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FREE RADICAL SCAVENGING AND ANTIPROLIFERATION OF PEPTIDE FROM CHICKEN FEATHER MEAL

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อนุมูลอิสระคืออะตอมหรือสารประกอบที่ประกอบด้วยอิเล็กตรอนโดดเดี่ยว สามารถทำปฏิกิริยากับเซลล์และโมเลกุลต่าง ๆ ในร่างกาย อนุมูลอิสระเหล่านี้ไม่สามารถถูกกำจัดและอาจก่อให้เกิดโรคหลายโรค ยกตัวอย่างเช่น โรคหลอดเลือดหัวใจตีบ โรคมะเร็ง โรคสมองเสื่อม และโรคไขข้ออักเสบ เป็นต้น ระบบป้องกัน ยับยั้ง หรือขจัดอนุมูลอิสระในร่างกายเป็นระบบหนึ่งที่มีความสำคัญต่อการขจัดอนุมูลอิสระเหล่านี้ให้หมดไป ดังนั้นจึงทำการศึกษาและเตรียมเพปไทด์จากขนไก่ป่นโดยการย่อยด้วยเอนไซม์จากจุลินทรีย์ ได้แก่ แอลคาเลส ฟลาโวไซม์ และนิวเทรส และศึกษาความสามารถในการขจัดอนุมูลอิสระพบว่าเพปไทด์จากขนไก่ป่นที่ถูกย่อยด้วยเอนไซม์นิวเทรส ความเข้มข้นร้อยละ 5 มีความสามารถในการขจัดอนุมูลอิสระสูงที่สุดเมื่อวิเคราะห์ความสามารถในการขจัดอนุมูลอิสระด้วยวิธี DPPH และ ABTS radical scavenging activity โดยแสดงค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งอนุมูลอิสระได้ร้อยละ 50 (IC_{50}) เท่ากับ 16.45±0.23 และ 9.34±0.08 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ต่อมาคัดแยกเพปไทด์ที่ได้ตามขนาดโมเลกุล ได้แก่ 10 5 3 และ 0.65 กิโลดาลตัน ด้วยเทคนิคอัลตราฟิลเตรชันและวิเคราะห์ความสามารถในการขจัดอนุมูลอิสระพบว่าเพปไทด์ที่มีขนาดโมเลกุลน้อยกว่า 0.65 กิโลดาลตัน (เพปไทด์ F5) สามารถขจัดอนุมูลอิสระเมื่อวิเคราะห์ด้วยวิธี DPPH และ ABTS radical scavenging activity ได้สูงที่สุดโดยแสดงค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งอนุมูลอิสระได้ร้อยละ 50 (IC_{50}) เท่ากับ 1.72±0.04 และ 0.42±0.02 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ จากนั้นนำเพปไทด์ที่มีขนาดน้อยกว่า 0.65 กิโลดาลตัน มาทำให้บริสุทธิ์ด้วยเทคนิคเจลฟิลเตรชันโครมาโตกราฟีโดยใช้คอลัมน์ Superdex® 75 พบว่าเพปไทด์ F52 มีความสามารถในการขจัดอนุมูลอิสระได้สูงที่สุดเมื่อวิเคราะห์ด้วยวิธี DPPH และ ABTS radical scavenging activity โดยแสดงค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งอนุมูลอิสระได้ร้อยละ 50 (IC_{50}) เท่ากับ 7.35±0.30 และ14.79±0.24 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ต่อมานำเพปไทด์ F52 ที่ได้มาทำให้บริสุทธิ์และพิสูจน์เอกลักษณ์ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงและเทคนิคแมสสเปกโตรเมตรี ตามลำดับ พบว่าเพปไทด์ F52 สามารถแยกได้เป็นเพปไทด์ 3 ชนิด ได้แก่ Phe-Asp-Asp-Arg-Gly-Arg-X (FDDRGRX หรือ F521 มีขนาดโมเลกุลคือ 875 ดาลตัน) Val-Thr-Leu-Ala-Val-Thr-Lys-His (VTLAVTKH หรือ F522 มีขนาดโมเลกุลคือ 868 ดาลตัน) และ Val-Ser-Glu-Ile-X-Ser-Ile-Pro-Ile-Ser (VSEIXSIPIS หรือ F523 มีขนาดโมเลกุลคือ 1055 ดาลตัน) และมีความเข้มข้นต่ำสุดที่สามารถยับยั้งอนุมูลอิสระได้ร้อยละ 50 (IC_{50}) เท่ากับ 32.01±2.17 22.52±4.87 และ 59.66±5.89 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ เมื่อวิเคราะห์ความสามารถในการขจัดอนุมูลอิสระด้วยวิธี DPPH radical scavenging activity นอกจากนี้ได้มีการนำเพปไทด์ F52 มาศึกษาความสามารถในการป้องกันการถูกทำลายของดีเอ็นเอจากพลาสมิด 3 ชนิด ได้แก่ pKS pUC19 และ pBR322 ด้วยอนุมูลไฮดรอกซี พบว่าเพปไทด์ F52 มีความสามารถในการป้องกันไม่ให้ดีเอ็นเอถูกทำลายไป และจากการศึกษาความสามารถในการยับยั้งการเพิ่มจำนวนเซลล์มะเร็งของเพปไทด์ F52 ด้วยวิธี MTT พบว่าเพปไทด์ F52 มีความสามารถในการยับยั้งการเพิ่มจำนวนเซลล์มะเร็งลำไส้ SW620 ได้สูงที่สุด อีกทั้งยังไม่เป็นพิษต่อเซลล์ โดยแสดงค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งเซลล์มะเร็งได้ร้อยละ 50 (IC_{50}) เท่ากับ 26.37±2.87 ไมโครกรัมต่อมิลลิลิตร นอกจากนี้เมื่อนำเพปไทด์ F52 ความเข้มข้นร้อยละ 0.5 (ปริมาณโปรตีนเท่ากับ 0.31 ไมโครกรัมต่อมิลลิลิตร) มาบ่มกับเซลล์มะเร็งลำไส้ SW620 พบว่าที่เวลา 24 และ 48 ชั่วโมง เพปไทด์ F52 ความเข้มข้นร้อยละ 0.5 (ปริมาณโปรตีนเท่ากับ 0.31 ไมโครกรัมต่อมิลลิลิตร) มีความสามารถในการชักนำให้เซลล์มะเร็งลำไส้ SW620 เกิดการตายแบบอะพอพโทซิสโดยใช้ชุดสีย้อม FITC Annexin V Apoptosis Detection Kit with PI และวิเคราะห์เปอร์เซ็นต์การตายแบบอะพอพโทซิสด้วยเทคนิคโฟลไซโทเมทรี นอกจากนี้ยังพบการเพิ่มขึ้นของการทำงานของเอนไซม์แคสเปส 3 และ 8 อีกด้วย ดังนั้นจึงสามารถสรุปได้ว่าเพปไทด์ F52 ที่เตรียมได้จากขนไก่ป่นสามารถใช้เป็นสารต้านอนุมูลอิสระและต้านการเพิ่มจำนวนเซลล์มะเร็งแหล่งใหม่ที่ได้จากธรรมชาติเพื่อนำไปประยุกต์ใช้ในด้านการพัฒนายาต่อไป

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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PICHAMON JEAMPAKDEE: FREE RADICAL SCAVENGING AND ANTIPROLIFERATION OF PEPTIDE FROM CHICKEN FEATHER MEAL. ADVISOR: ASSOC. PROF. APHICHART KARNCHANATAT, Ph.D., 89 pp.

A free radical is an atom or compound, which contains a lone pair electron reactive to cells and molecules in the human body. The free radical cannot be eliminated and plays an important role in many diseases such as coronary thrombosis, cancer, Alzheimer's, and arthritis. The antioxidant system in the body is one of the mechanisms that respond to free radicals. In this research peptides from chicken feather meal protein hydrolysates with microbial proteases (Alcalase®, Flavourzyme® and Neutrase®) were prepared and their antioxidant activities determined. Peptide fractions derived from chicken feather meal hydrolyzed by 5% Neutrase®, shown the highest DPPH and ABTS radical scavenging activity with IC_{50} values of 16.45±0.23 and 9.34±0.08 µg/mL, respectively. Peptide fractions were fractionated using molecular weight cut-offs of 10, 5, 3 and 0.65 kDa membranes and their antioxidant properties further analyzed. Of the fractions, MW < 0.65 kDa (F5 fraction) exhibited high levels of free radical scavenging activities towards DPPH and ABTS with IC_{50} values of 1.72±0.04 and 0.42±0.02 µg/mL, respectively. The F52 fraction from Superdex® 75 column presented the highest scavenging activities on DPPH and ABTS radicals with IC_{50} values of 7.35±0.30 and 14.79±0.24 µg/mL, respectively. The F52 fraction was purified using RP-HPLC and separated into three fractions (F521, F522, and F523). All fractions exhibited very strong DPPH radical scavenging activities (32.01±2.17, 22.52±4.87 and 59.66±5.89 µg/mL), and all fractions were identified by mass spectrometry as Phe-Asp-Asp-Arg-Gly-Arg-X (FDDRGRX, 875 Da), Val-Thr-Leu-Ala-Val-Thr-Lys-His (VTLAVTKH, 868 Da) and Val-Ser-Glu-Ile-X-Ser-Ile-Pro-Ile-Ser (VSEIXSIPIS, 1,055 Da), respectively. Furthermore, the F52 fraction could protect hydroxyl radical-induced DNA damage as shown in pKS, pUC19, and pBR322. In addition, as concerns cancer, the F52 fraction possessed high antiproliferative activity in human SW620 colon cancer cell lines using an MTT assay with IC_{50} values of 26.37±2.87 µg/mL, and 0.5% of the F52 fraction (protein content as 0.31 µg/mL) could induce apoptosis as measured by an FITC Annexin V Apoptosis Detection Kit with PI using flow cytometry and increased caspase 3 and 8 activities in SW620 cells for 24 and 48 h. Hence, F52 fraction could be used as a new natural antioxidant and source of antiproliferative activities for drug development.

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LIST OF ABBREVIATION

A	Absorbance
Amax	Maximum absorbance
Abs	Absorbance
ABTS	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
AIF	Apoptosis-inducing factor
ALA	Alpha-lipoic acid
APAF-1	Apoptotic protease activating factor 1
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
bp	Base pair
BSA	Bovine serum albumin
CAT	Catalase
CFCs	Chlorofluorocarbons
$Cl^{\bullet}$	Atomic chlorine radical
CO	Carbon monoxide
CO_2	Carbon dioxide
$CO_3^{-\bullet}$	Carbonate radical
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
DR4	TRAIL-R1
DR5	TRAIL-R2
ESI-Q-TOF MS	Electrospray ionization quadrupole time of flight mass spectrometry
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
H_2O	Water
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
$HOO^{\bullet}$	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
$LO^{\bullet}$	Lipid alkoxyl radical
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid peroxide
M	Molar
MEM	Eagle's Minimum Essential Medium
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
NSS	Normal saline
O_2	Oxygen
$O_2^{-\bullet}$	Superoxide radical
$^1O_2$	Singlet oxygen
O_3	Ozone
$OH^{\bullet}$	Hydroxyl radical
PBS	Phosphate buffered saline solution
PI	Propidium iodide
PUFAs	Polyunsaturated fatty acids
$RO^{\bullet}$	Alkoxyl radical
$RO_2^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high performance liquid chromatography
rpm	Round per minute
SE	Standard error
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
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µL	Microliter
µm	Micrometer or micron
µM	Micromolar
/	Per
%	Percentage
:	Ratio

CHAPTER I
INTRODUCTION

One of the gravest diseases for human beings is cancer. This ranges from breast cancer, lung cancer, liver cancer and colon cancer to gastric cancer, among others. Cancer is defined as a group of cells in the human body which seem unable to control growth rate. Cells undergo more divisions and abnormal transcription or translation caused by free radicals damaging the cell or tissue. Free radicals are unstable atoms or molecules composed of unpaired electrons in the outer orbital that are highly reactive and attack other molecules. Other molecules become new free radicals after being attacked by free radicals and so a chain reaction begins. However, free radicals are scavenged by the enzymatic antioxidant defense systems in the human body, for example, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST), in order to maintain balance in the body. Nevertheless, when the body produces too many free radicals this can result in oxidative stress, causing many chronic diseases including Alzheimer's disease, atherosclerosis or heart disease, arthritis, emphysema, aging, wrinkling and particularly cancer. As a result, the human body needs to receive antioxidants from other sources. Various causes behind the occurrence of free radicals include cholesterol from food, food preservatives and pesticides, ultraviolet light (UV light), chemotherapy and radiation, environmental pollution and smoking (Sharma, 1995). Furthermore, the rate of birth in several countries, for instance in Asia, is increasing rapidly bringing about greater pollution being released into the atmosphere – carbon dioxide (CO_2) from waste burning, carbon monoxide (CO), sulfur dioxide (SO_2), chlorofluorocarbons (CFCs) and nitrogen oxides (NO) produced by many industries and motor vehicles. Pollution is viewed as a cause of free radicals in the form of positive, negative and neutral charges that cause cell damage. Moreover, cancer remains one of the deadliest diseases for humanity, with no effective prevention or cure at present for several types of cancers.

An antioxidant is a substance that scavenges the free radical by scavenging free radicals directly, chelating free radicals, or by metal chelation to inhibit the reaction of free radicals and enzyme production (Sies *et al.*, 1992). The antioxidant can be

classified into two groups consisting of enzymatic antioxidants comprising superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST), and non-enzymatic antioxidant. However, non-enzymatic antioxidants are separated into two types: synthetic antioxidants and natural antioxidants comprising carotenoids, vitamin C (ascorbic acid), vitamin A (retinol) and vitamin E (alpha tocopherol). Synthetic antioxidants include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) (Cai *et al.*, 2015). Although the human body with its enzymatic antioxidant defense systems plays an important role in eliminating reactive oxygen species (ROS), including superoxide radical, hydrogen peroxide (H_2O_2), hydroxyl radical and singlet oxygen ($^1O_2$) produced by electron transport chain, to scavenge free radicals from oxidation biomolecules packed with lipid, protein and DNA, the body produces extremely free radicals with enzymatic antioxidants appearing inadequate. Subsequently, antioxidants must be derived from other sources (Li *et al.*, 2016) including antioxidants from natural products.

Natural products are in demand from many people, especially with increasing health consciousness. Organic products as well as supplements that are chemical free have become a preferred choice. Thailand is abundant with natural resources, especially antioxidant herbs and fruits. These include khaminkhruea (*Arcangelisia flava* (L.) Merr.) (Keawpradub *et al.*, 2005), star gooseberry (*Phyllanthus acidus* (L.) Skeels), kaempfer (*Boesenbergia rotunda* (L.) Mansf.), malacca tree (*P. emblica* L.) and grape (*Vitis vinifera* L.) (Prohmhirangul, 2011). In addition, many experiments have reported that protein hydrolysate or bioactive peptides have high antioxidant activity (Najafian and Babji, 2015). In particular, the antioxidant peptide is composed of 2-20 amino acids as components (Meisel and FitzGerald, 2003). Furthermore, the side chain of the amino acid causes the specific activity, including antioxidant activity, of protein hydrolysates or bioactive peptides (Je *et al.*, 2007). The antioxidant peptide also contains aromatic amino acids including phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) (Intarasirisawat *et al.*, 2012). These aromatic amino acids seem to be able to facilitate in donating a protons to free radicals and scavenging all free radicals (Wang and de Mejia, 2005). Additionally, the imidazole group in the histidine (His) and thiol group in cysteine (Cys) also scavenges free radicals (Elias *et al.*, 2008). Recently, the global

market as well as many researchers has paid greater attention to peptide drugs in developing new drugs with high efficiency and less side effects.

Protein hydrolysates or bioactive peptides are free amino acids or short chains of peptide hydrolyzed by enzymatic or chemical hydrolysis and may exert various physiological functions, such as controlling optimal pH, temperature, time, etc. (Pokora *et al.*, 2013; Samaranayaka and Li-Chan, 2011; Taherzadeh and Karimi, 2007). Chemical hydrolysis, using acid or base solutions, incurs low costs in production. Nevertheless, this chemical hydrolysis is limited in applications in food or pharmaceutical products, and it is difficult to control the degradation of protein leading to the unstable quality of the product. On the other hand, enzymatic hydrolysis the peptide bond by protease and water molecules. When the peptide bond is split there is an increase in the free carboxylic and amino groups leading to greater solubility and change in the protein structure. Additionally, there is high specificity as well as a mild reaction (dos Santos *et al.*, 2011; Edens *et al.*, 2002; Kim *et al.*, 2009; Mahmoud, 1994; Moure *et al.*, 2001; Taheri *et al.*, 2013).

With agriculture being the country's major activity, there exist several related industries in Thailand, including the chicken industry. Chicken feather meal is one of the by-products of the chicken industry in Thailand. Chicken feather meal is also used for producing animal food. Moreover, chicken feather meal may bring about allergic reactions in humans. Nevertheless, as previously reported, chicken feather meal is a rich source of protein including important amino acids, for example, the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp). These aromatic amino acids have benzene rings in their structures with strong antioxidant activity. In addition, there have been several reports on small molecules and their antioxidant activity, with few reports focusing on peptide antioxidant. Accordingly, chicken feather meal is the focus of this study on peptide antioxidant and antiproliferative activity.

Thus, natural products are an interesting and suitable choice for people as an alternative to chemical drugs and for those looking for new sources of antioxidants for health benefits. For this study, developing new bioactive peptides from a natural treatment, such as chicken feather meal for drug development, is a new method with the potential to cure several types of cancers.

CHAPTER II
LITERATURE REVIEW

2.1 Free radicals

A free radical is an unstable atom or molecule composed of an unpaired electron at the outer orbital and which is highly reactive and attacks by donating to or accepting an electron from other molecules for transforming into a stable molecule or non-radical state (Gutowski and Kowalczyk, 2013). These other molecules become new free radicals, after being attacked by free radicals, and so begin a chain reaction. Three forms of free radicals packed with positive, negative and neutral charges include pyridinyl radical ($NAD^{+\bullet}$), superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$) and hydroperoxyl radical ($HOO^{\bullet}$) (Hermes-Lima, 2004). Moreover, the major types of free radicals in the biological system, which are precursors of the production of many free radicals, comprise reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS), for example, hydroxyl radical ($OH^{\bullet}$), hydroperoxyl radical ($HOO^{\bullet}$), superoxide radical ($O_2^{-\bullet}$), alkoxyl radical ($RO^{\bullet}$), peroxyl radical ($RO_2^{\bullet}$), carbonate radical ($CO_3^{-\bullet}$), nitric oxide radical ($NO^{\bullet}$), nitrogen dioxide radical ($NO_2^{\bullet}$ and $NO_2^{-\bullet}$) and atomic chlorine radical ($Cl^{\bullet}$). Furthermore, non-radical species, including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) and lipid peroxide (LOOH), are also precursors of several free radicals in life forms. However, non-radical species have lower reactivity than reactive radical species due to their high stability (Pham-Huy *et al.*, 2008). The reaction of a free radical can be divided into the three following steps: initiation (the production of a free radical), propagation (the changing of a free radical into another free radical) and termination (the stopping of the reaction of the combination between two free radicals and the generation of a stable molecule). In the initiation step, the production of free radicals by bond homolysis (the breaking bond of the molecule because of a weak bond such as the disulfide bond and the rate of reaction being low), photolysis (the breaking bond of the molecule from light energy absorption such as ultraviolet light (UV light), for instance the breaking bond of H_2O_2 to $OH^{\bullet}$), radiolysis (such as lipid peroxidation in living cells) and redox (such as metal ions). In the propagation step, the changing of the position of an unpaired electron to

generate a new free radical and the process of reaction can be classified into three types: packed with atoms or group transfer (involving pull hydrogen), electron transfer (electron transfers from negative or neutral charges to non-radical molecule such as lipid peroxidation) and addition of radicals (such as lipid peroxidation). Lipid peroxidation occurs from the oxidation chain reaction of an unsaturated fatty acid by a free radical. Then lipid peroxides (LOOH) are generated before the terminal reaction, and lipid oxidation easily occurs with lipids in the cell membrane that contain a lipid bilayer. After that the properties of cell membranes change resulting in the loss of function of enzymes and receptors in the cell membrane, thereby causing several diseases. Also, the termination step comprises the three processes of the homolinking and cross-linking of radicals, radical scavenging, and electron transfer (Hudson, 1990; Roberfroid and Calderon, 1995). Many causes can result in the greater occurrence of free radicals including cholesterol, pesticides, UV light, chemotherapy, radiation, environmental pollution and smoking (Sharma, 1995). In addition, the rate of birth is at present increasing rapidly and causing greater amounts of pollution. For example, chemicals are released into the environment such as carbon dioxide (CO_2) from waste burning, carbon monoxide (CO), sulfur dioxide (SO_2), chlorofluorocarbons (CFCs) and nitrogen oxides (NO) produced by various industries and motor vehicles. These seem to be some of the most important causes behind the appearance of free radicals. Biomolecules in living cells including protein, lipid and nucleic acid can be destroyed by free radicals such as ROS and this may then lead to mutations that cause various diseases and also cell damage. ROS are highly reactive molecules that can be produced by electron-transport chain and during respiration (about 0.1-0.2% of the oxygen consumed by aerobic cells is converted to ROS) which are necessary for the normal functioning of cells consisting of redox signaling and resistance to pathogens (Fridovich, 2004; Lushchak, 2011; Muñoz *et al.*, 2000). Besides, ROS can stimulate denaturing reactions in many cellular components, which creates oxidative stress and results in many diseases (Leanderson *et al.*, 1997). Also, if the body produces to many free radicals – oxidative stress – this also causes many chronic diseases, such as Alzheimer's, atherosclerosis or heart disease, arthritis, emphysema, aging, wrinkling, inflammation and particularly cancer because the DNA in the nucleus is transformed and mutated (Sharma, 1995).

In the human body enzymatic and non-enzymatic endogenous antioxidant defense systems play a key role in eliminating ROS, including $O_2^{-\bullet}$, $OH^{\bullet}$, H_2O_2 and singlet oxygen ($^1O_2$) produced by electron transport chain, to scavenge free radicals from oxidation biomolecules packed with lipid, protein and DNA. Nevertheless, if the body receives extremely free radicals or is in a state of oxidative stress, the enzymatic and non-enzymatic antioxidants are insufficient and must be acquired by dietary antioxidants or exogenous antioxidants from outside of the body (Li *et al.*, 2016). Hence, the search for antioxidants from other sources, especially natural sources, is of real interest and significance.

2.2 Antioxidants

An antioxidant is a substance which can prevent, scavenge or inhibit the free radical by eliminating free radicals directly, scavenging metal chelation, thereby acting as the catalyst of oxidation reaction for stopping chain-breaking to prevent the generation of free radicals and inhibit the enzyme involved in catalyzing free radical production (Sies *et al.*, 1992). Moreover, antioxidants at lower concentration are able to delay the oxidation of the substrate and stop oxidative chain reactions by removing free radical intermediates (Halliwell and Gutteridge, 1990). There are three categories of antioxidant: primary, secondary and tertiary antioxidants. The primary antioxidant prevents oxidant formation while the secondary antioxidant acts as the scavenger of ROS and the tertiary antioxidant repairs the oxidized molecules passing sources of antioxidants such as dietary or exogenous antioxidants (Gutteridge and Halliwell, 1994). The antioxidant defense system in the human body controls free radicals and maintains balance. These systems can be classified into two groups: endogenous and exogenous antioxidants (Bouayed and Bohn, 2010). Reactive free radicals generation in living cells is controlled by endogenous antioxidant systems including enzymatic, non-enzymatic or transition metals. Furthermore, exogenous antioxidants such as dietary source can also defend against free radicals and maintain balance in living cells. Thus, antioxidants are important substances for normal biological oxidant processes and functions (Shafaq, 2012).

2.2.1 Endogenous antioxidants

An endogenous antioxidant is an antioxidant produced by the body of a living organism. Endogenous antioxidants are packed with superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) – the primary antioxidant enzymes in the body – and these are able to inactivate the ROS into intermediates (Shu, 1998). Additionally, endogenous antioxidants consist of glutathione reductase (GR), glucose-6-phosphate dehydrogenase, glutathione-S-transferase (GST) and ubiquinone – the secondary antioxidant enzymes – and these can detoxify ROS directly (Gale, 2001; Vertuani *et al.*, 2004). Therefore, the endogenous antioxidant comprises primary and secondary antioxidants. The primary antioxidant is water-soluble (for example, ascorbate, glutathione (GSH), uric acid, etc.) and lipid soluble (for example, tocopherols, ubiquinols and carotenoids, etc.). The endogenous antioxidant can be divided into two types, namely, enzymatic and non-enzymatic antioxidants (Bouayed and Bohn, 2010).

2.2.1.1 Enzymatic antioxidants

Enzymatic antioxidants are mainly packed with superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione-S-transferase (GST), thioredoxin reductase and glucose-6-phosphate dehydrogenase (Cai *et al.*, 2015). SOD is an enzyme that can detoxify a superoxide radical ($O_2^{-\bullet}$) and transform the most reactive free radicals to H_2O_2. CAT and GPX are enzymes involved in peroxide detoxification, in which CAT acts against H_2O_2 and GPX against H_2O_2 and ROOH. GR is an enzyme involved in glutathione regeneration. Thioredoxin reductase is an enzyme involved in protecting against protein oxidation. Also, glucose-6-phosphate dehydrogenase is an enzyme involved in NADPH regeneration (Bouayed and Bohn, 2010).

2.2.1.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants are composed of glutathione (GSH), alpha-lipoic acid (ALA), uric acid, NADPH, coenzyme Q, albumin,

ferritin, metallothioneine, melatonin, bilirubin and L-carnitine (Bouayed and Bohn, 2010). A non-enzymatic antioxidant is a scavenger of ROS and RNS (Shafaq, 2012).

2.2.2 Exogenous antioxidants

Exogenous antioxidants are those received from out of the body by diet and supplements, for example, food, vegetables, fruits, grains, herbs, spices and vitamins. Exogenous antioxidants derived from natural sources, such as flavones, isoflavones, flavonoids, anthocyanins, isocatechins, catechins, coumarins, lignans, esculatin and gallic acid are called phytochemicals, which are the phenolic and polyphenol compounds (Aqil *et al.*, 2006; Dempster *et al.*, 1995; Shafaq, 2012; Wu *et al.*, 1999). An exogenous antioxidant is composed of dietary, synthetic and protein hydrolysate antioxidants.

2.2.2.1 Dietary antioxidants

Dietary antioxidants are mainly divided into the three basic classes of polyphenols, carotenoids and some nutrients. Polyphenols are the majority of dietary antioxidants and also secondary plant metabolites. Polyphenols are composed of flavonoids, involving flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols, and non-flavonoid polyphenolics, including phenolic acids (lignans and stilbenes). Polyphenols have an aromatic benzene ring in their structure, which exhibits antioxidant activity resulting from the donation of hydrogen or an electron to free radicals, and these free radicals are added to the stable molecule. However, polyphenols do not become a new free radical and the aromatic benzene ring remains stable due to the delocalization of unpaired electrons around the aromatic ring. Additionally, polyphenols can chelate the prooxidant transition metal ions such as ferrous (Fe^{2+}) involved in the reaction of free radical production because Fe^{2+} is able to react with oxidation with the oxygen in the atmosphere and generate the superoxide radical ($O_2^{-\bullet}$) that is the precursor of many free radicals, for example, $OH^{\bullet}$, alkoxyl radical ($RO^{\bullet}$). Moreover, polyphenols can also

scavenge lipid alkoxyl ($LO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$) radicals. Sources that have many polyphenols include potato, plums, leafy vegetables, whole grain products and coffee. Carotenoids are synthesized in the chloroplasts of plants. Furthermore, bacteria and several fungi also synthesize the carotenoids. Carotenoids are pigments with several conjugated double bonds to delocalize unpaired electrons. In addition, the main structure of carotenoids is that of a tetraterpene that may conjugate with five or six membered rings at the terminal chain, which show antioxidant activity and mostly lipid-soluble antioxidants except crocin, which is a water-soluble carotenoid. The major mechanism of carotenoids' antioxidant activity is radical scavenging and also quenching excited triplet states of singlet oxygen ($^1O_2$). Carotenoids can be classified into the two groups of oxygen containing carotenoids and non-oxygen containing carotenoids. Oxygen containing carotenoids is packed with oxocarotenoids or xanthophylls such as alpha- and beta-cryptoxanthin, lutein and zeaxanthin, while non-oxygen carotenoids contain alpha-, beta- and gamma-carotene and lycopene. Carotenoids are found in colored fruits and vegetables, algae as well as some nutrients such as vitamins C and E. Vitamin C or ascorbic acid is a water-soluble antioxidant. Vitamin C in the human body is primarily present in the form of ascorbate anions and this will transform into monodehydroascorbate (by loss of an electron) and dehydroascorbate (by loss of hydrogen), respectively after its sequential oxidation. These are relatively stable radicals, and the reaction is reversible. Vitamin C can be used for scurvy prevention owing to it being a cofactor in the hydroxylation of proline (Pro) to hydroxyproline. Besides, vitamin C can also be used in the structure of collagen and other tissues. Vitamin C exhibits a protective effect against oxidative injury within the cytosol and also protects mitochondria, which are the targets of the oxidative attacks by ROS. Vitamin C is a powerful reducing agent and exhibits antioxidant activity by scavenging radicals directly such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and peroxyl radical ($RO_2^{\bullet}$), mediating electron transfer to ascorbate-dependent

peroxidases or regenerating membrane bound vitamin E that has been oxidized by lipid peroxyl radicals ($LOO^{\bullet}$). Thus, vitamin C is able to indirectly limit lipid peroxidation in cell membranes. Moreover, vitamin C is also a promoter of the antioxidant efficiency of vitamin E by changing the alpha-tocopherol radical ($TO^{\bullet}$) to alpha-tocopherol (TOH). Sources of vitamin C include bell peppers and citrus fruits (such as oranges). Vitamin E is a lipid soluble antioxidant. Vitamin E has an aromatic ring in its structure, which is a phenolic antioxidant and can stabilize the unpaired electron of free radicals by electron delocalization around the aromatic ring. In the human body, vitamin E is the major membrane bound antioxidant or lipophilic chain-breaking antioxidant that exhibits the ability to inhibit lipid peroxidation. Vitamin E is a necessary antioxidant for membrane protection against the oxidative damage of lipids either by quenching singlet oxygen ($^1O_2$) or by intercepting directly free radical intermediates, for instance, hydroxyl radical ($OH^{\bullet}$), lipid radical ($L^{\bullet}$), lipid alkoxyl radical ($LO^{\bullet}$) and lipid peroxyl radical ($LOO^{\bullet}$) by donating the hydrogen generated during lipid oxidation, terminating this chain reaction of peroxidation (Bouayed and Bohn, 2012; Cai *et al.*, 2015; Samee, 2005).

2.2.2.2 Synthetic antioxidants

A synthetic antioxidant is able to prevent the oxidative stress that plays a key role in the triggering of many chronic diseases, similar to natural antioxidants. Most synthetic antioxidants are small molecules with the structure developed from natural antioxidants such as vitamin E and polyphenol. Synthetic antioxidants include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ) and propyl gallate, which usually are supplemented in food products to delay lipid peroxidation. Furthermore, a synthetic antioxidant is profitable and efficient. On the other hand, synthetic antioxidants in food products aid against toxic and hazardous effects on human health. Thus, the search for natural antioxidants is of much importance and interest (Cai *et al.*, 2015; Hettiarachchy *et al.*, 1996; Ito *et al.*, 1985; Rice-Evans and Diplock, 1993; Vajragupta *et al.*, 2006).

2.2.2.3 Antioxidative protein hydrolysate

Antioxidative protein hydrolysate is a protein from natural sources, including shellfish such as oysters (*Crassostrea gigas*), fin fish, peanut kernels, rice bran, sunflower protein, alfalfa leaf protein, frog skin, egg-yolk protein and algae protein waste (Qian *et al.*, 2008[a]; Sarmadi and Ismail, 2010). It is derived from enzymatic proteolysis through antioxidant activity to produce bioactive peptide. A bioactive peptide is several linked amino acids purified from hydrolysate (Sarmadi and Ismail, 2010). The antioxidant activity of protein hydrolysates or peptides can be evaluated by many methods including DPPH and ABTS radical scavenging activity assay. Additionally, bioactive peptides also possess antihypertensive and immune-modulatory activities (Umayaparvathi *et al.*, 2014). Furthermore, it has been reported that bioactive peptides exhibit a protective effect against cell damage from ROS through the induction of genes. The parent protein, which is not hydrolyzed or released by enzymatic hydrolysis, will remain in inactive form and may not perform various physiological functions (Sarmadi and Ismail, 2010). Bioactive peptides usually contain 3-20 amino acid residues in their structure with a molecular mass of often less than 6000 Da (Sun *et al.*, 2004). In addition, the antioxidant activity of bioactive peptides depends on their structure, amino acid composition (such as Tyr, Trp, Met, Lys, Cys and His exhibit antioxidant activity) and sequence (Pihlanto-Leppälä, 2000) as presented in Table 2.1. Aromatic amino acids such as Phe, Tyr and Trp have aromatic rings in their structure, which are able to donate protons to an unpaired electron of free radicals and these free radicals transform into new stable molecules whereas these aromatic amino acids can maintain their own stability due to their resonant structure which exhibits the radical scavenging activity (Rajapakse *et al.*, 2005[b]). Moreover, the hydrogen donating, lipid peroxyl radical trapping and/or the metal ion-chelating ability are associated with the imidazole group in His (Chan *et al.*, 1994; Rajapakse *et al.*, 2005[b]). The thiol group (SH group) in Cys are radical scavengers because they can interact with

radicals directly (Patterson and Rhoades, 1988; Qian *et al.*, 2008[b]). Likewise, hydrophobic amino acids such as Gly are able to attack with hydrophobic radical species and hydrophobic PUFAs owing to the hydrophobic amino acids in the peptide sequence being able to increase the solubility of the peptides in lipids (Chen *et al.*, 1998; Qian *et al.*, 2008[a]; Suetsuna and Chen, 2002). Besides, acidic amino acid (such as Asp) and basic amino acid (such as His) like carboxyl and amino groups in the side chains of peptide are the chelator metal ions and hydrogen donor (Qian *et al.*, 2008[b]; Suetsuna *et al.*, 2000). Nevertheless, the combination of peptides results in greater exposure to antioxidant activity than the individual peptide (Chen *et al.*, 1998). As previously reported, antioxidant peptides with MW higher than 1500 Da and less than 500 Da will have low antioxidant activity; the strong antioxidant activity of peptides should be in the MW range between 500-1500 Da and low MW usually has greater antioxidant activity than high MW (Li *et al.*, 2008). Similarly, antioxidant peptides from foods are assumed to be harmless and healthy compounds with low molecular weight, low cost, highly active and easily absorbed (Sarmadi and Ismail, 2010). The advantages of antioxidant peptides compared with the enzymatic antioxidants include the simpler structure, greater stability in different situations, no hazardous immunoreaction, and exhibit nutritional and functional properties (Hattori *et al.*, 1998; Xie *et al.*, 2008). Finally, the bioactive peptides can be used as the basic compound for functional foods, nutraceuticals, dietary supplements and pharmaceuticals (Sarmadi and Ismail, 2010).

Table 2. 1 Antioxidant activity of protein hydrolysate or peptide

Source of protein	Enzyme	Peptide sequence	Reference
Grass carp (*Ctenopharyngodon idella*) skin	Alcalase®	Pro-Tyr-Ser-Phe-Lys (640.74 Da) Gly-Phe-Gly-Pro-Glu-Leu (618.89 Da) Val-Gly-Gly-Arg-Pro (484.56 Da)	Cai *et al.* (2015)
Bluefin leatherjacket (*Navodon septentrionalis*) skin	Alcalase®	Gly-Ser-Gly-Gly-Leu (389.41 Da) Gly-Pro-Gly-Gly-Phe-Ile (546.63 Da) Phe-Ile-Gly-Pro (432.52 Da)	Chi *et al.* (2015[b])
Bluefin leatherjacket (*Navodon septentrionalis*) head	Papain	Trp-Glu-Gly-Pro-Lys (615.69 Da) Gly-Pro-Pro (269.33 Da) Gly-Val-Pro-Leu-Thr (485.59 Da)	Chi *et al.* (2015[c])
Blood clam (*Tegillarca granosa*) muscle	Neutrase®	Trp-Pro-Pro (398.44 Da)	Chi *et al.* (2015[a])
Oyster (*Saccostrea cucullata*)	Protease from *Bacillus cereus* SU12	Leu-Ala-Asn-Ala-Lys (515.29 Da) Pro-Ser-Leu-Val-Gly-Arg-Pro-Pro-Val-Gly-Lys-Leu-Thr-Leu (1432.89 Da) Val-Lys-Val-Leu-Leu-Glu-His-Pro-Val-Leu (1145.75 Da)	Umayaparvathi *et al.* (2014)
Round scad (*Decapterus maruadsi*) muscle	Alcalase®	His-Asp-His-Pro-Val-Cys (706.8 Da) His-Glu-Lys-Val-Cys (614.7 Da)	Jiang *et al.* (2014)
Ostrich (*Struthio camelus*) egg white	Trypsin	Leu-Thr-Glu-Gln-Glu-Ser-Gly-Val-Pro-Val-Met-Lys (1317.65 Da)	Tanzadehpanah *et al.* (2012)

Source of protein	Enzyme	Peptide sequence	Reference
Sweet potato (*Ipomoea batatas* (L.) Lam.)	Alcalase®	Tyr-Tyr-Ile-Val-Ser (643.2 Da)	Zhang *et al.* (2014)
Patin (*Pangasius sutchi*) myofibrillar protein	Papain	Phe-Val-Asn-Gln-Pro-Tyr-Leu-Leu-Tyr-Ser-Val-His-Met-Lys (1739 Da)	Najafian and Babji (2015)

2.3 Protein hydrolysate

Protein hydrolysate as the protein cleavage of the peptide bond is hydrolyzed by chemicals or enzymes and generates varying sizes of peptides (Rustad, 2003; Tavano, 2013). These protein hydrolysates or peptides are mixtures mainly composed of free amino acids or a short chain of peptides and may utilize various physiological functions, such as control optimal pH, temperature, time, etc. (Pokora *et al.*, 2013; Samaranayaka and Li-Chan, 2011; Taherzadeh and Karimi, 2007). The advantage of chemical hydrolysis, using acid or alkali treatment or fermentation, is its low cost production. Nevertheless, there are limitations in applications in food or pharmaceutical products. Moreover, with chemical hydrolysis it is difficult to control the degradation of protein, which brings about an unstable quality product and yields a product with modified amino acids. For instance, 6M HCl is used to hydrolyze protein to determine the amino acids at 110ºC for more than 24 h. The result is that it can destroy Trp and the use of alkaline hydrolysis can decrease Cys, Arg, Thr, Ser, Ile and/or Lys content. In contrast, enzymatic hydrolysis causes the peptide bond degradation of protein by protease with water molecules. When the peptide bond is split the free carboxylic and amino group is increased leading to greater solubility and changing the protein structure (such as the sequence and composition of amino acids). Additionally, enzymatic hydrolysis has many advantages including that it can be performed under mild conditions, does not need extreme environments required by chemical treatments, high substrate specificity, has not side reactions and the nutritional value of the protein source is not destroyed or lost (de Castro and Sato, 2015; dos Santos *et al.*, 2011; Edens *et al.*, 2002; Kim *et al.*, 2009; Mahmoud, 1994; Moure *et al.*, 2001; Taheri *et al.*, 2013; Tavano, 2013). The proteases, peptidases, proteolytic enzymes or peptide bond hydrolysates which are used to hydrolyze protein to generate the protein hydrolysates or peptides have an active site, and the structure around the active site can determine the way the substrate binds with the site of the protease due to its high specificity of substrate with only a few amino acid residues involved in the active site of protease. Hence, the specificity of a protease is able to determine the position of an enzyme that hydrolyzes the peptide bond. Proteases can be categorized into two modes of action – exopeptidase and endopeptidase – based on the interaction of the substrate. Exopeptidases are the protease that acts near the terminal of polypeptide chains.

Exopeptidases that act on the peptide bonds from the N-terminal are called aminopeptidases and those that act on peptide bonds from the C-terminal are called carboxypeptidases. Endopeptidases are proteases that act randomly on the peptide bond at the middle of the peptide substrates. The peptide substrate runs through the whole length of the active site of an endopeptidase framework and is cleaved in the middle of the molecule. Differences in the specificity of both proteases tend to be indicators for predicting the products from the protease selection to hydrolyze protein sources. The same protein source is able to produce several protein hydrolysates or peptides using different proteases. Proteases can be derived from three sources: animal, plant and microbial such as trypsin, pepsin, pancreatin, chymotrypsin, papain, Alcalase®, Flavourzyme® and Neutrase®. Furthermore, the proteases are activated under several conditions and controlled by pH and temperature as shown in Table 2.2. Therefore, different enzymes or proteases can release various bioactive fractions in a variety of smaller peptides and free amino acids due to the different abilities of enzyme specificity, mechanisms of action and the optimal conditions of hydrolysis based on the enzyme's site of activity on the protein (Ambigaipalan *et al.*, 2015; Clemente, 2000; de Castro and Sato, 2015; Kim *et al.*, 2013[a]; Nimalaratne *et al.*, 2015; Samarakoon and Jeon, 2012; Tavano, 2013).

Table 2. 2 Proteases, characteristics and optimal conditions

Protease	Hydrolysis reaction	Source of origin	Optimal conditions	
			pH	Temperature (°C)
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

2.4 DPPH radical scavenging activity assay

Antioxidant activity as the concentration of antioxidants, usually reported in an IC_{50} value, gives the effective concentration of an antioxidant substance (such as peptide fraction) that is required to scavenge 50% of radical activity (Bursal and Gülçin, 2011), needed to provide a specified rate or extent of reaction. The DPPH radical scavenging activity assay is one of the methods used to determine the antioxidant activity of antioxidant substances that are able to reduce free radicals by measuring the antioxidative properties of substances could donate a hydrogen atom to free radicals or as the free radical scavengers with characteristic absorption at 517 nm. DPPH radical is a synthetic stable nitrogen free radical, which is more stable than the highly reactive and transient peroxyl and hydroxyl radicals that related with lipid peroxidation, and have deep purple color. The DPPH radical scavenging activity assay is based on electron transfer and involves color oxidant reduction. When the DPPH radical is scavenged by a free radical scavenger, it accepts an electron or a hydrogen radical. The deep purple color of DPPH is then reduced (the degree of color change is associated with the level of antioxidant activity), and the absorbance is reduced and transformed into a stable molecule. The DPPH radical scavenging activity assay should be applied in a hydrophobic antioxidant system due to the DPPH radical being hydrophobic and dissolving in organic solvents such as methanol and ethanol solutions. The advantages of DPPH radical scavenging activity assay include it being quick, easy to use, highly accurate and convenient for application (Ambigaipalan *et al.*, 2015; Blois, 1958; de Castro and Sato, 2015; Floegel *et al.*, 2011; Fontoura *et al.*, 2014; Schaich *et al.*, 2015; Tanzadehpanah *et al.*, 2012; Vajragupta *et al.*, 2006).

2.5 ABTS radical scavenging activity assay

The ABTS radical scavenging activity assay is the method for determining the antioxidative properties of antioxidant substances by measuring the potential of an antioxidant to inhibit the formation of a blue-green ABTS cation radical ($ABTS^{+\bullet}$) at an absorption of 734 nm. The ABTS radical scavenging activity assay based on electron transfer and hydrogen atom transfer also involves color oxidant reduction. When the substances exhibit antioxidant activity on the ABTS radical scavenging activity assay, the blue-green $ABTS^{+\bullet}$ decreases. $ABTS^{+\bullet}$ is a stable free radical. The advantages of

ABTS radical scavenging activity assay are that it is quick, easy to use, highly accurate and convenient for application, similar to the DPPH radical scavenging activity assay. However, the ABTS radical scavenging activity assay can be applied in both hydrophobic or lipophilic and hydrophilic antioxidant systems owing to the ABTS cation radical being a water-soluble structure. Furthermore, compared with the DPPH radical scavenging activity assay, the ABTS radical scavenging activity assay is based on the generation of a blue-green $ABTS^{+\bullet}$ by potassium persulphate while DPPH already exists in the form of free radicals in organic solvent (Ambigaipalan *et al.*, 2015; Blois, 1958; Floegel *et al.*, 2011; Fontoura *et al.*, 2014; Schaich *et al.*, 2015; Tanzadehpanah *et al.*, 2012; Vajragupta *et al.*, 2006).

2.6 DNA damage

DNA is the cellular macromolecule and a sensitive bio-target that can be damaged by free radicals, especially ROS, when the level of ROS is more than the antioxidant defense system efficiency or ROS-mediated oxidative stress, leading to gene mutation and causing several chronic diseases (Martinez *et al.*, 2003; Shackelford *et al.*, 1999). DNA is able to be broken into the following three forms: supercoiled (SC), linear (LC) and open circular (OC). These are exposed to ROS including hydroxyl radicals that are induced or generated by the Fenton reaction ($Fe^{2+}+H_2O_2$) and bring about formation change. The hydroxyl radical is the most reactive free radical in biological tissues because it can react with biomolecules such as protein and DNA easily and lead to cell damage (Cacciuttolo *et al.*, 1993). From previous research, it has been reported that protein hydrolysate exhibits a protective effect on DNA damage that is induced by the hydroxyl radical based on the Fenton reaction (Lee *et al.*, 2012). Mutagenesis and carcinogenesis is also implicated in DNA damage (Kittiphattanabawon *et al.*, 2013). Therefore, the protein hydrolysate shows antioxidant activity and exhibits a protective effect against DNA damage that induced by hydroxyl radical are very importance and necessary for using to primary prevention of many diseases (Lee *et al.*, 2012) and might prevent cellular damage caused by hydroxyl radical thus should be also used as the functional food ingredients and antimutagenic agents.

2.7 Antiproliferative activity

Cancer is a disease resulting from a group of normal cells changing owing to many causes such as free radicals that lead to the growth of cells in the body that cannot be controlled. Cancer is one of the major causes behind human deaths (Kaufmann and Earnshaw, 2000). For example, there are approximately 12.7 million new cases of cancer a year. In addition, about 7.6 million people died from cancer in 2008, based on GLOBOCAN 2008 (Samarakoon and Jeon, 2012). Accordingly, research into new natural sources of antioxidant and antiproliferative activity related to anticancer activity that is more effective and less toxic is of great importance in drug development. Much research has studied the antiproliferative or cytotoxic activity of protein hydrolysate and using an MTT assay to ascertain the ability of protein hydrolysate to decrease the growth rate of cancer cell lines and screen for anticancer activity (Chi *et al.*, 2015[a]; Umayaparvathi *et al.*, 2014). The MTT assay is a colorimetric assay and has several advantages, including that it is packed with rapidly, its precision and that it lacks any radioisotopes (Mosmann, 1983). The MTT molecule has a yellow color. The MTT assay is based on the detection of mitochondrial reductase activity in living cells, not dead cells. If the protein hydrolysate is able to reveal antiproliferative activity that could reduce cancer cell lines, the yellow MTT will not be converted to purple due to the reaction being active only in the active mitochondria of living cells and the number of viable cells is directly proportionate with absorbance (Umayaparvathi *et al.*, 2014).

2.8 Apoptosis

The pattern of cell death can be separated into the two main forms of apoptosis and necrosis as shown in Figure 2.1. Apoptosis is a programmed cell death, which is a natural occurring and evolution conserved process together with plasma membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation, organelle fragmentation, DNA digestion by endonucleases and cytoplasm fragmentation, or apoptotic body formation. These bodies are disposed by phagocytosis and then degraded by adjacent cells, particularly without the accompaniment of cellular inflammation. Nevertheless, necrosis is non-programmed or unregulated cell death involving the sudden loss of membrane. Cells will die when experiencing accidental or random DNA degradation. Accidents, for instance, burning, intake of poison or

overdose of drugs damage the adjacent cells, which results in inflammation and swelling that leads to the immune system activation (Chen *et al.*, 2016; Martin and Henry, 2013; Su *et al.*, 2015). Apoptosis is usually a preferable means of cancer cell death during cancer treatments due to it being one of the key defense mechanisms of cell death in response against the formation and progression of cancer (cancer therapies) (Kim *et al.*, 2013[b]; Zheng *et al.*, 2013). In contrast, if the body loses pro-apoptotic signals or receives anti-apoptotic signals, this can bring about cancer initiation and the cancer treatment fails. Form previous research, it has been reported that protein hydrolysate effectively induces the apoptotic cells detected by flow cytometry and used Annexin V and propidium iodide (PI) double staining to count the percentage of apoptotic cells, which Annexin V will bind with the phosphatidyl serine that express on the outer layer of the cytomembrane and was used to determine the amount of apoptotic cells while PI was used to indicate the amount of necrotic cells. From these studies, it seems likely that the hydrophobic properties of protein hydrolysate play a vital role in anticancer activity. Hence, this study on the antioxidant and antiproliferative protein hydrolysate derived from natural sources that can induce apoptosis is of great interest and importance for anticancer drug development (Chen *et al.*, 2016; Chi *et al.*, 2015[a]; Kim *et al.*, 2013[b]).

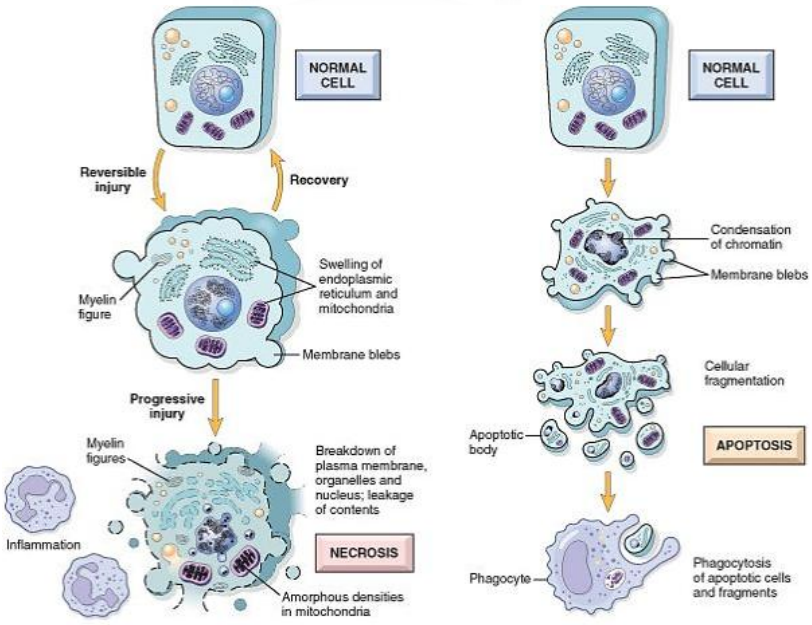


Figure 2. 1 The different morphologies between necrosis (left) and apoptosis (right) (Goland, 2014)

2.8.1 The mechanisms of apoptosis

There are two main signaling systems of the apoptosis mechanism in mammals, namely, the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. Both pathways are activated in an independent manner but are linked as shown in Figure 2.2. In addition, the morphological features of apoptosis are also closely involved with cysteinyl aspartate proteinases or caspases (Siddik, 2013).

2.8.1.1 Extrinsic death receptor apoptotic pathway

When the specific death-inducing ligands are packed with tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligands (TRAILs) and FasL ligands bind with their cognitive transmembrane death receptors on the cell surface containing TNF receptor type I (TNF-R1), TRAIL-R1 or TRAIL-R2 (DR4 or DR5) and Fas (also known as Apo-1 or CD95), respectively. The extracellular apoptotic signals are then transduced into cells and the extrinsic death receptor apoptotic pathway or extrinsic pathway can be activated. Later on, after the binding of specific death-inducing ligands and transmembrane death receptors, oligomerization of the receptor will be induced and this permits the intracellular extension of the receptor to enlist the specific TNF or TRAIL receptor-associated death domain (TRADD) and Fas-associated death domain (FADD) adapter protein. After that, the binding of procaspase 8 is allowed and the death-inducing signaling complex (DISC) formed that authorizes the autoactivation of procaspase 8 to the initiator caspase 8. Following this, downstream effector caspases, including caspases 3, 6 and 7 are activated and target cellular structures leading to cell death.

2.8.1.2 Intrinsic mitochondrial apoptotic pathway

The intrinsic pathway of apoptosis is activated by stress stimulation such as oxidative stress and DNA damage that can be generated within the cell and also related to the critical change, which is to increase the permeability and release proapoptotic biochemicals and protein in the outer membrane of the mitochondria. When the body

receives various stimuli indicating DNA damage, the specialized damage recognition proteins will recognize and transduce stress signals during a series of protein-protein interactions. This results in the cytochrome c being released by mitochondria into the cytosol together with the second mitochondria-derived activator of apoptosis (SMAC) (also known as the direct inhibitor of apoptosis protein (IAP) binding protein with low pI called DIABLO) and the serine protease HTRA2 (Omi). Moreover, these protein functions are coordinated to activate or enhance the potential of the effector caspase. Firstly, when the cytochrome c is released into the cytosol, this will promote the binding with the adapter protein APAF-1 and employment of procaspase 9 to form the apoptosome structure that affects the formation of active caspase 9. Then caspase 9 will convert procaspases 3, 6 and 7 to active forms. Additionally, the mitochondria may also release the apoptosis-inducing factor (AIF), which can translocate to the nucleus and induce apoptosis in a caspase-independent manner. Likewise, caspase 8 in the extrinsic pathway can cleave BID, which is a member of the BCL-2 family and then release the t-BID product that could translocate to the mitochondria and regulate the process of releasing cytochrome c into the cytosol and activate the intrinsic apoptosis pathway.

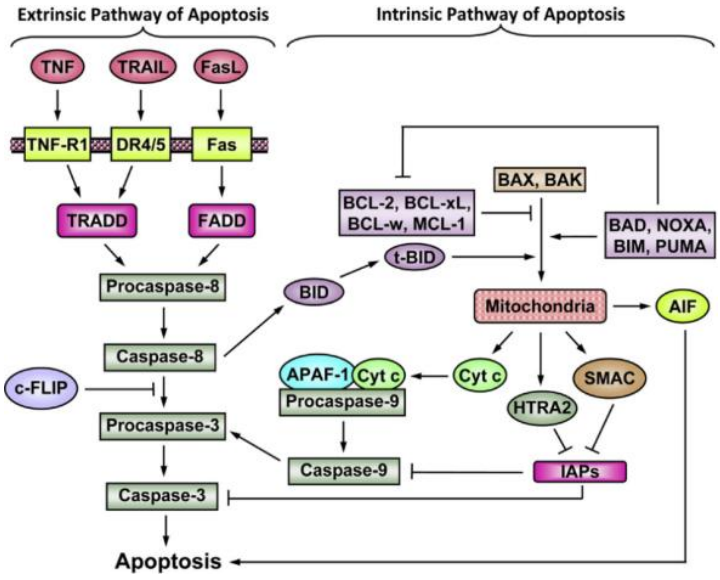


Figure 2. 2 The extrinsic and intrinsic pathways of apoptosis

2.8.2 The caspase activity

Apoptosis is the cell death process regulated by a series of signal cascades, which comprise an important homeostatic mechanism for maintaining balance between cell division and cell death and also conserves an appropriate number of cells in the body (Indran *et al.*, 2011; Zheng *et al.*, 2013). Moreover, the activation of different caspase cascades are related to the extrinsic and intrinsic apoptosis pathways. There are 14 types of caspases that are usually present in inactivate form within cells and when they receive cell death signaling such as stress signals, they will be activated through a series of cleavage reactions. Although apoptosis usually depends on caspases, it is able to also occur in a caspase-independent manner (Siddik, 2013). From previous research, it has been found that protein hydrolysate is able to induce apoptosis associated with caspase 3 and 8 activation and that it could be concluded that caspase-dependent cell death is involved in the apoptosis process. Thus, research into the activation of the caspase of protein hydrolysate is necessary so as to discover novel anticancer protein hydrolysate (Zheng *et al.*, 2013). Caspases comprise a family of intracellular cysteine proteases with specificity for aspartic acid residues involved in apoptosis and can be divided into two groups: initiators (such as caspase 8) which are normally present in the form of inactive procaspase monomers, and effectors (such as caspase 3) which will generally be cleaved by initiator caspases. Caspase 3 is the common effector in apoptosis pathways and plays a key and special role as executioner in the active form that causes the cleaving and breaking down of various cellular components involved in DNA repair and regulation such as membrane blebbing, disassembly of the structure of the cell and DNA fragmentation, resulting in cell death (Ausbacher *et al.*, 2012; Ji *et al.*, 2011). At the beginning, caspase 8 remains in inactive form and procaspase 8 can be activated by a membrane-associated protein and mitochondria-dependent activation. After the cell receives the stress signal, caspase 8 can stimulate the extrinsic cascades in the apoptotic process (Chen *et al.*, 2012[b]). Hence, caspase 3 and 8 activations are able to indicate that apoptosis has entered an irreversible stage (Sui *et al.*, 2016).

2.9 Chicken feather meal

Protein hydrolysates derived from animal by-products such as feathers reveal many potential applications in food technology and are a particularly good source of amino acids owing to these peptides being able to increase nutraceuticals for food and pharmaceutical preparations. Feathers are the most abundant protein source and are renewable and inexpensive raw materials. Furthermore, feathers are the cause of environmental pollution and may cause allergies in humans. Nevertheless, feather meal hydrolysate is used as a supplement in animal feed and nitrogen fertilizer. Additionally, feather meal hydrolysate is an interesting source of amino acids for pigs. Besides, feather hydrolysate is hydrolyzed by microbial protease present in antioxidant activity. Also, from a previous study, chicken feather meal was found to be a protein rich source containing important aromatic amino acids, for example, phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) as shown in Table 2.3. These aromatic amino acids have benzene rings in their structures, which have strong antioxidant activity. In addition, several reports also focus on small molecules which have antioxidant activity and there have been few on peptide antioxidants. Hence, the protein hydrolysate derived from chicken feather meal is interesting for use as a new protein source that may exhibit antioxidant activity (Fontoura *et al.*, 2014; Martínez-Alvarez *et al.*, 2015; Reddy *et al.*, 2013).

Table 2. 3 Amino acid content of chicken feather meal

Amino acid profile	Content (%)
Alanine	3.80
Arginine	6.30
Glycine	6.82
Aspartic acid	5.70
Valine	5.85
Cysteine	2.90
Glutamic acid	10.60
Leucine	6.46
Isoleucine	3.94

Histidine	0.59
Threonine	3.96
Proline	8.37
Lysine	1.45
Methionine	0.67
Hydroxyproline	Not detected
Serine	7.84
Phenylalanine	4.03
Hydroxylysine	Not detected
Tyrosine	1.10
Tryptophan	0.28

CHAPTER III
MATERIALS AND METHODS

3.1 Preparation of protein hydrolysate or peptide

Chicken feather meal, received from Betagro Public Company (Thailand), was dried at 60ºC in hot air oven and spun by churn and then was selected for a particle size by mesh 150 micron. After that, pre-treated sample, 0.5 g of chicken feather meal was stirred in 10 mL of 20 mM phosphate buffer with 150 mM NaCl, pH 7.2 at 4ºC for overnight. Later on, the mixture was hydrolyzed by 0, 1, 2.5 and 5% Alcalase® (EC 3.4.21.62), Flavourzyme® (EC 3.4.11.1), and Neutrase® (EC 3.4.24.28) at 50ºC in shaker (150 rpm), pH 8 for Alcalase® and pH 7 for Flavourzyme® and Neutrase®, for 4 h. The reaction was stopped by heating at 80-90ºC for 10-15 min. Next, the mixture was centrifuged at 12,000 rpm for 10 min and the supernatant was collected and kept at 4ºC. The experimental design was completely randomized design that was conducted in triplicates (Torres-Fuentes *et al.*, 2011).

3.2 Determination of protein contents

Bradford assay was used to determine protein contents of peptides derived from chicken feather meal. The Bradford assay, a colorimetric protein assay, was measuring the concentration of total protein in the sample, which based on an absorbance shift of the dye Coomassie Brilliant Blue G-250. The dye occurred in two forms including cationic (unbinding) in red-brown or green color and anionic (the binding of the dye with the protein molecules) in blue color. The principle of this assay was binding of the dye Coomassie Brilliant Blue G-250 with protein molecules consisting of basic amino acid residues; arginine, lysine and histidine or aromatic amino acid to form the formation, protein-dye complex, and changed color from red-brown to blue color under acidic conditions. Under acidic condition, the dye was doubly protonated to the ionizable groups on protein caused of the protein's native state disruption and revealed the hydrophobic pockets that shown in red-brown cationic form (Amax = 470 nm). These pockets in the tertiary protein structure bound non-covalently to the non-polar region of the dye through van der Waals forces, located the positive amine groups in

closeness with the negative charge of the dye. The bond was further strengthened by the ionic interaction. And then when the dye bound with protein, this protein-dye complex was converted to a stable unprotonated, which presented in blue form (Amax = 595 nm). This blue protein-dye complex formation was detected at 595 nm. The advantages of this assay was easy and wide to use owing to its sensitivity, rapidity of the dye binding process which approximately complete in 2 min, color stability for 1 h, suitability, lack of need for a UV-capable spectrophotometer and adaptability to 96-well plates. Moreover, this assay could be detected in microgram quantities of protein. However, several compounds, for instance; large amounts of dye detergents involving sodium dodecyl sulfate, Triton X-100 and commercial glassware detergents might interfere with this assay (Bradford, 1976).

Bradford assay, first, prepared the standard protein at 0, 0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 mg/mL bovine serum albumin (BSA) concentration. Then, pipetted 60 µL of each concentration of BSA into each microtube. Next, added 600 µL of Bradford reagent, Bradford working buffer, into each microtube and then mixed these solutions by vortex. Later on, pipetted 200 µL of these solutions into 96-well plates and measured an absorbance at 595 nm using a microplate reader spectrophotometer. To generate the standard curve of BSA. In the same way, pipetted 60 µL of peptide samples derived from chicken feather meal into microtube and then added 600 µL of Bradford reagent. Mixed all solutions and pipetted 200 µL of these solutions into 96-well plates. After that, measured an absorbance at 595 nm using a microplate reader spectrophotometer and calculated protein contents by compared with the standard curve of BSA (Bollag *et al.*, 1996).

3.3 Antioxidant activity assay

3.3.1 DPPH radical scavenging activity assay

The DPPH radical scavenging activity assay was determined and modified with the method described by Tanzadehpanah *et al.* (2012). First, 0.004 g of DPPH was dissolved in 100 mL of methanol to prepare 100 µM DPPH radical solution and then 100 µM DPPH radical solution was added to each sample in the ratio of 1:4 (80 µL of sample and 320 µL of DPPH radical solution) and incubated in the dark room at room temperature for 15

min. After that, centrifuged this solution at 1,300 rpm for 5 min. Later on, pipetted 100 µL of each solution into 96-well plates and measured an absorbance at 517 nm using a microplate reader spectrophotometer. 0.1 mg/mL of ascorbic acid was used as a positive control. The percentage of radical scavenging was calculated as follows:

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

where Abs of control was an absorbance of the control (no sample), Abs of blank was an absorbance of the deionized water, Abs of sample was an absorbance of the chicken feather meal hydrolysates (peptide samples) and Abs of background was the color absorbance of the samples. The IC_{50} values (i.e., the concentration chicken feather meal hydrolysate required to inhibit the antioxidant activity by 50%) were 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 error of the triplicate data.

3.3.2 ABTS radical scavenging activity assay

The ABTS radical scavenging activity assay was determined and modified with the method described by Cai *et al.* (2004). ABTS cation radical was generated by mixing ABTS solution (7 mM) and potassium persulphate (2.45 mM) in the ratio of 1:1 in the dark room at room temperature for 12 h. Next, diluted the ABTS cation radical solution to have the value of 0.7±0.02 at the absorbance of 734 nm. Then, mixed with the solution of peptides in the ratio of 1:30 (25 µL of sample and 750 µL of ABTS cation radical solution) and incubated in the dark room at room temperature for 10 min and then measured an absorbance at 734 nm using a microplate reader spectrophotometer. 1 mg/mL of ascorbic acid was used as a positive control. The percentage of radical scavenging was calculated as follows:

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

where Abs of control was an absorbance of the control (no sample), Abs of blank was an absorbance of the deionized water, Abs of sample was an absorbance of the chicken feather meal hydrolysates (peptide samples) and Abs of background was the color absorbance of the samples. The IC_{50} values (i.e., the concentration chicken feather meal hydrolysate required to inhibit the antioxidant activity by 50%) were 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 error of the triplicate data.

3.4 Isolation and purification

3.4.1 Ultrafiltration

Ultrafiltration was a purification technique by membrane filtration process. The principle of this technique was separated sample based on molecular weight cut-off (MWCO) by pore size of membrane and used pressure for driving the sample through these pore size.

Antioxidant peptides derived from chicken feather meal were partial purified by ultrafiltration technique using membrane with a 10, 5, 3, and 0.65 kDa MW cut-off. Peptides were passed membranes filtration packing with five fractions which were collected; MW ≥ 10 kDa, MW 5-10 kDa, MW 3-5 kDa, MW 0.65-3 kDa and MW < 0.65 kDa.

3.4.2 Gel filtration chromatography

Gel filtration chromatography or size exclusion or molecular sieve chromatography was a purification technique by column based on porosity of beads or gel matrix which was made from polymers in column to separate sample by size, mass and shape of sample. The sample that bigger than porosity of bead would be eluded first.

The antioxidant purified peptide fraction, the highest antioxidant activity from molecular weight cut-off by ultrafiltration (MW < 0.65 kDa or F5 fraction), was further fractionated by a Superdex® 75 in a column (0.8 cm diameter × 60 cm length) of gel filtration chromatography, which pre-

equilibrated with deionized water and used an automatic liquid chromatography system (AKTA prime Amersham Pharmacia Biotech, Sweden). The column was directed in downward flow at room temperature. Then, 5 mL of peptide fractions were eluted with deionized water at a flow rate of 0.5 mL/min and then 5 mL of each eluted peptide fractions were collected (F51 and F52 fractions) and detected by an absorbance at 280 nm.

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

3.5.1 Preparation plasmid from *E.coli*

Three types of plasmid including pKS, pUC19 and pBR322 were used for this experiment. First, streaked plate *E.coli* containing with pKS, pUC19 and pBR322 plasmid in LB agar (2% agar composing of peptone, yeast extract, NaCl and agar powder) consisting of antibiotic, ampicillin, to select only these plasmids. Second, incubated *E.coli* plate at 37ºC for overnight to separate single colony. Third, inoculated a single colony about 2-3 colonies from *E.coli* plate into 5 mL of LB broth (was added 5 µL of ampicillin). Next, incubated at 37ºC in shaker (250 rpm) for overnight. Kept the *E.coli* culture containing with pKS, pUC19 and pBR322 plasmid at -80ºC by pipetted 1 mL of *E.coli* culture and mixed with 500 µL of 100% glycerol.

3.5.2 Plasmid DNA purification

First, the lysate preparation, transferred 1.5 mL of *E.coli* culture containing with pKS, pUC19 and pBR322 plasmid to a microcentrifuge tube and centrifuged for 30 sec (14,000×g). Then, poured off the supernatant and kept the cell pellet and did it at the same way two times. Later on, added 200 µL of resuspension buffer (was added RNAse) to the cell pellet after that resuspended the cells by pipetting in and out or by gentle vortexing and incubated at room temperature for 5 min. Then, added 250 µL of lysis solution to the cell suspension and mixed the contents by gently inverting the tube several times (must not vortex, as this will shear the genomic DNA). This step, the suspension should become clear and viscous as the cells began

to lyse. Continued mixing until the mixture became clear. If necessary, allowed the solution to incubate at room temperature provided the total incubation time is no more than 5 min. The step was also critical for the denaturation of cellular proteins and genomic DNA. Next, added 350 µL of binding solution and immediately mixed by inverting the tube several times. This step, the solution will become turbid as insoluble particles from denatured materials start to form. After that, centrifuged for 10 min (14,000×g) to clarify the lysate and then an insoluble pellicle will be collected on the bottom of the centrifuge tube. Second, the binding to column, obtained a spin column assembled with a collection tube, and then transferred the lysate into the spin column. Ensured that none of the white particulates from the previously step were transferred onto the column. Cap the column and centrifuged the unit for 1 min (14,000×g). After centrifugation, separated the column from its collection tube and then removed the flow through and reassembled the spin column with its collection tube. Third, the washing bound DNA, applied 600 µL of wash solution (was added 32 mL of 96 to 100% ethanol) to the column and centrifuged for 1 min (14,000×g). After that, removed the flow through and reassembled the spin column with its collection tube. Next, spun the column for 2 min in order to thoroughly dry the column and then removed the collection tube. Fourth, the elution of clean DNA, assembled the spin column (with DNA bound to the column) with a fresh 1.5 mL elution tube. Later on, added 50 µL of elution buffer to the center of column, it was important to place the elution buffer directly over the column's membrane and not on the side of the column, as this will decrease the DNA recovery. Then, Let standing at room temperature for 1 min and then centrifuged the column for 2 min (14,000×g). Finally, kept the pure plasmid DNA in -20ºC.

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

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

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

DNA damage was induced by hydroxyl radical based on the Fenton reaction using and modifying the method described by Sheih *et al.* (2009). Step one, 3 µL of pure plasmid DNA (pKS 2,961 bp, pUC19 2,686 bp and pBR322 4,361 bp in the DNA concentration of 113.5, 45.5 and 30.7 ng/µL, respectively and protein content was 0.056, 0.068 and 0.073 mg/mL, respectively) was mixed with 4 µL of 0.0536, 0.0268, 0.0134, 0.0067 and 0.0034 mg/mL antioxidant purified peptide fraction from gel filtration chromatography (F52 fraction) after that let stand at room temperature for 20 min. Step two, 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. Step three, 5 µL of the solution was mixed with 2 µL of loading dye (purple dye (6X)). Step four, pipetted the solution and loading dye into a well of 1% w/v of an agarose gel and ran in 1x TAE buffer at 100 V for 30 min. Later on, brought an agarose gel to stain in ethidium bromide for 10 min. Next, washed an agarose gel with water. Finally, checked DNA band by UV light. Plasmids band will appear about 2,000-5,000 bp.

3.6 Antiproliferative activity assay

3.6.1 Cancer cell culture

Five types of human cancer cell lines composing of BT474 (human ductal breast carcinoma ATCC no. HTB 20), CHAGO-K1 (human undifferentiated lung carcinoma ATCC no. HTB 168), 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 for an antiproliferative assay. Moreover, one type of human normal cell line consisting of WI-38 (human diploid lung fibroblast ATCC no. CCL 75) was used for an antiproliferative assay to compare with the human cancer cell lines. First, cancer cell lines were cultured in 25 cm^2 culture flask using

RPMI 1640 medium containing with 10% fetal calf serum (FCS) while normal cell lines were cultured in 25 cm^2 culture flask using MEM medium containing with 10% FCS. Then, cancer cell line and normal cell line cultures were incubated at 37ºC with 5% CO_2 in CO_2 incubator.

3.6.2 Antiproliferative activity

MTT assay (Mosmann, 1983) was used to determine the antiproliferative or cytotoxicity of antioxidant purified peptide fraction from gel filtration chromatography (F52 fraction) on human cancer cell lines and human normal cell lines including BT474 (breast cancer), CHAGO-K1 (lung cancer), HEP-G2 (liver cancer), KATO-III (gastric cancer), SW620 (colon cancer) and WI-38 (lung fibroblast normal cell). WI-38 was used for comparing the antiproliferative activity with human cancer cell lines after was treated by F52 fraction. The principle of the MTT assay based on the ability of active mitochondrial of living cell. When the MTT molecule in form of tetrazolium rings (yellow form) combined with the mitochondrial reductase which was an enzyme on metabolically active mitochondria of living cell in the healthy cells will transform to the insoluble formazan crystals (purple form). The advantages of MTT assay packed with easy to use, high throughput and not expensive.

The method was modified from the method of Pérez *et al.* (2014). First, trypsinization human cancer cell lines (BT474, CHAGO-K1, HEP-G2, KATO-III and SW620) and normal cell lines (WI-38) by 0.05% trypsin-EDTA. Second, HEP-G2 and SW620 cells were diluted and plated at 0.5 x 10^4 cell/well/200 µL in 96-well plates while BT474, CHAGO-K1, KATO-III and WI-38 cells were diluted and plated at 1 x 10^4 cell/well/200 µL in 96-well plates to control the final OD_{540} value at 1. Next, pipetted 200 µL of cell suspensions into 96-well plates and then incubated at 37ºC with 5% CO_2 in CO_2 incubator for 24 h. After that, removed the cell culture medium and added F52 fraction in the concentration of 19.55, 9.77, 4.89, 2.44, 1.22, 0.61, 0.31, 0.15, 0.08 and 0.04 µg/mL with each well (200 µL of F52 fraction/well) and then incubated at 37ºC with 5% CO_2 in CO_2 incubator for 72 h. Later on, added 5 mg/mL MTT in NSS solution (10 µL of 5 mg/mL

MTT in NSS solution/well) and mixed gently by shaking and then incubated at 37ºC with 5% CO_2 in CO_2 incubator for 4 h. Then, 100% DMSO solution was added into 96-well plates (150 µL/well) to dissolve the insoluble purple formazan crystals. Finally, measured an absorbance at 540 nm using a microplate reader spectrophotometer which absorbance was directly proportional to the number of viable cells. The percentage of cell viability was calculated following:

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

where Abs of sample was an absorbance of antioxidant purified peptide fraction from gel filtration chromatography (F52 fraction) and Abs of control was an absorbance of the control (no sample) that was 100% cell survival. All data were shown as the half inhibitory concentration (IC_{50}) in termed of the tested sample concentrations that inhibit 50% of cell growth. The IC_{50} values (i.e., the concentration of peptide required to inhibit the cancer cell growth by 50%) were 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 error of the triplicate data.

3.7 Apoptosis analysis

3.7.1 Apoptosis by flow cytometer

Membrane alteration was used to define the pattern of cell death in apoptosis form (Elmore, 2007). The membrane alteration was detected by dual staining packing with FITC conjugated Annexin V and propidium iodide (PI). The dual staining could identify the pattern of cell death composing of early apoptosis, late apoptosis and necrosis and then used flow cytometer for separating cells. Flow cytometer was used to separate cells to a single cell for measuring fluorescence intensity and calculating the amount of apoptotic cells. Later on, determined the percentage of apoptotic cell using version 7 of FlowJo program to establish the boundary of cells and divide

into 4 quadrants to identify the pattern of cell death including normal cells, early apoptosis, late apoptosis and necrosis. Annexin V and PI were membrane impermeable (Casciari *et al.*, 2001) thus normal cells were not stained by annexin V and PI. Nevertheless, Annexin V and PI stained early apoptotic and necrotic cells, respectively while dual staining stained late apoptotic cells. Apoptosis was counted by measuring phosphatidylserine externalization using a flow cytometer (Pérez *et al.*, 2014) which Annexin V was used to label the phosphatidylserine externalization on the outer plasma membrane of apoptotic cells (Hsu *et al.*, 2009). In early apoptotic cells or initiation of apoptotic cells, phospholipid phosphatidylserine which a membrane molecule that trans-located from the intracellular side of the membrane to the extracellular side which Annexin V had a natural affinity (Casciari *et al.*, 2001). In late apoptotic and necrotic cells, the integrity of the plasma and nuclear membranes decreased and allowed PI passing through the membranes and staining with DNA within nucleus or intercalating into nucleic acids.

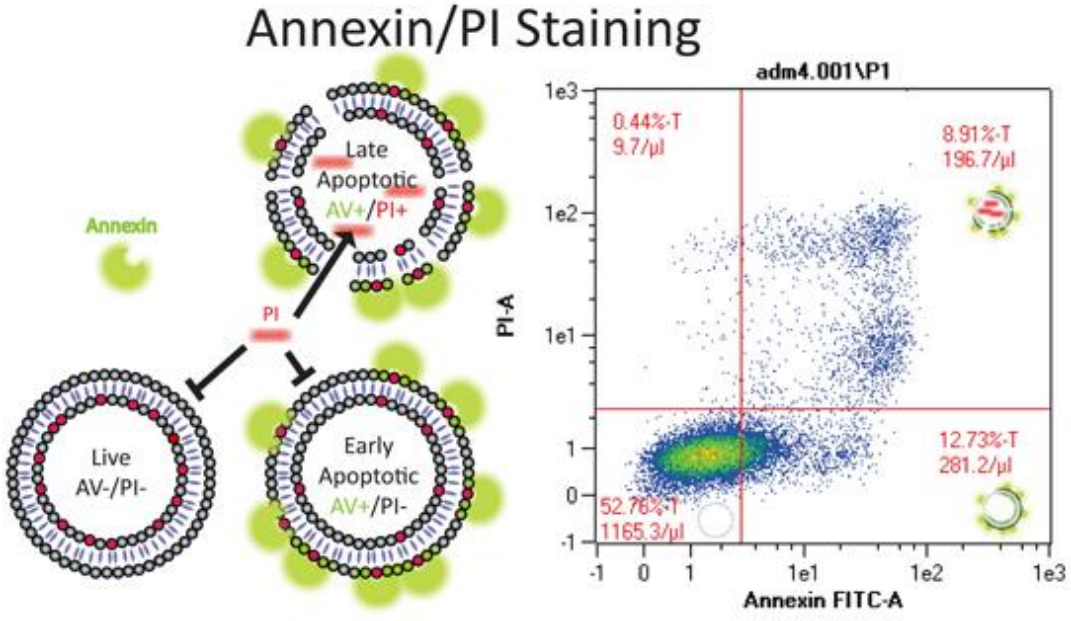


Figure 3. 1 Annexin V and propidium iodide (PI) staining (Hospital, 2016)

Human cancer cell line was SW620, the highest antiproliferative activity that was treated by F52 fraction sample from gel filtration chromatography, was stained with trypan blue to select the living cells in suspension. The number of living cells in suspension was counted in duplicate using a hemocytometer. Then, cultured SW620 in 25 cm^2 culture flask at a density of cells were 5×10^5 cell/flask/5 mL of RPMI 1640 medium containing with 10% FCS. Later on, SW620 cultures were incubated at 37ºC with 5% CO_2 in CO_2 incubator. After 24 h, SW620 cultures were treated with various percentage of F52 fraction (protein content was 62 µg/mL) containing with 0.25%, 0.5%, 1% and 2% which had protein contents were 0.15, 0.31, 0.62 and 1.24 µg/mL, respectively and incubated at 37ºC with 5% CO_2 in CO_2 incubator for 24 h. Moreover, SW620 cultures were treated by 0.5 µg/mL doxorubicin which used as a positive control and negative control was untreated cells. Then, SW620 cells of each treatment were harvested by scrapper and then centrifuged at 2,500 rpm for 5 min. Next, washed cells twice by 5 and 0.5 mL of 20 mM PBS buffer containing with 1% FCS and then centrifuged at 3,000 rpm 4ºC for 5 min. After that, the cells were analyzed by FITC Annexin V Apoptosis Detection Kit with PI. Firstly, for untreated cells, suspended cells with 300 µL of Annexin V binding buffer and kept on ice whereas for the other treatments, suspended cells with 100 µL of Annexin V binding buffer and then added 5 µL of FITC conjugated Annexin V solution and incubated on ice in the dark room for 10 min. Secondly, added 100 µL of Annexin V binding buffer and mixed with 10 µL of PI solution. Thirdly, pipetted 200 µL of each treatment into 96-well plates. Finally, analyzed the pattern of cell death using flow cytometer, measured fluorescence intensity with the FL-1H channel detecting FITC (Chung *et al.*, 2010), with cytomics EC500 MPL and then analyzed the data by version 7 of FlowJo software. Next, the highest percentage or protein content of F52 fraction for inducing apoptotic cells that was 0.5% of F52 fraction (protein content was 0.31 µg/mL) was selected for this assay and incubated with SW620 cells for 24, 48 and 72 h.

3.7.2 Caspase 3 and 8 activities assay

Caspases (Cysteine-requiring Aspartate proteases) were a family of proteases, which belong to a highly conserved family of cysteine proteases with specificity for aspartic acid residues found in their substrates. Caspases mediated cell death and were important to the process of apoptosis.

3.7.2.1 Preparation of cell lysates from apoptotic cells

Induced apoptosis in cell suspension of SW620 cultures in 25 cm^2 culture flask at a density of cells were 1×10^6 cell/flask/5 mL of RPMI 1640 medium containing with 10% FCS by addition of 0.5% of F52 fraction sample which had the protein content was 0.31 µg/mL and then incubated at 37ºC with 5% CO_2 in CO_2 incubator for 24, 48 and 72 h. Furthermore, untreated cells and 0.5 µg/mL doxorubicin treatments were used as a negative and positive control, respectively which were treated with SW620 cultures and incubated at 37ºC with 5% CO_2 in CO_2 incubator for 24, 48 and 72 h. Then, harvested cells all of treatments by scrapper after that centrifuged cells at 2,500 rpm for 5 min. Next, washed the cell pellets once with 1 mL of 20 mM PBS buffer and centrifuged cells at 600×g for 5 min at 4ºC. Later on, removed the supernatant completely by gentle aspiration. After that, suspended the cell pellets in 1x lysis buffer at a concentration of 100 µL per 1×10^6 cells and incubated the cells on ice for 15-20 min and then centrifuged the lysed cells at 16,000 to 20,000×g for 10-15 min at 4ºC. Finally, transferred the supernatants that was cell lysates to the new 1.5 mL tubes and kept the cell lysates at -70ºC.

3.7.2.2 Caspase 3 activity assay

Caspase 3 was a member of the CED-3 subfamily of caspases and was one of the critical enzymes of apoptosis. In addition, caspase 3 plays a central role in mediating nuclear apoptosis involving chromatin condensation, DNA fragmentation and cell blebbing.

Caspase 3 was determined by Caspase 3 Colorimetric Assay Kit which the caspase 3 colorimetric assay 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 could calculated from the absorbance values at 405 nm because pNA had a high absorbance at 405 nm or calculated from a calibration curve prepared with defined pNA solution.

Six conditions which required for this assay packed with non-induced cells (non-treated cell lysates), inhibitor-non-induced cells, induced cells (treated cell lysates), inhibitor-induced cells, caspase 3 positive control, inhibitor-caspase 3 positive control (for measuring the nonspecific hydrolysis of the substrate) and reagent blank (negative control). Step one, placed 5 µL of cell lysate or 5 µg/mL caspase 3 positive control into 96-well plates. Step two, added 90, 85 and 75 µL of 1x assay buffer 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. Step three, added 200 µM caspase 3 inhibitor to the appropriate wells. Later on, added 10 µL of 2 mM caspase 3 substrate to each well and mixed gently by shaking to start the reaction. Additionally, try to avoid forming bubbles in the well. And then, covered the 96-well plates and incubated at 37ºC for 70 to 90 min or continued the incubation overnight if signal, yellowish color that was visualized by the naked eyes, was too low. Next, measured an absorbance at 405 nm and considered the results using a pNA calibration curve. Lastly, calculated the caspase 3 activity in µmol pNA released per min per mL of cell lysate or positive control based on the formula:

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

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

3.7.2.3 Caspase 8 activity assay

Caspase 8 was localized at the top of the hierarchy of the caspase cascade and was a member of the upstream or initiatior family of caspases. The activation of the proenzyme was triggered by the aggregation of protein, which leaded to auto- or transprocessing. Caspase 8 activated downstream caspases (3, 6 and 7) that cleave key cellular substrates and lead to apoptotic death of the cells.

Caspase 8 was determined by Caspase 8 Colorimetric Assay Kit which the caspase 8 colorimetric assay 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 by caspase 8. The concentration of the pNA released from the substrate could calculated from the absorbance values at 405 nm because pNA had a high absorbance at 405 nm or calculated from a calibration curve prepared with defined pNA solution.

Six conditions, which required for this assay, composed of non-induced cells (non-treated cell lysates), inhibitor-non-induced cells, induced cells (treated cell lysates), inhibitor-induced cells, caspase 8 positive control, inhibitor-caspase 8 positive control (for measuring the nonspecific hydrolysis of the substrate) and reagent blank (negative control). Step one, placed 10 µL of cell lysate or 10 µg/mL caspase 8 positive control into 96-well plates. Then, added 90, 80 and 78 µL of 1x assay buffer 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. After that, 25 µM caspase 8 inhibitor was added to the appropriate well and ensure that all the cells were mixed gently to avoid bubble formation. Later on, let stand at room temperature for 5 min. Next, 10 µL of 2 mM caspase 8 colorimetric substrate was added into each well of all the conditions to start the reaction. Finally, covered the 96-well plates and measured an absorbance at 405 nm (t = 0) and then read every 5 min until

60 min. Calculated the caspase 8 activity as nmol of pNA released per min per mL based on the formula:

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

where V was volume of sample in mL, d was dilution factor, t was reaction time in minutes, $A_{1\ nmol}$ was absorbance of 1 nmol of pNA in the well, A_t was absorbance at time t minutes and A_0 was absorbance at zero time.

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

RP-HPLC was isolation and purification technique by column. The principle of this technique was separated sample based on polar or non-polar related with polar or non-polar of the stationary phase.

The antioxidant purified peptide fraction, the highest antioxidant activity from gel filtration chromatography (F52 fraction), was filtrated through 0.45 µm of nylon filtration membrane and further separated by RP-HPLC on a Luna 5U C18 100A (4.6 mm × 250 mm) column with a gradient of 88% in elute B; 0.1% trifluoroacetic acid (TFA) and 12% in elude C; 70% acetonitrile (ACN) containing with 0.05% trifluoroacetic acid (TFA) at a flow rate of 0.7 mL/min. The peptide peaks were eluded monitoring at 280 nm. After that, the antioxidant peptide peaks were collected and the purity antioxidant peptides were analyzed by mass spectrometer.

3.9 Mass spectrometry

Identification an accurate molecular mass and amino acid sequence of the antioxidant purified peptide was accomplished by ESI-Q-TOF mass spectrometry. The ESI-Q-TOF mass spectrometry involved electrospray ionization and quadrupole time of flight mass spectrometer. The ESI-Q-TOF mass spectrometry instrument was calibrated peptide chain in mass range 50-25,000 m/z. *De novo* sequencing and mascot evaluated all collected ESI-Q-TOF mass spectrometry data. The principle of *de novo* sequencing was using the difference of mass between two fragment ions to calculate mass of amino acid residue on the peptide chain. The mass could be determined the residue. Definition of *de novo* peptide sequencing was peptide

sequencing without database of the amino acid sequence. While, mascot was comparing the peptide sequence with the database.

3.10 Statistics

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

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Antioxidant activity assay of protein hydrolysate or peptide

Protein hydrolysates or peptides obtained from chicken feather meal were hydrolyzed by the microbial proteases Alcalase®, Flavourzyme® and Neutrase®. The free radical scavenging activities of peptides derived from chicken feather meal were evaluated by DPPH and ABTS radical scavenging activity assays as shown in Tables 4.1 and 4.2. The results indicated that the peptide hydrolysates acquired through microbial proteases could reveal the antioxidant activities and 5% Neutrase® was an effective protease that could release effective antioxidant peptides from chicken feather meal with the IC_{50} values of 16.45±0.23 and 9.34±0.08 µg/mL, respectively. The peptide derived from bluefin leatherjacket (*Navodon septentrionalis*) which was hydrolyzed by 2% Neutrase® (w/w) had IC_{50} values of 9.105 mg/mL after determining the antioxidant activity by DPPH radical scavenging activity assay (Chi *et al.*, 2015[b]). Moreover, the peptide derived from brown seaweed (*Scytosiphon lomentaria*) which was hydrolyzed by 0.1% Neutrase® exhibited a strong scavenging activity on DPPH radical of 74.03% (Ahn *et al.*, 2004). In addition, the results implied that the different types of enzymes influenced the antioxidant effects of peptides from chicken feather meal because of the activities of the hydrolyzed pattern of enzymes on the peptide bond, namely, the Flavourzyme® was endo- and exoprotease while the Alcalase® and Neutrase® were endoprotease (Kim *et al.*, 2013[a]). Furthermore, the activity increased with greater concentrations of the enzyme. Therefore, peptides from chicken feather meal using 5% Neutrase® was selected and prepared for follow-up studies.

4.1.1 DPPH radical scavenging activity assay

Table 4. 1 The IC_{50} value of the DPPH radical scavenging of peptide hydrolysate from chicken feather meal

Enzyme	DPPH radical scavenging activity (IC_{50}) (µg/mL)			
	Control	1%	2.5%	5%
Alcalase®	269.13 ± 10.73^{c}	68.30 ± 2.56^{b}	61.71 ± 11.05^{b}	56.76 ± 1.46^{b}
Flavourzyme®	121.43 ± 10.19^{cc}	50.86 ± 4.52^{bb}	31.66 ± 0.78^{aabb}	20.84 ± 0.67^{aa}
Neutrase®	96.63 ± 4.09^{D}	42.60 ± 0.48^{C}	27.63 ± 1.25^{B}	16.45 ± 0.23^{A}

*All data are presented by mean ± standard error of triplicate results. $^{b\text{-}c,\ aa\text{-}cc,\ A\text{-}D}$ 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 with IC_{50} = 26.97±0.34 µg/mL.

4.1.2 ABTS radical scavenging activity assay

Table 4. 2 The IC_{50} value of the ABTS radical scavenging of peptide hydrolysate from chicken feather meal

Enzymes	ABTS radical scavenging activity (IC_{50}) (µg/mL)			
	Control	1%	2.5%	5%
Alcalase®	42.89 ± 1.87^{b}	12.92 ± 0.05^{a}	12.86 ± 0.08^{a}	9.97 ± 0.31^{a}
Flavourzyme®	64.72 ± 2.28^{cc}	19.07 ± 2.19^{bb}	14.88 ± 0.33^{aabb}	10.16 ± 0.19^{aa}
Neutrase®	60.18 ± 2.51^{C}	18.97 ± 1.16^{B}	13.60 ± 0.06^{AB}	9.34 ± 0.08^{A}

*All data are presented by mean ± standard error of triplicate results. $^{a\text{-}b,\ aa\text{-}cc,\ A\text{-}C}$ values with same letters indicate no significant difference for each group of samples at the same protease ($p > 0.05$).

**ascorbic acid as a positive control with IC_{50} = 98.44±3.79 µg/mL.

4.2 Isolation and purification of the ultrafiltration of antioxidant peptide from 5% Neutrase® treatment

Antioxidant peptide, obtained from 5% Neutrase® treatment, was fractionated by ultrafiltration with 0.65, 3, 5, and 10 kDa MWCO and five different MW fractions

of ≥ 10 kDa (F1), 5-10 kDa (F2), 3-5 kDa (F3), 0.65-3 kDa (F4) and < 0.65 kDa (F5) were prepared. As shown in Table 4.3, the < 0.65 kDa or F5 fraction had the highest DPPH and ABTS radical scavenging activities with IC_{50} values of 1.72±0.04 and 0.42±0.02 µg/mL, respectively. The strong free radical scavenging activity of the < 1 kDa peptide fractions was comparable to the bluefin leatherjacket skin (Chi *et al.*, 2015[b]), bluefin leatherjacket head (Chi *et al.*, 2015[c]) and blood clam (Chi *et al.*, 2015[a]). It could be concluded that low MW peptides were required for the high antioxidant activity because they could be more effective in their interaction with radicals interfering in the oxidizing process (He *et al.*, 2013; Wang *et al.*, 2012). Thus, the lowest MW peptide fraction in < 0.65 kDa could possess high antioxidant activity and be selected for follow-up studies. This study used ascorbic acid as a positive control.

Table 4. 3 DPPH and ABTS radical scavenging activities of peptide hydrolysate from chicken feather meal derived from 5% Neutrase® and its ultrafiltration membrane fractions

Fraction samples	**Cut off (kDa)**	**Radical scavenging activity (IC_{50}) (µg/mL)**	
		DPPH	**ABTS**
F1	≥ 10	22.33±1.27[d]	3.14±0.14[A]
F2	10-5	9.46±0.44[c]	1.73±0.08[A]
F3	5-3	4.77±0.12[b]	1.33±0.02[A]
F4	3-0.65	1.99±0.01[a]	0.45±0.02[A]
F5	< 0.65	1.72±0.04[a]	0.42±0.02[A]
	Ascorbic acid	26.97±0.34[e]	98.44±3.79[B]

*All data are presented by mean ± standard error of triplicate results. [a-e, A-B] values with same letters indicate no significant difference for each group of fraction samples at the same radical scavenging activity assay (IC_{50}) ($p > 0.05$).

4.3 Purification on Superdex® 75 gel filtration chromatography of antioxidant purified peptide of fraction F5

Based on the antioxidant activities, the F5 as < 0.65 kDa peptide fraction was further fractionated into two fractions, F51 and F52 fractions, to purify using gel filtration chromatography with a Superdex® 75 column. F51 and F52 fractions were collected and read an absorbance at 280 nm (Figure 4.1). The IC_{50} values of DPPH and ABTS radical scavenging activities of F52 fraction were 7.35±0.30 and 14.79±0.24 µg/mL, respectively which was 6.17 and 12.81 µg/mL more than the F51 fraction respectively as shown in Table 4.4. Consequently, F52 fraction was selected for the next steps of purification by RP-HPLC, identification by mass spectrometry and used for studying DNA damage and antiproliferative activity. This calculation used ascorbic acid as a positive control.

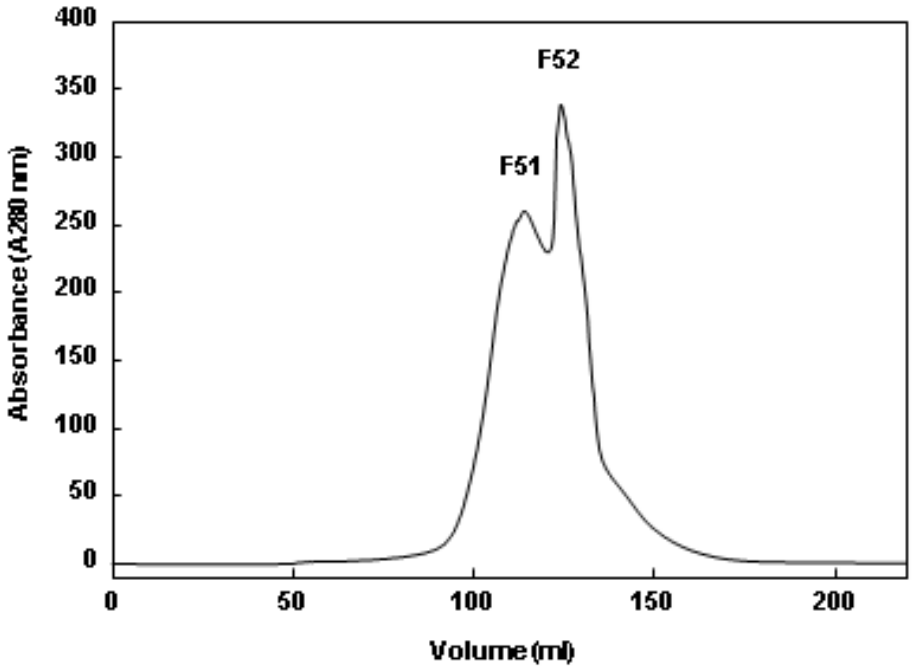


Figure 4. 1 Gel filtration chromatography of peptide fraction < 0.65 kDa on a Superdex® 75

Table 4. 4 DPPH and ABTS radical scavenging activities of purification using gel filtration chromatography of fraction < 0.65 kDa peptide hydrolysate from chicken

feather meal derived from 5% Neutrase® on Superdex® 75 gel filtration chromatography

Fraction samples	Radical scavenging activity (IC_{50}) (µg/mL)	
	DPPH	ABTS
F51	13.52 ± 0.27^{b}	27.60 ± 1.63^{B}
F52	7.35 ± 0.30^{a}	14.79 ± 0.24^{A}
Ascorbic acid	26.97 ± 0.34^{c}	98.44 ± 3.79^{C}

*All data are presented by mean ± standard error of triplicate results. $^{a\text{-}c,\ A\text{-}C}$ values with same letters indicate no significant difference for each group of fraction samples at the same radical scavenging activity assay (IC_{50}) ($p > 0.05$).

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

Many human conditions and diseases including aging, inflammation, and particularly cancer diseases involve ROS and other free radicals (Lee *et al.*, 2012). In addition, the hydroxyl radical (an ROS) could cause oxidative stress in cells which generally leads to DNA damage (Kittiphattanabawon *et al.*, 2013) due to the fact that the one of the sensitive bio-molecule targets for ROS-mediated oxidative damage is DNA (Martinez *et al.*, 2003). Three forms of plasmid DNA consist of supercoiled, linear and open circular DNA. In this study, three types of plasmids were used, namely, pKS, pUC19 and pBR322 and the DNA protective effect of F52 was assessed on the hydroxyl radical. The plasmids were induced by the Fenton reaction to generate DNA strand breakages which could be broken into three forms (supercoiled, linear and open circular DNA). The varying concentrations of antioxidant purified peptide of F52 fraction with 0.0536, 0.0268, 0.0134, 0.0067 and 0.0034 mg/mL were used for protective hydroxyl radical-induced DNA damage (lane 4-8). All of the concentrations could protect hydroxyl radical-induced DNA damage except for that of 0.0034 mg/mL which was similar to plasmid DNA and the induced DNA damage by hydroxyl radical generated from the Fenton reaction (lane 3). According to Figures 4.2 and 4.3, the

results reveal that supercoiled DNA decreased and converted to linear and open circular DNA (lane 4-5) and completely converted to linear and open circular DNA (lane 6-7) in pKS and pUC19 plasmid DNA when compared with the pKS and pUC19 plasmid control DNA (lane 2). Moreover, as shown in Figure 4.4, supercoiled DNA decreased while linear DNA increased in pBR322 plasmid DNA when compared with the pBR322 plasmid control DNA (lane 2). According to the research of Sheih *et al.* (2009), supercoiled DNA can completely convert to another form when the DNA is treated with purified peptide. Furthermore, the research of Lee *et al.* (2012) reported that the concentrations of purified peptide at 0.125, 0.25, 0.5 and 0.1 mg/mL could protect hydroxyl radical-induced DNA damage. From the results, it seems likely that the 0.0536, 0.0268, 0.0134 and 0.0067 mg/mL concentrations of antioxidant purified peptide of F52 fraction could be more effective in protecting against the hydroxyl radical generated from Fenton reaction and induced DNA damage. This could be applied in an antiproliferative assay owing to the antioxidant activities of the F52 fraction and protective effect on DNA damage. Additionally, the result indicated that the ability of 5% Neutrase® to hydrolyze chicken feather meal to derive peptide could inhibit oxidative damage to DNA.

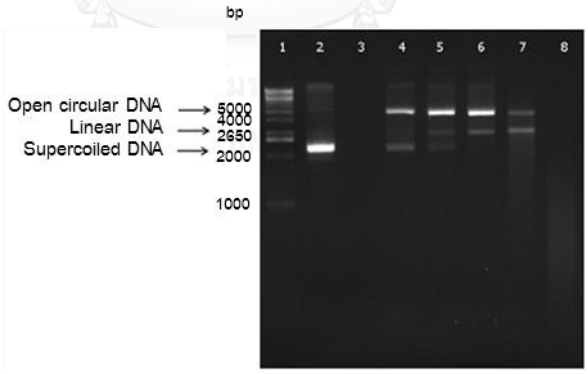


Figure 4. 2 Agarose gel electrophoresis patterns of pKS plasmid DNA broken by hydroxyl radical generated from the Fenton reaction with the pKS plasmid DNA receiving the protective effect of the antioxidant purified peptide of F52 fraction at concentrations of 0.0536, 0.0268, 0.0134, 0.0067 and 0.0034 mg/mL on the hydroxyl radical-induced oxidation of pKS plasmid DNA. Lane 1: marker 1 kbp; Lane 2: pKS plasmid DNA 2,961 bp (DNA = 113.5 ng/µL and protein content = 0.056 mg/mL); Lane 3: pKS plasmid DNA with $FeSO_4$ 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 the F52 fraction

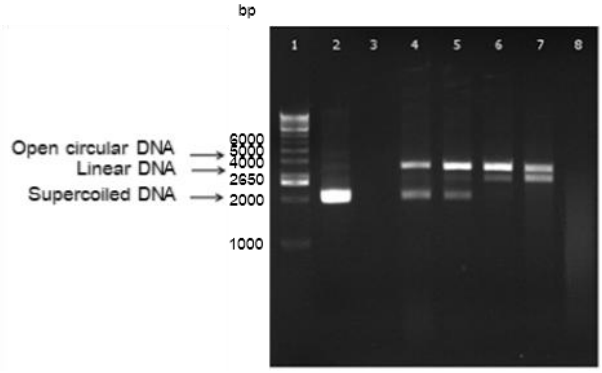


Figure 4. 3 Agarose gel electrophoresis patterns of pUC19 plasmid DNA broken by hydroxyl radicals generated from the Fenton reaction with the pUC19 plasmid DNA receiving the protective effect of the antioxidant purified peptide of F52 fraction at concentrations of 0.0536, 0.0268, 0.0134, 0.0067 and 0.0034 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 F52 fraction

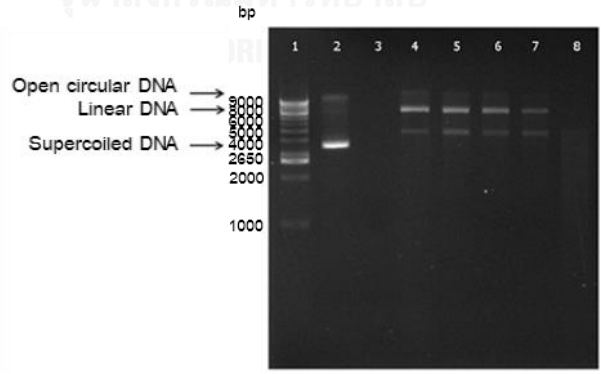


Figure 4. 4 Agarose gel electrophoresis patterns of pBR322 plasmid DNA broken by hydroxyl radicals generated from the Fenton reaction with the pBR322 plasmid DNA receiving the protective effect of the antioxidant purified peptide of F52 fraction at concentrations of 0.0536, 0.0268, 0.0134, 0.0067 and 0.0034 mg/mL on hydroxyl radical-induced oxidation of pBR322 plasmid DNA. Lane 1: marker 1 kbp; Lane 2: pBR322 plasmid DNA 4,361 bp (DNA = 30.7 ng/µL and protein content = 0.073

mg/mL); Lane 3: pBR322 plasmid DNA with $FeSO_4$ 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 the F52 fraction

4.5 Antiproliferative activity assay

Normal tissue normally performs an anti-proliferation role against cell death or cell proliferation to maintain balance in living organisms. On the other hand, cell proliferation cannot control the cell division that would induce tissue proliferation and might become cancerous (Chi *et al.*, 2015[a]). Thus, antiproliferative activity assay is one of the effective methods in anticancer or patterns of cell death in apoptosis form that requires further study. In this experiment, WI-38 was used as a human normal fibroblast cell line and five human cancer cell lines comprising BT474, Chago-K1, Hep-G2, KATO-III and SW620 were treated with an antioxidant purified peptide F52 fraction at different concentrations, incubated for 72 h and quantified using the MTT assay. As shown in Table 4.5, the F52 fraction exhibited a significantly higher and stronger antiproliferative activity on SW620 cells at IC_{50} values of 26.37±2.87 µg/mL than BT474, Chago-K1, Hep-G2, KATO-III whose IC_{50} values were 33.39±0.43, 32.47±0.48, 32.52±0.78 and 32.53±2.44 µg/mL, respectively. Many researchers reported that the antioxidant purified peptide exhibited an antiproliferative activity on cancer cells and could induce cell apoptosis (Chi *et al.*, 2015[a]; Hsu *et al.*, 2011). Conversely, the F52 fraction had no cytotoxicity effect in the human normal fibroblast cell line (WI-38). Hence, the results suggest that the antioxidant purified peptide of the F52 fraction might be used to examine apoptosis induction for drug development in cancer, especially colon cancer (SW620) inhibition. The SW620 cell line was selected for the followed-up studies in apoptosis analysis.

Table 4. 5 The antiproliferative activity of the antioxidant purified peptide of fraction < 0.65 kDa peptide hydrolysate from chicken feather meal derived from 5% Neutrase® on Superdex® 75 gel filtration chromatography

Type of cancer cell lines	Antiproliferative activity (IC_{50}) (µg/mL)
BT474	33.39 ± 0.43^{b}
Chago-K1	32.47 ± 0.48^{b}
Hep-G2	32.52 ± 0.78^{b}
KATO-III	32.53 ± 2.44^{b}
SW620	26.37 ± 2.87^{a}
WI-38 (Normal fibroblast cell line)	38.64 ± 0.10^{c}

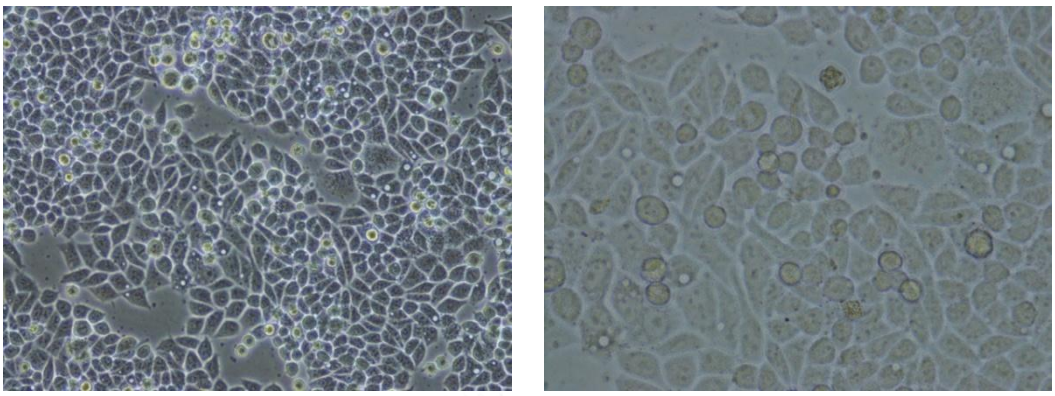
*All data are presented by mean ± standard error of triplicate results. $^{a-c}$ values with the same letters indicating no significant difference for each group of fraction samples at the same antiproliferative activity (IC_{50}) ($p > 0.05$).

4.6 Apoptosis by flow cytometer

Apoptosis or programmed cell death is a cellular phenomenon when the cell receives external or internal factors such as disease, toxin, mitochondria or activation of death receptors and morphogenesis development. The physical and biochemical changes of the apoptosis process involve plasma membrane blebbing, mitochondrial membrane potential loss, DNA fragmentation and caspases activation. The antioxidant purified peptide from gel filtration chromatography (F52 fraction, 62 µg/mL), which had high antiproliferative activity on the human colon cancer cell line SW620 using MTT assay was used to induce apoptotic cells on SW620 cells. Doxorubicin is one anticancer drugs commonly used in the clinic for cancer treatments and can induce apoptosis through activation of the intrinsic pathway of the apoptosis process, initiated at the mitochondria (Wesselborg *et al.*, 1999). Therefore, doxorubicin was used as a positive control in this experiment. The morphology of SW620 cells is shown in Figure 4.5. FITC Annexin V Apoptosis Detection Kit with PI was used to determine the apoptotic cells and calculate the percentage of apoptotic cells using a flow cytometer. Annexin V was a marker of apoptotic cells that bind with the phosphatidylserine (PS) at the outer layer of the plasma membranes, while PI was an indicator of the necrotic cells that bind with the DNA within the nucleus.

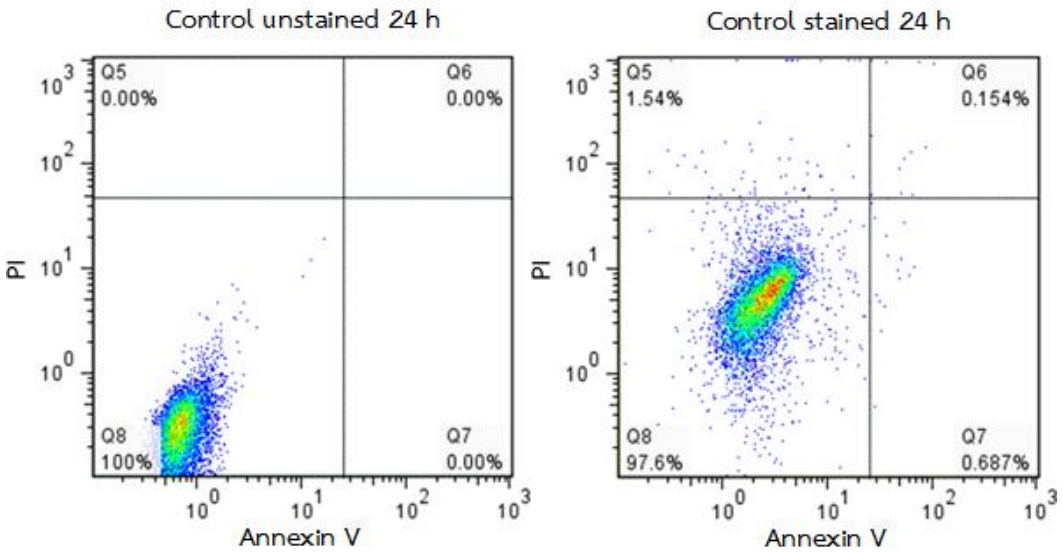
The effect of various concentrations of F52 fraction, with 0.25%, 0.5%, 1% and 2% of the F52 fraction (protein contents were 0.15, 0.31, 0.62 and 1.24 μg/mL, respectively), were treated with SW620 cells for 24 h as shown in Figure 4.6. It can be concluded that the F52 fraction at a concentration of 0.5% (protein content was 0.31 μg/mL) can induce the highest early apoptotic cells. The 0.5% F52 fraction (protein content was 0.31 μg/mL) could induce early apoptotic cells to 1.970%, while more than 0.25, 1 and 2% F52 fractions (protein contents were 0.15, 0.62 and 1.24 μg/mL, respectively) induced early apoptotic cells at 1.100, 0.603 and 0.738%, respectively. Additionally, the 0.5% F52 fraction (protein content was 0.31 μg/mL) can induce early apoptotic cells higher than SW620 with RPMI+10%FCS (negative control) treatment at 1.283%, and lower than 0.5 μg/mL doxorubicin (positive control) treatment at 0.51%. Consequently, the 0.5% F52 fraction (protein content was 0.31 μg/mL) was selected for studying the effect of various times on SW620 cells. As shown in Figures 4.7 and 4.8, the 0.5% F52 fraction (protein content was 0.31 μg/mL) was treated with SW620 cells for 24 h could induce early apoptotic cells, late apoptotic cells and necrotic cells at 1.13±0.50, 0.36±0.15 and 4.06±0.77%, respectively. For 48 h, the 0.5% F52 fraction (protein content was 0.31 μg/mL) could induce early apoptotic cells, late apoptotic cells and necrotic cells at 1.14±0.62, 0.46±0.07 and 3.64±0.29%, respectively. Also, for 72 h, the 0.5% F52 fraction (protein content was 0.31 μg/mL) could induce early apoptotic cells and necrotic cells at 0.19±0.14 and 6.43±0.76%, respectively. It seems likely that the 0.5% F52 fraction (protein content was 0.31 μg/mL) treated with SW620 cells for 24 and 48 h could induce apoptosis in SW620 cells and this suggests that 24 and 48 h were effective times for anticancer drug development for cancer treatment. Chen *et al.* (2012[b]) reported that Baicalin, a flavone derivative isolated and purified from the dry root of *Scutellaria*, at the concentration of 200 μM had antiproliferative activity on SW620 cells and could increase apoptotic cells through Annexin V positive signal in SW620 cells. Pérez *et al.* (2014) found that the leaves of *llex laurina* and *l. paraguariensis* displayed an inhibitory effect on SW620 cell viability at the IC_{50} values of 115 and 133.4 μg/mL, respectively and could induce 25% and 30% early apoptotic cells of SW620, respectively after 48 h. In addition, Ji *et al.* (2011) reported on the

induction of apoptosis in the SW620 cells of GLAI, which was a triterpene-enriched extract from the fruiting bodies of *Ganoderma lucidum*.

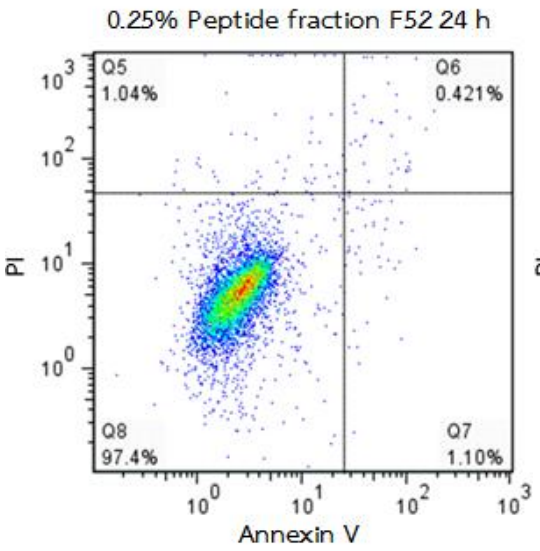


A **B**

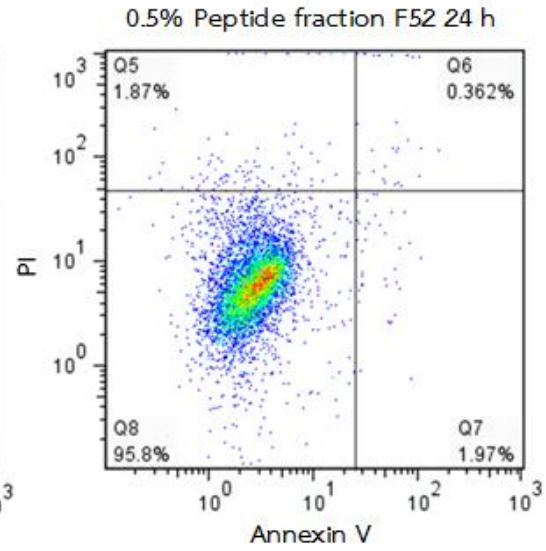
Figure 4. 5 Morphology of SW620 cell line cultures incubated at 37ºC with 5% CO_2 in CO_2 incubator for 72 h at actual magnification (A) 20x and (B) 40x



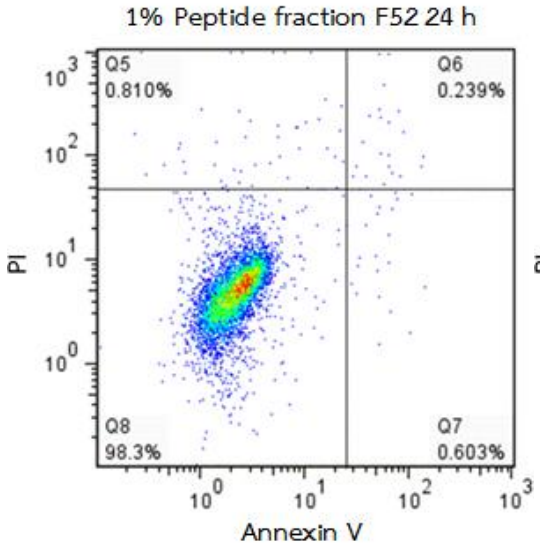
A **B**


0.25% Peptide fraction F52 24 h
Q5
1.04%
Q6
0.421%
Q8
97.4%
Q7
1.10%
PI
Annexin V

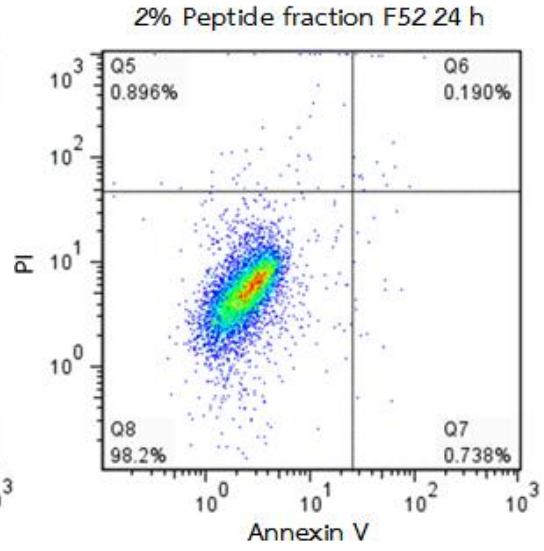
C


0.5% Peptide fraction F52 24 h
Q5
1.87%
Q6
0.362%
Q8
95.8%
Q7
1.97%
PI
Annexin V

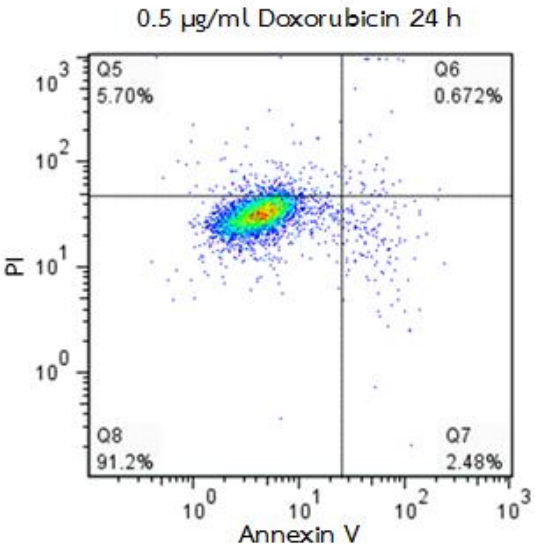
D


1% Peptide fraction F52 24 h
Q5
0.810%
Q6
0.239%
Q8
98.3%
Q7
0.603%
PI
Annexin V

E

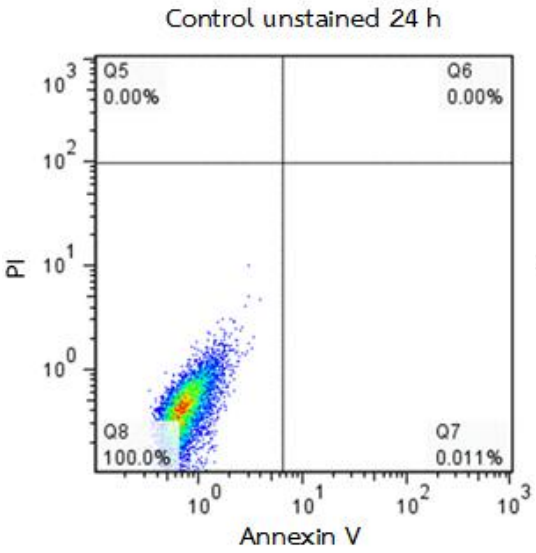

2% Peptide fraction F52 24 h
Q5
0.896%
Q6
0.190%
Q8
98.2%
Q7
0.738%
PI
Annexin V

F

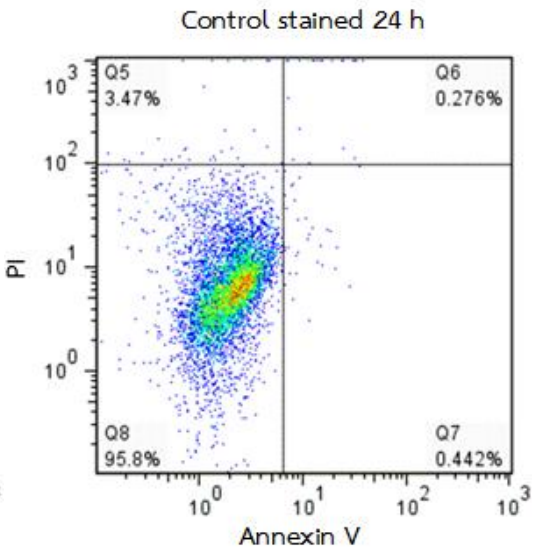


G

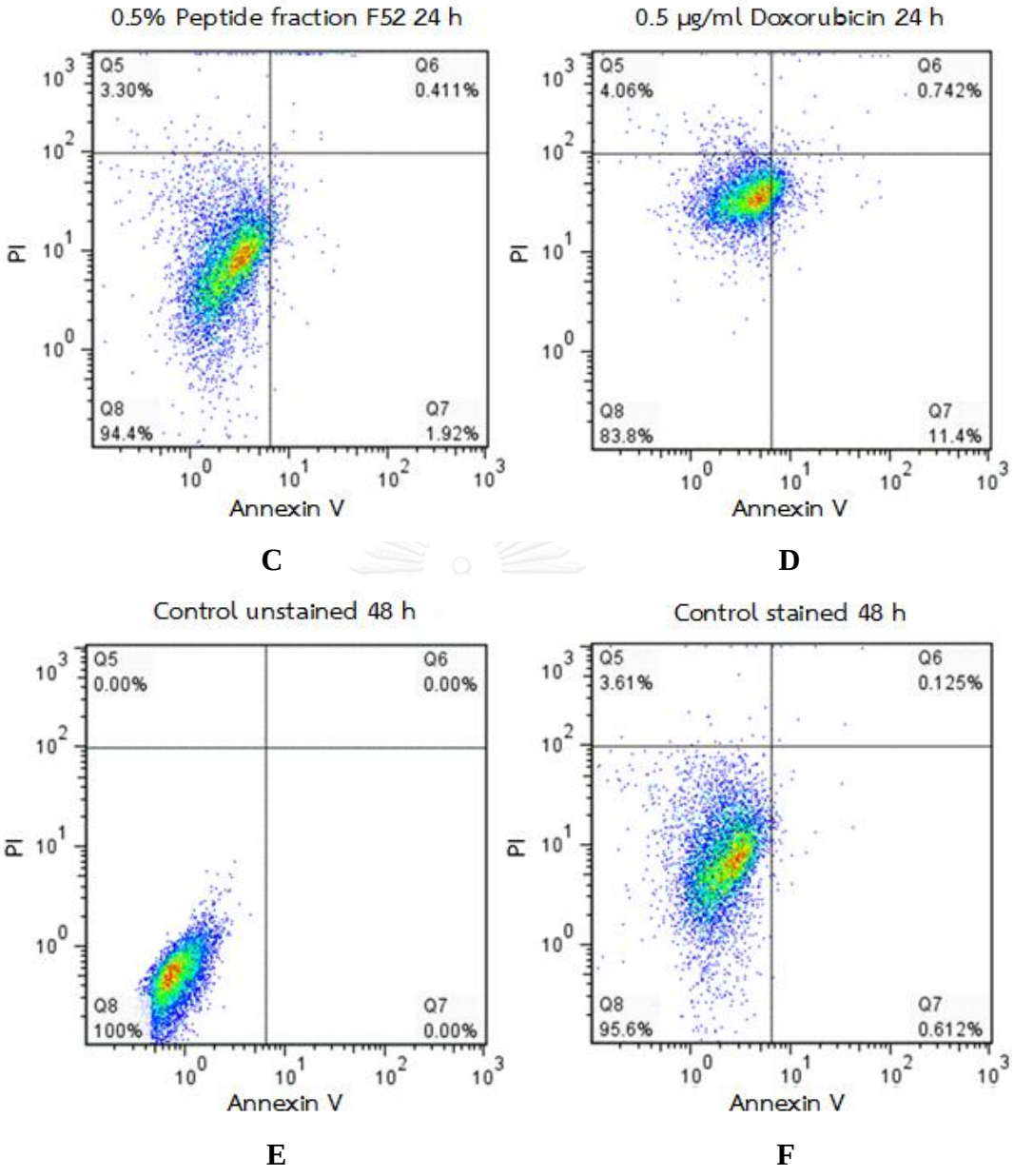
Figure 4. 6 Flow cytometry analysis of SW620 cells treated with (A) RPMI+10%FCS and not labeled with Annexin V-FITC and PI (blank control) (B) RPMI+10%FCS (negative control) (C) 0.25% (D) 0.5% (E) 1% (F) 2% concentrations of F52 peptide fraction which had protein contents were 0.15, 0.31, 0.62 and 1.24 µg/mL, respectively (G) 0.5 µg/mL doxorubicin (positive control) 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

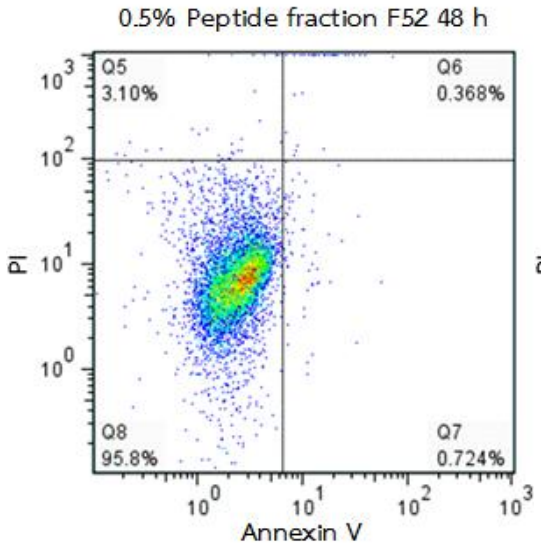


A

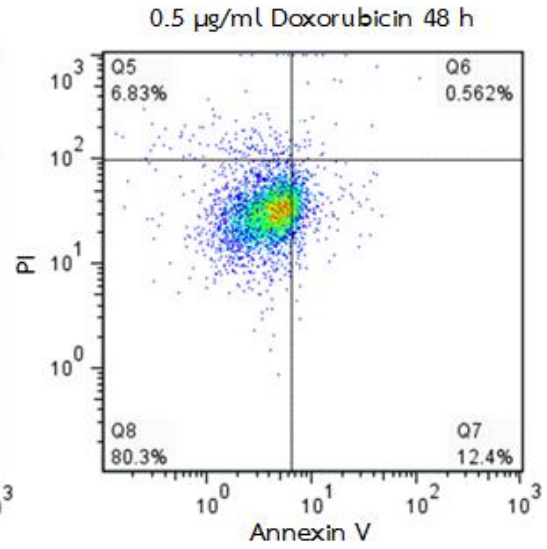


B

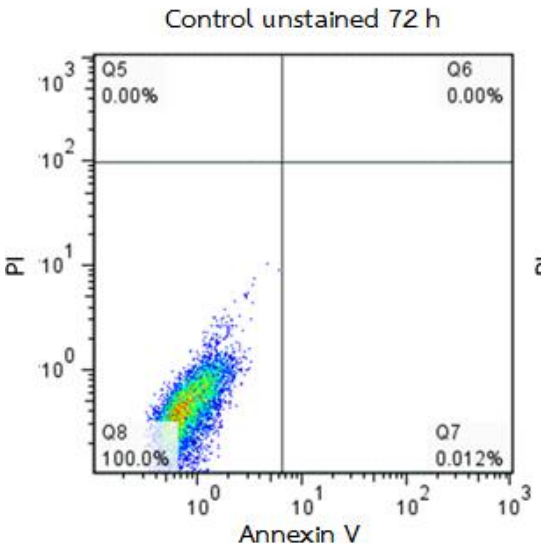

0.5% Peptide fraction F52 24 h
Q5
3.30%
Q6
0.411%
Q8
94.4%
Q7
1.92%
PI
Annexin V
C
0.5 µg/ml Doxorubicin 24 h
Q5
4.06%
Q6
0.742%
Q8
83.8%
Q7
11.4%
PI
Annexin V
D
Control unstained 48 h
Q5
0.00%
Q6
0.00%
Q8
100%
Q7
0.00%
PI
Annexin V
E
Control stained 48 h
Q5
3.61%
Q6
0.125%
Q8
95.6%
Q7
0.612%
PI
Annexin V
F


0.5% Peptide fraction F52 48 h
Q5
3.10%
Q6
0.368%
Q8
95.8%
Q7
0.724%
PI
Annexin V

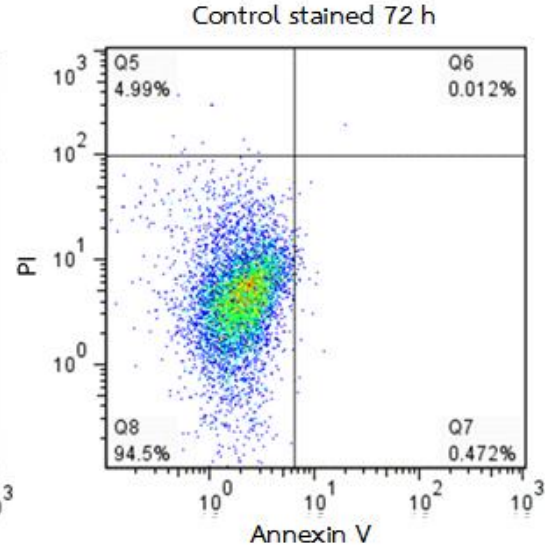
G


0.5 µg/ml Doxorubicin 48 h
Q5
6.83%
Q6
0.562%
Q8
80.3%
Q7
12.4%
PI
Annexin V

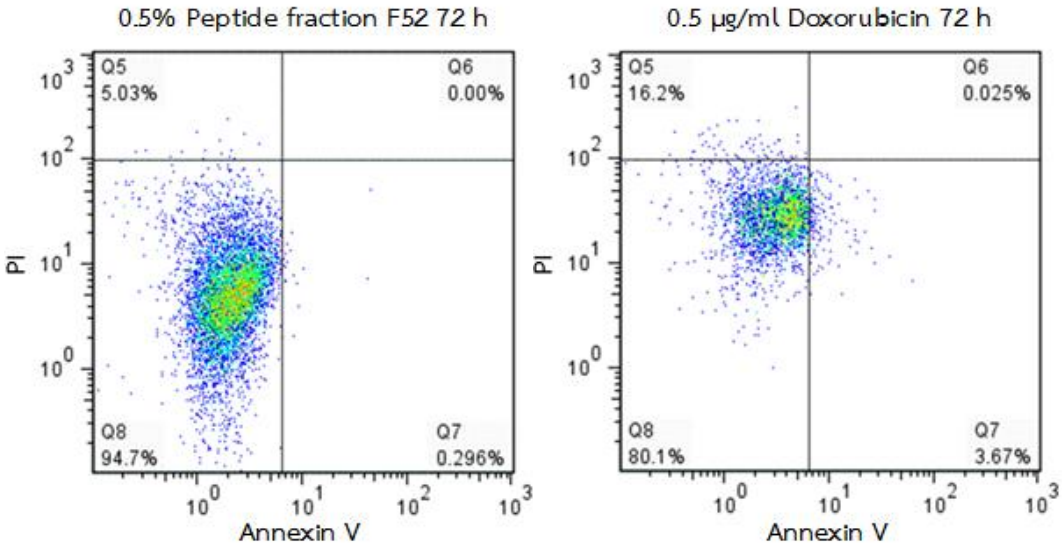
H


Control unstained 72 h
Q5
0.00%
Q6
0.00%
Q8
100.0%
Q7
0.012%
PI
Annexin V

I

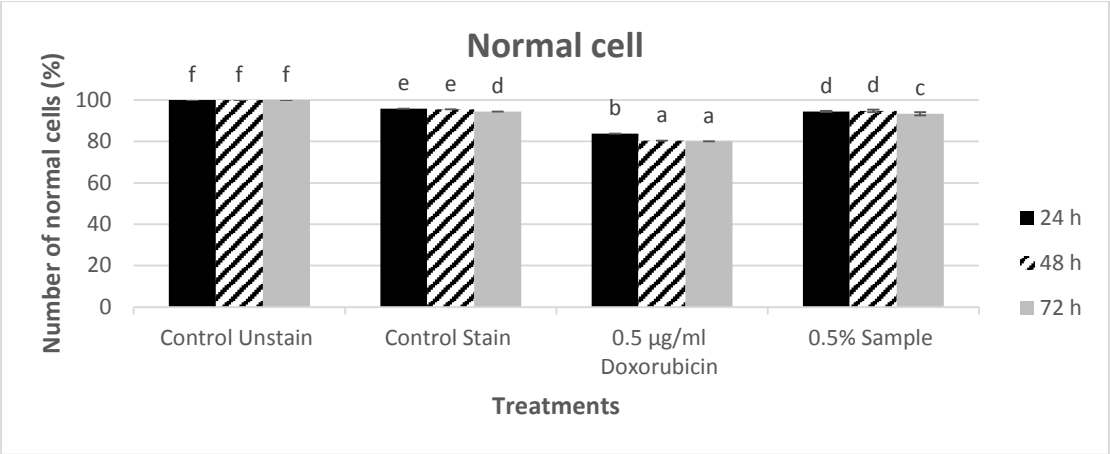

Control stained 72 h
Q5
4.99%
Q6
0.012%
Q8
94.5%
Q7
0.472%
PI
Annexin V

J

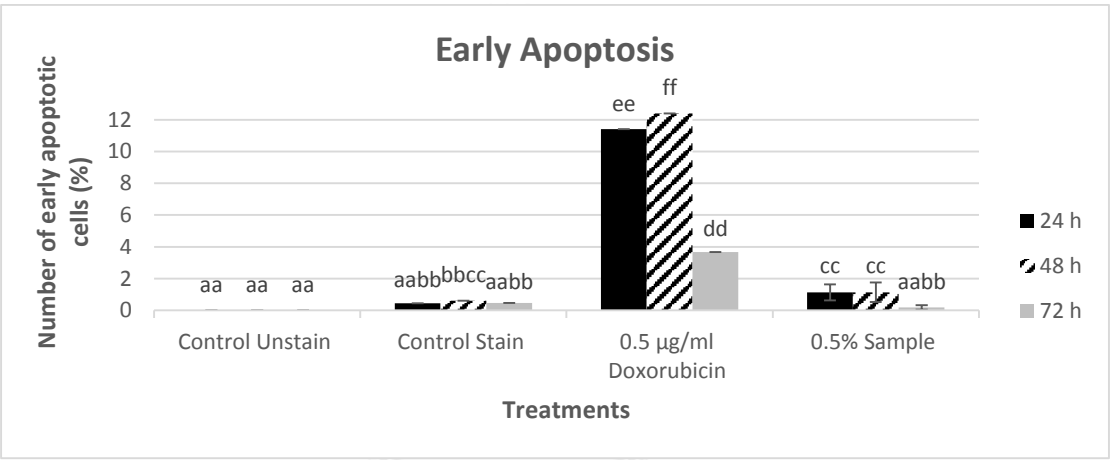


K **L**

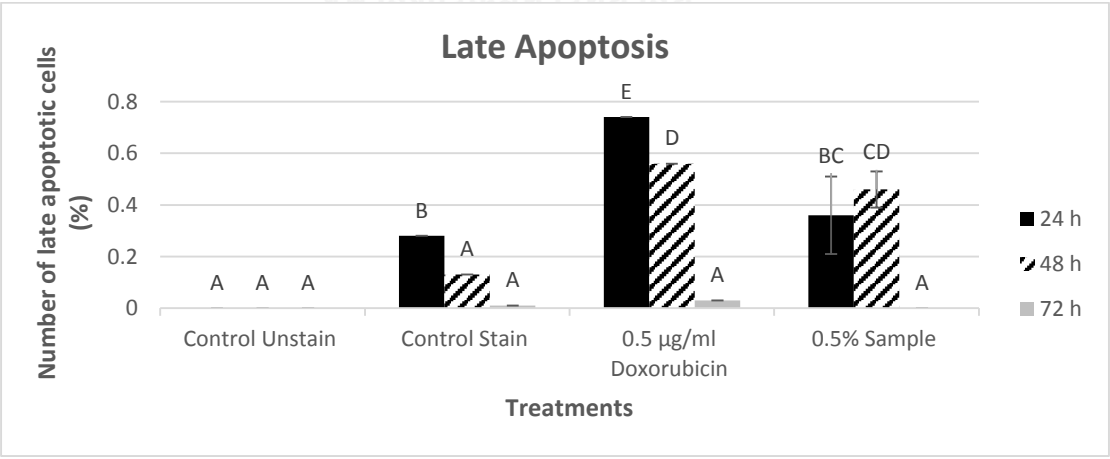
Figure 4. 7 Flow cytometry analysis of SW620 cells treated with (A) RPMI+10%FCS and not labeled with Annexin V-FITC and PI (blank control) (B) RPMI+10%FCS (negative control) (C) 0.5% concentration of F52 peptide fraction which had protein content was 0.31 µg/mL (D) 0.5 µg/mL doxorubicin (positive control) and labeled with Annexin V-FITC and PI for 24 h (E) RPMI+10%FCS and not labeled with Annexin V-FITC and PI (blank control) (F) RPMI+10%FCS (negative control) (G) 0.5% concentration of F52 peptide fraction which had protein content was 0.31 µg/mL (H) 0.5 µg/mL doxorubicin (positive control) and labeled with Annexin V-FITC and PI for 48 h (I) RPMI+10%FCS and not labeled with Annexin V-FITC and PI (blank control) (J) RPMI+10%FCS (negative control) (K) 0.5% concentration of F52 peptide fraction which had protein content was 0.31 µg/mL (L) 0.5 µg/mL doxorubicin (positive control) 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 cells (%)
100
80
60
40
20
0
f f f
e e d
b a a
d d c
Control Unstain
Control Stain
0.5 µg/ml Doxorubicin
0.5% Sample
Treatments
24 h
48 h
72 h

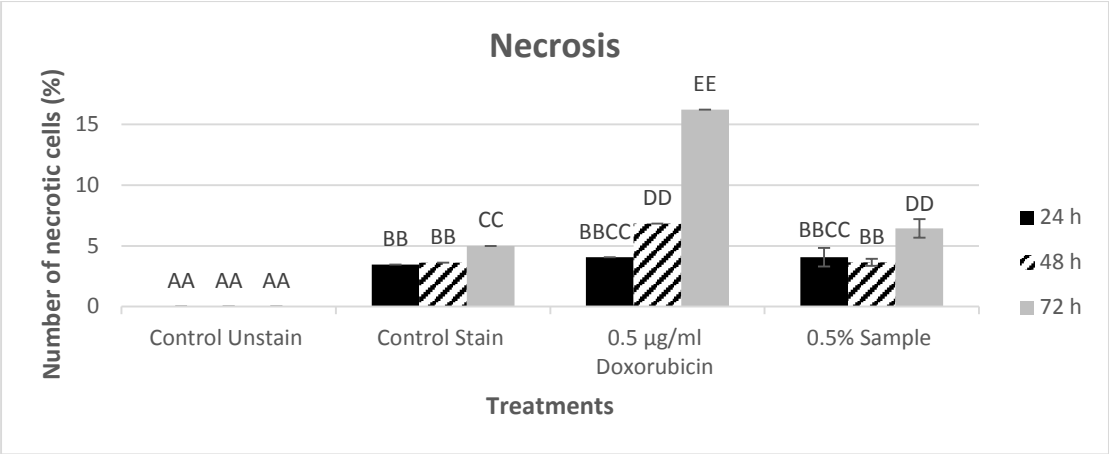
A


Early Apoptosis
Number of early apoptotic cells (%)
12
10
8
6
4
2
0
aa aa aa
aabb bbcc aabb
ee ff dd
cc cc aabb
Control Unstain
Control Stain
0.5 µg/ml Doxorubicin
0.5% Sample
Treatments
24 h
48 h
72 h

B


Late Apoptosis
Number of late apoptotic cells (%)
0.8
0.6
0.4
0.2
0
A A A
B A A
E D A
BC CD A
Control Unstain
Control Stain
0.5 µg/ml Doxorubicin
0.5% Sample
Treatments
24 h
48 h
72 h

C



D

Figure 4. 8 The percentage of the number of SW620 cells using 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

4.7 Caspase 3 and 8 activities assay

The 0.5% F52 fraction at the concentration of 0.31 µg/mL from gel filtration chromatography was selected for analyzing caspases 3 and 8 activities in cell lysates of SW620 cells for 24, 48 and 72 h. Caspase 3 is a key caspase in the proapoptotic cascade and plays a pivotal role in various forms of apoptosis (Ichikawa *et al.*, 2012). Caspase 8 is a common factor that leads to the apoptosis process (Nagata, 1997). Thus, these caspases can be used as an alternative assay for apoptosis analysis. Caspase 3 activity was determined using a Caspase 3 Colorimetric Assay Kit. As presented in Table 4.6, the values of caspase 3 activity for 0.5% F52 fraction (protein content was 0.31 µg/mL) at 24, 48 and 72 h were $2.00\times10^{-5}\pm0.25\times10^{-5}$, $4.80\times10^{-5}\pm0.40\times10^{-5}$ and $2.30\times10^{-5}\pm0.30\times10^{-5}$ µmole pNA/min/mL, respectively. The results imply that the 0.5% F52 fraction (protein content was 0.31 µg/mL) at 48 h could induce the highest activity of caspase 3. Likewise, the 0.5% F52 fraction (protein content was 0.31 µg/mL) at 48 h, as shown in Table 4.6, could exhibit the most effective caspase 8 activity at the value of $12.32\times10^{-5}\pm0.25\times10^{-5}$ µmole pNA/min/mL. Caspase 8 activity was determined using a Caspase 8 Colorimetric Assay Kit. From Table 4.6, the 0.5% F52 fractions (protein content was 0.31 µg/mL) at 24 and 72 h exhibiting caspase 8 activity were

$3.99\times10^{-5}\pm0.57\times10^{-5}$ and $0.08\times10^{-5}\pm0.08\times10^{-5}$ µmole pNA/min/mL, respectively. From both results (caspase 3 activity as well as caspase 8 activity), the 0.5% F52 fraction (protein content was 0.31 µg/mL) on SW620 cells lysate is able to induce greater caspase 3 and 8 activities at 48 h (17.12×10^{-5} µmole pNA/min/mL) than 24 h (5.99×10^{-5} µmole pNA/min/mL) and 72 h (2.38×10^{-5} µmole pNA/min/mL), respectively. It can also be concluded that the effect of caspases 3 and 8 activities of 0.5% F52 fraction (protein content was 0.31 µg/mL) on SW620 cells lysate shown the highest values for 48 h. Furthermore, the activities of caspases 3 and 8 were effective at 48 h, similar to apoptosis analysis using flow cytometry. Hence, apoptosis induction could be analyzed at 48 h and from the apoptosis analysis by flow cytometry and caspase 3 and 8 activities, it can be assumed that the most efficient time of apoptosis induction of the 0.5% F52 fraction (protein content was 0.31 µg/mL) on SW620 cells was 48 h, which was the period most able to induce apoptosis in SW620 cells through the creation of caspase 3 and 8 activities. Finally, it can be concluded that the apoptosis process related to caspase 3 and 8 production. Also, if the percentage of apoptotic cells increased, the caspase 3 and 8 activities also increased. Previous research has reported that Baicalin (200 µM) can increase caspase 3 and 8 activities in SW620 cells for 48 h, which induced apoptosis (Chen *et al.*, 2012[b]). Additionally, GLAI (the triterpene-enriched extract from *G. lucidum*) possessed caspase 3 activity in SW620 cells after being treated with 10, 25 and 50 µg/mL of GLAI for 24 h (Ji *et al.*, 2011).

Table 4. 6 Caspase 3 and 8 activities of the 0.5% F52 fraction (protein content was 0.31 µg/mL) from gel filtration chromatography on Superdex® 75

Treatments	**Caspase 3 activity (µmole pNA/min/mL)**	**Caspase 8 activity (µmole pNA/min/mL)**
Positive control	$7.20\times10^{-5}\pm0.81\times10^{-5}$ de	$91.01\times10^{-5}\pm0.04\times10^{-5}$ E
Negative control	$2.63\times10^{-5}\pm0.83\times10^{-5}$ a	$1.46\times10^{-5}\pm0.07\times10^{-5}$ B
0.5% F52 fraction for 24 h	$2.00\times10^{-5}\pm0.25\times10^{-5}$ a	$3.99\times10^{-5}\pm0.57\times10^{-5}$ C
0.5% F52 fraction for 48 h	$4.80\times10^{-5}\pm0.40\times10^{-5}$ bc	$12.32\times10^{-5}\pm0.25\times10^{-5}$ D
0.5% F52 fraction for 72 h	$2.30\times10^{-5}\pm0.30\times10^{-5}$ a	$0.08\times10^{-5}\pm0.08\times10^{-5}$ A

*All data are presented by mean ± standard error of triplicate results. $^{a\text{-}e,\ A\text{-}E}$ values with the same letters indicate no significant difference for each group of treatments at the same caspase 3 and 8 activities, respectively (IC_{50}) ($p > 0.05$).

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

The higher antioxidant purified peptide of F52 fraction from a Superdex® 75 gel filtration chromatography was filtrated through 0.45 µm nylon filtration membrane and further separated by RP-HPLC. Figure 4.9 shows that the RP-HPLC profile had three peaks for F521, F522 and F523 which were eluted from the F52 fraction by gel filtration chromatography. The three peaks were collected and referred to as F521, F522 and F523 fractions and then the antioxidant activity determined by DPPH and ABTS radical scavenging activities assay. The results, as shown in Table 4.7, reveal that the three fractions exhibited antioxidant activity when analyzed using DPPH radical scavenging activity assay. The antioxidant activity of the F522 fraction was higher than F521 and F523 fractions, respectively. The IC_{50} values of the F521, F522 and F523 fractions were 32.01±2.17, 22.52±4.87 and 59.66±5.89 µg/mL, respectively with protein contents of 3.12, 2.76 and 2.39 µg/mL, respectively. However, the antioxidant activity of the three fractions could not be detected by ABTS radical scavenging activity assay. DPPH is a free radical that accepts an electron or a hydrogen radical and then becomes a stable molecule, whereas the ABTS radical is reduced when antioxidants combine with hydrogen-donating or chain-breaking properties. Besides, there are many reasons leading to the different behavior of DPPH and ABTS radical scavenging activities assays including the different stereoselectivity of radicals, the different stoichiometry of reactions between the antioxidant compounds in hydrolysates with DPPH and ABTS radicals, the fact that specific peptides are capable of reacting and suppressing different radicals, and the solubility and diffusibility of the radicals (Rival *et al.*, 2001; Zhu *et al.*, 2008). The results indicate that higher activity with the DPPH method does not necessarily mean the ability to scavenge ABTS radical increases. This experiment used ascorbic acid as a positive control.

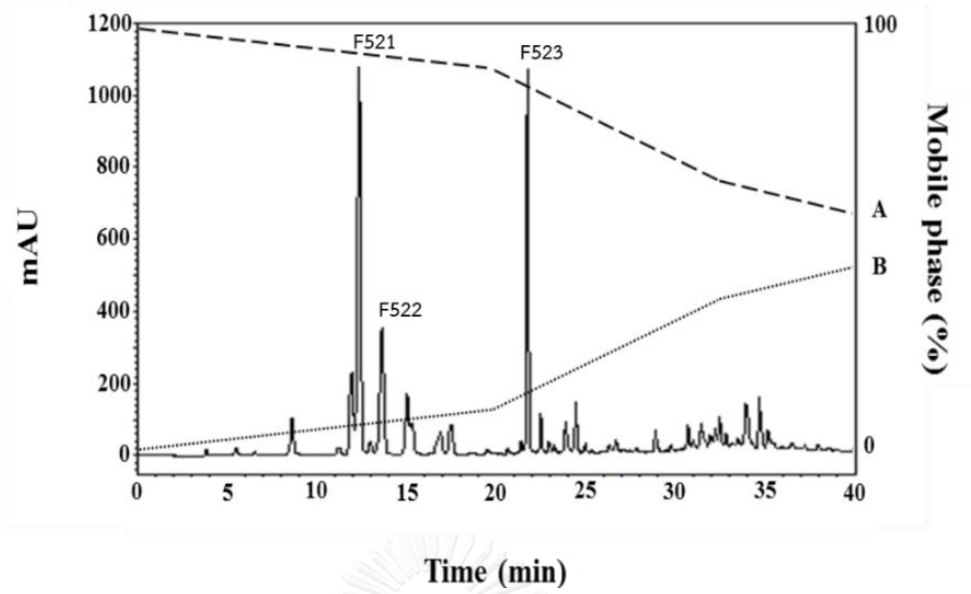


Figure 4. 9 RP-HPLC profile of antioxidant purified peptide of F52 fraction from gel filtration chromatography

Table 4. 7 DPPH and ABTS radical scavenging activities of F521, F522 and F523 fractions, from fraction < 0.65 kDa (F52 fraction) peptide hydrolysate from chicken feather meal derived from 5% Neutrase® using gel filtration chromatography purified and separated on RP-HPLC

Fraction samples	**Radical scavenging activity (IC_{50}) (µg/mL)**	
	DPPH	**ABTS**
F521	32.01±2.17[a]	>3.12 µg/mL
F522	22.52±4.87[a]	>2.76 µg/mL
F523	59.66±5.89[b]	>2.39 µg/mL
Ascorbic acid	26.97±0.34[a]	98.44±3.79

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

4.9 Mass spectrometry

The identification of peptide sequences and molecular masses of antioxidant purified peptide consisting of F521, F522 and F523 fractions from RP-HPLC were characterized using MS/MS experiments which were performed on a Q-TOF mass spectrometer equipped with ESI source. As shown in Table 4.8 the amino acid sequences of the F522 fraction were analyzed using *de novo* peptide sequencing as Val-Thr-Leu-Ala-Val-Thr-Lys-His (VTLAVTKH, 868 Da). However, F521 and F523 could not be analyzed by *de novo* peptide sequencing and were submitted to a database search against SwissPlot database using Mascot software and also searched by the BLAST database of NCBI to be Phe-Asp-Asp-Arg-Gly-Arg-X (FDDRGRX, 875 Da) and Val-Ser-Glu-Ile-X-Ser-Ile-Pro-Ile-Ser (VSEIXSIPIS, 1055 Da), respectively. Previous research reported that molecular size, amino acid sequence and composition play an important role in the antioxidant properties (Chen *et al.*, 2012[a]). Thus, from this study, F521 and F522 fractions with 7 to 8 amino acid residues could scavenge significantly higher on the DPPH radical scavenging activity assay than the F523 fraction which had 10 amino acid residues. Similarly, it has been reported that peptides with 2-10 amino acids might affect bioactivities more than their parent native proteins or large polypeptides (Zhu *et al.*, 2013). In addition, the indole group and benzene ring of aromatic amino acids packed with Phe, Tyr, Trp and His could donate protons to electron deficient radicals. After that, ROS becomes stable during the radical scavenging process while aromatic amino acids can keep their own stability through resonance structure (Rajapakse *et al.*, 2005[b]). Hence, it could be suggested that the peptide sequence with the aromatic amino acid (Phe, Tyr and Trp) had strong antioxidant activity (Hernández-Ledesma *et al.*, 2005; Sarmadi and Ismail, 2010). Furthermore, antioxidant peptides composed of many hydrophobic amino acids with non-polar aliphatic groups packed with Val, Pro, Tyr, Trp, Leu, Ile, Ala, Lys and Met have been reported to have high antioxidant activity (Elias *et al.*, 2008; Guo *et al.*, 2009; Rajapakse *et al.*, 2005[a]). This is due to the hydrophobic amino acid residues in the peptide sequences being able to enhance the solubility of peptides at the water-lipid interface and enable access to scavenging free radicals generated at the lipid phase (Ranathunga *et al.*, 2006), for instance, hydrophobic radical species and hydrophobic polyunsaturated fatty acids (hydrophobic PUFAs) such as omega-6 and omega-3 (Chen

et al., 1998; Qian *et al.*, 2008[a]; Suetsuna and Chen, 2002). Additionally, a hydrogen donor involving Gly has been reported to have high antioxidant activity. Likewise, the SH group in Cys is a radical scavenger with an independently important antioxidant action owing to its direct interaction with radicals (Qian *et al.*, 2008[b]). Moreover, the position of the amino acid in the sequence plays a key role in the antioxidant activity of peptides (Rajapakse *et al.*, 2005[b]), for example, His at the N-terminal was an effective metal ion chelator, His at C-terminal was an effective scavenger against various radicals (Chen *et al.*, 1998) and tripeptides with Trp or Tyr at the C-terminal as strong radical scavengers (Saito *et al.*, 2003). Accordingly, the F521 (FDDRGRX) fraction could have DPPH radical scavenging activity at the IC_{50} value of 32.01±2.17 µg/mL because it possesses Phe which was an aromatic amino acid in its sequence. From the result, the amino acid sequences of F522 (VTLAVTKH) and F523 (VSEIXSIPIS) fractions contained hydrophobic amino acid such as Val and Pro in their sequences; therefore, the F522 and F523 fractions could have antioxidant activity. Additionally, the F522 fraction obtained His in its sequence which could indicate that the F522 fraction has high antioxidant activity.

Table 4. 8 Identification of antioxidant purified peptides by ESI-QUAD TOF mass spectrometry

Fraction samples	**Sequences**	**Organisms**	**Mass (Da)**	**Query cover (%)**	**Identity (%)**	**Accession**
F521 (Mascot)	FDDRGRX	phosphoenolpyruvate carboxykinase (GTP), mitochondrial precursor (*Gallus gallus*)	875	85	100	NP_990801.1
		matrin-3 (*Gallus gallus*)	875	71	100	NP_989478.1
		PREDICTED: rapamycin-insensitive companion of mTOR isoform X2 (*Gallus gallus*)	875	71	100	XP_015133019.1
		PREDICTED: rapamycin-insensitive companion of mTOR isoform X1 (*Gallus gallus*)	875	71	100	XP_003643026.1
		PREDICTED: protein shisa-4 isoform X3 (*Gallus gallus*)	875	71	100	XP_015154282.1

Fraction samples	Sequences	Organisms	Mass (Da)	Query cover (%)	Identity (%)	Accession
F522 (*De novo*)	VTLAVTKH	protein FAM179A (*Gallus gallus*)	868	87	100	NP_001264726.1
		PREDICTED: 60 kDa SS-A/Ro ribonucleoprotein (*Gallus gallus*)	868	87	86	XP_422201.1
		PREDICTED: cilia- and flagella-associated protein 43 isoform X3 (*Gallus gallus*)	868	87	86	XP_015144202.1
		PREDICTED: cilia- and flagella-associated protein 43 isoform X2 (*Gallus gallus*)	868	87	86	XP_015144201.1
		PREDICTED: cilia- and flagella-associated protein 43 isoform X1 (*Gallus gallus*)	868	87	86	XP_015144200.1

Fraction samples	Sequences	Organisms	Mass (Da)	Query cover (%)	Identity (%)	Accession
F523 (Mascot)	VSEIXSIPIS	deltex 2, partial (*Gallus gallus*)	1055	90	80	AAF65193.1
		PREDICTED: E3 ubiquitin-protein ligase DTX4 (*Gallus gallus*)	1055	90	80	XP_015142322.1
		PREDICTED: 4-aminobutyrate aminotransferase, mitochondrial (*Gallus gallus*)	1055	80	75	XP_414940.2
		MHC class II M beta chain 1 precursor (*Gallus gallus*)	1055	70	86	BAG69422.1
		PREDICTED: N-myc-interactor (*Gallus gallus*)	1055	90	67	XP_015145346.1

CHAPTER V
CONCLUSION

Results from this study shown that the peptide hydrolysates prepared from chicken feather meal using 5% Neutrase® could produce peptides with antioxidative activities. The activity assessment for fractions separated by membrane ultrafiltration shown that low molecular weight peptides (MW < 0.65 kDa; F5 fraction) were important for their antioxidant activities. MW < 0.65 kDa was further separated using gel filtration chromatography into two fractions which were collected (F51 and F52 fractions). The F52 fraction exhibited strong free radical scavenging effects on the DPPH and ABTS radical method. In addition, the protective abilities of the peptide hydrolysates (F52 fraction) in DNA damage were also tested. The results revealed that when the amounts of the F52 fraction increased, DNA damage by hydroxyl radicals tended to decrease. Based on these results, the peptides obtained from chicken feather meal by enzymatic hydrolysis could be used as potential natural antioxidants to enrich the antioxidant properties of functional and fresh foods, and neutraceuticals. Furthermore, the F52 fraction was used to determine the antiproliferative activity on normal and cancer cell lines. The results indicated that the F52 fraction was not cytotoxic on normal and cancer cell lines and had the highest antiproliferative activity effect on the colon cancer cell line (SW620 cell). After that, the F52 fraction was used to study the induction of apoptosis that used flow cytometry and the caspase 3 and 8 activities analysis method. The results revealed that the F52 fraction could induce the highest apoptotic cells and caspase 3 and 8 activities on SW620 cell for 48 h. Hence, from all the results, it can be concluded that the peptides obtained from chicken feather meal by enzymatic hydrolysis may also be used for the development of anticancer drugs.

The further experiment, for apoptosis analysis, should studying about the period of time for inducing apoptotic cells of colon cancer cell lines (SW620 cell) with peptide fraction F52 was 2, 4, 6 and 8 h. Moreover, should study about the key of enzyme that exhibit in the intrinsic mitochondrial apoptosis pathway including caspase 9 activation to confirm the ability of peptide fraction F52 in both of extrinsic death receptor and intrinsic mitochondrial apoptosis pathways for inducing apoptotic cells because the

research of caspase 3 and 8 activities as reveal only the extrinsic death receptor apoptotic pathway.

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APPENDIX

APPENDIX A

Buffer and reagents preparation

1. 20 mM Phosphate buffer, pH 7.2

20 mM KH_2PO_4	2.721 g
20 mM K_2HPO_4	3.483 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.

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

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 mix this solution.
3. Pipette 200 µL of this solution into 96-well plates.
4. Shake and read an absorbance at 595 nm after 2 min but before 1 h.

3. DPPH solution and protocol

100 μM DPPH	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 for 15 min in the dark room at room temperature.
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 7 mM ABTS (solution A)

Dissolve 0.096 g ABTS in 25 mL of deionized water.

4.2 2.45 mM potassium persulfate (solution B)

Dissolve 0.016 g potassium persulfate in 25 mL of deionized water.

4.3 ABTS solution

Mix solution A and solution B in the dark room at room temperature for 12 h. Before using, 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 10 min in the dark room 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.

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.

7. DNA damage

7.1 2 mM $FeSO_4$

Dissolve $FeSO_4 \cdot 7H_2O$ 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

Standard curve for determine the protein concentration by Bradford method

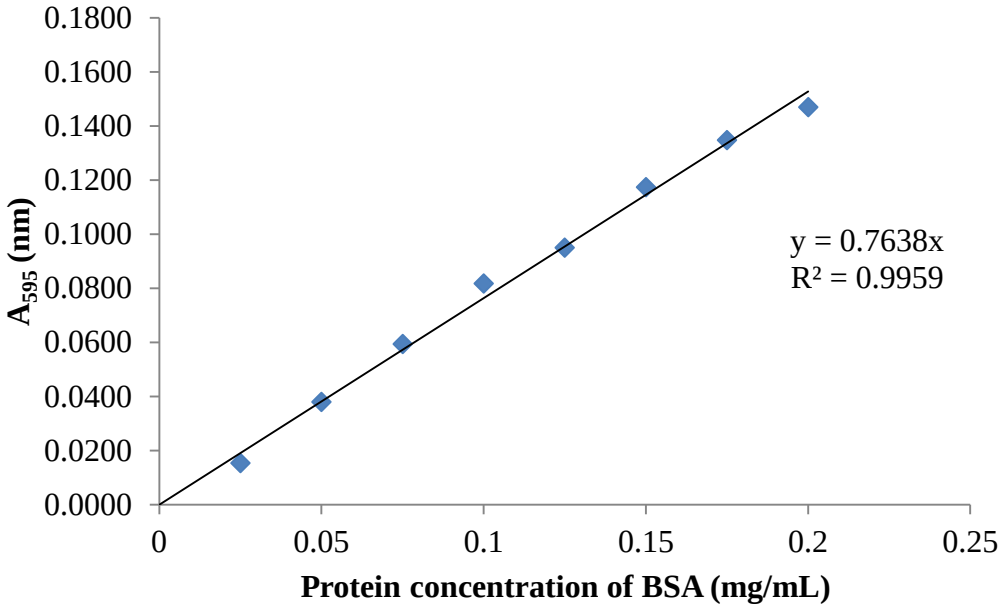

Figure B. 1 Standard curve of BSA concentration at the absorbance of 595 nm

APPENDIX C

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 D

Mass spectrum analysis

Amino acid sequence of F522 fraction: VTLAVTKH

Intensity coverage:	100.0% (6284144 cnts)
Sequence coverage MS:	0.0%
Sequence coverage MS/MS:	0.0%
pI (isoelectric point):	0.0
Parent mass:	868.611
Mass error:	0.085
MH+ (mono):	868.526
MH+ (avg):	869.042
Threshold (a.i.):	0.000
Tolerance (Da):	0.500
Number of peaks:	72
Above Threshold:	72
Assigned peaks:	10
Not assigned peaks:	62

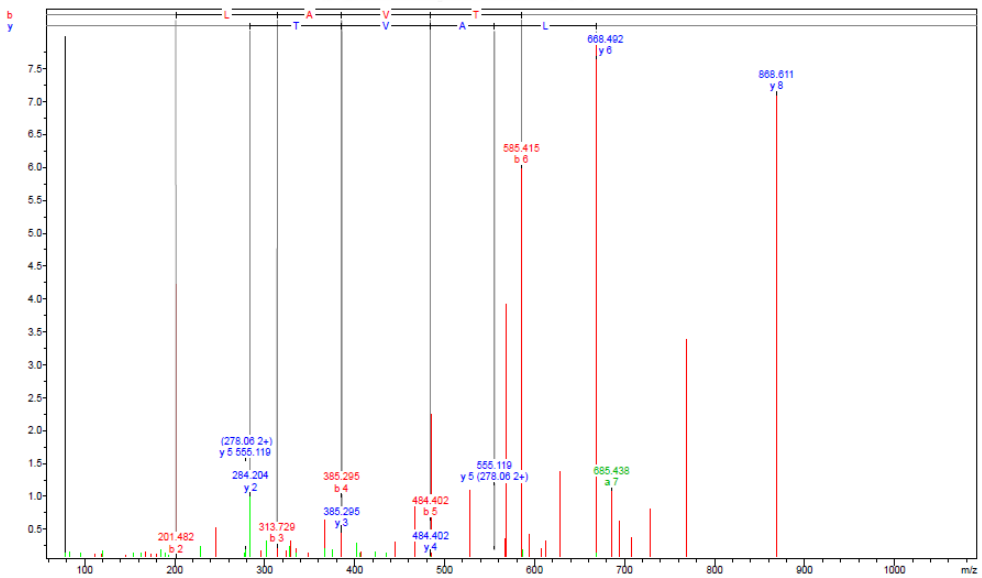

Figure D. 1 Identification of amino acid sequence and molecular mass of the antioxidant purified peptide of F522 fraction from RP-HPLC. MS/MS experiments were performed on a Q-TOF mass spectrometer equipped with ESI source. Sequencing

of antioxidant purified peptide was acquired over the m/z range 100-1000. Mass spectrum analysis of antioxidant purified peptide of F522 fraction as VTLAVTK

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