µL of each treatment was pipetted into 12 x 75mm round bottom polystyrene test tubes. Finally, the cells were analyzed with a flow cytometer. Data analysis was performed with a BD FACSCalibur Flow Cytometer.

3.8.2 Caspase 3, 8 and 9 activities assay

Caspases (Cysteine-requiring Aspartate protease) were a family of proteases that important process of program cell death (Apoptosis).

3.8.2.1 Preparation of cell lysates from apoptotic cells

First, the number of living cells in suspension was counted using 5 µL of living cells mixed with 5 µL of trypan blue added to the hemocytometer andthe amount of living cells counted (trypan blue was not absorbed). Then, the Chago-k1 was cultured in a RPMI 1640 medium containing 10% FCS added to a 35 x 10 mm sterile round cell culture dish in a 5×105 cell/mL/dish. After that, the Chago-k1 cultures were incubated at 37 ºC with 5% CO_2 in a CO_2 incubator. After 24 h, the Chago-k1 cultures were treated with 1 mL of purified peptide fraction F51 (protein content was 42.58 µg/mL). In the same way, Chago-k1 cultures were treated with 1 mL of 0.5 µg/mL doxorubicin used as a positive control and 1 mL of RPMI 1640 medium containing 10% FCS which was used as a negative control. Then, this was incubated at 37 ºC with 5% CO_2 in a CO_2 incubator for 24, 48 and 72 hours. Afterward, each treatment of Chago-k1 cells was collected and centrifuged at 2,500 rpm for 5 minutes and then the precipitate collected. Next, the cells were washed twice by 500 µL of 20 mM PBS buffer containing 1% FCS and centrifuged again at 4 °C, 2,500 rpm for 5 minutes. Then, the precipitate was collected and 100 µL added in a 1x lysis buffer at a concentration of 100 µL per 1×106 cells. Subsequently, the cells were suspended and incubated on ice for 15-20 minutes. Later, the lysed cells were centrifuged at 4 °C, 2,500 rpm for 5 minutes. Finally, the supernatants that had cell lysates were transferred to the new 1.5 mL eppendorf tubes and the cell lysates kept at -70 ºC until use.

3.8.2.2 Caspase 3 activity assay

Caspase 3 was a member of the CED-3 subfamily of caspases and is one of the critical enzymes in apoptosis. Moreover, caspase 3 includes chromatin condensation and DNA fragmentation as cell blebbing. Caspase 3 was determined by a Caspase 3 Colorimetric Assay Kit based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA) to Ac-DEVD and releases the p-nitroaniline (pNA) moiety by caspase 3. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm (pNA had high absorbance at 405 nm) or calculated from a calibration curve prepared with a defined pNA solution.

$$\text{Ac-DEVD-pNA} \xrightarrow{\text{Caspase 3}} \text{Ac-DEVD + pNA}$$

The conditions required for caspase 3 activity include non-induced cells (non-treated cell lysates), induced cells (treated cell lysates), caspase 3 positive control and reagent blank (negative control). The reaction scheme for the 96 well plate microassay method was replicated three times. Firstly, 5 µL of non-induced and induced cell lysate were pipetted into each of the 96-well plates. Secondly, 85 µL of 1x assay buffer was added into each of the wells for non-induced cells, induced cells and caspase 3 positive control except for reagent blank, of which 90 µL of 1x assay buffer was added into each of the wells. Thirdly, 5 µg/mL of caspase 3 was added into the well as a caspase 3 positive control. Next, 10 µL of 2 mM caspase 3 substrate was added to each well for non-induced cells, induced cells, caspase 3 positive control and reagent blank, respectively. Then, this was mixed gently and incubated at 37 ºC for 70 to 90 minutes. Lastly, absorbance was measured at 405 nm and the results calculated using a p-

nitroaniline calibration curve. The unit of caspase 3 activity in µmole pNA released per min per mL of cell lysate was calculated.

$$\text{Activity (µmole pNA / min /mL)} = \frac{\text{µmole pNA} \times d}{t \times V}$$

Where d was dilution factor, V was volume of sample in mL and t was reaction time in minutes.

3.8.2.3 Caspase 8 activity assay

Caspase 8, also known as Mch5, MACH and FLICE, is localized at the top of the hierarchy of the caspase cascade and was a member of the upstream or initiator family of caspases. Caspase 8 exists in the cell as an inactive proenzyme of 55 kDa. It is converted to an active form, including 18 and 12 kDa subunits, upon its recruitment to the cytoplasmic domain of activated death receptors such as Fas, via the adaptor protein FADD. The activation of the proenzyme was triggered by the aggregation of protein, which led to auto- or transprocessing. Caspase 8 activated downstream caspases (3, 6 and 7) that cleave key cellular substrates and lead to the apoptotic death of the cells.

The caspase 8 colorimetric assay was based on the hydrolysis of the peptide substrate Acetyl-Ile-Glu-Thr-Asp p-nitroaniline (Ac-IETD-pNA) to Ac-IETD and releases the p-nitroaniline (pNA) moiety. Caspase 8 activity was determined by a Caspase 8 Colorimetric Assay Kit.

The conditions required for caspase 8 activity include unknown sample, caspase 8 positive control and reagent blank (negative control). The reaction scheme for the 96 well plate microassay method was replicated three times. Firstly, 10 µL of unknown sample was pipetted into each of the 96-well plates. Secondly, 80 µL of 1x assay buffer

was added into each of the wells for the unknown sample and caspase 3 positive control except the reagent blank, of which 90 µL of 1x assay buffer was added into each of the wells. Thirdly, 10 µg/mL of caspase 8 was added into the well for caspase 8 positive control. Next, 10 µL of 2 mM caspase 8 substrate was added to each well for the unknown sample, caspase 3 positive control and reagent blank, respectively. Then, this was mixed gently and incubated at 37 ºC for 5 minutes. Lastly, the absorbance at 405 nm (t = 0 and 60 minutes) was measured and the results calculated using a p-nitroaniline calibration curve. The caspase 8 activity was calculated as nmoles of p-nitroaniline released per min per mL for the unknown sample.

$$\text{Activity (nmole pNA / min /mL)} = \frac{(A_t - A_0) \times d}{(A_{1\ \text{nmole}}) \times t \times V}$$

Where A_t was absorbance at time t minutes, A_0 was absorbance at zero time, d was dilution factor, $A_{1\ \text{nmole}}$ was absorbance of 1 nmole of pNA in the well, t was reaction time in minutes (60 minutes) and V was volume of sample in mL.

3.8.2.4 Caspase 9 activity assay

Caspase 9 is an initiator caspase encoded by the CASP9 gene. CASP9 has been identified in mammalian cells. The aspartic acid specific protease caspase-9 has been linked to the mitochondrial death pathway. Once initiated caspase-9 cleaves procaspase-3 and procaspase-7 which cleave several cellular targets including poly ADP ribose polymerase. It is activated during apoptosis. Caspase 9 colorimetric activity assay kit provides a simple for assaying the activity of caspase that recognizes the LEHD. Caspase 9 activity assay was based on the spectrophotometric detection of the chromophore pNA after cleavage from the labeled substrate Ac-LEHD-pNA. An absorbance of the free pNA was measured at 405 nm.

The conditions required for caspase 9 activity included test sample, substrate blank and buffer blank. The reaction scheme for the 96 well plate microassay method was replicated three times. First, 10 µL of test sample was pipetted into each of the 96-well plates. Second, 60 µL of DI water was added into each of the wells for test samples except for the substrate blank and buffer blank, of which 70 and 80 µL of DI water was added into each of the wells, respectively. Third, 10 µL of caspase 9 substrate was added into the well for the test sample and substrate blank. Next, 20 µL of 5x assay buffer was added to each well for the test sample, substrate blank and buffer blank. Then, this was mixed gently and incubated at 37 ºC for 120 minutes. Lastly, absorbance was measured at 405 nm and the results calculated using a p-nitroaniline calibration curve. The fold-increase in caspase 9 activity was determined by comparing the OD from the induced apoptotic sample with the level of the uninduced control. The unit of caspase 9 activity in µmole pNA released per min per mL of cell lysate was calculated.

$$\text{Activity (µmole pNA / min /mL)} = \frac{\text{µmole pNA} \times d}{t \times V}$$

Where d was dilution factor, V was volume of sample in mL and t was reaction time in minutes.

3.9 Reverse phase high performance liquid chromatography (RP-HPLC)

RP-HPLC is a technique in analytical chemistry used to separate each component in a mixture and purify the selected fraction to sub-fractions by column. The principle of RP-HPLC separates each component in a mixture based on thepolar of mobile phase related to the non-polar of the stationary phase. The purified peptide fraction F51 which had the highest antioxidant activity from gel filtration chromatography was filtrated through 0.45 µm of nylon filtration membrane and

separated by RP-HPLC on a Luna 5U C18 100A (4.6 mm × 250 mm) column with a gradient of 88% of elute B consist 0.1% trifluoroacetic acid (TFA) and 12% of elude C consisting of 70% acetonitrile (ACN) with 0.05% TFA at a flow rate of 0.7 mL/min. The peaks of purified peptide fraction F51 were eluded and monitored at 280 nm. After that, sub-fractions of purified peptide fraction F51 were collected and the antioxidant activity then determined.

3.10 Mass spectrometry

Mass spectrometry is used in many different fields and applied to pure samples as well as complex mixtures. In this study, the molecular mass and amino acid sequence of the purified peptide was determined by ionization source; electrospray, mass analyzer; quadrupole-time of fight, detector; and microchannel plate detector also known as ESI-Q-TOF mass spectrometry. The ESI-Q-TOF mass spectrometry instrument calibrated the peptide chain in the mass range of 50-25,000 m/z. The results of the peptide amino acid sequence from ESI-Q-TOF mass spectrometry were determined by de novo sequencing and mascot. The principle of de novo sequencing is the commitment of fragment ions from a mass spectrum to calculate the mass of amino acid residue on the peptide chain while Mascot is a powerful search engine which uses mass spectrometry data to identify proteins from primary sequence databases.

3.11 Statistical analysis

Each experiment was conducted in triplicate. The results were mean ± standard deviation. The statistical analyses were performed using the SPSS program. All data were analyzed by one-way analysis of variance (ANOVA), and the means were compared using Duncan's new multiple range test. Differences were considered significant at $p < 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Amino acid composition of *A. hygrometricus*

The amino acid composition of *A. hygrometricus* is shown in Table 4. 1 (mg/100mg dry weight). Glutamic acid consists of the highest contents of essential amino acid (0.75 mg/100 mg). The presence of hydrophobic amino acid, such as Gly, Val, Leu and Pro, could be important in the antioxidant activity.

Table 4.1 Amino acid composition of *A. hygrometricus* (mg/100 mg dry weight)

Amino acid Profile	Results (%)
Alanine	0.43
Arginine	0.15
Glycine	0.19
Aspartic Acid	0.29
Valine	0.13
Cystine	0.04
Glutamic Acid	0.75
Leucine	0.17
Isoleucine	0.08
Histidine	0.04
Threonine	0.16
Proline	0.13
Lysine	0.14
Methionine	0.03
Serine	0.15
Phenylalanine	0.07
Tyrosine	0.06
Tryptophan	0.01

4.2 Antioxidant activities

The antioxidant activity of the protein hydrolysates was determined using DPPH and ABTS radical scavenging activities. Table 4.2 shows that the peptide hydrolysates of *A. hygrometricus* prepared by 1% Alcalase® had the highest DPPH and ABTS radical scavenging activities with IC_{50} values of 13.40 ± 0.48 µg/ml and 5.53 ±

0.59 µg/ml, respectively. The results showed that the different types and concentration of enzymes are influenced on the antioxidant effects of peptides from *A. hygrometricus* because of the activities of enzymes on the peptide bond. Alcalase® and Neutrase® were endoprotease which digests internal peptide bond while Flavourzyme® was both types of endoprotease and exoprotease that digests terminal peptide bond to release amino acids. Alcalase® was an enzyme that has many preferential cleavage site such as Phe, Trp, Tyr, Glu, Met, Leu, Ala and Ser (I. W. Y. Cheung, 2007). Therefore, peptides from *A. hygrometricus* using 1% Alcalase® was selected and prepared for follow-up studies.

Table 4.2 Antioxidant activity of peptide hydrolysates from *A. hygrometricus*

Enzyme	%	IC_{50} (µg/ml)	
		DPPH	ABTS
Alcalase®	1	13.40 ± 0.48^{a}	5.53 ± 0.59^{A}
	2.5	17.71 ± 0.25^{ab}	6.10 ± 0.90^{A}
	5	20.98 ± 1.32^{bc}	6.36 ± 0.23^{ABC}
Neutrase®	1	14.28 ± 0.92^{a}	12.69 ± 0.26^{BC}
	2.5	33.24 ± 3.09^{fg}	6.04 ± 0.41^{A}
	5	14.37 ± 0.47^{a}	5.91 ± 0.68^{A}
Flavourzyme®	1	17.96 ± 1.98^{ab}	14.32 ± 0.22^{CD}
	2.5	25.64 ± 2.42^{cde}	11.00 ± 0.70^{B}
	5	17.38 ± 3.35^{ab}	12.75 ± 0.78^{BC}

∗ $^{a\text{-}g,\ A\text{-}D}$ values shown are the mean ± standard deviation of triplicate determinations and separation within column by Duncan's new multiple range test at $p \leq 0.05$

4.3 Isolation of peptides from *A. hygrometricus* protein hydrolysates

1% Alcalase® was fractionated according to molecular weights using ultrafiltration with four different molecular weight cut-off (MWCO) membranes (10, 5, 3 and 0.65 kDa). The results revealed that fractions of MWCO less than 0.65 kDa (F5) had the highest DPPH and ABTS radical scavenging activities with IC_{50} values of 4.84±0.26 µg/ml and 1.49±0.39 µg/ml, respectively. As shown in Table 4. 3, fractions

of MWCO less than 0.65 kDa were separated by gel filtration chromatography. Similar to other studies, the low MW peptides required for the high antioxidant activity because of the low MW peptides could have more effective interaction with radicals interfering in the oxidizing process (Chi, Wang, et al., 2015).

Table 4.3 The IC_{50} values of DPPH and ABTS radical scavenging activities prepared from 1% Alcalase digestion of *A. hygrometricus* peptide hydrolysates by ultrafiltration

Fractions	Molecular weight cut off (kDa)	IC_{50} (µg/ml)	
		DPPH	ABTS
F_1	>10	38.09 ± 2.89^{d}	14.87 ± 0.61^{D}
F_2	10-5	16.78 ± 1.55^{c}	4.83 ± 0.33^{C}
F_3	5-3	11.19 ± 1.54^{b}	3.60 ± 0.19^{B}
F_4	3-0.65	8.19 ± 0.71^{ab}	2.40 ± 0.12^{A}
F_5	< 0.65	4.84 ± 0.26^{a}	1.49 ± 0.39^{A}
Ascorbic acid	-	33.27 ± 0.32^{d}	147.73 ± 0.49^{E}

∗ $^{a\text{-}d, A\text{-}E}$ values shown are the mean ± standard deviation of triplicate determinations and separation within column by Duncan's new multiple range test at $p\leq 0.05$

4.4 Purification of antioxidant purified peptide of fraction F5 by gel filtration chromatography

The purified peptide of fraction F5 was separated by gel filtration chromatography into two sub-fractions (F51 and F52) as shown in Figure 4. 1. In addition, Table 4. 4 showed the highest DPPH and ABTS radical scavenging activities of the purified peptide fraction F51 with IC_{50} values at 32.47±1.16 µg/ml and 15.58±0.73 µg/ml, respectively. Thus, the purified peptide fraction F51 was selected for further study.

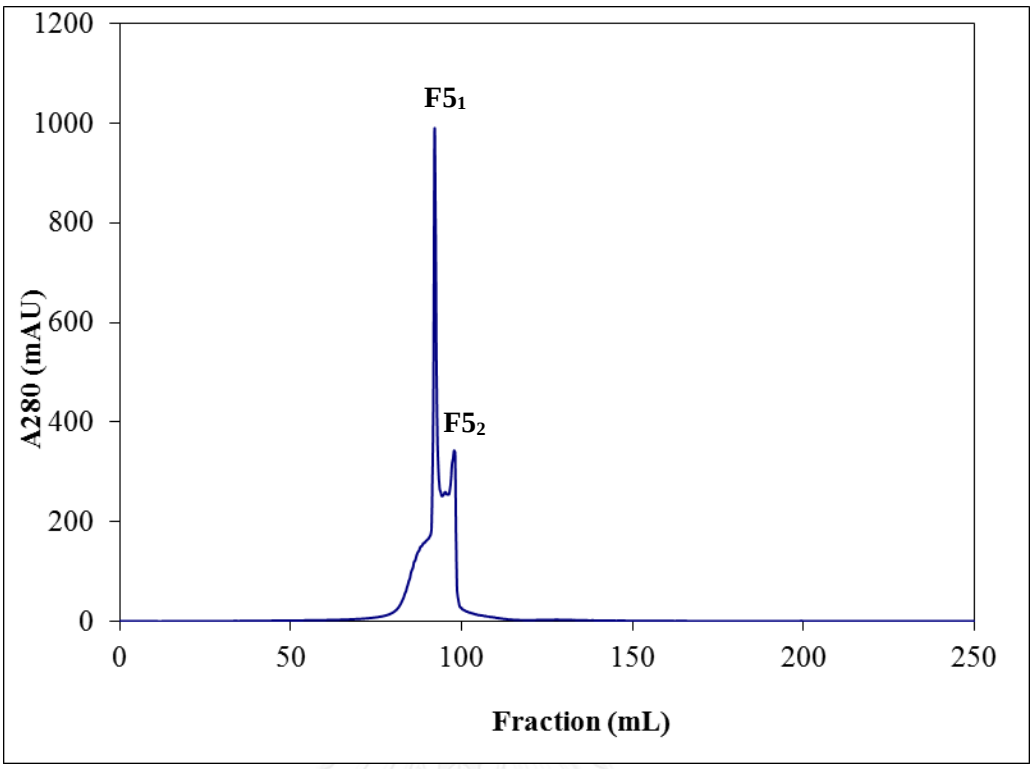


Figure 4.1 Elution profile of peptide hydrolysates (F5) from *A. hygrometricus* by gel filtration chromatography. The hydrolysate was eluted on a Superdex G 75 column.

Table 4.4 Antioxidant activity of the fractionated (F5) was separated by gel filtration chromatography using a Superdex G 75 column

Peak	IC_{50} (µg/ml)	
	DPPH	ABTS
$F5_1$	32.47 ± 1.16^{a}	15.58 ± 0.73^{A}
$F5_2$	36.84 ± 0.28^{b}	39.05 ± 2.30^{B}
Ascorbic acid	33.27 ± 0.32^{a}	147.73 ± 0.49^{C}

∗ $^{a\text{-}b,\ A\text{-}C}$ values shown are the mean ± standard deviation of triplicate determinations and separation within column by Duncan's new multiple range test at $p \leq 0.05$

4.5 Protective effect of the purified peptide ($F5_1$) against hydroxyl radical-induced DNA damage

Protective effect of the purified peptide fraction $F5_1$ was investigated by various concentrations against hydroxyl radical-induced DNA damage. The results showed the purified peptide fraction $F5_1$ (Lane 4-5) also protected supercoiled DNA strand in three plasmid DNA (pBR322, pUC19 and pKS). As shown in Figure 4.2 – 4.4. The findings

indicated that DNA could be protected by purified peptide ($F5_1$). Similar to other studies, the hydroxyl radicals generating system was based on the Fenton reaction (Fe^{2+} + H_2O_2). The super coil DNA was completely converted to the open circular (OC) form due to the hydroxyl radical damage. The purified peptide from algae had the protective capacity increased with increasing peptide concentrations in oxidation-induced DNA damage. It could be conclude that the purified peptide from algae can protect hydroxyl radical induced damage (Sheih et al., 2009).

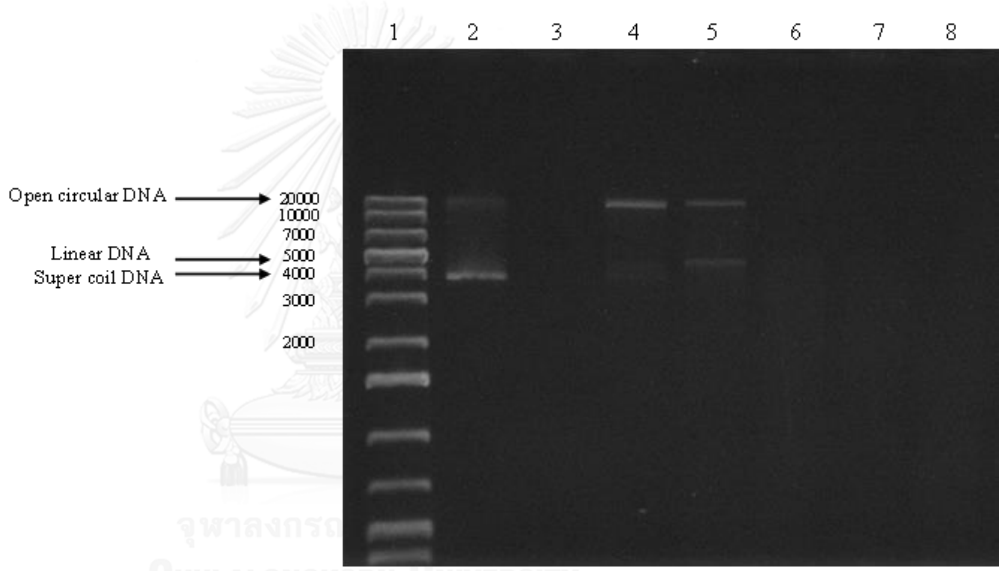


Figure 4.2 Protective effect of the purified peptide against hydroxyl radical-induced DNA damage of pBR322 plasmid DNA. Lane 1: marker 1 kbp, Lane 2: pBR322 plasmid DNA 4,361 bp, Lane 3: $FeSO_4$ and H_2O_2 treatment (as DNA damage control), Lane 4-8: pBR322 plasmid DNA with $FeSO_4$ and H_2O_2 treatment in the presence of purified peptide at concentrations of 0.0536, 0.0268, 0.0134, 0.0067 and 0.0034 mg/mL.

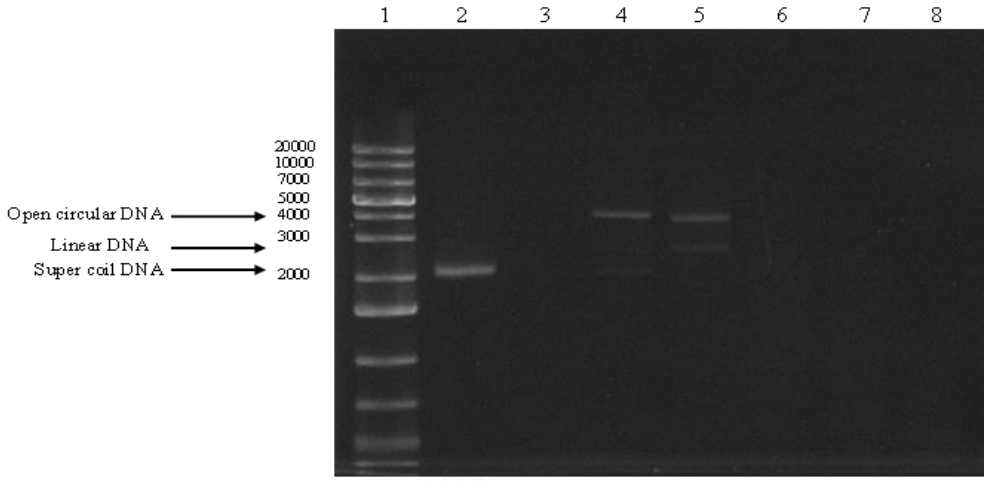


Figure 4.3 Agarose gel electrophoresis patterns of pUC19 plasmid DNA broken by hydroxyl radicals generated from the Fenton reaction with the pUC19 plasmid DNA receiving the protective effect of the antioxidant purified peptide of $F5_1$ fraction at concentrations of 0.0536, 0.0268, 0.0134, 0.0067 and 0.0034 mg/mL on hydroxyl radical-induced oxidation of pUC19 plasmid DNA. Lane 1: marker 1 kbp; Lane 2: pUC19 plasmid DNA 2,686 bp (DNA = 45.5 ng/µL and protein content = 0.068 mg/mL); Lane 3: pUC19 plasmid DNA with $FeSO_4$ and H_2O_2 treatment (as DNA damage control); Lane 4-8: pUC19 plasmid DNA with $FeSO_4$ and H_2O_2 treatment in the presence of the $F5_1$ fraction

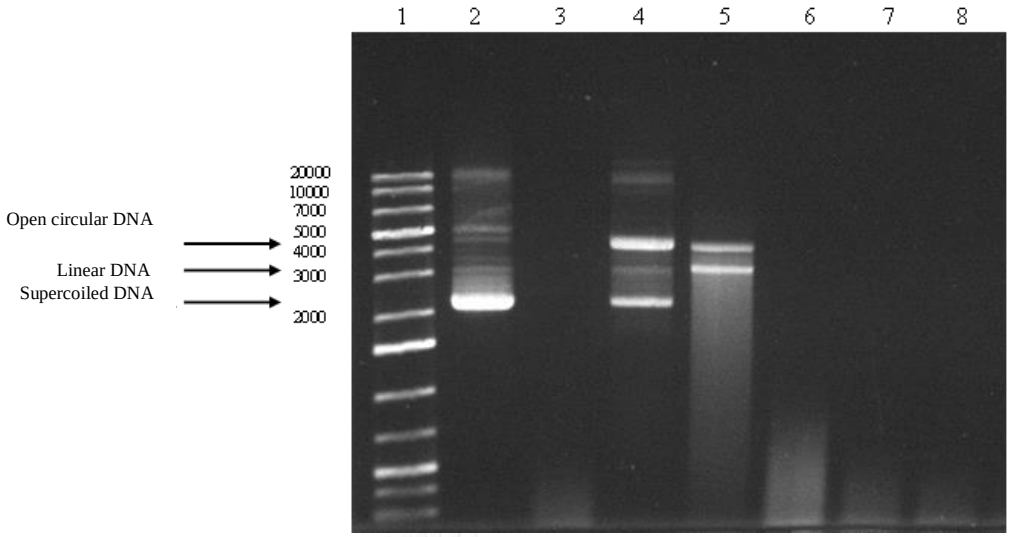


Figure 4.4 Protective effect of the purified peptide $F5_1$ against hydroxyl radical-induced DNA damage of pKS plasmid DNA. Lane 1: marker 1 kbp, Lane 2: pKS plasmid DNA 2,958 bp, Lane 3: $FeSO_4$ and H_2O_2 treatment (as DNA damage control), Lane 4-8: pKS plasmid DNA with $FeSO_4$ and H_2O_2 treatment in the presence of purified peptide at concentrations of 0.0536, 0.0268, 0.0134, 0.0067 and 0.0034 mg/mL.

4.6 Antiproliferative activity

The antiproliferative activity from purified peptide $F5_1$ could be determined using an MTT assay to assess the cellular cytotoxicity caused by any protein hydrolysates to decrease the growth rate of cancer cell lines and screen for anticancer activity. In this research, WI-38 was used as a human normal fibroblast cell line and five human cancer cell lines including Hep-G2, SW620, BT474, Chago-k1 and KATO-III were treated with purified peptide fraction $F5_1$ for 24, 48 and 72 hours. The result of MTT assay were shown in Table 4.5. The purified peptide fraction $F5_1$ exhibited the highest antiproliferative activity on Chago-k1 at IC_{50} values of 1.56±0.19 µg/mL. In addition, Hep-G2, SW620, BT474 and KATO-III had IC_{50} values of 4.05 ± 0.38,

5.76±0.52, 1.96±0.20 and 2.84±0.19µg/mL, respectively. Thus, Chago-k1 cell line was selected for determine apoptosis.

Table 4.5 The antiproliferative activity of the purified peptide ($F5_1$) using MTT assay

Cells	IC_{50} (µg/mL)
Breast cancer (BT474)	1.96 ± 0.20^{a}
Lung cancer (Chago-k1)	1.56 ± 0.19^{a}
Liver cancer (Hep-G2)	4.05 ± 0.38^{c}
Gastric cancer (KATO-III)	2.84 ± 0.19^{b}
Colon cancer (SW620)	5.76 ± 0.52^{d}
Normal cell (Wi-38)	2.49 ± 0.07^{b}

*Values shown are the mean ± standard deviation of triplicate determinations and separation within column by Duncan's new multiple range test at $p \leq 0.05$

4.7 Apoptosis analysis

4.7.1 Apoptosis by flow cytometer

The purified peptide fraction $F5_1$ (protein content was 42.58 µg/mL) from gel filtration chromatography that determined by MTT assay had the highest antiproliferative activity on the human lung cancer cell line Chago k-1 was used to induce apoptotic cells. The morphology of Chago k-1 cells was shown in Figure 4.5. Furthermore, the purified peptide fraction $F5_1$ was treated with Chago-k1 cells for 24, 48 and 72 hours. At 24 hours the purified peptide fraction $F5_1$ could induce early apoptotic cells, late apoptotic cells and necrotic cells at 21.85±0.74, 17.69±2.25 and 0.05±0.06%, respectively. At 48 hours, the purified peptide fraction $F5_1$ could induce early apoptotic cells, late apoptotic cells and necrotic cells at 14.12±0.30, 21.65±1.14 and 0.11±0.03%, respectively. At 72 hours, the purified peptide fraction $F5_1$ could induce early apoptotic cells, late apoptotic cells and necrotic cells at 32.75±1.86, 21.33±1.09 and 0.04±0.01%, respectively. The results of apoptosis using flow cytometer as show in Figure 4.6-4.9 and bar graph in Figure 4.10, Living cell was normal cell that not stained by Annexin V and PI. Meanwhile, early apoptotic cell was

only stained with Annexin V due to phospholipids called phosphatidylserines flipped to the outer leaflet of a cell membrane and binds with Annexin V. In addition, late apoptotic cell was stained with both Annexin V and PI while necrotic cell was only stained with PI because of their membranes was ruptured.

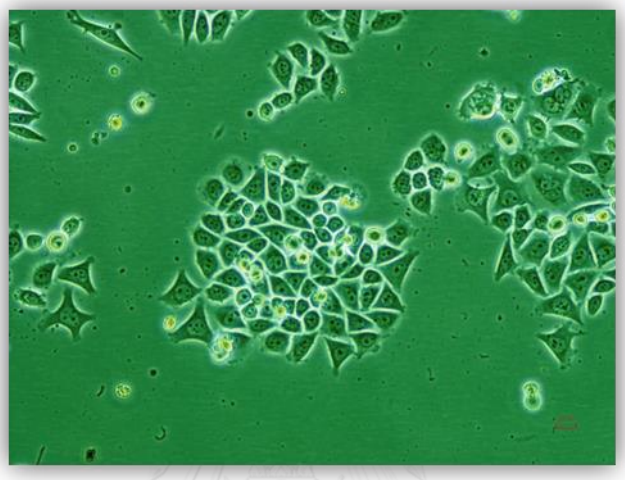


Figure 4.5 Morphology of Chago-k1 cell line cultures incubated at 37 ºC with 5% CO_2 in CO_2 incubator at actual magnification 20x.

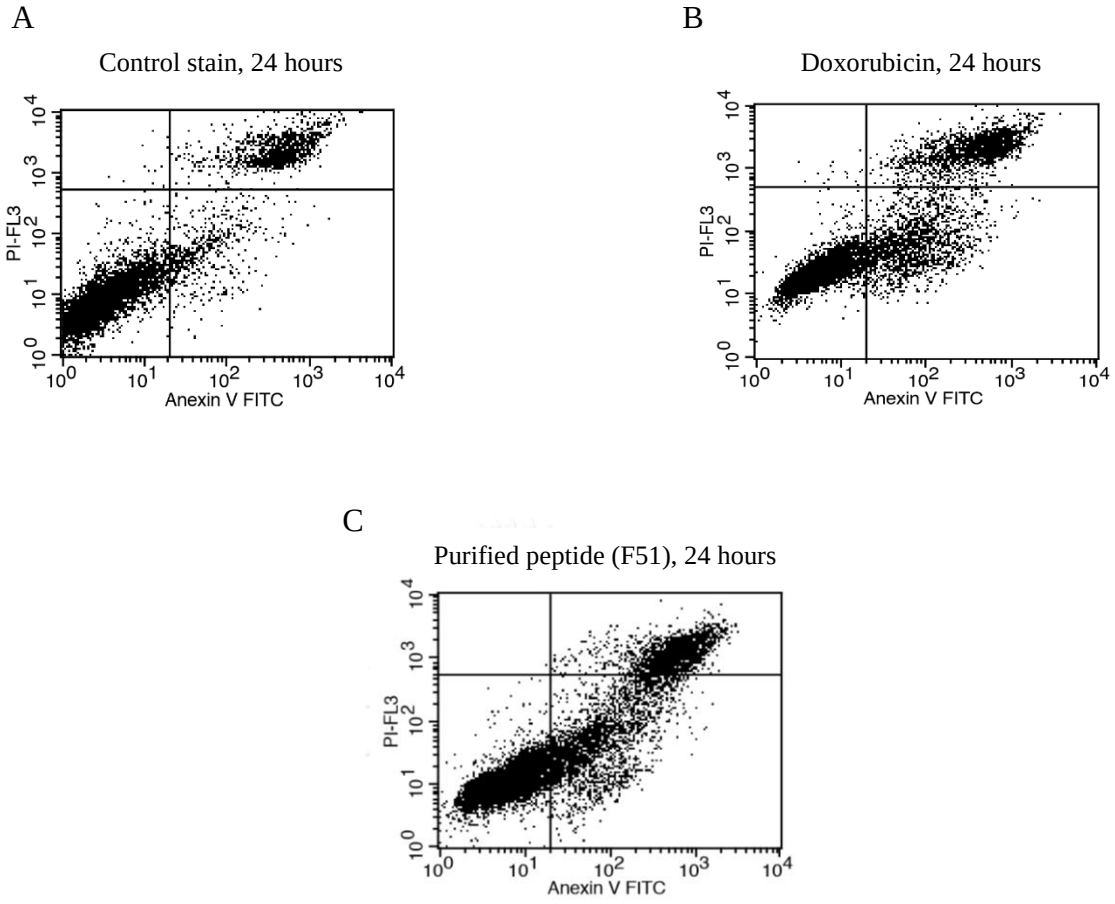


Figure 4.6 Flow cytometry analysis of Chago-k1 cells treated at 24 hours with (A) RPMI+10% FCS labeled with Annexin V-FITC and PI (negative control) (B) 0.5 µg/mL doxorubicin labeled with Annexin V-FITC and PI (positive control) (C) the purified peptide fraction $F5_1$(protein content was 42.58 µg/mL) labeled with Annexin V-FITC and PI, Quadrants: Lower left was the normal or live cells; Lower right was the early apoptotic cells; Upper left was the necrotic cells; Upper right was the late apoptotic cells.

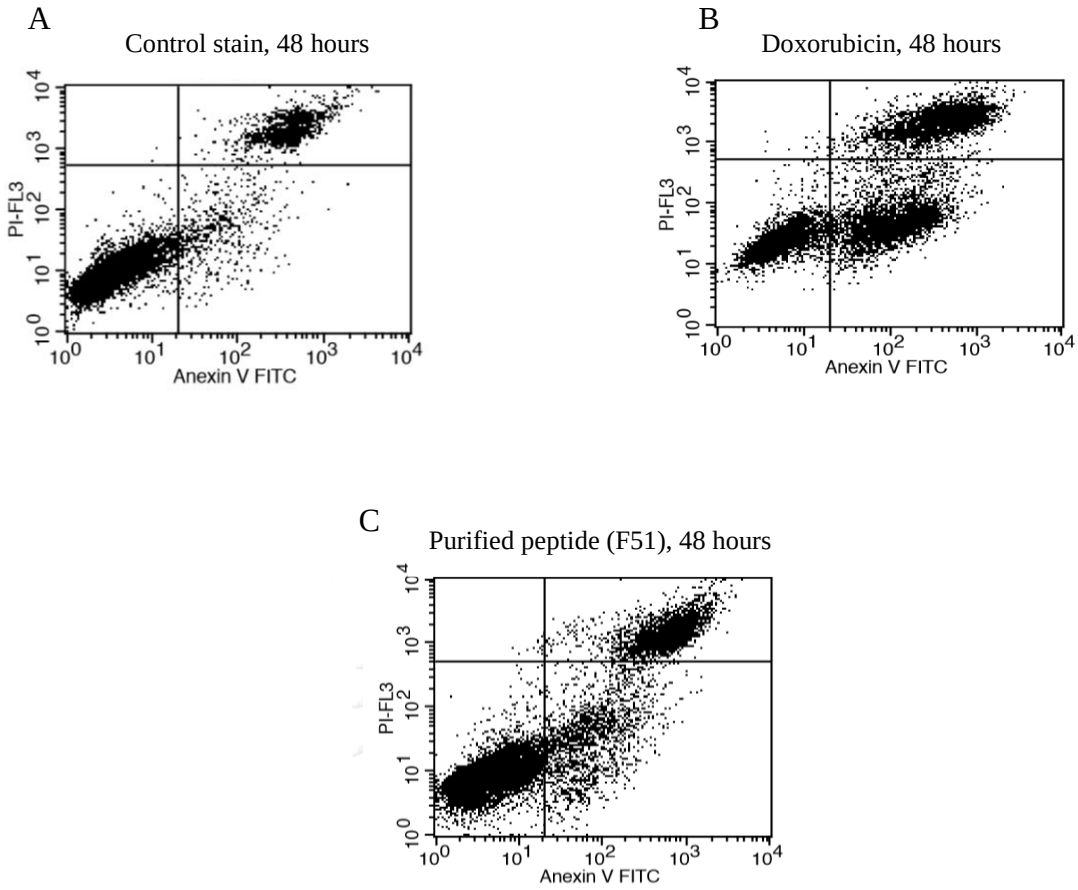


Figure 4.7 Flow cytometry analysis of Chago-k1 cells treated at 48 hours with (A) RPMI+10% FCS labeled with Annexin V-FITC and PI (negative control) (B) 0.5 µg/mL doxorubicin labeled with Annexin V-FITC and PI (positive control) (C) the purified peptide fraction $F5_1$(protein content was 42.58 µg/mL) labeled with Annexin V-FITC and PI, Quadrants: Lower left was the normal or live cells; Lower right was the early apoptotic cells; Upper left was the necrotic cells; Upper right was the late apoptotic cells.

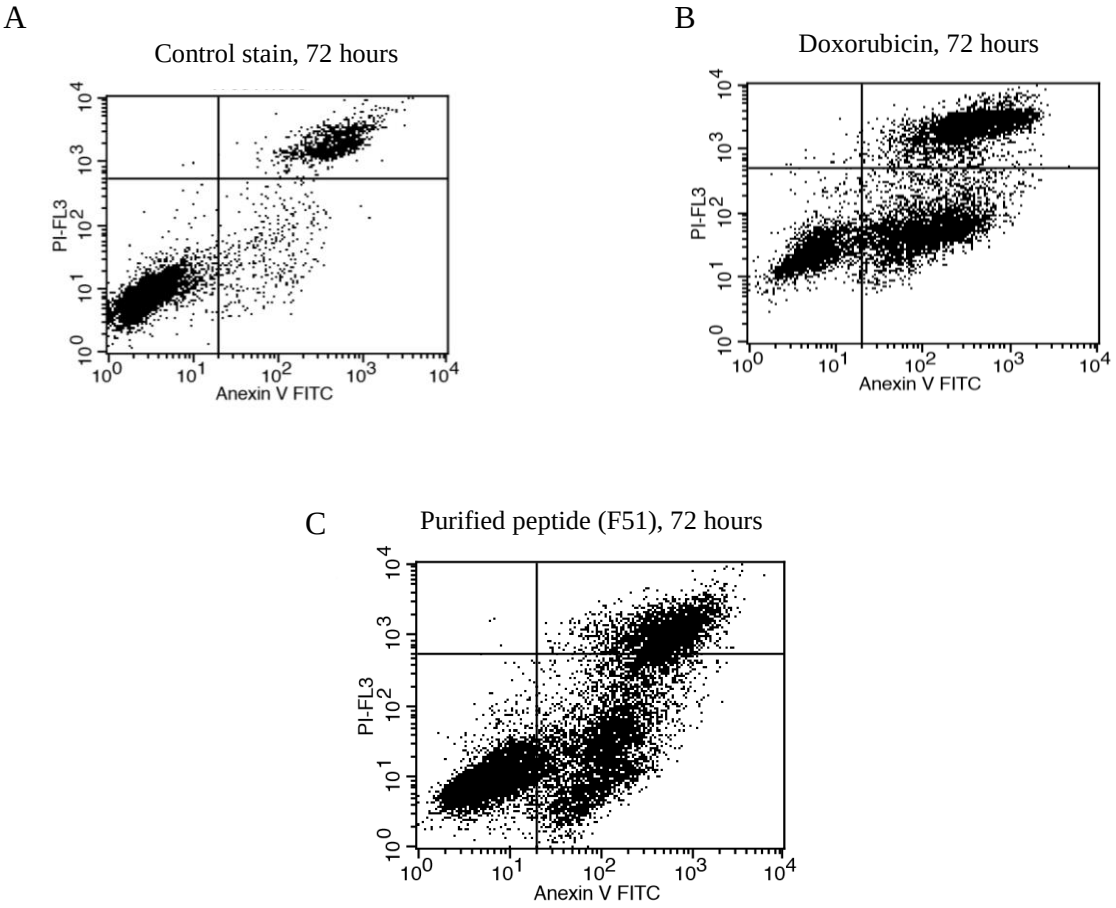
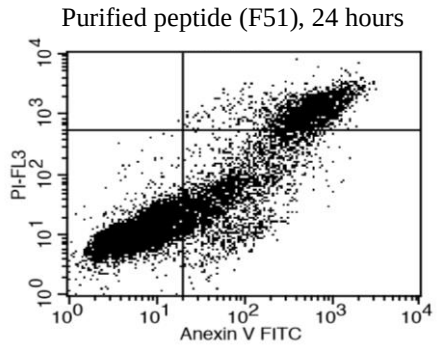
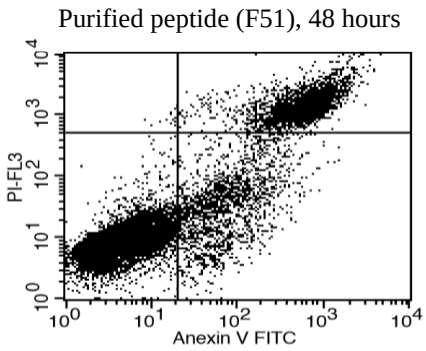


Figure 4.8 Flow cytometry analysis of Chago-k1 cells treated at 72 hours with (A) RPMI+10% FCS labeled with Annexin V-FITC and PI (negative control) (B) 0.5 µg/mL doxorubicin labeled with Annexin V-FITC and PI (positive control) (C) the purified peptide fraction $F5_1$(protein content was 42.58 µg/mL) labeled with Annexin V-FITC and PI, Quadrants: Lower left was the normal or live cells; Lower right was the early apoptotic cells; Upper left was the necrotic cells; Upper right was the late apoptotic cells.

A



B



C

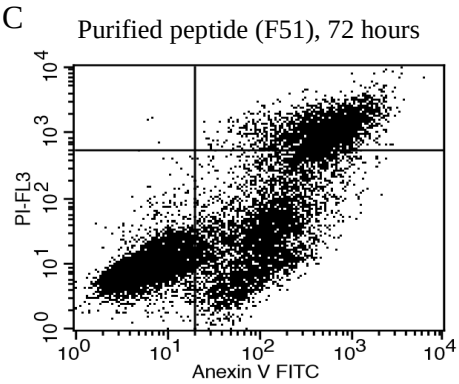


Figure 4.9 Flow cytometry analysis of Chago-k1 cells treated with purified peptide fraction $F5_1$ (protein content was 42.58 µg/mL) labeled with Annexin V-FITC and PI at 24 (A), 48 (B) and 72 (C) hours, Quadrants: Lower left was the normal or live cells; Lower right was the early apoptotic cells; Upper left was the necrotic cells; Upper right was the late apoptotic cells.

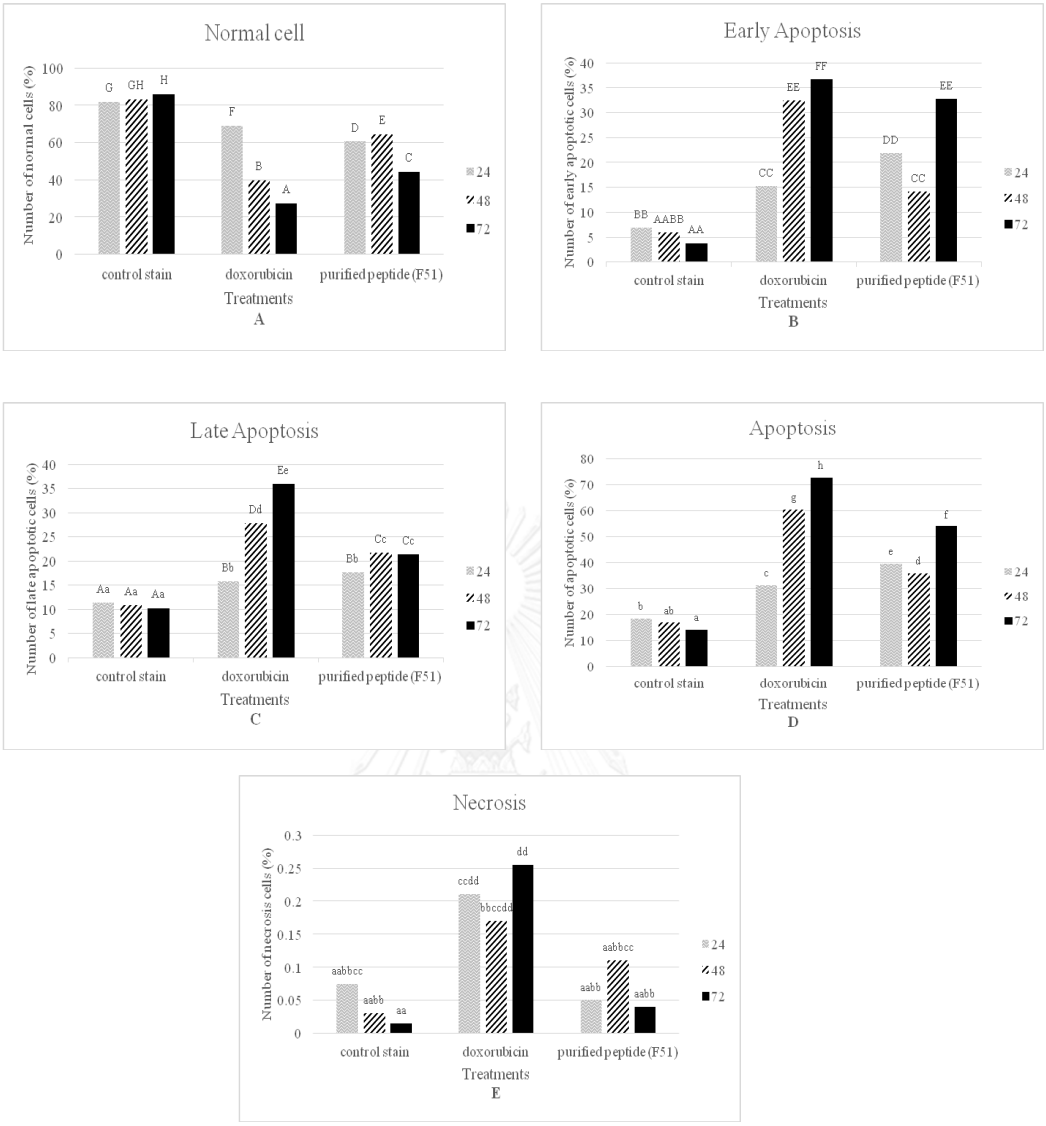


Figure 4.10 The number of Chago-k1 cells (%) that treated with RPMI+10% FCS labeled with Annexin V-FITC and PI (negative control), 0.5 µg/mL doxorubicin labeled with Annexin V-FITC and PI (positive control) and the purified peptide fraction $F5_1$(protein content was 42.58 µg/mL) for 24, 48 and 72 hours determined by flow cytometer which compared in bar graph (A) normal cells, (B) early apoptotic cells, (C) late apoptotic cells, (D) apoptosis cells and (E) necrotic cells. All data shown are the mean ± standard deviation of triplicate determinations and separate by Duncan's new multiple range test at $p \leq 0.05$.

4.7.2 Caspase 3, 8 and 9 activity assay

The purified peptide fraction $F5_1$ (protein content was 42.58 µg/mL) from gel filtration chromatography was selected for analyzed caspases 3, 8 and 9 activities in Chago-k1 cell lysates for 24, 48 and 72 hours using caspase colorimetric assay kit. As the results in Table 4.6, the values of caspase 3 activity from purified peptide fraction $F5_1$ (protein content was 42.58 µg/mL) at 24, 48 and 72 hours were $5.25\times10^{-2} \pm 3.80\times10^{-3}$, $3.91\times10^{-2} \pm 2.36\times10^{-3}$, $6.59\times10^{-2} \pm 2.51\times10^{-2}$ µmole pNA/min/mL, respectively. The results indicated that the purified peptide fraction $F5_1$ at 72 hours had the highest caspase 3 activity. Moreover, the values of caspase 8 activity from purified peptide fraction $F5_1$ (protein content was 42.58 µg/mL) at 24, 48 and 72 hours were $6.44\times10^{-3} \pm 8.58\times10^{-4}$, $6.35\times10^{-3} \pm 6.47\times10^{-4}$, $9.61\times10^{-3} \pm 7.47\times10^{-4}$ µmole pNA/min/mL, respectively. The results indicated that the purified peptide fraction $F5_1$ at 72 hours had the highest caspase 8 activity. In addition, the values of caspase 9 activity from purified peptide fraction $F5_1$ (protein content was 42.58 µg/mL) at 24, 48 and 72 hours were $4.55\times10^{-4} \pm 1.25\times10^{-5}$, $3.83\times10^{-4} \pm 1.45\times10^{-5}$, $5.79\times10^{-4} \pm 1.98\times10^{-5}$ µmole pNA/min/mL, respectively. The results indicated that the purified peptide fraction $F5_1$ at 72 hours had the highest caspase 9 activity. In the same way, the other researcher reported that human lung cancer cells (H460) with peptides extracted from *L. squarrosulus* results in a significant depletion of c-FLIP, an inhibitor of death receptor-activated caspase cascade which was associated with the augmentation of cleaved-caspase-8. The result of the activity of caspase-8 was little higher than those of caspase-9. However, these results conclude that both intrinsic and extrinsic pathways contribute to peptides extracted from *L. squarrosulus* induced apoptosis (Prateep et al., 2017).

Table 4.6 Caspase 3 and 8 activities of the purified peptide fraction $F5_1$ (protein content was 42.58 µg/mL)

Treatments	Caspase 3 activity (µmole pNA/min/mL)	Caspase 8 activity (µmole pNA/min/mL)
Peptide ($F5_1$) for 0 hour (control)	$9.06\times10^{-4} \pm 1.37\times10^{-3a}$	$2.20\times10^{-3} \pm 2.44\times10^{-4A}$
Peptide ($F5_1$) for 24 hours	$5.25\times10^{-2} \pm 3.80\times10^{3bc}$	$6.44\times10^{-3} \pm 8.58\times10^{-4B}$
Peptide ($F5_1$) for 48 hours	$3.91\times10^{-2} \pm 2.36\times10^{-3b}$	$6.35\times10^{-3} \pm 6.47\times10^{-4B}$
Peptide ($F5_1$) for 72 hours	$6.59\times10^{-2} \pm 2.51\times10^{-2c}$	$9.61\times10^{-3} \pm 7.47\times10^{-4C}$

*$^{a-c, A-C}$ values shown are the mean ± standard deviation of triplicate determinations and separation within column by Duncan's new multiple range test at $p \leq 0.05$

Table 4.7 Caspase 9 activity of the purified peptide fraction $F5_1$ (protein content was 42.58 µg/mL)

Treatments	Caspase 9 activity (µmole pNA/min/mL)
Peptide ($F5_1$) for 0 hour (control)	$2.79\times10^{-4} \pm 5.31\times10^{-6a}$
Peptide ($F5_1$) for 24 hours	$4.55\times10^{-4} \pm 1.25\times10^{-5c}$
Peptide ($F5_1$) for 48 hours	$3.83\times10^{-4} \pm 1.45\times10^{-5b}$
Peptide ($F5_1$) for 72 hours	$5.79\times10^{-4} \pm 1.98\times10^{-5d}$

*Values shown are the mean ± standard deviation of triplicate determinations and separation within column by Duncan's new multiple range test at $p \leq 0.05$

4.8 RP-HPLC

The purified peptide fraction $F5_1$ (protein content was 42.58 µg/mL) from gel filtration chromatography was selected for HPLC analysis and purification of peptides. The results showed the RP-HPLC profile that had five sub-fraction consist of $F5_1$-1, $F5_1$-2, $F5_1$-3, $F5_1$-4 and $F5_1$-5 in Figure 4.11. And then determined the antioxidant activity by DPPH and ABTS radical scavenging activities assay. The results of antioxidant activity as shown in Table 4.8, for DPPH assay, percent inhibition of sub-fractions $F5_1$-1, $F5_1$-2, $F5_1$-3, $F5_1$-4 and $F5_1$-5 were 79.59 ± 4.24, 154.10 ± 5.94, 196.30 ± 1.84, 64.60 ± 5.73 and 69.21 ± 3.97 µg/mL, respectively. For ABTS assay, percent

inhibition of sub-fractions $F5_1$-1, $F5_1$-2, $F5_1$-3, $F5_1$-4 and $F5_1$-5 were 98.11 ± 9.75, 182.55 ± 5.44, 261.85 ± 5.73, 105.36 ± 8.55 and 106.75 ± 3.61 µg/mL, respectively. In addition, protein contents of sub-fractions $F5_1$-1, $F5_1$-2, $F5_1$-3, $F5_1$-4 and $F5_1$-5 were 7.69, 10.87, 13.60, 9.30 and 11.63 µg/mL, respectively. In this results, the value of antioxidant from purified peptide sub fraction $F5_1$-1 to $F5_1$-5 had decreased because of low protein content in each sub fraction.

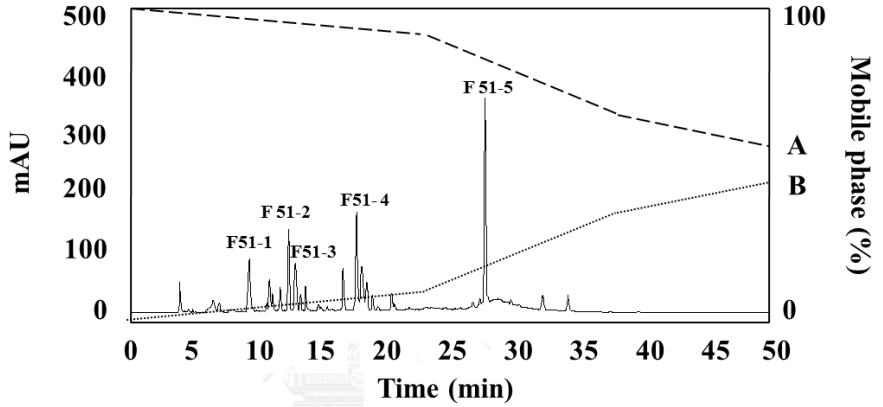


Figure 4.11 RP-HPLC profile of purified peptide fraction $F5_1$ from gel filtration chromatography

Table 4.8 Percent inhibition of DPPH and ABTS radical scavenging activities of purified peptide fractions $F5_1$-1 to $F5_1$-5 separated by RP-HPLC

Fraction	Percent inhibition (%)	
	DPPH	ABTS
F51-1	79.59 ± 4.24^{c}	98.11 ± 9.75^{A}
F51-2	154.10 ± 5.94^{d}	182.55 ± 5.44^{C}
F51-3	196.30 ± 1.84^{e}	261.85 ± 5.73^{D}
F51-4	64.60 ± 5.73^{b}	105.36 ± 8.55^{A}
F51-5	69.21 ± 3.97^{b}	106.75 ± 3.61^{A}
Ascorbic acid	33.27 ± 0.32^{a}	147.73 ± 0.49^{B}

∗$^{a\text{-}e,\ A\text{-}D}$ values shown are the mean ± standard deviation of triplicate determinations and separation within column by Duncan's new multiple range test at $p \leq 0.05$

4.9 Mass spectrometry

The purified peptide sub-fraction $F5_1$-1 to $F5_1$-5 from RP-HPLC were identified molecular mass and characterized using MS/MS (ESI-Q-TOF). The results that showed in Table 4.9, amino acid sequences of sub-fraction $F5_1$-1, $F5_1$-4 and $F5_1$-5 were analyzed using *de novo* peptide sequencing as Val-Thr-Thr-His-Lys-Thr-Val-Thr-Lys-His (VTTHKTVTKH, 1152 Da), Thr-Leu-Ser-Asn-Leu-Gly-Leu-Val-Leu-Val-His (TLSNLGLVLVH, 1165 Da) and Leu-Thr-Asn-Ser-Pro-Trp-Ala-His-Ala (LTNSPWAHA, 996 Da), respectively. On the other hand, fraction $F5_1$-2 and $F5_1$-3 were analyzed using Mascot software because it could not be analyzed by *de novo* peptide sequencing. After that, fraction $F5_1$-2 and $F5_1$-3 were searched by BLAST database of NCBI as Met-Pro-His-His-Tyr-Thr-Ile-Phe-Ala-Leu-Gly-Thr-Gln-Ser-Arg-Pro-Ser (MPHHYTIFALGTQSRPS, 1942 Da) and Pro-Ser-Arg-Gly-Ser-Glu-Arg-Ala-Arg-Ala (PSRGSERARA, 1086 Da), respectively. The results indicated that amino acid compositions and peptide sequences also influence the antioxidant activity of a peptide. The sequences of purified peptide fractions $F5_1$-1 to $F5_1$-5 had amino acid such as His, Phe, Tyr, Trp, Val and Pro it mean that their sequences had high antioxidant activity. Moreover, many researcher had reported that antioxidant peptides composed of many hydrophobic amino acids with non-polar aliphatic groups packing with Val (V), Pro (P), Tyr (Y), Trp (W), Leu (L), Ile (I), Ala (A), Lys (K) and Met (M) have high antioxidant activity (Girjal et al., 2012).

Table 4.9 Identification of purified peptides fraction $F5_1$-1 to $F5_1$-5 by ESI-Q-TOF mass spectrometry

Fractions	Sequence	Organism	Mass (Da)	Accession number
$F5_1$-1	VTTHKT-VTKH	hypothetical protein PSTG_14153 [Puccinia striiformis f. sp. tritici PST-78]	1152	KNE92432.1
De novo		hypothetical protein VP01_1143g1 [Puccinia sorghi]	1152	KNZ63440.1
		hypothetical protein PTTG_08683 [Puccinia triticina 1-1 BBBD Race 1]	1152	OAV88127.1
		hypothetical protein PGTG_03382 [Puccinia graminis f. sp. tritici CRL 75-36-700-3]	1152	XP_003321845.2
		related to RAD54-DNA-dependent ATPase of the Snf2p family [Ustilago bromivora]	1152	SAM84384.1

Fractions	Sequence	Organism	Mass (Da)	Accession number
$F5_1$-2	MPHHY- TIFALG- TQSRPS	hypothetical protein WG66_7774 [Moniliophthora roreri]	1942	KTB39648.1
Mascot		hypothetical protein WG66_11463 [Moniliophthora roreri]	1942	KTB35960.1
		hypothetical protein WG66_900 [Moniliophthora roreri]	1942	KTB46525.1
		hypothetical protein WG66_10729 [Moniliophthora roreri]	1942	KTB36694.1

Fractions	Sequence	Organism	Mass (Da)	Accession number
		hypothetical protein PHLCEN_12122 [Phlebia centrifuga]	1942	OKY67860.1
$F5_1$-3	PSRGSE-RARA	Prefoldin domain containing protein [Rhodotorula toruloides NP11]	1086	XP_016274245.1
Mascot		hypothetical protein M407DRAFT_192181 [Tulasnella calospora MUT 4182]	1086	KIO33436.1
		hypothetical protein AURDEDRAFT_175666 [Auricularia subglabra TFB-10046 SS5]	1086	XP_007356642.1

Fractions	Sequence	Organism	Mass (Da)	Accession number
		hypothetical protein EXIGLDRAFT_155417 [Exidia glandulosa HHB12029]	1086	KZW03682.1
		hypothetical protein PHLGIDRAFT_128602 [Phlebiopsis gigantea 11061_1 CR5-6]	1086	KIP05879.1
$F5_1$-4	TLSNLG LVLVH	quinidine resistance protein-like protein [Coprinellus disseminatus]	1165	AAZ14930.1
De novo		hypothetical protein PUNSTDRAFT_133899 [Punctularia strigosozonata HHB-11173 SS5]	1165	XP_007383410.1

Fractions	Sequence	Organism	Mass (Da)	Accession number
		hypothetical protein A1Q1_04148 [Trichosporon asahii var. asahii CBS 2479]	1165	XP_014178000.1
		hypothetical protein A1Q2_02055 [Trichosporon asahii var. asahii CBS 8904]	1165	EKD03638.1
		ATPase subunit 6, partial (mitochondrion) [Bondarzewia guaitecasensis]	1165	AGO64392.1

Fractions	Sequence	Organism	Mass (Da)	Accession number
$F5_1$-5	LTNSPW-AHA	ras GEF [Lentinula edodes]	996	GAW03078.1
De novo		hypothetical protein HYPSUDRAFT_142836 [Hypholoma sublateritium FD-334 SS-4]	996	KJA20077.1
		hypothetical protein A4X03_g6434 [Tilletia caries]	996	OAJ13372.1
		OPT superfamily oligopeptide transporter [Auricularia subglabra TFB-10046 SS5]	996	XP_007342842.1
		related to MAP kinase pathway-interacting protein [Serendipita indica DSM 11827]	996	CCA68748.1

CHAPTER V

CONCLUSION

From the results in this study, the peptide hydrolysates derived from *A. hygrometricus* using 1% Alcalase® was exhibited strong free radical scavenging effects on the DPPH and ABTS radical method. The peptide hydrolysates with 1% Alcalase® was separated using ultrafiltration, the results shown that the smallest molecular size of peptide hydrolysate (MW < 0.65 kDa; F5) could have the highest antioxidant activities. The peptide hydrolysate fraction F5 was purified using gel filtration chromatography, the results showed that fraction $F5_1$ was the most active peptide than the others. The fraction $F5_1$ could protect plasmid DNA (pBR322, pUC19 and pKS) against hydroxyl radical-induced DNA damage. In addition, fraction $F5_1$ was used for determine the antiproliferative activity by MTT assay on normal and cancer cell lines. The results showed that purified peptide fraction $F5_1$ was not cytotoxic on normal and cancer cell lines and the lung cancer cell line (Chago-k1 cell), and had the highest antiproliferative activity. Moreover, fraction $F5_1$ was used for determine the induction of apoptosis that used flow cytometry and the caspase 3, 8 and 9 activities analysis method. Caspase 3 and 8 activities were reveal about the extrinsic death receptor apoptotic pathway caspase 9 activity was used to confirm about the ability of purified peptide fraction $F5_1$ with extrinsic death receptor and intrinsic mitochondrial apoptosis pathways for inducing apoptotic cells. The results indicated that purified peptide fraction $F5_1$ could induce the highest apoptotic cells and caspase 3, 8 and 9 activities on Chago-k1 cell at 72 hours. Furthermore, the peptide fraction $F5_1$ was purified using RP-HPLC and collected sub-fraction $F5_1$-1 to $F5_1$-5. The result was found to possess the highest antioxidant activity on fraction $F5_1$-5. Last, sub-fraction $F5_1$-1 to $F5_1$-5 was identify

amino acid sequence using MS/MS (ESI-Q-TOF). The results showed that amino acid compositions on peptide sequences. The sequences of purified peptide fractions $F5_1$-1 to $F5_1$-5 had amino acid such as His, Phe, Tyr, Trp, Val and Pro in their sequences which important in the antioxidant activity. The results indicated that peptides derived from *A. hygrometricus* by enzymatic hydrolysis could be a nutraceuticals and potential natural antioxidant for drug development.

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APPENDIX

APPENDIX A

Buffer and reagents preparation

1. 20 mM Phosphate buffer, pH 7.2 (1000 mL)

20 mM KH_2PO_4	2.721 g
20 mM K_2HPO_4	3.484 g
150 mM NaCl	8.766 g
Deionized water	1,000 mL

Protocal: Mix 1000 mL of deionized water with all reagents. Adjust the pH to 7.2 by 5M KOH or HCl.

2. Bradford solution and protocol

2.1 Bradford stock solution

95% Ethanol	100 mL
88% Phosphoric acid	200 mL
Serva Blue G	350 mg

2.2 Bradford working buffer (500 mL)

Bradford stock solution	30 mL
Deionized water	425 mL
95% Ethanol	15 mL
88% Phosphoric acid	30 mL

Note: Before using, Bradford working buffer must be filtered through the Whatman No.1 paper. Bradford working buffer is kept in a brown glass bottle at room temperature.

Bradford's protocol: 1. Pipette 60 µL of sample into 1.5 mL microtube.

2. Add 600 µL of Bradford working buffer and mixed this solution.

3. Pipette 200 µL of this solution into 96-well plates.

4. Shake and read an absorbance at 595 nm

3. DPPH solution and protocol (100 mL)

100 µM DPPH (MW = 394.32)	0.004 g
Methanol	100 mL

DPPH protocol: 1. Pipette 80 µL of sample into 1.5 mL microtube.

2. Add 320 µL of DPPH solution and mix this solution and then incubate in the dark at room temperature for 10 minutes.

3. Centrifuge at 1,300 rpm for 5 min.

4. Pipette 100 µL of this solution into 96-well plates.

5. Shake and read an absorbance at 517 nm.

4. ABTS solution and protocol

4.1 solution A: 7 mM ABTS (MW = 548.68)

Dissolve 0.096 g ABTS in 25 mL of deionized water.

4.2 solution B: 2.45 mM potassium persulfate (MW = 270.32)

Dissolve 0.0166 g potassium persulfate in 25 mL of deionized water.

4.3 ABTS solution

Mix solution A and solution B in the dark at room temperature for 12 hours before using and then dilute ABTS solution with distilled water to obtain an absorbance value of 0.7±0.02 at 734 nm.

ABTS protocol: 1. Pipette 25 µL of sample into 1.5 mL microtube.

2. Add 750 µL of ABTS solution and mix this solution and then incubate for 15 min in the dark at room temperature.

3. Pipette 200 µL of this solution into 96-well plates.

4. Shake and read an absorbance at 734 nm.

5. LB agar for *E.coli*

Peptone	1 g
Yeast extract	0.5 g
NaCl	1 g
Agar powder	2 g

Protocol: Mix all reagents with 100 mL of deionized water and sterile at 121°C for 15 min.

6. LB broth for *E.coli*

Peptone	1 g
Yeast extract	0.5 g
NaCl	1 g

Protocol: Mix all reagents with 100 mL of deionized water and sterile at 121°C for 15 minutes.

7. DNA damage

7.1 2 mM $FeSO_4$

Dissolve FeSO4·7H2O 0.0278 g in 50 mL of deionized water.

7.2 30% H_2O_2

Protocol: 1. Pipette 3 µL of DNA plasmid into PCR tube.

2. Add 4 µL of sample and incubate for 20 min at room temperature.

3. Add 3 µL of 2 mM $FeSO_4$.

4. Add 3 µL of 30% H_2O_2 and mix this solution and then incubate at 37ºC for 30 min.

5. Check DNA bands by 1% agarose gel electrophoresis.

8. MTT solution

5 mg/mL MTT solution

MTT	5 mg
Deionized water	1 mL

Protocol: Dissolve 5 mg MTT with 1 mL of deionized water.

9. Mobile phase in RP-HPLC analysis

9.1 Eluent B: 0.1% Trifluoroacetic acid (TFA), 1,000 mL

Add 1 mL of TFA into 999 mL of double deionized water followed by filtration using a cellulose acetate membrane.

9.2 Eluent C: 70% Acetronitrile containing 0.05% TFA, 500 mL

Step one, filtrate 350 mL of Acetronitrile followed by filtration using a PTFE membrane. Step two, add 150 mL of 0.05% TFA in double deionized water (add 75 µL of TFA into 150 mL of double deionized water) followed by filtration using a cellulose acetate membrane into 350 mL of acetronitrile was filtrated.

APPENDIX B

Amino acid abbreviations and structures

Amino acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid or aspartate	Asp	D
Cysteine	Cys	C
Glutamic acid or glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX C

Mass spectrum analysis

Amino acid sequence of purified peptide fraction F5$_1$-1: VTTHKTVTKH

Intensity coverage:	100.0% (26199911 cnts)
Sequence coverage MS:	0.0%
Sequence coverage MS/MS:	0.0%
pI (isoelectric point):	0.0
Parent mass:	1151.724
Mass error:	0.070
MH+ (mono):	1151.654
MH+ (avg):	1152.327
Threshold (a.i.):	0.000
Tolerance (Da):	0.500
Number of peaks:	18
Above Threshold:	18
Assigned peaks:	11
Not assigned peaks:	7

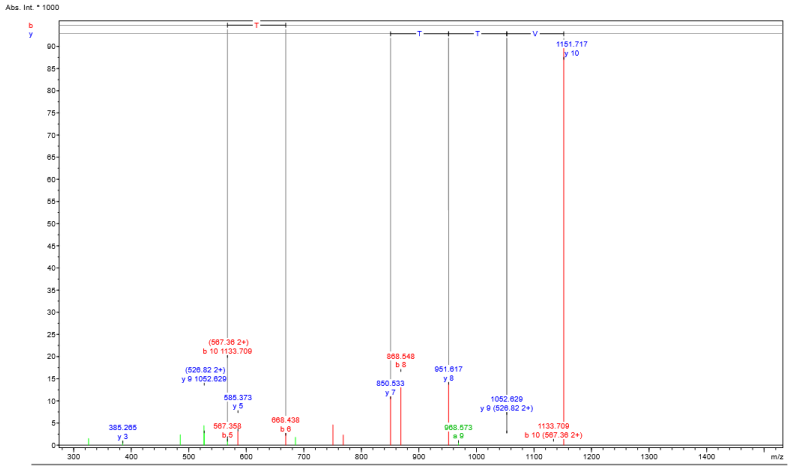

Figure C. 1 Identification of amino acid sequence and molecular mass of the purified peptide fraction F5$_1$-1 from RP-HPLC. MS/MS experiments were performed on ESI-Q-TOF mass spectrometer. Sequencing of antioxidant purified peptide was acquired over the m/z range 300-1400. Mass spectrum analysis of purified peptide fraction F5$_1$-1 as VTTHKTVTKH

Amino acid sequence of purified peptide fraction F5$_1$-4: TLSNLGLVLVH

Intensity coverage:	100.0% (32920251 cnts)
Sequence coverage MS:	0.0%
Sequence coverage MS/MS:	0.0%
pI (isoelectric point):	0.0
Parent mass:	1165.734
Mass error:	0.040
MH+ (mono):	1165.694
MH+ (avg):	1166.393
Threshold (a.i.):	0.000
Tolerance (Da):	0.500
Number of peaks:	19
Above Threshold:	19
Assigned peaks:	8
Not assigned peaks:	11

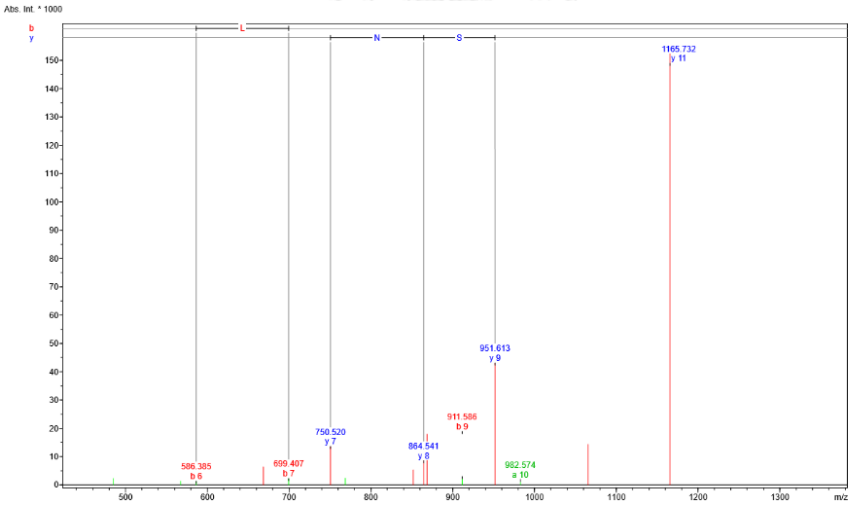

Figure C. 2 Identification of amino acid sequence and molecular mass of the purified peptide fraction F5$_1$-4 from RP-HPLC. MS/MS experiments were performed on ESI-Q-TOF mass spectrometer. Sequencing of antioxidant purified peptide was acquired over the m/z range 500-1300. Mass spectrum analysis of purified peptide fraction F5$_1$-4 as TLSNLGLVLVH

Amino acid sequence of purified peptide fraction F5$_1$-5: LTNSPWAHA

Intensity coverage:	100.0% (82842018 cnts)
Sequence coverage MS:	0.0%
Sequence coverage MS/MS:	0.0%
pI (isoelectric point):	0.0
Parent mass:	996.589
Mass error:	0.099
MH+ (mono):	996.490
MH+ (avg):	997.087
Threshold (a.i.):	0.000
Tolerance (Da):	0.500
Number of peaks:	15
Above Threshold:	15
Assigned peaks:	8
Not assigned peaks:	7

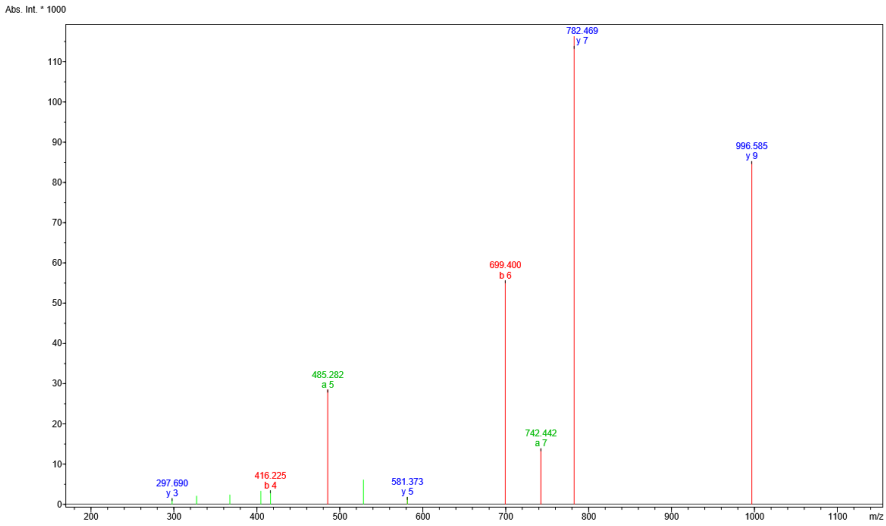

Figure C. 3 Identification of amino acid sequence and molecular mass of the purified peptide fraction F5$_1$-5 from RP-HPLC. MS/MS experiments were performed on ESI-Q-TOF mass spectrometer. Sequencing of antioxidant purified peptide was acquired over the m/z range 200-1100. Mass spectrum analysis of purified peptide fraction F5$_1$-5 as LTNSPWAHA

APPENDIX D

Standard curve for determine the protein concentration by Bradford method

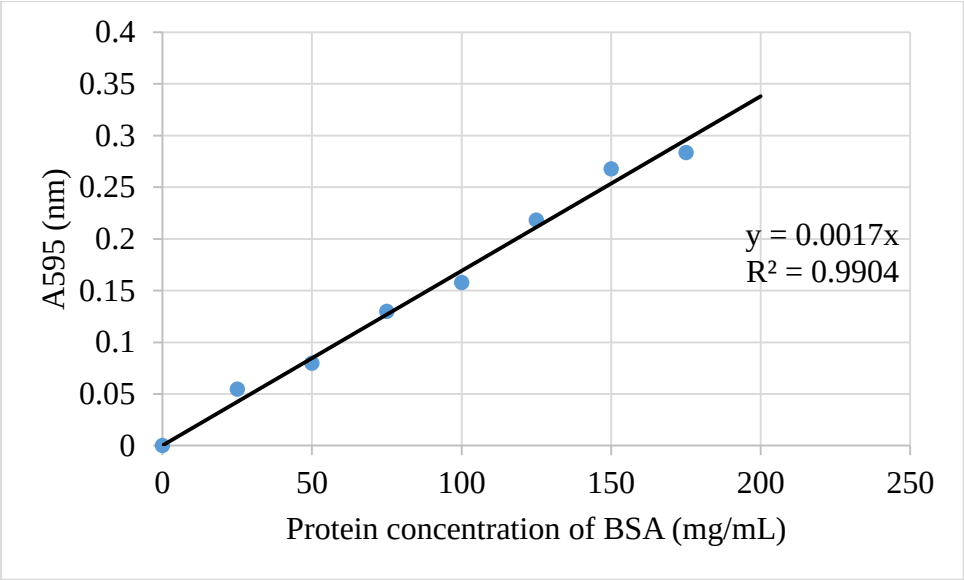

Figure D. 1 Standard curve of BSA concentration at the absorbance of 595 nm

VITA

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Academic presentations;

1. Tunyatarinun, N., Reamtong, O., Sangtanoo, P., Saisavoey, T. and Karnchanatat, A. 2017. Free radical scavenging properties and DNA damage protecting of peptide hydrolysate derived from edible mushroom Astraeus hygrometricus. The Pure and Applied Chemistry International Conference 2017 (PACCON2017): Green Convergence on Chemical Frontiers, February 2-3, 2017, Centra Government Complex Hotel and Convention Centre, Bangkok, Thailand.