ฤทธิ์ขจัดอนุมูลอิสระและยับยั้งการเพิ่มจำนวนเซลล์มะเร็งของเพปไทด์จากเห็ดหัวลิง (*Hericium erinaceus*)

นางสาวธนิยา สังข์ทิตนุ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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FREE RADICAL SCAVENGING AND ANTIPROLIFERATIVE ACTIVITIES OF PEPTIDE FROM MONKEY'S HEAD MUSHROOM (*Hericium erinaceus*)

Miss Taniya Sangtitanu

A Thesis Submitted in Partial Fulfillment of the Requirements
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ธนิยา สังข์ทิตนุ : ฤทธิ์ขจัดอนุมูลอิสระและยับยั้งการเพิ่มจำนวนเซลล์มะเร็งของเพปไทด์จากเห็ดหัวลิง (*Hericium erinaceus*) (FREE RADICAL SCAVENGING AND ANTIPROLIFERATIVE ACTIVITIES OF PEPTIDE FROM MONKEY'S HEAD MUSHROOM (*Hericium erinaceus*)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. อภิชาติ กาญจนทัต, 83 หน้า.

อนุมูลอิสระจัดได้ว่าเป็นหนึ่งสาเหตุสำคัญของโรคต่างๆในระบบของเซลล์ ทำให้เกิดภาวะเครียดเมื่อได้รับสารต้านอนุมูลอิสระไม่เพียงพอ จึงมีความจำเป็นที่จะได้รับสารต้านอนุมูลอิสระจากแหล่งอื่น ในการศึกษาครั้งนี้ได้ทำการเตรียมเพปไทด์จากเห็ดหัวลิงโดยทำการย่อยด้วยเอนไซม์โปรติเอส คือ แอลคาเลส ฟลาโวไซม์ และนิวเทรส ที่ความเข้มข้นร้อยละ 1 2 และ 5 และศึกษาความสามารถในการขจัดอนุมูลอิสระ ซึ่งพบว่าเพปไทด์จากเห็ดหัวลิงที่ถูกย่อยด้วยเอนไซม์แอลคาเลสที่ความเข้มข้นร้อยละ 1 มีความสามารถในการขจัดอนุมูลอิสระสูงที่สุดเมื่อทำการวิเคราะห์ด้วยวิธี DPPH ABTS และ NO radical scavenging activity จากนั้นทำการคัดแยกเพปไทด์ตามขนาดโมเลกุลด้วยเทคนิคอัลตราฟิลเตชั่น พบว่าเพปไทด์ที่มีขนาดโมเลกุลน้อยกว่า 0.65 กิโลดาลตัน สามารถขจัดอนุมูลอิสระได้ที่สุด จากนั้นนำเพปไทด์ที่มีขนาดนั้นน้อยกว่า 0.65 กิโลดาลตัน มาทำให้บริสุทธิ์ด้วยเทคนิคเจลฟิวเตรชันโครมาโตรกราฟี โดยสามารถแยกเพปไทด์ได้เป็นสองส่วน (F1 และ F2) พบว่าเพปไทด์ F1 มีความสามารถในการขจัดอนุมูลอิสระได้ดี จึงนำ F1 ที่ได้มาทำให้บริสุทธิ์และพิสูจน์เอกลักษณ์ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงและเทคนิคแมสสเปกโตเมตรี สามารถแยกเพปไทด์ได้ 4 ส่วน (F11, F12, F13 และ F14) จากนั้นนำเพปไทด์ F1 มาศึกษาความสามารถในการยับยั้งการเพิ่มจำนวนของเซลล์มะเร็ง โดยวิธี MTT ซึ่งทดสอบกับเซลล์มะเร็ง 5 ชนิด พบว่าเพปไทด์ F1 มีความสามารถในการยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งปอดได้ดีที่สุด นอกจากนั้นเพปไทด์ F1ถูกนำมาศึกษาความสามารถในการป้องกันไม่ให้ดีเอ็นเอถูกทำลายจากพลาสมิด 3 ชนิด ได้แก่ pBR322 pKS และ pUC19 ด้วยอนุมูลไฮดรอกซี พบว่าเพปไทด์ F1 นั้นมีความสามารถในการป้องกันไม่ให้ดีเอ็นเอถูกทำลายไป นอกจากนี้เพปไทด์ F1 มาทดสอบความสามารถเหนี่ยวนำให้เซลล์เกิดการตายแบบอะพอพโทซิสด้วยเทคนิคโฟลไซโทเมทรี และทำการศึกษาการเพิ่มขึ้นของการทำงานของเอนไซม์แคสแปส 3 8 และ 9 ที่เวลา 24 48 และ 72 ชั่วโมง อีกด้วย ผลงานวิจัยนี้สามารถชี้ให้เห็นถึงความสำคัญของเห็ดหัวลิงในการเป็นแหล่งของเพปไทด์ที่มีฤทธิ์ขจัดอนุมูลอิสระ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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TANIYA SANGTITANU: FREE RADICAL SCAVENGING AND ANTIPROLIFERATIVE ACTIVITIES OF PEPTIDE FROM MONKEY'S HEAD MUSHROOM (*Hericium erinaceus*). ADVISOR: ASSOC. PROF. APHICHART KARNCHANATAT, Ph.D., 83 pp.

Free radicals have been identified as a major trigger of various syndromes in living systems. Oxidative stress occurs when free radicals are excessive and antioxidants are insufficient, resulting in the necessary consumption of additional antioxidants. Peptide derived from monkey's head mushroom seeds was hydrolysed using various concentrations (1, 2.5, and 5%) of proteases (Alcalase, Favourzyme, and Neutrase), and the 1% Alcalase hydrolysate exhibited the highest radical scavenging activities (DPPH, ABTS, and NO assay) compared to other hydrolysates. After ultrafiltration, Mw < 0.65 kDa showed the strongest activity. Then, Mw < 0.65 kDa was purified using gel filtration chromatography and separated into two fractions (F1, and F2), of which fraction F1 exhibited the highest activity and this was further purified by reversed phase high performance liquid chromatography. Four fractions (F11, F12, F13, and F14) from RP-HPLC were isolated. Thus, four antioxidant peptides were identified by quadrupole time-of-flight (Q-TOF) mass spectrometer. The cytotoxicity activity of the fraction F1 was determined by MTT assay in five cell lines. The results showed that F1 exhibited greater inhibition against the proliferation of Chago-K1 cell lines. Moreover, the fraction F1 has the protective activity of the hydroxy radical-induced DNA damage as shown in pBR322, pKS and pUC19. The apoptosis of F1 was measured by an FITC Annexin V Apoptosis Detection Kit with PI using flow cytometry and caspase 3, 8 and 9 activities were determined in Chago-K1 cells for 24, 48, and 72 hours. The findings indicate that monkey's head mushroom is a source of antioxidant peptides. Consequently, this suggests the importance of monkey's head mushroom as a source of antioxidant peptides.

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CONTENTS

Page

Page

LIST OF TABLE

LIST OF FIGURES

CHAPTER I
INTRODUCTION

Human beings are increasingly concerned about health and beauty. Consequently, research has increasingly been conducted to find substances effective in promoting the good health of the body. Antioxidants have received widespread attention, especially research on free radicals and antioxidants. (Lobo et al., 2010). The generation of reactive oxygen species (ROS) or free radicals such as superoxide anion radicals (O_2^-), hydroxyl radical ($HO^•$), and hydrogen peroxide (H_2O_2) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress (Zima et al., 2001) (Hudson, 2004). These radicals lead to oxidative stress in the human body, which plays a major role in heart diseases, malaria, neurodegenerative diseases, AIDS, cancer and in the aging process (Wahlqvist, 2013).

All organisms have antioxidant systems that are able to control and counter the onslaught of free radical mediated oxidative damage. Therefore, dietary sources have been recognized as safer and effective antioxidants in the context of their efficiency and nontoxicity. The intake of fruits and vegetables containing high amounts of antioxidative nutraceuticals has been associated with the balance of the free radical or antioxidant status, which helps to minimize the oxidative stress in the body and to reduce the risks of diseases (Ames et al., 1993). Recently, bioactive peptides with physiological properties and protein obtained from plant, animal, microbial and food sources have been investigated (Moller et al., 2008).

Bioactive peptides have physiological properties and in recent times several studies have been done on identifying and optimizing the isolation of biopeptides from both plant and animal sources (Korhonen and Pihlanto, 2003). These peptides are generated and in vitro from the proteolytic hydrolysis of food proteins. Enzymatic hydrolysis of proteins is one approach used to release bioactive peptides and is widely applied to improve the functional and nutritional properties of protein sources (Je et al., 2005). The biological activity of a peptide is widely recognized as being based on amino acid composition (Pownall et al., 2010). These bioactive peptides are 2-20 amino acid residues in length, although some have been reported to have more amino acid residues. The main players here include tyrosine, histidine, methionine, and tryptophan (Kristinsson and Rasco, 2000). Peptides could be used in the formulation of functional foods and nutraceuticals to prevent damage related to oxidative stress in human diseases. Moreover, natural antioxidants are desirable because they can be used at higher concentrations without any toxic side effects associated with the use of synthetic equivalents.

This research is of interest as mushrooms are used in medicine, pharmacy, food, and fermentation fields, as well. In particular, mushrooms are mostly used as therapeutic agents besides their nutritional properties (Palupi and Windrati, 2011). They are flavorful food, full of proteins, rich in vitamin B, rich in different minerals and have almost all essential amino acids. Mushrooms have been reported as useful in preventing diseases such as hypertension, hypercholesterolemia and cancer (Mujic et al., 2010). Recently, many studies have shown that polysaccharides from mushrooms have

substantial medicinal properties and no toxic side effects, unlike many existing chemotherapeutic anticancer drugs (Lee and Hong, 2010).

Monkey's head mushroom (*Hericium erinaceus*) is a basidiomycete belonging to the family Hericiaceae, order Russulales and class Agaricomycetes. In Japan, *H. erinaceus* is known as Yamabushitake. This mushroom is also known as "Lion's Mane","Monkey's Mushroom","Bear's Head","Hog's Head Fungus","White Beard", "Old Man's Beard", "PomPom" and "Bearded Tooth" in other parts of the world (Thongbai et al., 2015). So, this study focuses on new sources of protein hydrolysate from monkey's head mushroom. *H. erinaceus* contains various bioactive constituents such as polysaccharides proteins, lectin hericenones, erinacines, sterol, fatty acid and esters (He and Shen, 2015). This study, therefore, proposes that monkey's head mushroom might be the main source of protein hydrolysate. The objective of this study is to investigate the antioxidant scavenging activity and antiproliferative activities of protein hydrolysate obtained from the peptide of monkey's head mushroom. So, monkey's head mushroom hydrolysates are new peptides which are significantly bioactive and interesting.

CHAPTER II
LITERATURE REVIEW

2.1 Free radicals

Free radicals are molecules with an unpaired electron and are important intermediates in natural processes involving cytotoxicity, control of vascular tone, and neurotransmission. Free radicals are very unstable and react quickly with other compounds, and try to capture the needed electron to gain stability. A chain reaction thus begins. Once the process starts, it can cascade, and finally results in the disruption of a living cell (Sarma et al., 2010). There are several types of free radicals in living systems. Generally, the most important reactants in free radical biochemistry are aerobic cells, and the oxygen and its radical derivatives are superoxide and hydroxyl radicals. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed as byproducts of normal metabolism in aerobic organisms. ROS is a broader term; it includes many reactive species, e.g., superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$), alkyl radical, alkoxyl ($RO\bullet$) radicals, singlet oxygen (O) and semiquinone radical ($HQ^{\cdot}$), and ozone (O_3) (Tabel 1). They are mainly derived from reactive oxygen species (ROS) and reactive nitrogen species (RNS) and are generated in our body by various endogenous systems, exposure to different physicochemical states. ROS include free radicals like the superoxide anion ($O_2^{\cdot-}$), hydroperoxyl radical ($HO_2^{\cdot}$), hydroxyl radical ($^{\cdot}OH$), nitric oxide (NO), and other species like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid ($HOCl$) and peroxynitrite ($ONOO^-$). RNS is derived from NO

by reacting with $O_2^{\cdot-}$, and forming $ONOO^-$. Free radicals can adversely alter lipids, proteins and DNA and have been implicated in aging and a number of human diseases (Devasagayam et al., 2004; Halliwell, 2001).).

Free radicals can be derived from environmental sources including ultraviolet light, ionizing radiation, and pollutants such as paraquat and ozone. All of these sources of free radicals – both enzymatic and non-enzymatic – have the potential to inflict oxidative damage on a wide range of biological macromolecules. Free radicals could attack fatty acid side chains of intracellular membranes and lipoproteins. Free radicals have very short lives, e.g., in milli-, micro-, or nanoseconds, and readily react with lipids, DNA, and proteins causing damage and forming harmful products such as lipid peroxides and other lipid adducts. The consequent protein damage results in the loss of enzyme activity, while DNA damage can result in mutagenesis and carcinogenesis (Dupont et al., 1992).

Table 1 Reactive oxygen and nitrogen species of biological interest (Devasagayam et al., 2004).

Reactive species	Symbol	Reactivity / Remarks
Reactive oxygen species :		
Superoxide	$O_2^{\cdot-}$	Generated in mitochondria, in cardiovascular system and others
Hydroxyl radical	OH	Very highly reactive, generated during iron overload and such conditions in our body
Hydrogen peroxide	H_2O_2	Formed in our body by a large number of reactions and yields potent species like OH
Peroxyl radical	$ROO^{\cdot}$	Reactive and formed from lipids, proteins, DNA, sugars etc
Organic hydroperoxide	ROOH	Reacts with transient metalions to yield reactive species
Singlet oxygen	$^1O_2$	Highly reactive, formed during photosensitization and chemical reactions
Ozone	O_3	Present as an atmospheric pollutant, can react with various molecules, yielding $^1O_2$
Reactive nitrogen species:		
Nitric oxide	$NO^{\cdot}$	Neurotransmitter and blood pressure regulator, can yield potent oxidants during pathological states
Peroxynitrite	$ONOO^-$	Formed from $NO^{\cdot}$ and superoxide, highly reactive
Peroxynitrous acid	ONOOH	Protonated form of $ONOO^-$
Nitrogen dioxide	NO_2	Formed during atmospheric pollution

2.2 Antioxidant

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. To counteract the deleterious effects of oxidative stress, nature has endowed each cell with adequate protective antioxidant defenses which can be broadly categorized into enzymatic or non-enzymatic antioxidants based on their action in intracellular and extracellular compartments (Ji, 1999). It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals.

2.2.1 Endogenous antioxidants

Endogenous antioxidants constitute categorized into enzymatic and non-enzymatic antioxidants (Nimse and Pal, 2015).

2.2.1.1 Enzymatic antioxidants

Enzymatic antioxidants, such as superoxide dismutase, glutathione peroxidase and catalase are present in the body to prevent damage from ROS by catalyzing their conversion into a stable compound. The enzyme superoxide dismutase catalyses the conversion of superoxide to hydrogen peroxide, which is a long lived molecule that is readily diffusible through cell membranes. Hydrogen peroxide is then catalyzed by the enzymes catalase and glutathione peroxidase to oxygen and water, and this enables the survival of the cells.

Superoxide dismutase (SOD) (EC 1.15.1.1) is the antioxidant enzyme that catalyses the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. Peroxide can be destroyed by CAT or GPX reactions(Teixeira et al., 1998).

Catalase (CAT) (EC 1.11.1.6) is an enzyme responsible for the degradation of hydrogen peroxide. It is a protective enzyme present in nearly all animal cells. CAT reacts very efficiently with H_2O_2 to form water and molecular oxygen; and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity (Qujeq and Rezvani, 2007).

Glutathione peroxidase (GPx) is an enzyme that is responsible for protecting cells from damage arising from free radicals like hydrogen and lipid peroxides. GPx (EC 1.11.1.19) contains a single selenocysteine selenocysteine (Sec) residue in each of the four identical subunits, which is essential for enzyme activity (Speranza et al., 1993).

2.2.2 Exogenous antioxidants

Exogenous antioxidants are those received from outside of the body through diet and supplements, for example, food, vegetables, fruits, grains, herbs, spices and vitamins (Bouayed & Bohn, 2010).

2.2.2.1 Dietary antioxidants

Vitamin C, vitamin E, and beta-carotene are among the most widely studied dietary antioxidants. Vitamin C is considered the most important water-

soluble antioxidant in extracellular fluids. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Vitamin E, a major lipid-soluble antioxidant, is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation. Vitamin C has been cited as being capable of regenerating vitamin E (Traber and Packer, 1995).

Beta-carotene and other carotenoids are also believed to provide antioxidant protection to lipid-rich tissues. Research suggests beta-carotene may work synergistically with vitamin E. A diet that is excessively low in fat may negatively affect beta-carotene and vitamin E absorption, as well as other fat-soluble nutrients. Fruits and vegetables are major sources of vitamin C and carotenoids, while whole grains and high quality, properly extracted and protected vegetable oils are major sources of vitamin E (Sies and Stahl, 1995).

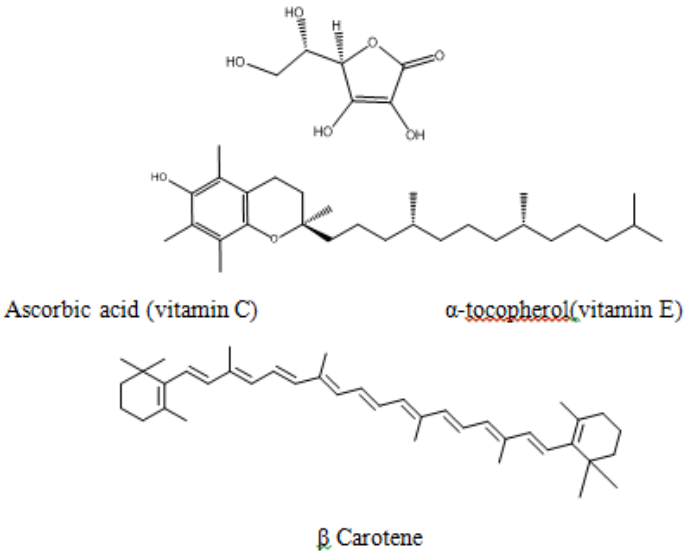


Figure 1 Dietary antioxidant

2.2.2.2 Synthetic antioxidants

Synthetic antioxidants are chemically synthesized petroleum based antioxidants[1] used primarily to "retard lipid oxidation"[2] in order to preserve and stabilize the refined oils and fats within a food product/food system. The four widely used synthetic antioxidants in the food industry are BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), PG (propyl gallate), and TBHQ (tert-butylhydroxyquinone) (Shahidi and Zhong, 2005)

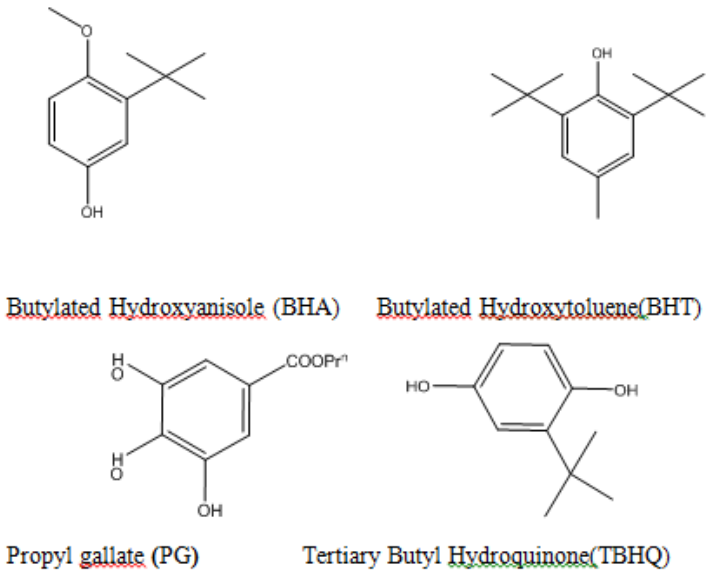


Figure 2 Synthetic antioxidants

2.2.2.3 Antioxidant *activity of* protein hydrolysates.

The findings of numerous studies have confirmed that protein hydrolysates possess antioxidant activity. Hence, enzymatically-modified proteins may serve as natural sources of antioxidants in food models to preserve these foods and to extend their shelf-life. The properties of the hydrolysates dependent on a number of factors including the type of protein and enzyme employed, the degree of hydrolysis (DH), the environmental conditions, and any substrate pretreatment. The capability of peptides to inhibit deleterious changes during lipid oxidation also depends on the presence of certain amino acid residues in the peptides. The main players here include tyrosine, histidine, methionine, and tryptophan. These amino acids are also capable of chelating pro-oxidant metal ions that the antioxidant properties of the hydrolysates dependent on the enzymes employ during hydrolysis. (Amarowicz, 2008). Therefore, the amino acid composition of peptides in protein hydrolysates is a critical factor in controlling the antioxidant activity of protein hydrolysates (Table 2).

Table 2 Protein hydrolysate from other sources in antioxidant activity.

Source of protein	Enzyme hydrolysis	Antioxidants	Results	Reference
Duck egg white	papain, trypsin, chymotrypsin, alcalase, and flavourzyme	DPPH	The higher the peptide content, the stronger the antioxidant activities in DEWHP.	(Y.-C. Chen et al., 2009)
Egg-white powder	alcalase, neutrase, flavourzyme, and protamex	DPPH, ABTS	Protein hydrolysates exhibited excellent antioxidant activities	(Cho et al., 2014)
Sunflower	pepsin, trypsin , chymotrypsin , alcalase and flavourzyme	Autoxidation, pyrogallol	Protein hydrolysate more antioxidant potency	(Taha et al., 2013)
Peanut	alcalase,	DPPH	Protein hydrolysate MW 3,000 - 5,000 kDa is the most antioxidant activity	(Jamdar et al., 2010)
Black Soybean	alcalase and flavourzyme	Reducing power and DPPH	Chickpea hydrolysate showed better antioxidant activity in the reducing power and DPPH	(Yust et al., 2012)

2.3 Protein hydrolysates

Protein hydrolysis of proteins involves the cleavage of peptide bonds to give peptides of varying sizes and amino acid composition. There are a number of types of hydrolysis: enzymatic, acid or alkali hydrolysis. Chemical hydrolysis is difficult to control and reduces the nutritional quality of products destroying L-form amino acids and producing toxic substances such as lysino-alanine (Wanasundara et al., 2002) as shown in Table 3. Enzymatic hydrolysis works without destroying amino acids and by avoiding the extreme temperatures and pH levels required for chemical hydrolysis, with the nutritional properties of the protein hydrolysates remaining largely unaffected. Production of protein hydrolysates in the food industry involves the use of digestive proteolytic enzymes from animals, plants and microorganisms which are regarded safe for human nutrition (see Table 4) (McCarthy et al., 2013).

Protein hydrolysis by enzymes includes proteases such as protease from animals (trypsin, pepsin and pancreatin), protease from plants (papain and protease), protease from microorganisms (alcalase, , flavourzyme and neutrase) under mind condition as shown in Table 5 For example, hydrolysates prepared from canola protein using flavourzyme (i.e., both with endo- and exo-peptidase activities) showed the highest antioxidant activity among all samples, whereas hydrolysates prepared with a combination of alcalase (i.e., an endopeptidase) and flavourzyme did not differ in antioxidant effectiveness from those generated by alcalase alone. Hydrolysates were prepared from whey protein isolate by pure enzyme (i.e., pepsin, papain, trypsin, chymotrypsin) The antioxidant activity was noted in the case of the hydrolysates

prepared from WHI using commercial crude enzymes (i.e., proteases from *Bacillus licheniformis*, *Aspergillus oryzae*, as well as *Bacillus protease* complex). Under the influence of alcalase (enzyme obtained from *Bacillus licheniformis*), an increase in activity for the reaction mixture was noted by the presence of cysteine-, methionine-, lysine-, and valine-containing protein fragments (Cumby et al., 2008) as shown in Table 5.

Table 3 The advantages and disadvantages of chemical hydrolysis

Advantages	**Disadvantages**
1. Low cost	1. Strong reaction
2. Reduce high molecular	2. Non-specific cleavage site
	3. Can damage activity
	4. Damage some amino acid

Table 4 The advantages and disadvantages of enzymatic hydrolysis

Advantages	**Disadvantages**
1. Not consumed during reaction	1. Instability
2. High conversion yield	2. High cost
3. Nontoxic and biodegradable	
4. High specificity	
5. Utilization in soft conditions	
6. Large-scale production	

Alcalase (E.C. 3.4.21.62) is an enzyme obtained from *Bacillus licheniformis* which contains several proteinases with various specificities. Its optimum pH for catalysis ranges from 6.5-8.5.

Flavourzyme (E.C. 3.4.11.1) is a kind of compound enzyme composed of the incision enzyme and the circumscribed enzyme from *Aspergillus oryzae*. Flavourzyme also acts as exopeptidase and endoprotease.

Neutrase (E.C. 3.4.24.28) is a protease produced by *Bacillus amyloliquefaciens* which is extensively used to break protein down into peptide. The optimal working conditions are 45-55 ºC and pH 5.5-7.5.

Pepsin (E.C. 3.4.23.1) is the predominant digestive protease in the gastric juice of vertebrates. The optimal pH for the activity of pepsin is 1.0-4.0. Pepsin can hydrolyze only peptide bonds. It does not hydrolyze non-peptide amide or ester linkages.

Trypsin (EC 3.4.21.4) is formed in the small intestine when its proenzyme forms and the trypsinogen produced by the pancreas is activated. Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. It is used for numerous biotechnological processes.

Table 5 Proteolytic enzymes, characteristics and optimum conditions

Enzyme	**Source of origin**	**Condition**	
		pH	**Temp (°C)**
Alcalase	*Bacillus licheniformis*	7.0	50
Flavourzyme	*Aspergillus oryzae*.	7.0	50
Neutrase	*Bacillus amyloliquefaciens*	7.0	50
Pepsin	Porcine gastric mucosa	2.0	37
Trypsin	Bovine, porcine or human pancreas	8.0	37
Papain	*Carica papaya* (papaya latex)	6.0	37

2.4 Bioactive peptides

Most dietary proteins demonstrating biological activity that have been investigated originate from milk (immunoglobulins, caseins, whey proteins). However, proteins from other animal sources as well as plant proteins have been reported to exert specific bioactivities. A broad spectrum of proteins shows physiological activity in the gastrointestinal tract. These activities range from the enhancement of nutrient absorption, inhibition of enzymes, enzyme activity, and growth stimulation to modulation of the immune system in defending against pathogens. A number of proteins have been suggested to facilitate the uptake of essential nutrients. For example, α- and β-caseins are thought to enhance calcium uptake by forming soluble casein

phosphopeptides during digestion; lactoferrin appears to facilitate iron uptake, whereas other proteins such as vitamin B12-binding protein (haptocorrin) and folate-binding protein improve the availability of vitamins. The bioactivities observed for a number of milk proteins such as immunoglobulins, vitamin- or mineral-binding proteins indicate that bioactive proteins are of importance for the development and protection of newborn mammals (Walther and Sieber, 2011).

2.5 Method for investigation of antioxidant activity

2.5.1 DPPH radical scavenging activity assay

DPPH is a stable radical of organic nitrogen, with a free electron, and is widely used to study the antioxidant activity of natural compounds. The DPPH radical is used to measure the ability of natural compounds to donate electrons or hydrogen to form a more stable compound. The DPPH radical is oil soluble and is stable in methanol and can act as an oxidizing substrate as well as the reaction indicator molecule. The DPPH assay method is easy, fast and important in measuring the activity of antioxidants compounds at a maximum absorbance of 515–520 nm.

Antioxidants donate hydrogen to radicals to form a stable molecule, and inhibit the formation of other toxic radicals. When the DPPH radical reacts with the hydrogen donor or free radical scavengers, it becomes reduced, causing change in the purple color and a decrease in absorption strength. Thus, the decrease in absorbance represents the DPPH scavenging activity. When the DPPH radicals accept an electron from antioxidants, they become a stable product. A low absorbance at 517 nm indicates strong DPPH scavenging activity(Molyneux, 2004)

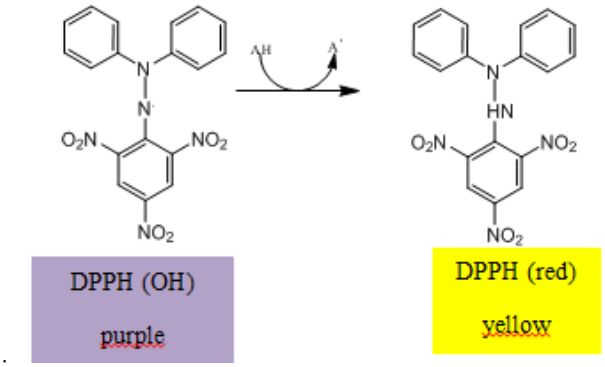


Figure 3 Structure of DPPH and its reduction by antioxidants

2.5.2 ABTS radical scavenging activity assay

This method involves the oxidation of 2, 2- azinobis- (3- ethylbenzothiazoline- 6-sulphonic acid). The principle behind the technique involves the reaction between ABTS and potassium persulfate to produce the ABTS radical cation, a blue green chromogen. In the presence of antioxidants, the colored radical is converted back to colorless ABTS, the absorbance of which is measured at 734 nm. The preformed radical mono-cation of 2, 2-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS•+) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen donating antioxidants (Re et al., 1999).

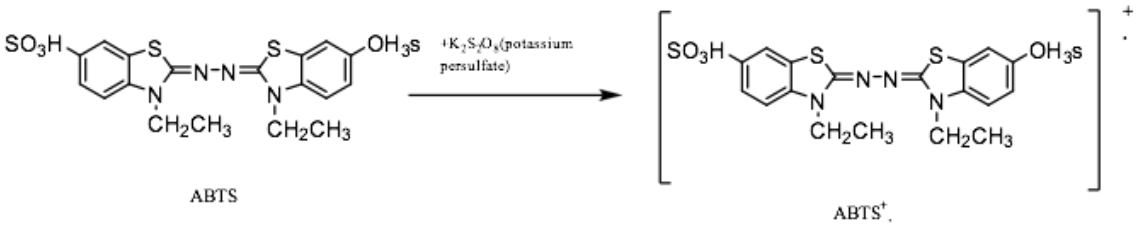


Figure 4 Structure of ABTS radical and its reduction by antioxidants

2.5.3 Nitric oxide radical scavenging activity

Nitric oxide scavenging activity can be estimated by the use of the Griess-Ilosvoy reaction. The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO Under aerobic conditions. The NO then reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of which can be determined using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to the reduced production of nitrite ions. In the presence of scavengers, the absorbance of the chromophore is evaluated at 5 40 nm.

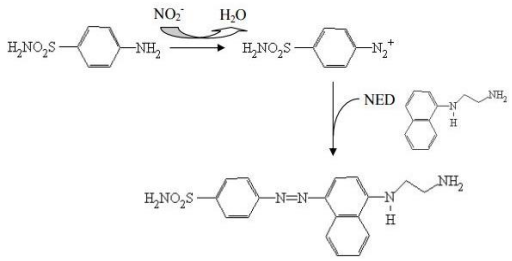


Figure 5 Structure of nitric oxide radicals and its reduction by antioxidants.

2.6 DNA damage

Oxidative damage to DNA is associated with many pathological conditions such as ischaemia/reperfusion, carcinogenesis, mutagenesis and ageing. One of the most reactive free radicals, hydroxyl radical (•OH), causes damage to DNA. The presence of oxidized DNA is known as a marker for ROS-mediated DNA damage. The most significant consequence of oxidative stress in the body is thought to be damage to DNA. DNA may be modified in a variety of ways, which can ultimately lead to mutations and genomic instability. (Hoeijmakers, 2009). This could result in the development of a

variety of cancers including colon, breast, and prostate. Here we discuss the various types of damage to DNA, including oxidative damage, hydrolytic damage, DNA strand breaks, and others. In this study, the hydroxyl radicals generating system was based on the Fenton reaction that makes the super coil (SC) form in DNA convert to an open circular (OC) form due to the hydroxyl radical damage. However, DNA treated with peptide was shown to have no damage. So, the purified peptide had the protective capacity in oxidation-induced DNA damage

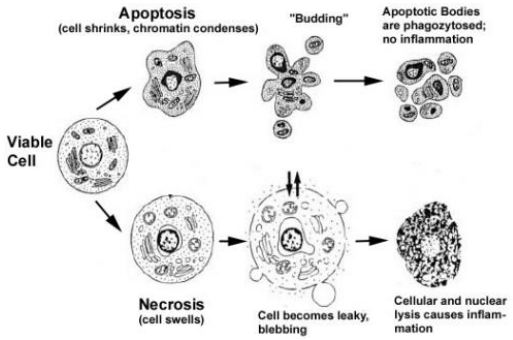
2.7 Antiproliferative Activities

Cancer is a complex multifactorial cell disease characterized by abnormal cellular proliferation (Giri et al., 2006). Cancer development and progression are dependent on the cellular accumulation of various genetic and epigenetic events, and it is an aberrant net accumulation of typical cells arising from excess proliferation, insufficient apoptosis, or a combination thereof (Abdul et al., 2009). Cancer development is normally caused by oncogene, tumor suppressor gene, and microRNA gene alterations (Burstein and Schwartz, 2008). Medicinal plants have a long history in both traditional and modern cancer treatments and have been used to treat human diseases for centuries (Conforti et al., 2008). Thus, it is possible that traditional medicinal plants can serve as potential sources for developing new drugs and more effective anti-cancer agents for future therapy (Caamal-Fuentes et al., 2011).

2.8 Apoptosis

Apoptosis or programmed cell death (PCD) plays a pivotal role in development, cancer, normal ageing and in neurological disorders such as Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease (Thompson, 1995). A common feature of many neurological diseases is the degeneration of neuronal cells. It is widely accepted that neuronal loss in such diseases occurs by the inappropriate activation of apoptotic cell-death pathways. Apoptosis is induced via two main routes involving either the mitochondria (the intrinsic pathway) or the activation of death receptors (the extrinsic pathway). Both pathways converge to induce the activation of caspases the final executioners of cell death, although, it should be noted that caspase-independent forms of apoptosis have been reported.

Apoptosis is a type of cell death regulated in an orderly way by a series of signal cascades under certain situations. It plays an essential role in regulating growth, development and immune response, and clearing redundant or abnormal cells in organisms. The induction and execution of apoptosis require the cooperation of a series of molecules including signal molecules, receptors, enzymes and gene regulating proteins. Figure 6 shows the apoptotic and necrotic cell death process.



.**Figure 6** Apoptotic and necrotic cell death process.

Table 6 Apoptosis is a morphological description of dying cells which contrasts with necrosis

Apoptosis	**Necrosis**
Physiological or pathological	Always pathological
Chromatin condensation	Chromatin disintegration
Asynchronous process in single cells	Occurs synchronously in multiple cells
Cell Shrinkage	Cell and nucleus swelling
Late loss of membrane integrity	Early loss of membrane integrity
Preservation of organelles and cell membranes	Disruption of organelles
Rapid engulfment by neighboring cells preventing inflammation	Rupture of cell and release of cellular contents
No inflammatory reaction	Inflammatory response
Biochemical hallmark: DNA fragmentation	

2.8.1 The mechanism of apoptosis

Apoptosis may be triggered by two major mechanisms: the binding of death ligands to death receptors in the extrinsic pathway or cytotoxicity that initiates the intrinsic "mitochondrial" pathway (Crow et al., 2004). Overall, these pathways converge to activate a series of cysteine aspartyl-specific proteases (caspases), which cleave key cellular proteins and dismantle the cell.

2.8.1.1 The extrinsic or death receptor pathway

The death-receptor mediated (extrinsic) apoptotic pathway is very similar to the intrinsic pathway. The only major difference is that apoptotic signaling is initiated through membrane-bound death receptors such as Fas and the tumour necrosis factor (TNF) receptor. In cancer cells, these death receptors can be down-regulated on the cell surface, desensitizing cancer cells to stimuli for extrinsic apoptosis. Much like the apoptosome, the death-inducing signaling complex (DISC) activates caspases of the extrinsic pathway, namely caspase-8 and caspase-10. DISC components are oligomers of Fas and Fas-associated death domain (FADD) proteins. Adaptors are recruited from DISC to bind domains on the procaspases and increase local caspase concentration. In these domains, the extrinsic equivalents of the CARDS in the intrinsic pathway, are called death effector domains (DEDs) (Green, 2011).

2.8.1.2 The intrinsic or mitochondrial receptor pathway

The intrinsic (or mitochondrial) pathway is tightly controlled by the opposing actions of the members of the BCL2 family. These proteins, which each harbor at least one BCL-2 homology (BH) domain, are divided into three functionally-distinct groups: (1) inhibitors of apoptosis, (2) antiapoptotic BCL2 members inhibit intrinsic apoptotic signals by constraining the proapoptotic proteins such as BAX and BAK, and (3) therefore, cytochrome c release (Koff et al., 2015).

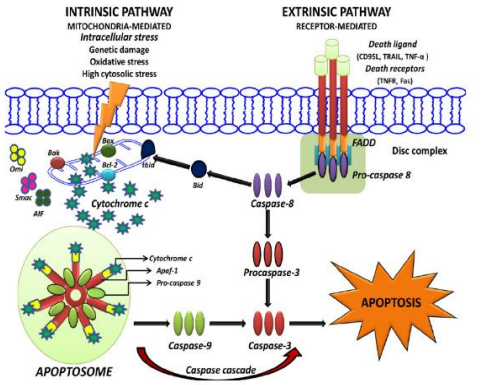


Figure 7 Extrinsic and intrinsic pathways involved in apoptosis

2.8.2 The caspase activity

Caspases are the proteases responsible for dismantling the cell in an ordered and histologically distinct process termed apoptosis. By cleaving critical proteins, caspases lead to the changes that characterize apoptosis both morphologically and biochemically, such as chromatin condensation, loss of cell adhesion, cell shrinkage, membrane blebbing, DNA fragmentation, and finally formation of apoptotic bodies, which stimulate their own engulfment by phagocytes.

The apoptotic caspases are classified as initiators or executioners, depending on their point of entry into the apoptotic cascade. The progress of apoptosis is regulated in an orderly way by a series of signal cascades under certain circumstances. The caspase-cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals. A group of intracellular proteases called caspases are responsible for the deliberate disassembly of the cell into apoptotic bodies during apoptosis. Caspases are present as inactive pro-enzymes that are activated by proteolytic cleavage. Caspases 8, 9 and 3 are situated at pivotal junctions in apoptosis pathways (Kogel and Prehn, 2013).

2.9 Monkey's head mushroom

Mushrooms have a long tradition of use in many Asian countries and have been used as food and as medicine. They are flavorful food, full of proteins, rich in vitamin B, rich in different minerals and have almost all essential amino acids. Mushrooms have been reported as useful in preventing diseases such as hypertension, hypercholesterolemia and cancer (Mujic et al., 2010).

Mushroom hunting for food is an age-old practice for the tribal population of north-eastern Indian states as mushrooms are considered highly nutritious. Lately, attention has been drawn to the second area of exploitation of this delicate organism because many of the mushrooms are reported to produce metabolites which are nutriceutical and pharmaceutical agents (Khan et al., 2013).

Hericium erinaceus, also called monkey's head mushroom or bearded tooth fungus, is an edible and medicinal mushroom belonging to tooth fungus. It is considered one of the best edible mushrooms and reported to have contain many nutriceutical and pharmaceutical agents. It contains neuroactive compounds, it has antioxidant and anti-inflammatory effects, and improves cognitive impairment. Also, the polysaccharides from this mushroom possess anti-tumor properties. *H. erinaceus* is rarely found in and around the forests of Japfu mountain in Kohima district usually during the months of October-November. Apart from its culinary, medicinal and nutriceutical values, this mushroom is collected by the local population out of curiosity due to its peculiarly beautiful appearance. This mushroom has become increasingly vulnerable to extinction following reports of its pharmaceutical and nutriceutical importance becoming known among the local population without proper management guidelines, and it thus requires urgent conservation attention (Wabang and Ajungla). Mushroom has a relatively high nutritive value. The fruitbodies of the lion's mane mushroom contain 57% carbohydrates, 3.52% fats, 7.81% fiber, 22.3% protein and 9.35% ash per dry matter (d.m.) (Mau et al., 2001)

2.9.1 Scientific classification

Kingdom	Fungi
Division	Basidiomycota
Subdivision:	Agaricomycotina
Class	Agaricomycetes
Order	Russulales
Family	*Hericiaceae*
Genus	*Hericium*
Species	*H. erinaceus*

Figure 8 Monkey's head mushroom (Hericium erinaceus)

Table 7 Bioactivity of Mushroom

Mushroom	Bioactivity	Results	References
H. erinaceus and *A. aegerita*	Antioxidant activity	The antioxidant activity of mushroom extracts highly depends on extract concentration,	(Mujic et al., 2010)
Grifola frondosa	Antioxidant activity	The results of this study showed that the peptide fractions with molecular weight less than 3000 Da were associated with higher antioxidant activity.	(DonG et al., 2015)
Volvariella volvaceae	protein hydrolysate	This protein hydrolysate has the potential for application as an ingredient in formulated diets.	(Palupi and Windrati, 2011)
Oyster mushroom	protein hydrolysate	The suitable timing to produce highest of %DH	(Banjongsinsiri et al., 2016)
Pleurotus ostreatus	Antioxidant activity	Oyster mushroom protein by papain resulted in fraction with good antioxidant activity.	(Meignanalakshmi et al. 2014)
Phellinus linteus	antioxidant	high bioactivity as measured by antioxidant	(Reis et al., 2014)
Oyster mushroom, Abalone mushroom and Shiitake mushroom	protein hydrolysates	protein hydrolysate showed the higher degree of hydrolysis	(Banjongsinsiri et al., 2016)

CHAPTER III
EXPERIMENTAL

3.1 Biological materials

Monkey's head mushroom was obtained from Marayat Farm Organic, Pathum Thani, Thailand. Five human tumor cell lines, BT474 (breast), Chago-K1 (lung), Hep-G2 (hepatoma), KATO-III (gastric), and SW620 (colon) were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand.

3.2 Chemicals

L-ascorbic acid, bovine serum albumin (BSA), 2, 2-Diphenyl-1-picryhydrazyl (DPPH), ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate)), curcumin from Curcuma longa (Turmeric), napthylethylenediamine dihydrochloride (NED), sodium nitroprusside (SNP), and BHT were purchased from Sigma Chemicals Co. (USA). Potassium dihydrogen phosphate (KH2PO4), potassium persulfate (K2S2O8) and methanol were purchased from Merck KGaA Darmstadt (Germany). Biomax® 5 and Biomax® 10 were sourced from Amersham Biosciences (Sweden). Protease G6 was obtained from Siam Victory Chemicals Co., Ltd (Thailand). All other biochemicals and chemicals used in the investigation were of analytical grade.

3.3 Preparation of peptide from monkey's head mushroom

Monkey's head mushroom mushroom (200 g.) was mixed in 4 L of phosphate buffer saline (PBS; 20 mM phosphate buffer with 0.15 M NaCl pH 7.2) using a mixer and subsequently stirred overnight at 4 ^{o}C (DonG et al., 2015). The samples were digested with alcalase, flavourzyme and neutrase at an enzyme-to-substrate ratio in various concentrations (1, 2.5 and 5%) for 4 h at 50 ^{o}C with shaking (150 rpm) respectively. After digestion, the enzyme was inactivated by boiling for 10 min and the undigested proteins were precipitated by centrifugation at 10,000 rpm for 15 min and the supernatant collected. The experimental design was a completely randomized design (CRD) with analysis conducted three times. The hydrolysate was kept at -20 ºC until use.

3.4 Protein concentration assay

The protein contents were determined using the Bradford method with bovine serum albumin as a standard protein (BSA) used as the standard with four different concentrations between 5-20 µg/ml to construct the calibration curve (Bradford, 1976). This method is used to measure the concentration of total protein in a sample. It comprises Bradford working buffer; 95% ethanol, 88% phosphoric acid, Bradford stock solution with bovine serum albumin as a standard protein in the range of sensitive: 0-200 µg/ml protein solution. 20 µl protein hydrolysate was mixed with 200 µl Bradford working buffer and incubated for 2 min. The absorbance was measured at 595 nm using a microplate reader spectrophotometer.

3.5 Total amino acid analysis

3.5.1 Acid hydrolysis

Five milliliters of HCl 6N was added (5 mg protein/mL HCl) and mixed. The tube was flushed with nitrogen for 1 min to remove the air. Hydrolysis was then carried out at 110 ^{o}C for 22 h. The internal standard (10 mL of 2.5 mM L-α -amino-*n*-butyric acid in HCl 0.1 M) was added and diluted with water to 250 mL. The solution was filtered with 0.20 µm filter and was then derivatized with 6 aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Flour reagent). It was then heated in a heating block at 55 ^{o}C, for 10 min. Heating converts a minor side product of tyrosine to a major mono-derivatized compound. The total amino acid content was determined by high performance liquid chromatography.

3.5.2 Chromatographic conditions

Chromatographic separation was carried out in a Waters Alliance 2695 with heater amino acid analysis Hypersil Gold column C18. The column was thermostatted at 35 ± 1 ^{o}C and the flow rate was 1.0 mL/min. The injection volume was 5 µL. Mobile phase A consisted of sodium acetate buffer pH 4.90 and 60% acetonitrile.

3.6 Determination of antioxidant activities

3.6.1 DPPH radical scavenging activity

The DPPH (2, 2-Diphenyl-1-picryhydrazyl) radical scavenging assay was determined with the method described (Tanzadehpanah et al., 2012) . 0.1 M DPPH

radical dissolved in 95% ethanol was added to each sample in the ratio of 1:4 for 15 minutes and absorbance measured at 517 nm. Ascorbic acid was used as a positive control.

3.6.2 ABTS radical scavenging activity

The ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate)) radical scavenging assay was determined with the method described (Cai et al., 2004). ABTS radical cation was generated by mixing ABTS solution (7 mM) and potassium persulphate (2.45 mM) in the ratio of 1:1 in the dark at room temperature for 12 hours. It was then mixed with a solution of peptides in the ratio of 1:30 for 10 minutes and absorbance measured at 734 nm. Ascorbic acid was used as a positive control.

3.6.3 Nitric oxide radical scavenging assay

Nitric oxide radical scavenging activity was measured using the method described (Govindarajan et al., 2003). Nitric oxide radicals were generated by sodium nitroprusside solution to produce nitrite ions which were measured by the Griess reaction. 25 µL of each protein hydrolysate at various dilutions were mixed with 10 mM Sodium nitroprusside in phosphate buffer (pH 7.2). The mixture was incubated at room temperature for 150 minutes. Then, 100 µL of 0.33% sulphanilamide was added in 20% acetic acid and let stand for 5 minutes for completing diazotization. 0.1% naphthylethylenediamine dichloride was added and then incubated at room temperature

for 30 minutes. The absorbance was measured at 546 nm. Curcumin was used as positive control.

3.7 Isolation and purification

3.7.1 Ultrafiltration

Peptides were partially purified by the ultrafiltration technique using a membrane with a 10, 5, 3, and 0.65 kDa MW Cut off (MWCO), and were collected. Their radical scavenging activities were calculated.

3.7.2 Gel filtration chromatography

The fraction that showed the highest radical scavenging activities from molecular weight cut-off by ultrafiltration (MW □ 0.65 kDa) was further fractionated with a Superdex 75 column (1.6 cm diameter × 15 cm length), which was pre-equilibrated with deionized water. The fractions were eluted with deionized water at a flow rate of 0.5 mL/min, and each eluted fraction was detected at 280 nm. Their radical scavenging activities were calculated.

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

The peptides solution after partial purification through gel filtration techniques was fractionated using a RP-HPLC on C_{18} column (Shimpak, 250 × 46 mm) with 0.1% trifluoroacetic acid (TFA) and a linear gradient of acetonitrile (0-70%) containing 0.05% TFA in water at a flow rate of 0.7 mL/min. The injection volume was 50 µL and the peaks were detected at 280 nm. Peaks were collected separately. The

purification procedures were repeated until enough samples were collected for sequence identification (mass spectrometry).

3.7.4 Mass spectrometry

Mass spectrometry is an analytical chemistry technique to identify the compounds or peptides present in a sample by measuring the mass-to-charge ratio (m/z) and abundance of gas-phase ions. The objective of this study was to identify the compounds in protein hydrolysate by LC-MS/MS Q-TOF data analyzed by de novo peptide sequencing.

3.8 Percentage inhibition

The percentage of radical scavenging was calculated as follows:

$$\frac{[\text{Abs control} - (\text{Abs sample} - \text{Abs background})]}{\text{Abs control} - \text{Abs blamk}} \times 100$$

where Abs control is the absorbance of the control (no sample), Abs sample is the absorbance of the monkey's head mushroom hydrolysates, Abs background is the color absorbance of the sample and Abs blank is the absorbance of the deionized water. The IC_{50} values (i.e., the concentration of monkey's head mushroom hydrolysate required to inhibit the antioxidant activity by 50%) was calculated using version 6 of the GraphPad Prism software. All of these tests were performed in triplicate and the values provided herein have been expressed as the mean values ± the standard deviation of the triplicate data.

3.9 Statistical analysis

Each experiment made triplicate repeats and the SPSS program was used to compare the difference in average. The results were shown as mean ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test at the 0.05 level.

3.10 DNA protection activity

3.10.1 Preparation plasmid from *E.coli*

The streaking plate *E.coli* containing plasmid pBR322, pKS and pUC19 in LB agar and antibiotic (ampicillin) needs to be in agar to maintain plasmid, with growth overnight at 37°C. A single colony from the streaked plate of *E.coli* containing plasmid pBR322, pKS and pUC19 was selected. The selected colony was inoculated with 5 ml of an LB broth containing ampicillin, incubated for 12-16 h at 37°C while shaking at 250 rpm. The *E.coli* culture was harvested by centrifugation at 13000 rpm in a microcentrifuge for 2 min at room temperature. The supernatant was decanted.

3.10.2 Plasmid DNA purification

1.5 mL of *E.coli* culture was transferred containing pKS, pUC19 and pBR322 plasmid to a microcentrifuge tube and centrifuged for 30 sec (14,000×g). 200 µL of resuspension buffer was added (and RNAse added) to the cell pellet. After that, the cells were resuspended by pipetting in and out or by gentle vortexing and incubated at room temperature for 5 min. 250 µL of lysis solution was added to the cell suspension

and the contents mixed by gently inverting the tube several times (it was not vortexed as this would shear the genomic DNA). The suspension became clear and viscous as the cells began to lyse. Mixing was continued until the mixture became clear. If necessary, the solution was allowed to incubate at room temperature provided the total incubation time was no more than 5 min. This step was also critical for the denaturation of cellular proteins and genomic DNA. 350 µL of binding solution was added and immediately mixed by inverting the tube several times. In this step, the solution would become turbid as insoluble particles from denatured materials started to form. After that, it was centrifuged for 10 min (14,000×g) to clarify the lysate and then an insoluble pellicle was collected on the bottom of the centrifuge tube. The supernatant was transferred to the spin column tube by decanting and centrifuged for 1 min. The flow-through was discarded and the column placed back into the same collection tube. 600 µL of wash solution was added to the spin column. It was centrifuged for 1 min and the flow-through discard. The column was placed into a 1.7 ml microcentrifuge tube. 10. 50 µL of elution buffer was added to the center of the spin column membrane to elute the plasmid DNA. This was incubated for 2 min at room temperature and centrifuged for 2 min with the pure plasmid DNA kept at -20ºC.

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

The DNA concentration and protein content of the pure plasmids DNA were measured by nanodrop technique using a Nanodrop 2000 spectrophotometer, Thermo Scientific.

3.10.4 Protection effect of the purified peptide on hydroxyl radical-induced DNA damage

DNA damage was induced by the hydroxyl radical based on the Fenton reaction using and modifying the method described by Sheih (Sheih et al., 2009). Briefly, 8 µl of pure plasmid DNA (pKS 2,961 bp, pUC19 2,686 bp and pBR322 4,361 bp in the DNA concentrations of 157.66, 24.81 and 33.52 ng/µL, respectively and protein content was 3.955, 0.59, and 0.686 mg/mL, respectively) was mixed with 4 µL of 0.00472, 0.00236, 0.0118, 0.0059, 0.0029 and 0.0015 µg/mL antioxidant purified peptide fraction from gel filtration chromatography (F1 fraction). After that, it was let stand at room temperature for 20 min. Next, 3 µL of 2 mM $FeSO^4$ and 3 µL of 30% H_2O_2 were added, respectively and then mixed and incubated at 37ºC for 30 min. Following this, 5 µL of the solution was mixed with 2 µL of loading dye (purple dye (6X)).Then, the solution and loading dye was pipetted into a well of 1% w/v of an agarose gel and run in a 1x TAE buffer at 100 V for 30 min. Later on, an agarose gel was stained in ethidium bromide for 10 min. Next, the agarose gel was washed with water. Finally, the DNA band was checked by UV light. Plasmids bands appear at about 2,000-5,000 bp.

3.11 Antiproliferative activity assay

The cytotoxicity assay for the in vitro antiproliferative activity of five different human malignant cell lines including BT474 (breast), Chago-K1 (lung), Hep-G2 (hepatoma), KATO-III (gastric), and SW620 (colon) was carried out tissue culture. The cells were kept in a complete medium composed of RPMI-1640 along with 2.0 mM L-glutamine and 10 % (v/v) FCS at 37 ºC under 5 % (v/v) CO_2 conditions. The cells were aspirated, trypsinized, and finally washed, prior to being seeded at a density of $5x10^3$ cells/µl immersed in 200 µl of complete medium in a 96-well plate and culture. They were then cultured for 1 day. Serial dilutions of the samples were then added into each well and incubated for 3 days. Afterwards, 10 µl of 3-[5,5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium] bromide solution (5 mg/ml) (MTT) was then poured into the wells and the samples incubated for 4 h prior to aspiration of the medium and washing with RPMI-1640 to remove any residual medium still left in the solution. Subsequently, 150 µl of DMSO was added into each well and then left for 30 min. The cell and its solution were then aspirated and the absorbance determined at 540 nm with a microplate reader. The percentage of cell viability was calculated as follows:

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

The concentration of the hydrolysate causing 50% inhibition of cancer cell growth was considered as IC_{50}

3.12 Apoptosis analysis

Apoptosis was assayed by Annexin V-FITC and propidium iodide (PI) reagent staining, followed by analysis with fluorescence-activated cell sorting (BioLegend Inc., Sandiego, CA, USA). The method described in the Annexin V-FITC/PI Detection Kit was followed. The Chago-k1 cells were seeded in 25 cm^2 flasks, at $1x10^5$ cells per flask, in a complete medium composed of RPMI-1640 with 2.0 mM L-glutamine, and 10 % (v/v) fetal calf serum (FCS) at 37 °C under 5 % (v/v) CO^2 conditions. After incubation overnight at 37 °C in 5 % CO^2, the cells were treated with protein hydrolysate and then incubated for 24, 48 and 72 h at 37 °C in 5 % CO^2. The positive control was the cells incubated with doxorubicin. The negative control was the cells incubated with RPMI 1640 containing 10% fetal calf serum. The cells were then harvested by trypsinization, and washed twice in cool phosphate buffer saline (PBS) pH 7.2 with 1 % fetal calf serum (FCS). The cell pellets were resuspended in 100 µl Annexin V Binding Buffer and 100 µl cell suspension were transferred to a 1.5 ml microcentrifuge tube. Then .5 µl of FITC Annexin V and 10 µl of propidium iodide solution were added into each tube. The cell suspension was vortexed and incubated in the dark for 10 min on ice . Then 200 µl of Annexin V binding buffer was added and the apoptosis was immediately measured by flow cytometry.

3.13 Caspase 3, Caspase 8 and Caspase 9 activity assay

3.13.1 Preparation of cell lysates from apoptosis cells

Apoptosis in Chago-k1 cells was induced in a density of $1x10^6$ cells by the addition of protein hydrolysates at a concentration of IC_{50} value, and then incubated at 37 °C in a 5% CO_2 for 24, 48 and 72 h. The induced cells and the control cells were harvested by trypsinization and washed with 20 mM cold PBS. Centrifugation was performed at 2,5000 x g for 5 min and the supernatant removed. The cell pellets were suspended in 1X lysis buffer 100 µl. The cells were incubated on ice for 15-20 minutes. The lysed cells were centrifuged at 2,500 for 5 min at 4 °C. The supernatants were transferred to new tubes and the cell lysates kept at -70ºC.

3.13.1.1 Caspase 3 activity assay

Caspase 3 was determined by Caspase 3 Assay Kit, Colorimetric. The caspase 3 colorimetric assay was based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-Nitroanililine (pNA) moiety. p- Nitroanililine has a high absorbance at 405 nm.The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

First, 5 µL of cell lysate caspase 3 positive control was placed into 96-well plates. 1X Assay Buffer 90, 85 and 75 µL were added into each of the wells for reagent blank, non-induced cells or induced cells or caspase 3 positive

control and inhibitor-non-induced cells or inhibitor-induced cells or inhibitor-caspase 3 positive control, respectively. The Caspase 3 inhibitor was added to the appropriate wells. Later, 10 µl of caspase 3 substrate was added to each well and mixed gently by shaking. This was then incubated at 37 °C for 70 to 90 minutes. The caspase activity was calculated as follows:

$$\text{Activity } (\mu\text{mol pNA / min/ mL}) = \frac{\mu\text{mol pNA} \times \mathbf{d}}{\mathbf{t} \times \mathbf{v}}$$

v = volume of sample in ml, d = dilution factor, t =reaction time in minutes

3.13.1.2 Caspase 8 activity assay

Caspase 8 was determined by Caspase 8 Assay Kit, Colorimetric. The assay is based on the hydrolysis of the peptide substrate Acetyl-Ile-Glu-Thr-Asp p-nitroaniline (Ac-IETD-pNA) by caspase 8 resulting in the release of a p-Nitroaniline (pNA) moiety . p- Nitroanililine. has absorbance at 405 nm. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

First,10 µl of Caspase 8 positive control was placed in 96 wells. 1x assay buffer 90, 80 and 78 µL were added into the well of reagent blank, caspase 8 positive control or non-induced cells or induced cells and inhibitor-caspase 8 positive control or inhibitor-non-induced cells or inhibitor-induced cells, respectively. Caspase 8 inhibitor was added to the appropriate wells and incubated at 5 min at room temperature.

Later, 10 µl of caspase 8 substrate was added and measured with absorbance at 405 nm. The caspase activity was calculated as follows:

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

v = volume of sample in ml, d = dilution factor, t = reaction time in minutes, A1 nmole = absorbance of 1 nmole of pNA in the well, At = absorbance at time t minutes, A0 = absorbance at zero time

3.13.1.3 Caspase 9 activity assay

Caspase 9 was determined by Caspase 9 Assay kit, Colorimetric. The assay is based on the spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate LEDH-pNA. p- Nitroanililine has absorbance at 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase 9 activity.

First, 20 µl of 5 X assay buffer added with DI H_2O 80, 70 and 60 µl was placed into the well of the buffer bank, substrate blank test sample and test sample inhibitor (optional), respectively. 10 µl was added into the well of the test sample. Later, caspase 9 substrate 10 µl was added into the well of the substrate blank and test sample. This was incubated for 2 h. at 37C and absorbance measured at 405 nm. The caspase activity was calculated as follows:

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

v = volume of sample in ml, d = dilution factor, t =reaction time in minutes

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Amino acid composition of monkey's head mushroom

Intact proteins, protein hydrolysates, individual peptides and amino acids have been reported to have significant antioxidant properties. Usually peptide fractions show greater antioxidant activity than intact proteins and amino acids. The antioxidant activities of protein digests vary depending on their peptide structure, i.e., size of the peptides and their amino acid sequences, which are influenced by the source of protein and conditions of hydrolysis involved (Chen et al., 1998). The relationship between hydrophobic amino acid and the reducing power of hydrolysates have been previously reported (Chen et al., 1998). The reducing power assay is used to evaluate the ability of a compound to donate an electron or hydrogen to fa ree radical thereby converting the radical to stable substances (Chen et al., 2012). Data concerning qualitative and quantitative amino acids composition is presented in **Table 8**. Amino acid composition indicates the nutritional quality of protein. Glutamic acid and aspartic acid were found to be the major non-essential amino acids in the samples tested. The results indicated that all essential amino acids, except S-containing types and tryptophan, are present in high amounts in this species. Moreover, protein showed the presence of phenylalanine, tyrosine, and histidine; these amino acids have also been reported to show antioxidant activity. Therefore, protein hydrolysates have antioxidant activity as well as high nutritive value because of the presence of important amino acids (Chen et al., 1998). The

results are comparable to those of earlier works (Gokavi et al., 2004), while there are no previous reports on the amino acid composition of monkey's head mushroom.

Table 8 Amino acid composition of monkey's head mushroom (mg/100mg)

Amino acid	Results (%)
Alanine	0.11
Arginine	0.30
Aspartic Acid	0.23
Cysteine	0.15
Glutamic Acid	0.42
Glycine	0.11
Histidine	0.08
Isoleucine	0.07
Leucine	0.14
Lysine	0.28
Methionine	0.03
Phenylalanine	0.05
Proline	0.10
Serine	0.11
Threonine	0.12
Tryptophan	0.05
Tyrosine	0.14
Valine	0.10

4.2 Antioxidant activities

The free radical scavenging activities of peptide from monkey's head mushroom were evaluated by DPPH, ABTS and NO assay. As shown in Table 9, the results indicate that 1% alcalase is an effective protease that can release effective antioxidant peptide from monkey's head mushroom with IC_{50} values of 31.54±1.86 4.94±0.07 and 54.62±0.40 µg/ml, by DPPH, ABTS and NO assay respectively. So, the preparation of peptide from monkey's head mushroom using 1% alcalase was selected. Monkey's head mushroom hydrolysate was fractionated using ultrafiltration with four molecular weight cut-off membranes (10, 5, 3, and 0.65 kDa). Among the fractions MW < 0.65 kDa showed the highest DPPH, ABTS, and NO radical scavenging activities with IC_{50} values of 0.28±.023, 18.73±2.90 and 20.56±2.10 µg/ml, respectively (Table 10). Several studies indicated that low molecular mass active peptides enriched by ultrafiltration could easily cross the intestinal barrier to exert biological effects and interact with targets (Sun et al., 2004), (Wang et al., 2013). It could be suggested that higher radical scavenging activities of MW < 0.65 kDa were due to its low molecular weight. MW < 0.65 kDa was separated into two sub-fractions (F1, and F2) as shown in Figure. 4.1, by Superdex-G-75. F1 showed the highest DPPH, ABTS, and NO radical scavenging activities with IC_{50} of 110.83±10.08, 5.73±0.67, and 23.65±5.51 µg/ml, respectively. Therefore, F1 was selected for follow-up stud

Table 9 The IC_{50} values of DPPH, ABTS, and NO radical scavenging of peptide hydrolysate from monkey's head mushroom

Enzyme		IC_{50} (µg/mL)		
		DPPH	ABTH	NO
Alcalase	1%	31.54±1.86[a]	4.94±0.07[a]	54.62±0.40[a]
	2.5%	38.17±2.24[a]	5.98±0.12[a]	73.82±1.23[a]
	5%	33.51±2.32[a]	5.93±0.32[a]	96.32±4.69[a]
Flavourzyme	1%	101.91±10.48[a]	29.79±5.49[a,b,c]	144.07±5.41[a]
	2.5%	95.41±11.45[a]	46.81±15.22[c]	66.74±0.90[a]
	5%	70.68±2.18[a]	13.25±0.58[a,b]	45.62±0.74[a]
Neutrase	1%	411.67±44.74[b]	114.13±3.98[d]	270.57±32.05[b]
	2.5%	143.70±1.93[a]	43.71±1.73[c]	271.97±38.28[b]
	5%	104.91±4.04[a]	38.15±2.83[b,c]	78.44±10.79[a]

*All data are presented by mean ± standard deviation of triplicate results. [a-d] values with the same letters indicate no significant difference for each group of samples at the same protease ($p > 0.05$).

**Ascorbic acid as a positive control for DPPH, and ABTS assay with IC_{50} 19.16±3.40, and 112.27±18.47 µg/ml, respectively.

***Curcumin as a positive control for NO assay with IC_{50} 259.40±107.15 µg/ml.

Table 10 Ultrafiltration separation of peptide hydrolysate from monkey's head mushroom prepared by 1% alcalase digestion and their free radical scavenging capacity determined by DPPH, ABTS, and NO assay.

Cut off (kDa)	IC_{50} (µg/mL)		
	DPPH	ABTH	NO
>10	501.90±121.08^{c}	119.47±9.64^{d}	297.00±8.26^{c}
10-5	252.73±101.63^{b}	64.99±4.27^{b}	162.47±19.06^{b}
5-3	82.97±10.49^{a,b}	51.20±10.83^{b}	120.80±11.13^{b}
3-0.65	65.33±5.78^{a,b}	29.46±1.50^{a}	209.47±64.63^{b,c}
□□0.65	0.28±.023^{a}	18.73±2.90^{a}	20.56±2.10^{a}

*All data are presented by mean ± standard deviation of triplicate results. $^{a-d}$ values with the same letters indicate no significant difference for each group of samples at the same assay ($p > 0.05$).

**Ascorbic acid as a positive control for DPPH, and ABTS assay with IC_{50} 26.97±0.34, and 98.35±3.87 µg/ml, respectively.

***Curcumin as a positive control for NO assay with IC_{50} 121.33±121.33 µg/ml.

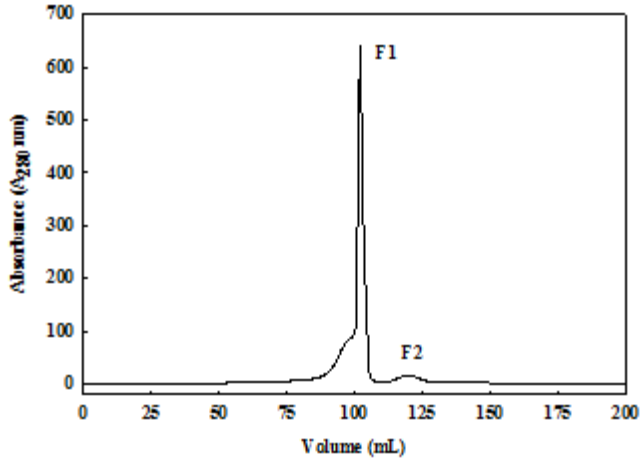


Figure 9 Gel filtration chromatography of MW < 0.65 kDa fraction from monkey's head mushroom hydrolysate on a Sephadex G-75. The results are presented as the mean ± SEM (n = 3); means with different lower case letters indicate significance between groups of P < 0.05.

4.3 RP-HPLC

The F1 fraction was separated by RP-HPLC and the four purified fractions (F11, F12, F13, and F14) were isolated (**Figure.10**). The purification procedures were repeated until enough samples were collected for further identification by mass spectrometry.

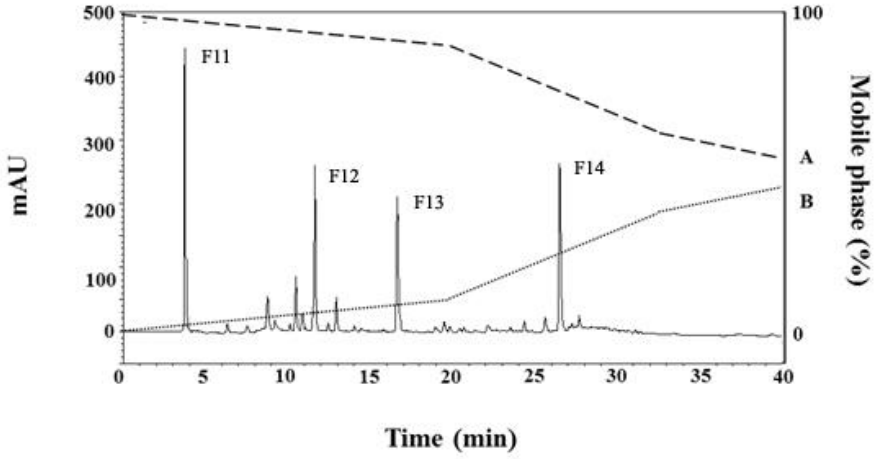


Figure 10 Ultraviolet chromatogram of reverse phase HPLC on a reversed phase C_{18} column of F1. Flow rate, 0.7 mL/min; monitoring absorbance, 280 nm, A: 0.1% (v/v) trifluoric acetic acid, B: 70% (v/v) acetonitrile in 0.05% (v/v) trifluoric acetic acid. Collected fractions were F11-F14.

4.4 Mass spectrometry

The F1 fraction was separated by RP-HPLC and four purified fractions (F11, F12, F13, and F14). The peptide sequences were identified by a LC-MS/MS Q-TOF mass spectrometer. The fraction F11 showed 1 peptide chain of protein hydrolysate from peak 1. The fraction F12 showed 2 peptide chains of protein hydrolysate from peak 2. The fraction F13 showed 1 peptide chain of protein hydrolysate from peak 3. The fraction F14 showed 3 peptide chains of protein hydrolysate from peak 4. All data were analyzed by de novo peptide sequencing. Table 4.4 shows the peptide sequence of protein hydrolysate. In previous research Try, Typ, Phe, His and Cys showed the highest

antioxidant activity (Lettera et al., 2010). Aromatic amino acids in peptides consisting of Phe, Tyr, and Trp could make active oxygen stable through direct electron transfer and very good radical scavenging activity.

Table 11 Peptide sequence of F1 identified by LC-MS/MS Q-TOF

Fraction	Sequence	Organism	Mass (kDa)	Query cover (%)	Identity (%)	Accession number
Peak 1	LVATCPHK	hypothetical protein HETIRDRAFT_434843 [*Heterobasidion irregulare* TC 32-1]	869	87	86	XP_009547665.1
Peak 2	TVNSPWHGA	hypothetical protein STEHIDRAFT_54890 [*Stereum hirsutum* FP-91666 SS1]	969	77	86	XP_007302357.1
	WVVYHVHLV	laccase [*Hericium coralloides*]	1151	88	63	BAQ25793.1
Peak 3	NSPAPRAR	hypothetical protein STEHIDRAFT_118696 [*Stereum hirsutum* FP-91666 SS1]	868	100	88	XP_007300670.1
Peak 4	WPVTTHK	manganese peroxidase 2 [*Hericium erinaceus*]	869	57	100	AFD50189.1
	VLAVTSNHK	ribonuclease T2 [*Hericium erinaceus*]	969	66	83	BAC21193.1
	LVAHRGPHAGH	ribonuclease T2 [*Hericium erinaceus*]	1152	18	100	BAC21193.1

4.5 Protection effect of the purified peptide on oxidation-induced DNA damage

The protection effect of the purified peptide on DNA damage was induced by hydroxyl radicals based on the Fenton reaction. The peptide protects the DNA of purified peptide hydrolysate from the protein hydrolyze of monkey's head mushroom with 1% alcalase. The results showed that the super coil (SC) form in the DNA was converted to the open circular (OC) form due to the hydroxyl radical damage based on the Fenton reaction ($Fe^{2+} + H_2O_2$). The purified peptide hydrolysate (F1) at 0.0472-0.0015 µg/ml (LAN 4-12) also protected the supercoiled DNA strand in the three plasmid DNAs of pBR322, pUC19 and pKS induced by the Fenton reaction. As shown in Figures 11, 12 and 13. Since a DNA is another major sensitive biotarget of oxidative damage, these results clearly explain the protective effect of the purified peptide against oxidative damage (Sheih et al., 2009).

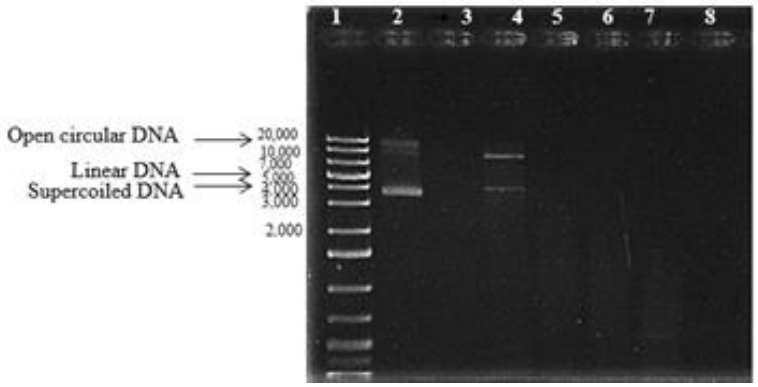


Figure 11 Protective effect of the F1 fraction on the hydroxyl radical-induced oxidation of pBR322 plasmid DNA. Lane 1: marker 1 kbp , Lane 2: pBR322 plasmid DNA 4,361 bp Lane 3: pBR322 plasmid DNA with $FeSO_4$ and H_2O_2 treatment (as DNA damage control) Lane 4-12: pBR322 plasmid DNA with $FeSO_4$ and H_2O_2 treatment in the presence of the F1 fraction at the concentrations of 0.0472, 0.0236, 0.0118, 0.0059, 0.00295 and 0.0015 µg/mL respectively.

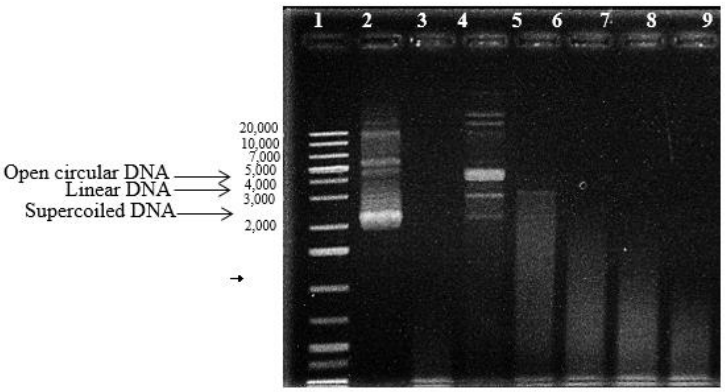


Figure 12 Protective effect of the F1 fraction on the hydroxyl radical-induced oxidation of pKS plasmid DNA. Lane 1: marker 1 kbp, Lane 2: pKS plasmid DNA 2,958 bp, Lane 3: pKS plasmid DNA with FeSO4 and H_2O_2 treatment (as DNA damage control), Lane 4-12: pKS plasmid DNA with $FeSO_4$ and H_2O_2 treatment in the presence of the F1 fraction at the concentrations of 0.0472, 0.0236, 0.0118, 0.0059, 0.00295 and 0.0015 µg/mL respectively.

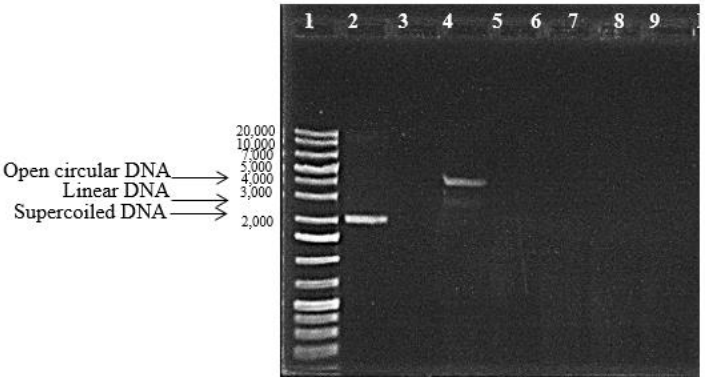
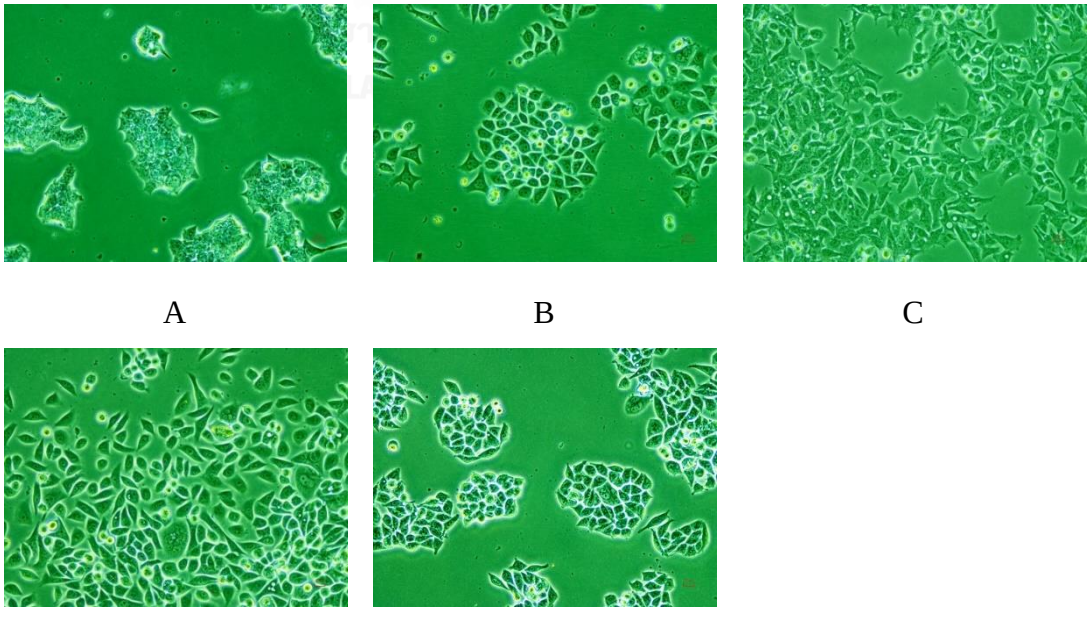


Figure 13 The protective effect of the F1 fraction on the hydroxyl radical-induced oxidation of pUC19 plasmid DNA. Lane 1: marker 1 kbp, Lane 2: pUC19 plasmid DNA 2,686bp, Lane 3: pUC19 plasmid DNA with $FeSO_4$ and H_2O_2 treatment (as DNA damage control), Lane 4-12: pUC19 plasmid DNA with $FeSO_4$ and H_2O_2 treatment in the presence of the F1 fraction at the concentrations of 0.0472, 0.0236, 0.0118, 0.0059, 0.00295 and 0.0015 µg/mL respectively

4.6 Cytotoxicity assay

The monkey's head mushroom protein hydrolysate was applied in a cytotoxicity assay of human malignant cell lines. The efficiency of antioxidant activity by radical scavenging was evaluated. Six human malignant cell lines were chosen for the investigation of cancer cells consisting of BT474 (breast), Chago-K1 (lung), Hep-G2 (hepatoma), KATO-III (gastric), and SW620 (colon). The purified peptide hydrolysate F1 with the most effective antioxidant activity was used to test the cytotoxicity assay with cancer cell lines (Figure 14). The monkey's head mushroom fraction showed significant anticancer activity against the five human malignant cell lines, BT474 (14.48±0.67 µg/ml), Chago-K1 (18 85±0.26 µg/ml), Hep-G2 (27.41±0.62 µg/ml), KATO-III (28.68±1.60dµg/ml) and SW620 (23.98±0.79µg/ml). These results show that Chago-K1 cells had the best anticancer activity (Table 12) (Varsano et al., 1998).



A B C

D E

Figure 14 Image from the microscope showing the morphology of cancer cell lines (A) BT474, (B) Chago, (C) HEP-G2, (D) KATO-III, (E) SW620 and (F) Wi-38

Table 12 The results of five different human malignant cell lines treated with monkey's head mushroom

Type of cancer cell lines	Antiproliferative activity (IC^{50}) (μg/mL)
BT474	14.48 ± 0.67^{b}
Chago-K1	8.85 ± 0.26^{a}
Hep-G2	27.41 ± 0.62^{d}
KATO-III	28.68 ± 1.60^{d}
SW620	23.98 ± 0.79^{c}

*All data are presented by mean ± standard error of triplicate results. $^{a-d}$ values with the same letters indicate no significant difference for each group of fraction samples at the same antiproliferative activity (IC_{50}) ($p > 0.05$).

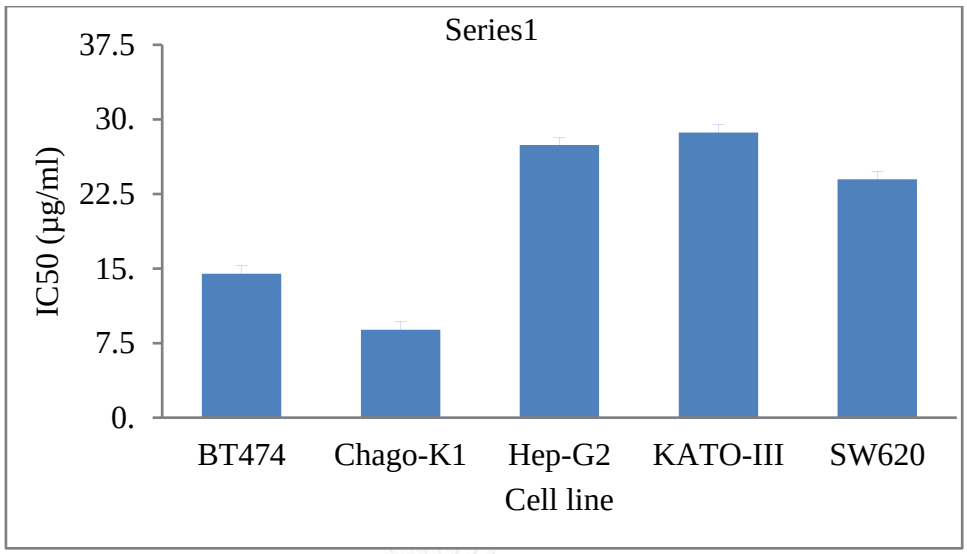
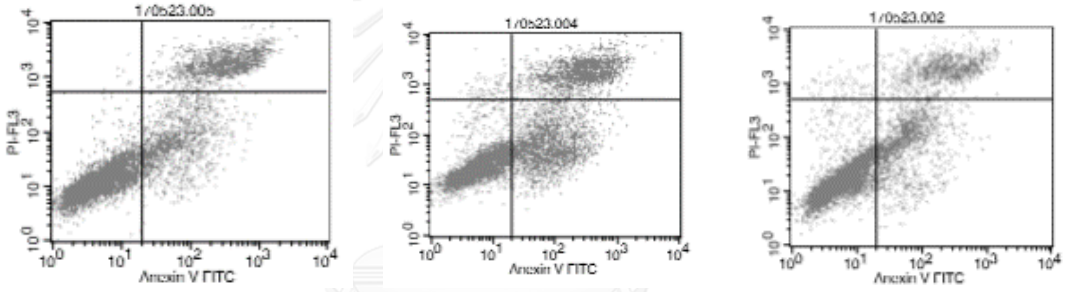


Figure 15 MTT assay of purified peptide hydrolysate (F1) on different cell lines

4.7 Apoptosis by flow cytometer

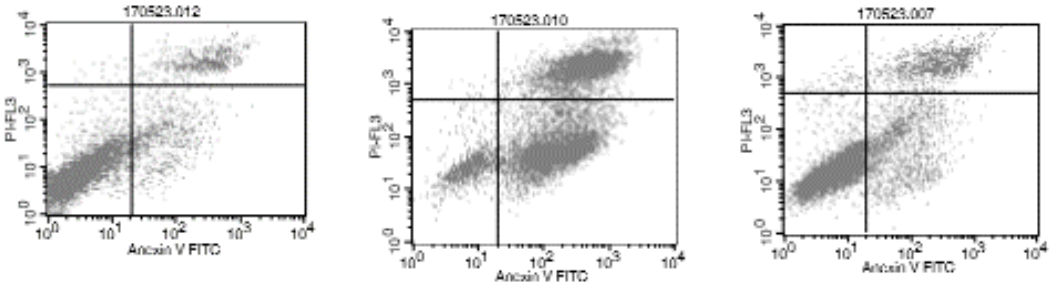
Apoptosis is the process of programmed cell death that occurs in multicellular organisms when cells are damaged by disease or toxic agents. The results showed that protein hydrolysates prepared from Chago-K1 cell viability. The effect of protein hydrolysates on the promotion of apoptosis in Chago-K1 cells was also investigated. Apoptosis cells stained with Annexin V-FITC and PI were classified as necrotic cells (the upper left quadrant; Annexin^{-}/PI^{+}), late apoptotic cells (the upper right quadrant; Annexin^{+}/PI^{+}), intact cells (the lower left quadrant; Annexin^{+}/PI^{-}) or early apoptotic cells (the lower right quadrant; Annexin^{+}/PI^{-}). Chago-K1 cells were cultured in RPMI-1640 medium for 24 h, and then treated with protein hydrolysate F1 for 24, 48 and 72 h as shown in Figures 16, 18 and 19. The percentages of cell death at the early apoptosis, late apoptosis and necrosis apoptosis stage are shown in Figure 19.

As shown in Figure 19, protein hydrolysate F1(protein content 139.63 µg/ml) treated with Chago-K1 cells for 24 h could induce early apoptotic cells, late apoptotic cells and necrotic cells at 17.28±2.64, 12.81±3.56 and 0.87±0.27 %, respectively. 48 h could induce early apoptotic cells, late apoptotic cells and necrotic cells at 14.25±0.79, 15.48±5.76 and 0.93±0.37 %, respectively. Also, 72 h could induce early apoptotic cells, late apoptotic cells and necrotic cells at 20.69±0.09, 22.88±0.60 and 1.35±0.31 %, respectively (Pattamadilok, 2016).



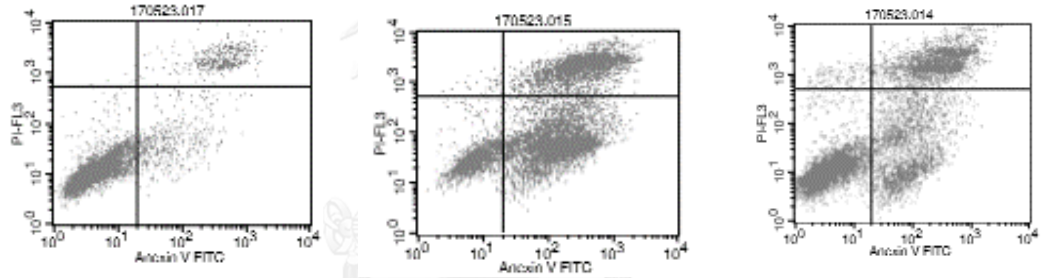
A B C

Figure 16 Flow cytometry analysis of Chago-K1 cells treated with (A) RPMI+10%FCS (negative control) (B) 0.5 µg/mL doxorubicin (positive control). (C) purified protein hydrolysate in F1 and labeled with Annexin V-FITC and PI for 24 h. Quadrants: Lower left – the normal or live cells; Upper left – the necrotic cells; Lower right – the early apoptotic cells; Upper right – the late apoptotic cells.



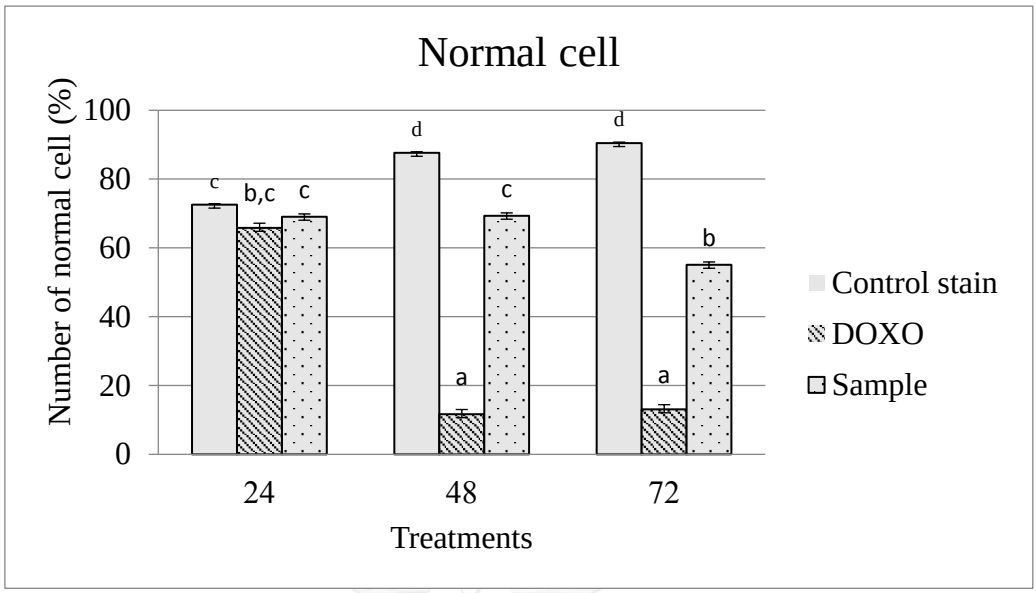
A B C

Figure 17 Flow cytometry analysis of Chago-K1 cells treated with (A) RPMI+10%FCS (negative control) (B) 0.5 µg/mL doxorubicin (positive control) (C) purified protein hydrolysate in F1 and labeled with Annexin V-FITC and PI for 48 h. Quadrants: Lower left – the normal or live cells; Upper left – the necrotic cells; Lower right – the early apoptotic cells; Upper right – the late apoptotic cells.

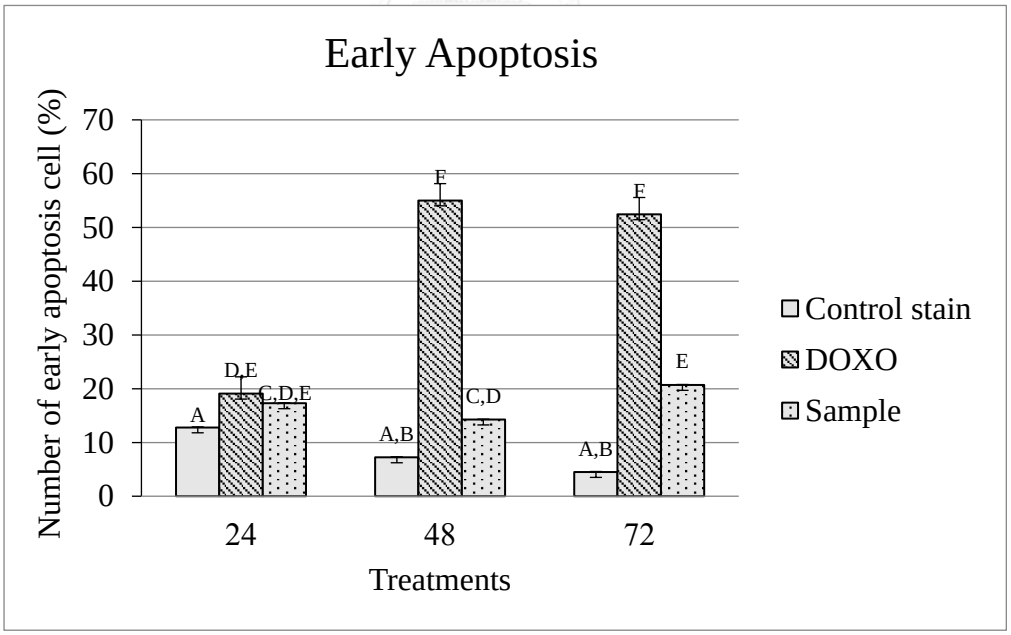


A B C

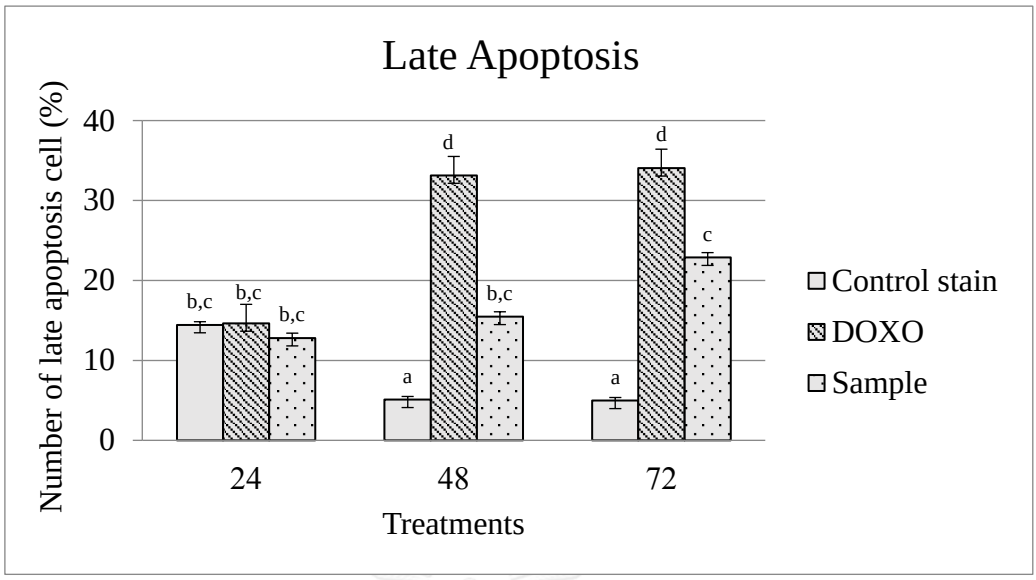
Figure 18 Flow cytometry analysis of Chago-K1 cells treated with (A) RPMI+10%FCS (negative control) (B) 0.5 µg/mL doxorubicin (positive control). (C) purified protein hydrolysate in F1 and labeled with Annexin V-FITC and PI for 72 h. Quadrants: Lower left – the normal or live cells; Upper left – the necrotic cells; Lower right – the early apoptotic cells; Upper right – the late apoptotic cells.


Normal cell
Number of normal cell (%)
100
80
60
40
20
0
c
b,c
c
d
a
c
d
a
b
24
48
72
Treatments
Control stain
DOXO
Sample

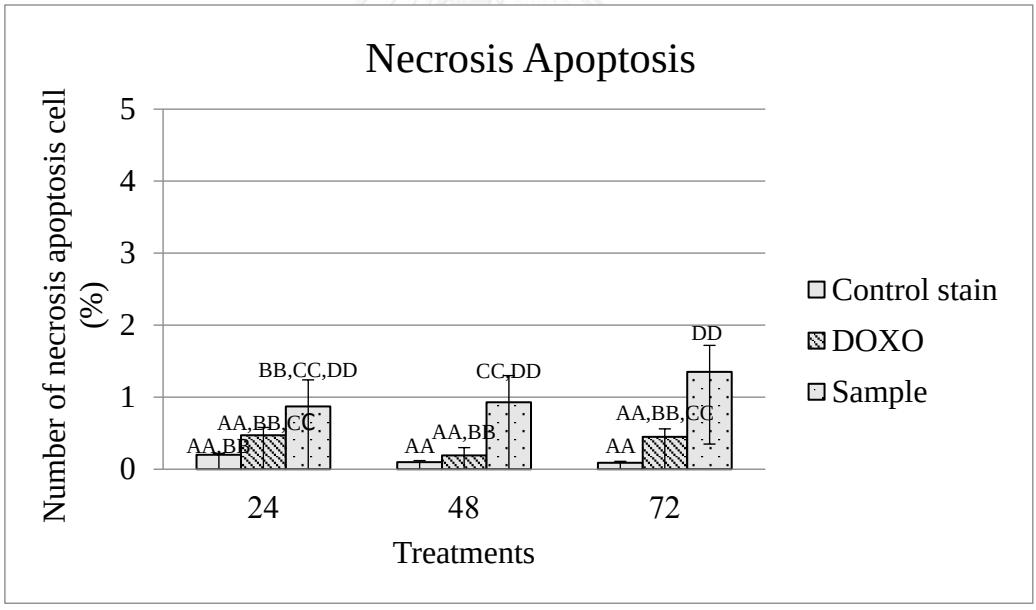
A


Early Apoptosis
Number of early apoptosis cell (%)
70
60
50
40
30
20
10
0
A
D,E
C,D,E
A,B
E
C,D
A,B
E
E
24
48
72
Treatments
Control stain
DOXO
Sample

B



C



D

Figure 19 The percentage of the number of Chago-k1 cells using the flow cytometry of four quadrants consisted of (A) normal or live cells, (B) early apoptotic cells, (C) late apoptotic cells and (D) necrotic cells after being treated for 24, 48 and 72 h. All data are presented as the mean ± standard error of triplicate results.

4.8 Caspase 3, 8 and 9 activities assay

F1 fraction at the concentration of 139.63 µg/mL from gel filtration chromatography was selected for analyzing caspases 3, 8 and 9 activities in cell lysates of Chago-k1 cells for 24, 48 and 72 h. Caspase 3 activity was determined using a Caspase 3 Colorimetric Assay Kit. As presented in **Table 13,** the values of caspase 3 activity for F1 fractions at 24, 48 and 72 h were $2.99\times10^{-4}\pm17.93\times10^{-3}$, $4.94\times10^{-4}\pm23.95\times10^{-3}$ and $1.37\times10^{-4}\pm5.91\times10^{-3}$µmole pNA/min/mL, respectively. The results imply that the F1 fraction at 24 h could induce the highest activity of caspase 3 as shown in **Table 13**. Caspase 8 activity was determined using a Caspase 8 Colorimetric Assay Kit. As seen from Table 13 , the F1 fractions at 24 and 72 h were $4.50\times10^{-5}\pm0.11\times10^{-3}$, $4.91\times10^{-5}\pm2.93\times10^{-4}$ and $1.63\times10^{-5}\pm2.93\times10^{-4}$µmole pNA/min/mL, respectively. Caspase 9 activity was determined using a Caspase 9 Colorimetric Assay Kit. As shown by Table 13, the F1 fractions at 24 and 72 h were $4.44\times10^{-6}\pm2.51\times10^{-7}$, $7.10\times10^{-6}\pm1.97\times10^{-7}$ and $1.55\times10^{-5}\pm9.02\times10^{-8}$ µmole pNA/min/mL

Table 13 Caspase 3, 8 and 9 activities of the F1 fraction (protein content was 139.63µg/mL) from gel filtration chromatography on Superdex 75

Treatments	Caspase 3 activity (µmole pNA/min/mL)	Caspase 8 activity (µmole pNA/min/mL)	Caspase 9 activity (µmolepNA/min/mL)
F1 fraction for 0 h	$9.621\times10^{-3}\pm12.04\times10^{-3}$	$2.38\times10^{-5}\times0.48\times10^{-3}$	$4.65\times10^{-6}\pm2.51\times10^{-7}$
F1 fraction for 24 h	$2.99\times10^{-4}\pm17.93\times10^{-3}$	$4.50\times10^{-5}\pm0.11\times10^{-3}$	$4.44\times10^{-6}\pm2.51\times10^{-7}$
F1 fraction for 48 h	$4.94\times10^{-4}\pm23.95\times10^{-3}$	$4.91\times10^{-5}\pm2.93\times10^{-4}$	$7.10\times10^{-6}\pm1.97\times10^{-7}$
F1 fraction for 72 h	$1.37\times10^{-4}\pm5.91\times10^{-3}$	$1.63\times10^{-5}\pm2.93\times10^{-4}$	$1.55\times10^{-5}\pm9.02\times10^{-8}$

CHAPTER V

CONCLUSION

The results of this study showed that protein hydrolysate derived from monkey's head mushroom had properties for antioxidant activities. The purified protein by ultrafiltration, gel filtration and RP-HPLC was purified crude peptide to low molecular weight peptides with the highest antioxidant activity. The purified peptide F1 also has significant protective effects on the DNA damage caused by hydroxyl radicals. Also, this peptide was applied to the antiproliferative activity against five cancer cell lines by MTT assay. The results showed the purified peptide (F1) had greater inhibition against the proliferation of Chago-k1 cell lines. Moreover, F1 could induce apoptosis in Chago-k1 cell line that showed caspase 3 activity, caspase 8 and caspase 9 activity in apoptosis cells. The best protein hydrolysate (F1) was purified by RP-HPLC, identified by LC-MS/MS Q-TOF mass spectrometer and its peptide sequence analyzed. Based on these results, the peptides obtained from monkey's head mushroom by enzymatic hydrolysis could be used as potential natural antioxidants to enrich the antioxidant properties of functional and fresh foods, and neutraceuticals.

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APPENDIX

APPENDIX A

Preparation of buffer and reagents

1. 20 mM Phosphate buffer pH 7.2

20mM KH_2PO_4	2.72 g/l
20mM K_2HPO_4	3.48 g/l
150mM NaCl	8.76 g
Deionized water	1000 ml

Protocal: Mix 1000 ml deionized water with all reagents. Adjust the pH to 7.2 with KOH

2. Bradford solution

- Bradford stock solution

95% Ethanol	100 ml
88% Phosphoric acid	200 ml
SERVA Blue G	350 g

- Bradford working buffer

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

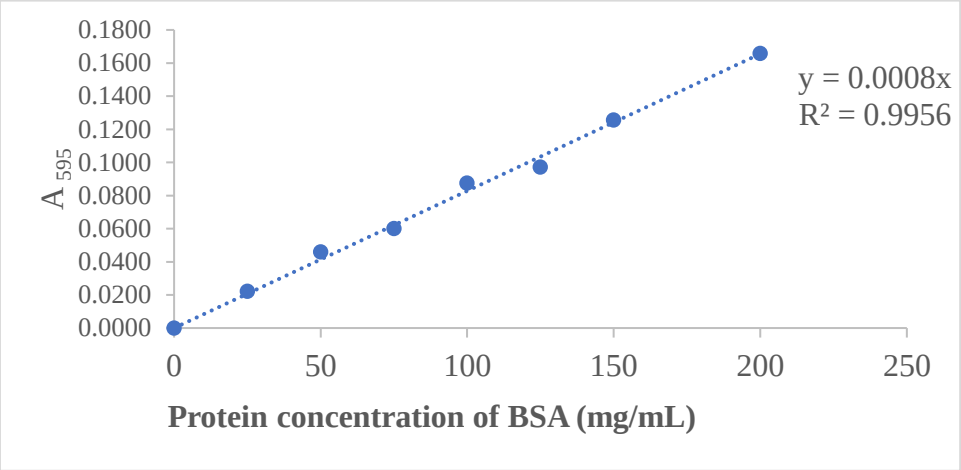
Note: Before using, Bradford working buffer must be filtered through the Whatman No.1 paper. It is stored in a brown glass bottle at room temperature.

Protocal: 1. Pipet protein solution into 96 well plates 20 µl.

2. Add Bradford working buffer 200 µl and shake.

3. Read A_{595} after 2 minutes but before 1 hour.

Standard curve for protein determination by Bradford method



3. DPPH solution

0.1 M DPPH	0.004 mg
Methanol	100 ml

Protocal: 1. Pipet protein solution into 96 well plates 80 µl.

2. Add DPPH solution 320 µl and incubate 20 min in the dark room.

3. Shake and Read the absorbance at 517 nm

4. ABTS solution

- 7 mM ABTS (solution A)

Dissolve 0.096 g ABTS in 25 ml deionized water.

- 2.45 mM potassium persulphate (solution B).

Dissolve 0.016 g potassium persulphate in 25 ml deionized water

- ABTS solution

Mix solution A and solution B in the dark room for 12 – 16 hours before using. Before use it, dilute ABTS solution with distilled water to obtain an absorbance value of 0.7 ± 0.02 at 734 nm

Protocal: 1. Pipet protein solution into 96 well plates 25 µl.

2. Add ABTS solution 750 µl and incubate 10 min in the dark room.

3. Shake and Read the absorbance at 734 nm

5. Nitric oxide method

- 0.1% (w/v) N- (1-Napthyl) ethylenediamine dihydrochloride (NED)

Dissolve 0.1 g NED in 100ml deionized water.

- 10 mM sodium nitroprusside (SND) in PBS pH 7.2

Dissolve 0.29 g Sodium nitroprusside in 100 ml Phosphate buffer pH 7.2 in the dark room.

- 0.33% (w/v) sulfanilamide in 20% acetic acid

Start with dissolve 0.33 g sulfanilamide in 100 ml in 5 % phosphoric acid (5 ml phosphoric acid in 95 ml distilled water.

Protocal : 1. Pipet protein solution into 96 well plates 25 µl.

2. Add SNP 25 µl and incubate 2.5 h

3. Add 0.33% (w/v) sulfanilamide 100 µl and incubate 5 min

4. Add 0.1% NED 100 µl and incubate 30 min

5. Shake and read the absorbance at 540 nm

6. Mobile phase in RP-HPLC analysis

- Eluent A: 0.1% trifluoroacetic acid (TFA), 1000 ml

start with add 1 ml TFA into 999 ml double deionized water followed by filtration using a cellulose acetate membrane.

- Eluent B: 70 % acetonitrile containing 0.05% TFA, 1000 ml

start with adding 300 ml 0.05% TFA in double deionized water into 700 ml 70% acetonitrile and

7. 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.

8. 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.

9. DNA damage

2 mM $FeSO_4$

Dissolve $FeSO_4 \cdot 7H_2O$ 0.0278 g in 50 mL of deionized water.

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.

10. MTT solution

5 mg/mL MTT solution

MTT	5 mg
Deionized water	1 mL

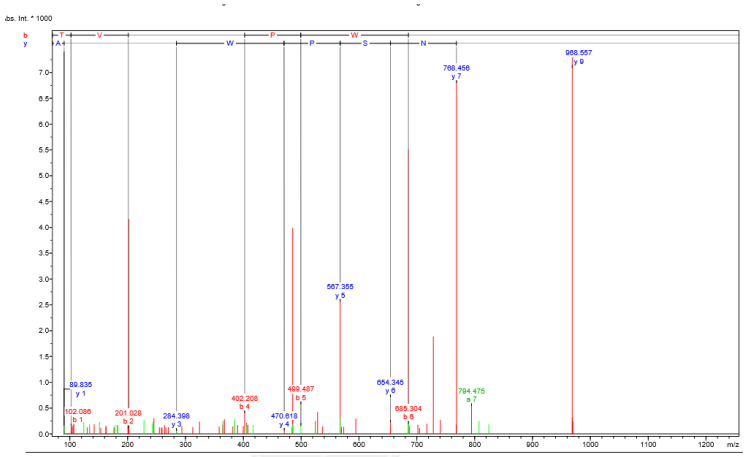
Protocol: Dissolve 5 mg MTT with 1 mL of deionized water.

APPENDIX B

PEAK 1

Sequence 11 LVATCPHK

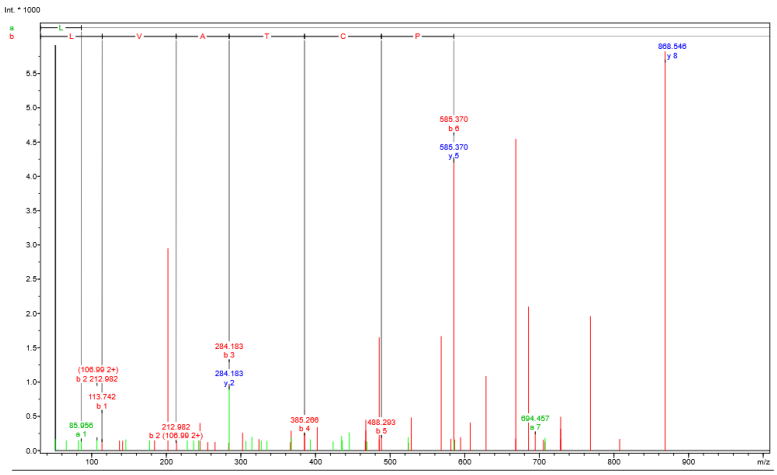
Parentmass: 868.545 Mass Error: 0.074 MH+ (mono): 868.471 MH+ (avg): 869.066 Threshold (a.i.): 0.000 Tolerance (Da): 0.500 Number of Peaks: 71 Above Threshold: 71 Assigned Peaks: 9 Not assigned Peaks: 62



PEAK 2

Sequence 12.1 TVNSPWHGA

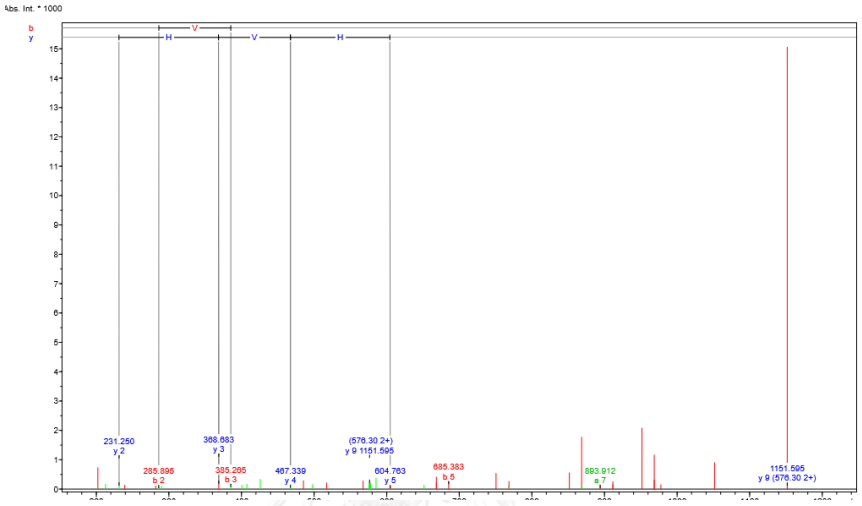
Parentmass: 968.558 Mass Error: 0.099 MH+ (mono): 968.459 MH+ (avg): 969.034 Threshold (a.i.): 0.000 Tolerance (Da): 0.500 Number of Peaks: 87 Above Threshold: 87 Assigned Peaks: 13 Not assigned Peaks: 74



PEAK 2

Sequence 12.2 WVVHVHV

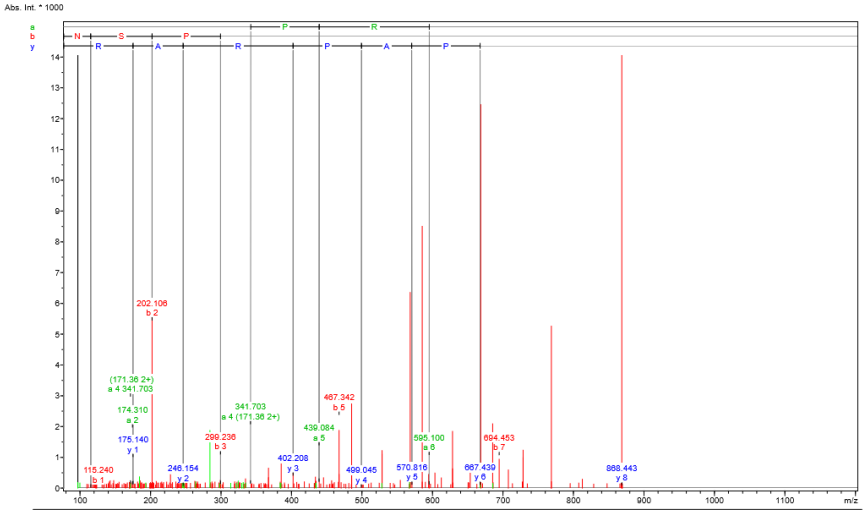
Parentmass: 1151.717 Mass Error: 0.080 MH+ (mono): 1151.637 MH+ (avg): 1152.370 Threshold (a.i.): 0.000 Tolerance (Da): 0.500 Number of Peaks: 55 Above Threshold: 55 Assigned Peaks: 9 Not assigned Peaks: 46



PEAK 3

Sequence 13 NSPAPA

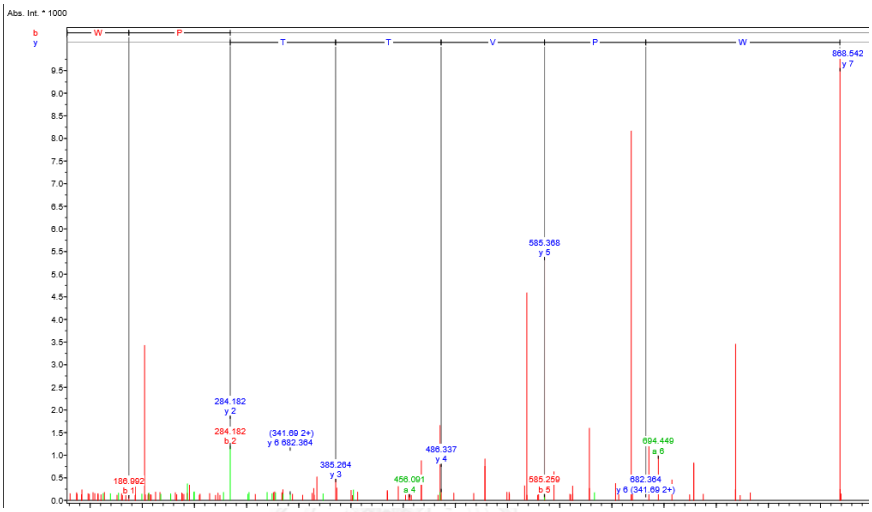
Parentmass: 868.542 Mass Error: 0.067 MH+ (mono): 868.475 MH+ (avg): 868.963 Threshold (a.i.): 0.000 Tolerance (Da): 0.500 Number of Peaks: 288 Above Threshold: 288 Assigned Peaks: 16 Not assigned Peaks: 27



PEAK 4

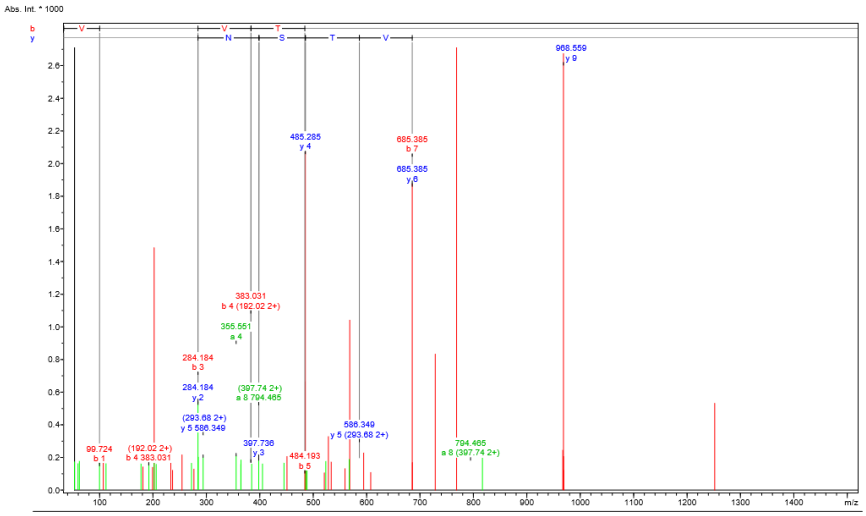
Sequence 14.1 WPVTTH

Parentmass: 868.541 Mass Error: 0.073 MH+ (mono): 868.468 MH+ (avg): 869.001 Threshold (a.i.): 0.000 Tolerance (Da): 0.500 Number of Peaks: 145 Above Threshold: 145 Assigned Peaks: 10 Not assigned Peaks: 135



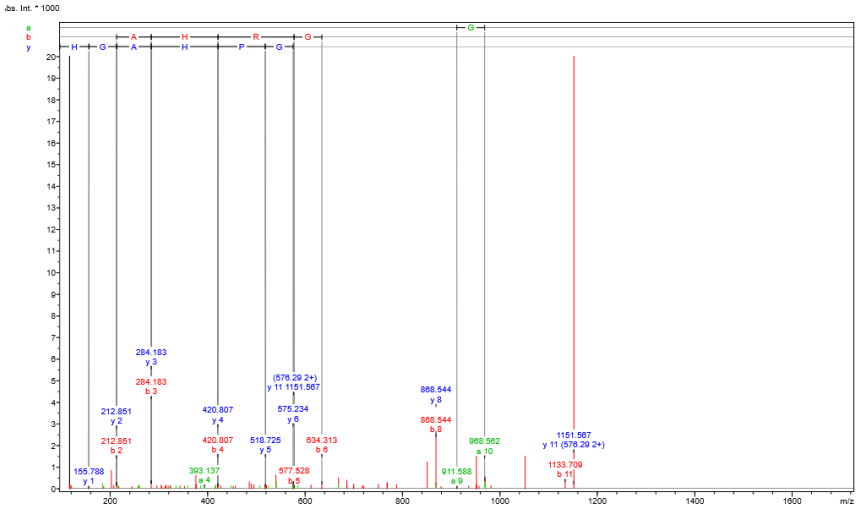
Sequence 14.2 VAVTSNH

Parentmass: 968.558 Mass Error: 0.005 MH+ (mono): 968.553 MH+ (avg): 969.118 Threshold (a.i.): 0.000 Tolerance (Da): 0.500 Number of Peaks: 54 Above Threshold: 54 Assigned Peaks: 11 Not assigned Peaks: 43



Sequence 14.3 VAHGPHAGH

Parentmass: 1151.716 Mass Error: 0.097 MH+ (mono): 1151.619 MH+ (avg): 1152.292 Threshold (a.i.): 0.000 Tolerance (Da): 0.500 Number of Peaks: 101 Above Threshold: 101 Assigned Peaks: 14 Not assigned Peaks: 87



APPENDIX C

Amino acid abbreviations and structures

Amino acid	Three-letter	One-letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	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

VITA

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Academic presentation

Sangtitanu, T., Chaisangsri, J., Saisavoey, T, Sangtanoo, P., and Karnchanatat, A. In vitro Antioidant activity of peptide hydrolysate from monkey's head mushroom Hericium erinaceus. The 11th International Symposium of the Protein Society of Thailand, 3-5th August 2016, Convention center, Chulabhorn Research Insititude Bangkok, Thailand. (Proceeding book)