เพปไทด์ที่มีฤทธิ์ทางชีวภาพที่ได้จากทริปซินไฮโดรไลซิสของไซยาโนแบคทีเรียชนิดเซลล์
เดี่ยว *Synechococcus* sp.
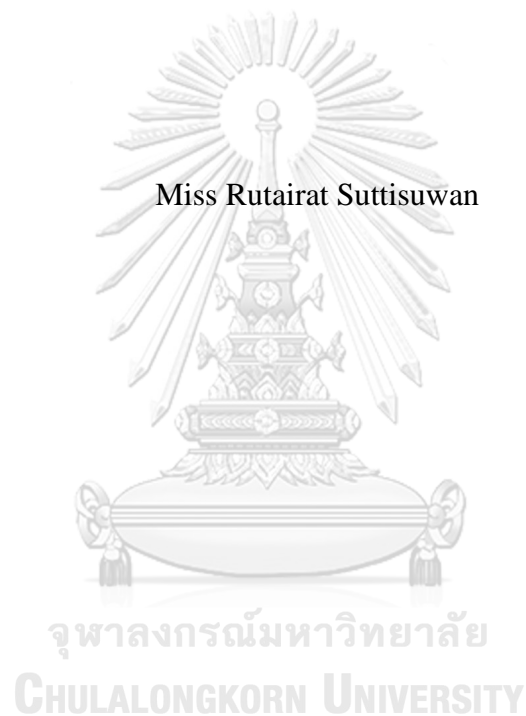
นางสาวฤทัยรัตน์ สุทธิสุวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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BIOACTIVE PEPTIDE DERIVED FROM TRYPSIN HYDROLYSIS OF

UNICELLULAR CYANOBACTERIUM *Synechococcus* sp.

Miss Rutairat Suttisuwan

จุฬาลงกรณ์มหาวิทยาลัย
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Thesis Title	BIOACTIVE PEPTIDE DERIVED FROM TRYPSIN HYDROLYSIS OF UNICELLULAR CYANOBACTERIUM *Synechococcus* sp.
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Field of Study	Biotechnology
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ฤทัยรัตน์ สุทธิสุวรรณ : เพปไทด์ที่มีฤทธิ์ทางชีวภาพที่ได้จากทริปซินไฮโดรไลซิสของไซยาโนแบคทีเรียชนิดเซลล์
เดี่ยว *Synechococcus* sp. (BIOACTIVE PEPTIDE DERIVED FROM TRYPSIN HYDROLYSIS OF
UNICELLULAR CYANOBACTERIUM *Synechococcus* sp.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.อภิชาติ กาญ
จนทัต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.ณัฏฐา ทองจุล, ผศ. ดร.สรัญญา พันธุ์พฤกษ์, 111 หน้า.

อนุมูลอิสระสามารถทำให้เกิดปฏิกิริยาการเปลี่ยนแปลงโครงสร้างโมเลกุลในองค์ประกอบต่างๆของเซลล์ซึ่งทำให้เกิด
ภาวะเครียดที่เกิดจากการออกซิเดชันและส่งผลให้มีความเสียหายในเนื้อเยื่อของร่างกาย อนุมูลอิสระสามารถทำให้เกิดโรคต่างๆได้
เช่น โรคหัวใจ โรคสมองเสื่อม การอักเสบ และโรคมะเร็ง งานวิจัยนี้มีจุดประสงค์เพื่อทำการตรวจสอบผลของเพปไทด์ที่มีฤทธิ์ทาง
ชีวภาพที่ได้จาก *Synechococcus* sp. ที่มีอายุ 21 วัน ต่อกิจกรรมการต้านอนุมูลอิสระ การต้านการอักเสบ และการต้านมะเร็ง โดย
โปรตีนไฮโดรไลเสทจาก *Synechococcus* sp. จากการย่อยโดยเอนไซม์ทริปซิน และนำไปทำให้บริสุทธิ์โดยใช้เทคนิคอัลตราฟิลเตร
ชันด้วยเมมเบรนที่มีขนาดโมเลกุล 10 5 และ 3 กิโลดาลตัน เพปไทด์ที่มีขนาดโมเลกุลน้อยกว่า 3 กิโลดาลตันแสดงค่า NO ABTS
และ DPPH radical scavenging activities ได้สูงที่สุด โดยแสดงค่าความเข้มข้นต่ำสุดที่สามารถกำจัดอนุมูลอิสระได้ร้อยละ 50 (IC_{50})
เท่ากับ 34.51±9.8, 11.54±0.3 และ 13.63±0.15 ไมโครกรัมโปรตีนต่อมิลลิลิตร ตามลำดับ จากนั้นนำเพปไทด์ที่มีขนาดโมเลกุลน้อย
กว่า 3 กิโลดาลตัน มาทำให้บริสุทธิ์ด้วยเทคนิคเจลฟิวเตรชัน พบว่า F_2 มีค่ากิจกรรมในการต้านอนุมูลอิสระได้ดีที่สุดเมื่อทำการ
ทดสอบด้วยวิธี NO และ ABTS โดยมีค่า IC_{50} เท่ากับ 7.68±0.64 และ 9.74±0.2 ไมโครกรัมโปรตีนต่อมิลลิลิตร ตามลำดับ จากนั้นนำ
F_2 ไปทำให้บริสุทธิ์ต่อด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง ทำให้สามารถคัดแยกเพปไทด์ออกมาได้ 4 ส่วน โดยเพป
ไทด์ที่แยกได้ในช่วงเวลานาทีที่ 30 – 40 (F_{2-4}) ได้ถูกคัดเลือกนำมาทำการวิเคราะห์เพื่อพิสูจน์เอกลักษณ์ของสาร โดยเทคนิค
แมสสเปกโตเมตรี พบว่าสามารถคัดแยกเพปไทด์ออกมาได้ 5 ชนิด ได้แก่ AILQSYSAGKTK มีขนาดโมเลกุล 1,265.69 ดาลตัน,
ALNKTHLIQTK มีขนาดโมเลกุล 1,265.74 ดาลตัน, LLVHAPVK มีขนาดโมเลกุล 875.55 ดาลตัน, IPDAHPVK มีขนาดโมเลกุล
875.48 ดาลตัน และ VVVLRDGAVQQLGTPR มีขนาดโมเลกุล 1,706.97 ดาลตัน F_{2-4} มีค่า DPPH และ NO radical scavenging
activities สูงที่สุดเมื่อทำการเปรียบเทียบกับเพปไทด์สังเคราะห์ ยิ่งไปกว่านี้เพปไทด์สังเคราะห์ AILQSYSAGKTK มีค่า ABTS
radical scavenging activities สูงสุด นอกจากนี้เพปไทด์ F_2 มีความสามารถในการป้องกันการถูกทำลายของดีเอ็นเอจากพลาสมิด
pBR322, pKS และ pUC19 ได้ เพปไทด์ F_2 ถูกนำมาศึกษาคุณสมบัติในการต้านการอักเสบและการต้านมะเร็ง โดยผลการวิจัยของการ
ต้านการอักเสบพบว่า F_2 ไม่มีความเป็นพิษต่อเซลล์แมคโครฟาจ และได้ทำการทดสอบการแสดงออกของยีนที่เกี่ยวข้องกับการ
อักเสบโดยวิธีการ RT-PCR และ qRT-PCR พบว่าสามารถลดการแสดงออกของยีน *iNOS, TNF-α, COX-1, COX-2* และ *IL-6* ได้
สำหรับฤทธิ์ในการต้านมะเร็งพบว่า F_2 สามารถยับยั้งการเกิดเซลล์มะเร็งได้ดีที่สุดในเซลล์มะเร็งลำไส้ SW 620 เมื่อทดสอบด้วยวิธี
MTT ซึ่งมีค่า IC_{50} เท่ากับ 106.58±21.46 ไมโครกรัมโปรตีนต่อมิลลิลิตร F_2 สามารถกระตุ้นให้เซลล์มะเร็งลำไส้เกิดการตายแบบอะ
พอพโทซิสหลังจากบ่มเป็นเวลา 24, 48 และ 72 ชั่วโมง โดยมีการทำงานของเอนไซม์แคสเปส 3 8 และ 9 สูงสุดในชั่วโมงที่ 72 จาก
ผลการวิจัยนี้กล่าวได้ว่าไซยาโนแบคทีเรียชนิดเซลล์เดี่ยว *Synechococcus* sp. มีความเป็นไปได้ที่จะนำมาใช้เพื่อพัฒนาสำหรับใช้เป็น
ยาต้านการอักเสบและยาต้านมะเร็งที่ได้มาจากธรรมชาติได้

สาขาวิชา เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต ....
ปีการศึกษา 2560	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
	ลายมือชื่อ อ.ที่ปรึกษาร่วม ....
	ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

5672885923 : MAJOR BIOTECHNOLOGY

KEYWORDS: PROTEIN HYDROLYSATE / ANTI-INFLAMMATION / ANTICANCER / APOPTOSIS / CYANOBACTERIUM

RUTAIRAT SUTTISUWAN: BIOACTIVE PEPTIDE DERIVED FROM TRYPSIN HYDROLYSIS OF UNICELLULAR CYANOBACTERIUM *Synechococcus* sp.. ADVISOR: ASSOC. PROF. APHICHART KARNCHANATAT, Ph.D., CO-ADVISOR: ASSOC. PROF. NUTTHA THONGCHUL, Ph.D., ASST. PROF. SARANYA PHUNPRUCH, Ph.D., 111 pp.

A free radicals can promote denaturing reactions in many cellular components, which create oxidative stress and it results to damage in body tissue. The free radicals can causing many diseases including heart diseases, Alzheimer, inflammation and cancer. This research aimed to investigate the effect of bioactive peptides derived from *Synechococcus* sp. cultured for 21 days on antioxidant, anti-inflammation and anticancer activities. The *Synechococcus* sp. protein hydrolysate was prepared by trypsin digest and purified by ultrafiltration with molecular weight cut off membranes of 10, 5 and 3 kDa. The MW <3 kDa fraction showed the highest NO, ABTS and DPPH radical scavenging activities with IC_{50} values of 34.51±9.8 µg protein/mL, IC_{50} 11.54±0.3 µg protein/mL and IC_{50} 13.63±0.15 µg protein/mL, respectively. The F_2 fraction from gel filtration chromatography showed the strongest NO and ABTS radical scavenging activities with IC_{50} values of 7.68±0.64 µg protein/mL and 9.74±0.2 µg protein/mL, respectively. The F_2 was purified by RP-HPLC to yield four fractions. The 30 – 40 min sub-fraction (F_{2-4}) was selected for further analysis by mass spectroscopy. Five isolated peptides with amino acid sequences of AILQSYSAGKTK; 1,265.69 Da, ALNKTHLIQTK; 1,265.74 Da, LLVHAPVK; 875.55 Da, IPDAHPVK; 875.48 Da and VVVLRDGAVQQLGTPR; 1,706.97 Da were identified. The F_{2-4} had higher DPPH and NO radical scavenging activity compared to the synthetic peptide. Moreover, AILQSYSAGKTK had the highest ABTS radical scavenging activity. Furthermore, the F_2 fraction protected oxidation-induced DNA damage in pBR322, pKS and pUC19 cells. F_2 fraction was selected to study anti-inflammatory and anticancer properties. Anti-inflammatory effect, F_2 fraction showed no cytotoxicity toward RAW264.7 macrophage cells. RT-PCR and qRT-PCR results showed that F_2 reduced gene expression of pro-inflammatory cytokines *iNOS*, *TNF-α*, *COX-1*, *COX-2* and *IL-6*. For anticancer activity, F_2 fraction showed high anticancer activities in the human colon cancer cells (SW620) according to cytotoxic activity (MTT assay), with IC_{50} values of 106.58±21.46 µg protein/mL. The F_2 fraction activated the apoptotic pathway in SW620 cells after treatment for 24, 48 and 72 hours. The highest activities of caspases 3, 8 and 9 were observed after treatment for 72 hours. These findings suggested that unicellular cyanobacterium *Synechococcus* sp. may be used to develop for natural anti-inflammation and natural anticancer drugs.

Field of Study: Biotechnology	Student's Signature _____
Academic Year: 2017	Advisor's Signature _____
	Co-Advisor's Signature _____
	Co-Advisor's Signature _____

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LIST OF ABBREVIATION

Abs	Absorbance
ABTS	2,2'-azinobis-(3 ethylbenzothiazoline-6-sulfonic acid)
BHA	Butyl hydroxyanisole
BHT	Butyl hydroxytoluene
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CO_2	Carbon dioxide
COX1	Cyclooxygenase 1
COX2	Cyclooxygenase 2
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
eNOS	Endothelial nitric oxide synthase
et al.	and others
FCS	Fetal calf serum
Fe^{2+}	Ferrous
FITC	Fluorescein isothiocyanate
H	Hydrogen
HCl	Hydrochloric acid
H_2O	water
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	Median inhibitory concentration, 50% maximum inhibition
IFN-γ	Interferon-gamma
IL-6	Interleukin-6

iNOS	Inducible nitric oxide synthase
kDa	Kilodalton
LPS	Lipopolysaccharide
mg	Milligram
mL	Milliliter
mM	Millimolar
mRNA	Messenger RNA
MS/MS	Tandem mass spectrometry
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MW	Molecular weight
MWCO	Molecular weight cut off
m/z	Mass to charge ratio
NaCl	Sodium chloride
NFkB	Nuclear factor-kappa B
nm	Nanometer
nNOS	Neuron nitric oxide synthase
NO	Nitric oxide
O_2	oxygen
$O_2\bullet-$	Superoxide anion
O.D.	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP-HPLC	Reverse phase High performance liquid chromatography
rpm	Round per minute
RPMI	Roswell park memorial institute
RT-PCR	Reverse transcription polymerase chain reaction

SOD	Superoxide dismutase
TBHQ	Tertiary butylthydroquinine
TE	Tris-EDTA
TFA	Trifluoroacetic acid
TLR-4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
UV	Ultraviolet
w/v	Weight by volume
v/v	Volume by volume
α	Alpha
β	Beta
°C	Degree Celsius
γ	Gamma
μg	Microgram
μL	Microliter
μM	Micromolar
/	Per
%	Percentage
:	Ratio

จุฬาลงกรณ์มหาวิทยาลัย

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CHAPTER I

INTRODUCTION

Nowadays, people pay more attention to health care, especially in the prevention and treatment of disease. They understand the importance of antioxidants which bind the free radicals in the body. Humans have a complex antioxidant system which protects body cells from damage caused by free radicals such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). Antioxidants can directly interact against free radicals and inhibit oxidation. There are many antioxidant agents including ascorbic acid, α-tocopherol, flavonoids, and β -carotene.

Reactive oxygen species (ROS) affect many substances in the human body, including fatty acids, proteins, and DNA. The generation of ROS or free radicals such as superoxide, hydroxyl radical, and hydrogen peroxide, during metabolism and other activities beyond the antioxidant capacity of a biological system, gives rise to oxidative stress. Oxidative stress plays a role in heart diseases, malaria, neurodegenerative diseases, AIDS, cancer, and the aging process. All organisms have antioxidant systems that are able to control and counter the onslaught of free radical mediated oxidative damage. Therefore, dietary sources have been recognized as safer and effective antioxidants in the context of their efficiency and nontoxicity. The intake of fruits and vegetables containing high amounts of antioxidative nutraceuticals has been linked to the balance of free radicals and antioxidant status, and helps to minimize the oxidative stress in the body beside to reduce the risks of disease.

Bioactive proteins and peptides have physiological properties. Recently several studies identified and optimized the isolation of biopeptides from both plant and animal sources. These peptides are generated both *in vivo* and *in vitro* from the proteolytic hydrolysis of food proteins. The enzymatic hydrolysis of proteins is one approach used to release bioactive peptides, and is widely applied to improve functional and nutritional properties of protein sources. The biological activity of a peptide is based on amino acid composition. Peptides could be used in the formulation of functional foods and nutraceuticals to reduce damage related to oxidative stress in human disease conditions. Moreover, natural antioxidants are preferable; they can be used at higher

concentrations, without any toxic side effects that might result from the use of synthetic equivalents.

Bioactive peptides are specific protein fragments, and in addition to act as amino acids and nitrogen sources, they have numerous potential physiological functions within the body (Harnedy and FitzGerald, 2012). Bioactive peptides can be naturally occurring biomolecules, produced by microbial fermentation or generated with a variety of different enzymes, such as commercially available enzymes or for instance gastro intestinal enzymes. The biologically active peptides or functional peptides (genuine or generated) can in addition to their nutritional value exert a physiological effect in the body (Vermeirssen *et al*., 2004). Thus, bioactive peptides are inactive or latent within the parent protein sequence, and can be released e.g. through either digestion with commercial enzymes, released during gastrointestinal digestion and during food processing in order to be in active form and thereby to exert an effect. Two factors determine the generated bioactive peptide: the primary sequence of the protein substrate and the specificity of the enzyme(s) which is used to generate the peptide (Harnedy and FitzGerald, 2012). The size of bioactive peptides is usually 2-20 amino acid residues in length, but longer residues have been found. The bioactive peptides can be absorbed by the intestine and be transported out intact in the circulatory system, where they exert physiological effects, or they may stay in the digestive tract to produce local effects (Erdmann *et al*., 2008). The ability of bioactive peptides to exert physiological effects in vivo depends on the peptides bioavailability, which is predominantly determined by the resistance to peptidase degradation of both the intestinal tract and serum, and its ability to intestinal absorption (Vermeirssen *et al*., 2004). The bioactive peptides have been detected in a wide range of food materials from plant and animal sources, fungi, microalgae (Sheih *et al*., 2009c) and macroalgae (Tierney *et al*., 2013).

The most widely used technique to produce bioactive peptides is by enzymatic digestion using various proteolytic enzymes. Methods include hydrolysis by plant, animal and bacterial proteases (Arihara, 2006). Commercial enzymes from bacterial and fungal sources as well as digestive enzymes such as chymotrypsin, pancreatin, trypsin and pepsin have been used (Ryan *et al*., 2011). Extraction of naturally occurring peptides is furthermore a method for obtaining bioactive peptides (Tierney *et al*., 2013).

So far the microbial fermentation of muscle proteins has not resulted in discovery of any bioactive peptides. In order to identify bioactive peptides following hydrolysis, the crude hydrolysates are assayed for various bioactivities and size fractionated. The size separated peptide fractions are then tested for bioactivities, and the fraction displaying the highest bioactivity is further purified with techniques as reverse phase high performance liquid chromatography. In order to identify individual peptide fractions a combination of HPLC and mass spectrometry (LC-MS) and protein sequencing are useful tools. Verification of the bioactivity can be done by repeating the assay with a synthetic version of the peptide of interest (Ryan *et al*., 2011).

Antioxidant is a molecule that inhibits the oxidation of other molecules which oxidation reactions can produce free radicals. The properties of antioxidant are different depending on mechanisms such as inhibit, chelate and scavenge (Strain and Benzie, 1999). In the cells, free radicals and reactive oxygen species (ROS) can be removed from cells by an enzyme-mediated system such as peroxidase, superoxide dismutase (SOD) and glutathione peroxidase and non-enzyme such as ascorbic acid (Sheih *et al*., 2009c). Two major but different mechanisms are known. Both mechanisms lead to a reduction of the radicals but differ in kinetics and propensity for side reactions. For hydrogen atom transfer, the antioxidants quench the free radicals by donating hydrogen whereas for single electron transfer the antioxidants transfer one electron to the radical (Prior *et al*., 2005). Free radicals lead to many degenerative disease conditions such as cancer, inflammation, atherosclerosis and diabetes (Suja *et al*., 2004). Typical antioxidants are natural and synthetic antioxidants. Natural antioxidants such as vitamin C, vitamin E, vitamin A and flavonoid (Packer *et al*., 1999). Synthetic antioxidants such as propyl gallate (PG), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Sheih *et al*., 2009c). The assay for determine antioxidant activities have many methods such as DPPH (2,2-diphenil-1-picrylhydrazyl), NO (nitric oxide) and ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (Kamiloglu *et al*., 2009). In order to retard e.g. lipid peroxidation synthetic antioxidants such as BHA and BHT are added to food products. However, the synthetic antioxidants are associated with some safety concerns and as a result of this, natural antioxidants such as bioactive peptides with no or little side-effects have gained interest due to their food quality prolonging abilities and potential health benefits. Antioxidant peptides have been found in different

foodstuffs such as milk (Clausen *et al*., 2009), egg (Davalos *et al*., 2004), wheat (Zhu *et al*., 2006), rice (Zhang *et al*., 2010) and fish (Kim *et al*., 2001). Peptides and protein hydrolysates derived from food have in research shown to exert antioxidant activities against enzymatic (lipoxygenase-mediated) and non-enzymatic peroxidation of lipids and fatty acids. The exact mechanism by which peptides display antioxidant activity is not fully understood, however the antioxidant properties has been suggested to be due to free radical scavenging, metal ion chelation and singlet oxygen quenching (Erdmann *et al*., 2008). The type, hydrophobicity and position of amino acids in the peptide are believed to play an essential role regarding antioxidant activity of a peptide. Amino acid residues such as cystein, histidine, leucine, methionine and tyrosine have been found to be associated with radical scavenging activity. These amino acids donate protons to electron deficient radicals and thereby enhancing radical scavenging activity (Harnedy and FitzGerald, 2012). Furthermore, many antioxidative peptides have the hydrophobic amino acid residues valine or leucine at the N-terminus at the peptide (Kim *et al*., 2001).

Inflammation has to significant of immune response of infection. Inflammation can lead to secured acute and chronic diseases by the uncontrolled production of pro-inflammatory cytokines, eicosanoids derived from arachidonic acid, reactive oxygen species and adhesion molecules. The four signs of inflammation to the injured tissue are pain, swelling, heat, and redness (Faro *et al*., 2014). The target of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair. The inflammatory process begins with chemical "alarms" a series of inflammatory chemicals that are released in the extracellular fluid. Injured tissue cells, phagocytes, lymphocytes, mast cells and blood proteins are all sources of inflammatory mediators, the most important of which are histamine, kinins, prostaglandins, complement, and lymphokines. Though some of these mediators have individual inflammatory roles as well, they all promote dilation of the small blood vessels in the vicinity of the injury. As more blood flows into the area local hyperemia occurs which accounts for the redness and the heat of the inflamed area. These chemicals also increase the permeability of local capillaries. Consequently, fluid containing proteins such as clotting factors and antibodies seeps from the bloodstream into the tissue spaces. This

is the cause of the local edema or swelling that in turn, presses on adjacent nerve endings, contributing to a sensation of pain. Pain also results from the release of bacterial toxins, lack of nutrition to the cells in the area, and the sensitizing effects of released prostaglandins and kinins. If the swollen and painful area is a joint, normal movement may be inhibited temporarily in order for proper healing and repair to occur (Faro *et al*., 2014; Scott and Pawson, 2000). Inducible nitric oxide synthase (iNOS) is involved in response of pro-inflammatory cytokines. iNOS is produced NO. NO has an over produced lead to cell death. So, lowing of NO has been proved to increase cell survival (Merrill *et al*., 1993). NO has been correlated with the inflammatory process (Lantz *et al*., 2007).

Cancer is one of the largest causes of death in many countries. Free radicals and reactive oxygen species (ROS) promote denaturing reactions in many cellular components, which create oxidative stress and it results of cancer diseases. It is happen when the cells in a part of the body start to grow out of control which cancer cell growth is different from normal cell growth. Anticancer is used a drugs which resistance at present. Bioactive compound is obtains from natural source reported best bio functional activities (Umayaparvathi *et al*., 2014). In Thailand has different cancer such as liver cancer, lung cancer, cervical cancer, breast cancer, oral cavity cancer, nasopharyngeal cancer, esophageal cancer, gastrointestinal cancer and thyroid cancer. Cancer pain occurs mostly in the later stages of many cancers. Cancer specialists have neglected cancer pain. A survey of the prevalence of cancer pain in all sites of those cancer patients admitted was approximately 62%. The statistics from Khon Kaen regarding cancer pain were comparable to those in other countries. In Thailand have ~21,645 cancer cases which had not received adequate pain care. The prescription of morphine, which is the best pain medicine, has been a significant problem in many of the hospitals (Vatanasapt *et al*., 2002).

Synechococcus sp. is a cyanobacteria (blue-green algae) which a prokaryotes capable of plant-type oxygen-evoling photosynthesis (Troshina *et al*., 2002). *Synechococcus* sp. is a unicellular cyanobacterium, In this study, *Synechococcus* sp. was isolated from Ao wong Duan, Koh Samet, Thailand. Extreme environment such as high salinity, low and high temperatures and drought can found this microorganism. Adaptations of *Synechococcus* sp. to environmental stress clause to microbes are

ubiquitous. *Synechococcus* sp. is composed of proteins, carbohydrates, lipids and other valuable components such as pigment, anti-oxidants, fatty acids, vitamin etc. The composition of cell walls composed of a peptidoglycan layer and an outer membrane. Outer membrane constituents include proteins, lipids and carotenoids (Woitzik *et al*., 1988). In 1994, Becker was reported about protein and carbohydrate content of *Synechococcus* sp. which has a protein 46-63% of cell dry weight and 15% carbohydrate. Nowadays, cyanobacteria have been used a natural products in terms of treat to cancer and HIV diseases (Mahdi and Fariba, 2012). Cyanobacteria have an important source of natural products with pharmaceutical activity.

   The objective of this study was to understand the peptides of *Synechococcus* sp. in term of free radical scavenging, anti-inflammation, and antiproliferative activities. Moreover this study was to provide a new knowledge for finding new bioactive peptide from *Synechococcus* sp. for medical or pharmaceutical application.

จุฬาลงกรณ์มหาวิทยาลัย

CHULALONGKORN UNIVERSITY
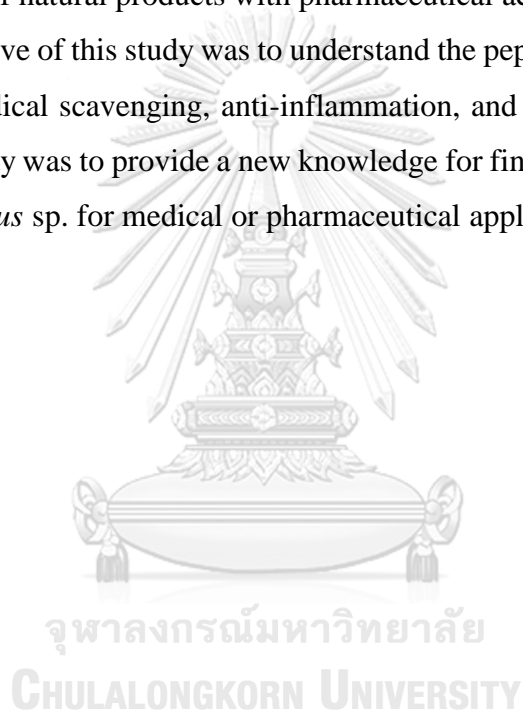
CHAPTER II

LITERATURE REVIEW

2.1 Peptide

A peptide is composed of a chain of two or more amino acids which can be derived from protein. In general, peptides derived from proteins by enzymatic hydrolysate have biological activity (Zhang *et al*., 2014), and are termed bioactive peptides. Biological activities cover many functions, such as antioxidant and antimicrobial (Park *et al*., 2017a). Bioactive peptides contain 3-20 amino acid residues, which are inactive in the sequence of the original protein. The antioxidant activity of peptides are based on the sequence and composition of the amino acids. One important factor that could affect antioxidant activity is the molecular weight of the peptides. Bioactive peptides with low molecular weights have been reported to have the highest antioxidant activity (Jiang *et al*., 2014a). Recently, bioactive peptides exhibiting antioxidant activity have become increasingly available from various sources, such as oyster (Umayaparvathi *et al*., 2014), round scad (Jiang *et al*., 2014a), sardinelle (Bougatef *et al*., 2010), sweet potato (Zhang *et al*., 2014), *Zizyphus jujube* fruit (Memarpoor-Yazdi *et al*., 2013), blue mussel (Je *et al*., 2005), egg white protein (Liu *et al*., 2015), *Anabenopsis* sp. (Asan-Ozusaglam *et al*., 2013), African yam bean seed (Ajibola *et al*., 2011), and defatted walnut (Gu *et al*., 2015). Additionally, peptides can be obtained from the hydrolyzation of chemicals including acids and alkalies. Peptides derived from chemical hydrolysis have the advantage of being low cost for production; however production is uncontrolled in the hydrolysis of the protein (de Castro and Sato, 2015).

2.2 Trypsin

Trypsin (EC 3.4.21.4) is an enzyme in the group of proteases. The molecular weight of trypsin is a 23.3 kDa. Trypsin is a serine protease present in the digestive systems of vertebrates. Produced in the pancreas as the inactive proenzyme trypsinogen. Trypsin consists of a single chain polypeptide of 223 amino acid residues. Trypsin is a part of the serine proteases S1 family. Trypsin is a globular protein and produced as an

inactive proenzyme (zymogen), trypsinogen within the acinar exocrine cells of the pancreas (Chen *et al*., 2009). Trypsin as an endoprotease which cleaves amide bonds within the protein chain further cleaves peptide on the C-terminal side (carboxyl side) of lysine and arginine. If a proline residue is on the carboxyl side of the cleavage site, cleavage will not occur. If an acidic residue is on either side of the cleavage site, the rate of hydrolysis has been show to slow. The optimum temperature for hydrolysis is in the pH range of 7 to 9 as shown in Figure 2.1 (Adler-Nissen,1993).
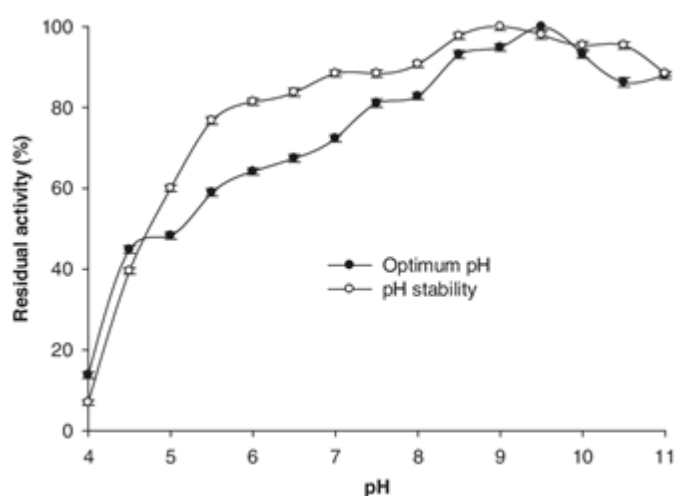
Figure 2.1 The optimum pH of trypsin
(Villalba-Villalba *et al*., 2013)

2.3 Free radicals

Free radicals are any species able to independently exist such as atoms or molecule that contain one or more unpaired electrons in the outer orbital. This unpaired electron gives a considerable degree of reactivity to the free radical. The ground state of the molecular oxygen has two unpaired electrons in its outer shell. Therefore, a radical species can occur by itself (Halliwell and Gutteridge, 2015). Uncoupled electrons are very reactive with adjoining molecules such as lipids, proteins, and carbohydrates and can cause cellular damage (Kuhn, 2003). In the biological system, free radicals are normally produced by electron transfer reactions. These free radicals can be deliberately or accidentally mediated by enzymatic or non-enzymatic reactions. The most biologically important free radicals are the radical derivatives of oxygen

known as ROS (Cheeseman and Slater, 1993). Free radicals are involved in the several human diseases, for example, Alzheimer's, heart disease, emphysema, arthritis, cancer and inflammation (Sharma *et al*., 1995). Many disease states occur from free radicals with the ubiquitous presence of oxygen in higher species, and diatomic oxygen can readily accept electrons to become oxygen derivatives. Oxygen play an essential role in aerobic life, the production of energy and the synthesis of a variety of significant compounds. These reactions - with iron, copper and other transition metals - play an essential role, and are generally bound in specific complexes within proteins. Disruption of these complex oxidative reactions can rapidly result in death. The oxygen molecule normally contains three unpaired electrons referred to as triplet oxygen. When triplet oxygen acts with transition metals and other compounds, partly decreased and highly active forms of oxygen can be potentially produced. The hydroxyl radical (OH·) is one of the most reactive (Cheeseman and Slater, 1993; Halliwell and Gutteridge, 2015). Furthermore, the biologically relevant free radicals derived from oxygen are superoxide anion (O_2^-), the perhydroxyl radical (protonated superoxide, HO_2) and free radical nitric oxide (Cuzzocrea *et al*., 2001). The process of the formation of reactive oxygen species (ROS) is shown in Figure 2.2.
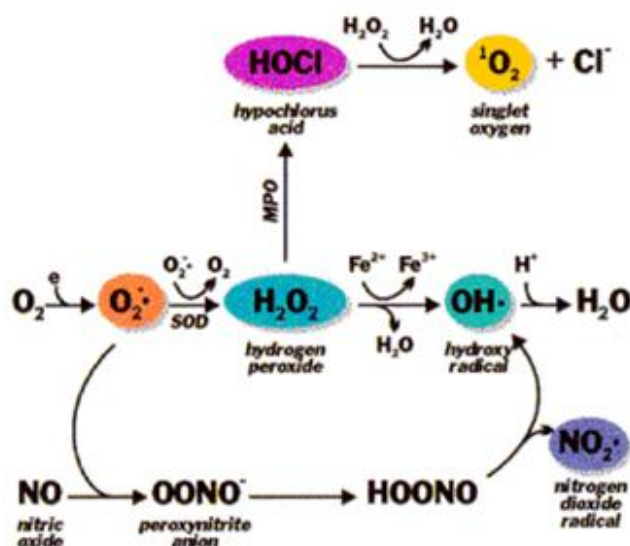
Figure 2.2 The process of the formation of reactive oxygen species (ROS)
(Middleton *et al*., 2000)

Table 2.1 The physiological properties of reactive oxygen species (ROS)

Radical	Name	Typical biological target
O_2-*	superoxide	enzymes
H_2O_2	hydrogen peroxide	unsaturated fatty acids
OH*	hydroxyl	all biomolecules
R*	R-yl	oxygen
RO*	R-oxyl	unsaturated fatty acids
ROO*	R-dioxyl (R-peroxyl)	unsaturated fatty acids
ROOH	hydroperoxide	unsaturated fatty acids
O_2	singlet molecular oxygen	H_2O
NO*	nitroxyl	several

2.4 Antioxidants

An antioxidant is a molecule capable of preventing or scavenging the oxidation reaction of other molecules. Antioxidants donate hydrogen atoms, chelate metal ions, scavenge free radicals, stop chain-breaking in the lipid peroxidation cycle and inhibit the free radical by eliminating free radicals directly (Sies, 1997). Antioxidant is a substance which can prevent oxidative stress in the body by neutralizing and removing free radicals (Bouayed and Bohn, 2010).

2.4.1 Types of antioxidants

Antioxidants are categorized into two types; enzymatic antioxidants and non-enzymatic antioxidants (Sies, 1997).

2.4.1.1 Enzymatic antioxidants

Enzymatic antioxidants are produced naturally in the body system to neutralize unstable oxidative species. The action occurs by transforming reactive oxygen species and reactive nitrogen species into stable compounds (Prior *et al*., 1998). Enzymatic antioxidants are important for cells in functions such as the repair of damaged DNA or protein, oxidized lipids and peroxides. They are classified as superoxide dismutase (SOD), catalase (CAT), and glutathione (Cemeli *et al*., 2009).

Superoxide dismutase: SOD is an enzyme that catalyzes the breakdown of the superoxide anion into oxygen and hydrogen peroxide. SOD is composed of metal ion cofactors including copper, zinc, iron and manganese. In humans, SOD is present in the cytosol and mitochondria (Reiter *et al*., 1997).

Catalase: CAT is an enzyme that catalyzes the decomposition of hydrogen peroxide into water and oxygen. The substrate for CAT is H_2O_2. CAT is localized to peroxisomes (Rhee *et al*., 2005).

Glutathione: Glutathione is composed of glutathione reductase, glutathione peroxidases and glutathione S-transferases. Glutathione is an enzyme that catalyzes the breakdown of hydrogen peroxide and organic hydroxides (Cai *et al*., 2015).

2.4.1.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants are important antioxidants for example ascorbic acid, vitamin A, vitamin E, glutathione, melatonin, bioflavonoids, uric acid, flavonoids, phenolic acid and tannins. Moreover, synthetic antioxidants such as butyl hydroxyanisole (BHA), butyl hydroxytoluene (BHT) and tertiary butylthydroquinine (TBHQ) comprise a group of non-enzymatic antioxidants able to prevent oxidative stress similar to enzymatic antioxidants (Cai *et al*., 2015; Wang *et al*., 2006; Warner *et al*., 2004).

2.4.2 Assay to detect antioxidant activity

2.4.2.1 DPPH free radical scavenging assay

DPPH is a stable free radical with maximal absorption at 515 nm. It loses this absorption when reduced by an antioxidant or a free radical species (Brand-Williams *et al*., 1995). The reaction mechanism and structure of DPPH are shown in the diagram below.
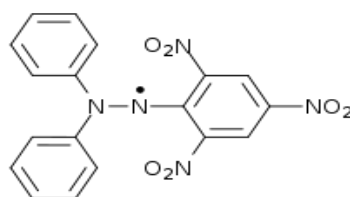
Figure 2.3 Chemical structure of DPPH

Where AH is the antioxidant and R· is the free radical species:

DPPH· + AH ⟶ DPPH-H + A·

DPPH· + R· ⟶ DPPH-R
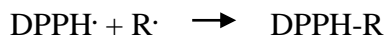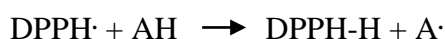
   The DPPH method allows for direct investigation of the ability for the extract or antioxidant to donate hydrogen and electrons to quench the DPPH radical. As the radical is quenched by antioxidants, the color of the solution changes from a deep purple to a light yellow and absorbance at 515 nm decreases. The DPPH method is widely used to determine the antioxidant activity of purified phenolic. However, the DPPH method also has its limitations. The most phenolic antioxidants react slowly with DPPH, reaching a steady state between 1-6 hours or longer. This suggests that antioxidant activity using DPPH should be evaluated over time (Bondet *et al*., 1997).

   2.4.2.2 ABTS free radical scavenging assay

   ABTS is another commonly used free radical to assess antioxidant activity in vitro. However, this free radical is foreign to biological systems. The structure of the ABTS free radical is shown in the diagram below.
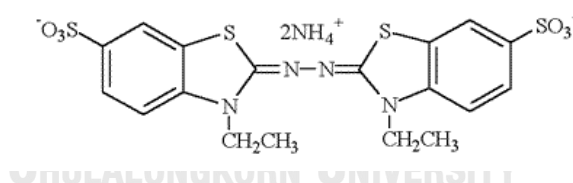
Figure 2.4 Chemical structure of ABTS

The reaction mechanism of ABTS is similar to the DPPH reaction shown above except the free radical is generated by ABTS : ABTS· + AH ⟶ ABTS-H + A·

         ABTS· + R· ⟶ ABTS-R
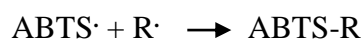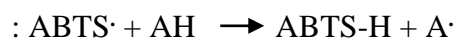
   The ABTS assay measures the relative ability of the antioxidant to scavenge the $ABTS^{\cdot+}$ generated in the aqueous phase, as compared with a Trolox (water soluble vitamin E analogue) standard. In this analysis, the antioxidant capacity is tested by reacting a test compound with ABTS solution resulting in the weakening of the color. The $ABTS^{\cdot+}$ is generated by reacting with a strong oxidizing agent (e.g., potassium

permanganate or potassium persulfate) with the ABTS salt. The reduction of the blue-green ABTS$^{.+}$ radical by hydrogen-donating antioxidant is measured by the suppression of its characteristic long wave absorption spectrum (Miller and Rice-Evans, 1997). The method is usually expressed as Trolox equivalent antioxidant capacity (TEAC). The advantage of using this method is that it is rapid and can be used over a wide range of pH values in both aqueous and organic solvent systems (Arnao *et al*., 2001).

      2.4.2.3 NO radical scavenging assay

Nitric oxide (NO) is an important bio regulatory molecule, which has a number of physiological effects, including the control of blood pressure, neural signal transduction, platelet function, and antimicrobial and antitumor activity (Jagetia *et al*., 2004). Nitric oxide also exhibits a toxic property after reaction with oxygen and superoxide radicals. The reaction products are able to cause substantial cellular damage (Vriesman *et al*., 1997). Nitric oxide is generated from sodium nitroprusside (SNP) and is measured by the Griess reagent. SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of the Griess reagent. Scavengers of NO complete with oxygen leading to the reduced production of NO (Green *et al*., 1982; Marcocci *et al*., 1994). The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine dichloride is read at 546 nm and refers to the absorbance of ascorbic acid, used as a positive control treated in the same way with the Griess reagent. The chemical reaction of NO in the Griess reagent system is shown in the diagram below. Many research has claimed that the peptide derived from organisms has the antioxidant activity as shown in Table 2.2.

Azo dye, λ = 548 nm
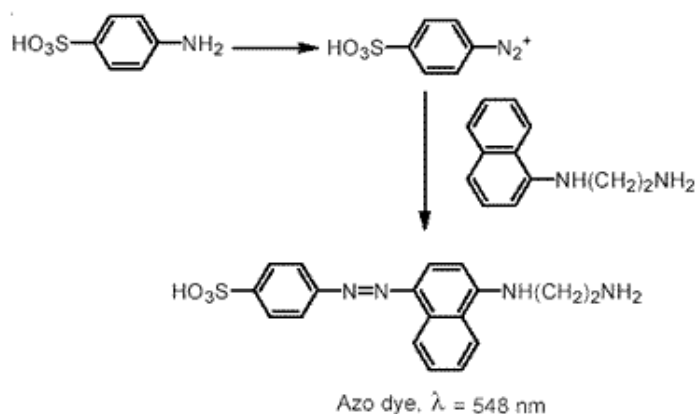
Figure 2.5 Chemical reaction of nitrite detection using the Griess reagent

Table 2.2 Literature reviews for antioxidant activity from the peptides of different organisms

Organism	Antioxidant Assay	References
Plant - Lemongrass (*Cymbopogon citratus*) - Walnut (*Juglans sigillata* Dode)	DPPH DPPH, ABTS, ORAC	(Balakrishnan *et al*., 2014) (Gu *et al*., 2015)
Animal - Sardinelle (*Sardinella aurita*) - Blue mussel (*Mytilus edulis*) - Egg white protein	DPPH DPPH, SOD, LPO, ORAC DPPH, ABTS, ORAC	(Bougatef *et al*., 2010) (Park *et al*., 2016; Wang *et al*., 2013) (Liu *et al*., 2015)
Cyanobacteria - *Oscillatoria agadhii*, *Anabaena sphaerica* - *Plectonema boryanum*, *Anabaena doliolum*, *Oscillatoria acuta*	DPPH DPPH	(El-Aty *et al*., 2014) (Singh *et al*., 2014)

2.5 Cyanobacteria

Cyanobacteria or blue-green algae comprise a group of bacteria which were the ancient colonizers of Earth and the photosynthetic ancestors of chloroplasts in eukaryotes including plants and algae. Cyanobacteria were culpable for oxygenating the Earth's atmosphere 2.5 billion years ago. Moreover, cyanobacteria were able to differentiate into specialized cell types called heterocysts and fix nitrogen, show gliding mobility, and tolerate a wide range of temperatures. Cyanobacteria can produce bioactive compounds, some of which have potential anti-microbial, anti-cancer and UV protectant properties. However, some parts of these bioactive compounds are highly toxic to wildlife and humans (Carmichael, 2008; Chorus and Bartram, 1999; Hudnell, 2010; Paerl and Huisman, 2008). Cyanobacteria can grow in fresh water, marine and terrestrial ecosystems but are more commonly found in hot springs, hyper saline, lakes, ponds, rivers and freezing environments (Fogg *et al*., 1973).

2.5.1 Structure of cyanobacteria

The structure of cyanobacteria is composed of a cell wall, plasma membrane, phycobilisomes, photosynthetic lamellae, ribosomes, protein granules, nucleoids and lipid droplets as shown in Figure 2.6. The inner layer of the cell wall is similar to the bacterial cell in the chemical composition built up from peptidoglycans. Cyanobacteria have pigment molecules as chlorophyll a and blue phycobiliproteins, allophycocyanin and phycocyanin.
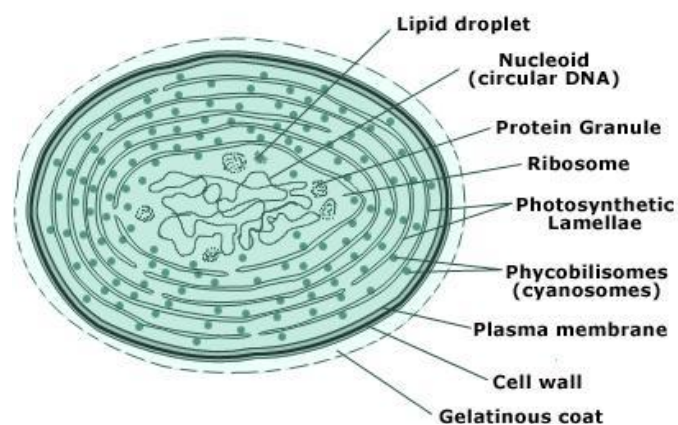
Figure 2.6 Cell structure of cyanobacteria

2.5.2 Classification of cyanobacteria

The cyanobacteria are classified within the kingdom Prokaryota, division Gracilicutes (Gram negative bacteria), class Photobacteria, subclass Oxyphotobacteria and order Cyanobacteriales. Cyanobacteriales. Singleton and Sainsbury (1987) (Singleton and Sainsbury, 1987) classified cyanobacteria by morphology into five sections.

Section 1: A group of unicellular cells comprising *Gloeobacter*, *Gloeocapsa*, *Gloeothece*, *Synechococcus*, *Synechocystis* and *Chamaesiphon*. These cyanobacteria reproduce by binary fission.

Section 2: A group of unicellular microorganisms covered by a fibrous layer. Some species reproduce by multiple fission including *Dermocarpa*, *Xenococcus* and some species reproduce both by multiple fission and binary fission such as *Chroococcidiopsis*, *Dermocarpella*, *Myxosarcina* and *Pleurocapsa*.

Section 3: A group of filamentous microorganisms. Each filament consists of trichomes with no branches and a lack of heterocytes and akinetes including *Spirulina*, *Oscillatoria*, *Pseudoanabaena*.

Section 4: A group of filamentous microorganisms with no branches and composed of heterocytes. Heterocysts are specialized nitrogen-fixing cells. Some species were created by akinetes. Reproduction occurs by the fragmentation of trichomes or germination of akinetes including *Calothrix*, *Nostoc*, *Scytonema*, *Anabaena*, *Cylindrospermum*.

Section 5: A group of filamentous microorganisms and composed of true branching. Reproduction in this group occurs by fragmentation and germination from akinetes such as *Fischerella*.

In this research, *Synechococcus* sp. was used for investigation. *Synechococcus* sp. is a unicellular cyanobacterium abundant in the world's oceans. Growth is generally limited especially the concentration of nutrients and trace metals such as iron and phosphorus. The photosynthetic pigment in *Synechococcus* sp. is composed of chlorophyll a and phycobilins (Waterbury *et al*., 1979). The scientific classification of *Synechococcus* as follows:

Kingdom : Bacteria
Phylum : Cyanobacteria
Order : Synechococcales
Family : Synechococcaceae
Genus : *Synechococcus*

Figure 2.7 *Synechococcus* sp. cells

2.5.3 Metabolic pathways of cyanobacteria

Cyanobacteria use light for the energy source to produce ATP and NDPH through photosynthetic electron transport. Photosynthetic is the biological process that converts solar energy to biomass and bioproducts. Central metabolic pathways of cyanobacteria as shown in Figure 2.8. Cyanobacteria play essential roles in global carbon, nitrogen and sulfur cycles in conjunction with their central carbon and energy metabolisms. Inorganic and organic carbon sources are utilize building cellular building blocks. The central carbon metabolism was the Calvin-Benson cycle, glycolysis, the pentose phosphate cycle, MEP pathway and the tricarboxylic acid cycle, which serves to synthesis 2-oxoglutarate (2OG) (Tang *et al*., 2011).
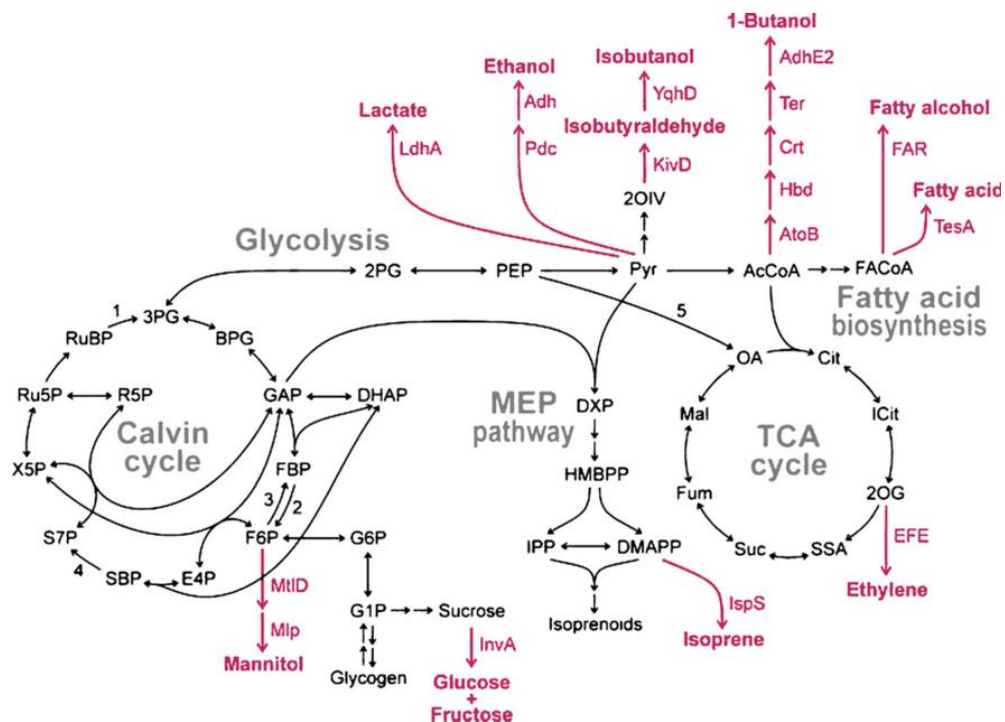
Figure 2.8 Central metabolic pathways of cyanobacteria
(Rosgaard *et al*., 2012)

2.5.4 Applications of cyanobacteria

Cyanobacteria have several properties as biologically active compounds including antibacterial, antifungal, antiviral, anticancer. Moreover, cyanobacteria are used in wastewater treatment, aquaculture, fertilizers, food, produce secondary metabolites such as vitamin, enzymes, toxins, exopolysaccharides and pharmaceuticals. Cyanobacteria have been used in nonbiodegradable petrochemical-based plastics because cyanobacteria can be produce polyhydroxyalkanoates.

2.5.4.1 Cyanobacterial bioactive compounds

Cyanobacteria are a new source of bioactive compounds, and those obtained tend to be lipopeptides. The bioactive compounds from cyanobacteria are described below.

1). Apratoxin A: Apratoxin is a secondary metabolite from cyanobacteria. It is a cytotoxic marine natural product. Apratoxin can induce G1-phase cell cycle arrest and apoptosis. This property can be developed for anticancer derivatives.

2). Borophycin: Borophycin has effective cytotoxicity in the human carcinoma cell. It is obtained from *Nostoc spongiaeforme* var. *tenue*.

3). Borophycin-8: Borophycin-8 is an antibiotic obtained from *Nostoc linckia*.

4). Cryptophycin: Cryptophycin is a compound with strong antifungal properties. The amount of production for cryptophycin relies on the present environmental conditions. It has the property to tumor cells, especially solid tumors including the brain, ovarian, colon, lung, breast, prostate and pancreas cancers. It is obtained from *Nostoc* sp.

5). Cryptophycin-8: Cryptophycin-8 is obtained from *Dunaliella* and *Spirulina*. It has an anticancer effect.

6). Calothrixin A: Calothrixin A is obtained from cell extracts of Calothrix. It can inhibit the growth of human HeLa cancer cells by inducing apoptotic death or working on the cell signaling via activation of the protein kinase-c family of singnaling enzymes.

7). Curacin A: Curacin A is obtained from *Lyngbya majusculata*. It has potential active properties against breast cancer.

8). Cyanobacterial drugs for AIDS: Cyanobacteria has a compound with antiviral activity. The antiviral cancer polysaccharides are spirulan and Ca-spirulan from *Spirulina* sp. It has the property of broad-spectrum activity against HIV-1, HIV-2, H and influenza. It can inhibit the reverse transcriptase activity of HIV-1.

9). Cyanovirin-N: Cyanovirin-N is isolated from *Nostoc ellipsosporum* which is 101 amino acids long and with 11 kDa polypeptide. It has a compound active against HIV and other lentiviruses. It can inhibit the measles virus and herpes simplex virus-6 in vitro.

10). Dolastatin 10: Dolastatin 10 is obtained from cyanobacteria such as *Dolabella auricularia* and *Symploca* sp. It is composed of four unique amino acids: dolavaline, dolaisoleucine, dolaproline and dolaphenine. It has the effect of an antiproliferative agent.

11). Dolastatin 15: Dolastatin 15 is a linear peptide with effect on various cancer cell lines with it being able to bind on the vinca alkaloid site on tubulin and block the transition into the M phase. It is a member of the dolastatin family.

12). Largazole: Largazole is received from cyanobacteria of the genus *Symploca*. It has anticancer properties and the potential to inhibit Class I histone deacetylases (HDACs).

13). Lyngbyatoxin A: Lyngbyatoxin A isolated from *Lyngbya majuscule* is an anti-inflammatory agent. Moreover, it exhibits anti-HIV activity with this property being isolated from *Lyngbya lagerhaimanii* and *Phormidium tenue*.

14). Microcolin A: Microcolin A is isolated from *Lyngbya majusculata* and can suppress the two-way murine mixed lymphocyte reaction.

15). Stypoldione: Stypoldione has the ability to inhibit a variety of biological processes such as cell division. It can bind covalently to the sulfhydryl groups of thiol through the addition of sulfur to position of C-4^0 of the quinone ring.

16). Symplocin A: Symplocin A is isolated from *Symploca* sp. It has the ability to act as an inhibitor of cathepsin E. It exhibits activities against H-460 lung cancer and breast carcinoma cell lines.

17). Scytovirin: Scytovirin is isolated from the aqueous extract of *Scytonema varium*. It can bind to the envelope glycoprotein of HIV (Vijayakumar and Menakha, 2015).

2.5.4.2 Cyanobacterial bioplastics (polyhydroxyalkanoates, PHAs)

PHAs are a biocompatible material and lipoidic material accumulated by microorganisms. *Spirulina platensis* and *Synechocystis* sp. PCC 6803 can accumulate PHA in the growth condition of phototrophic or mixotrophic. Cyanobacteria fixates CO_2 from the atmosphere to become PHA under nitrogen limiting conditions. In addition, cyanobacteria can accumulate poly(3-hydroxybutyrate) which it is one type of PHA. The strains of cyanobacteria accumulating PHA include *Chlorogloea fritschii*, *Gloeothece* sp., *Oscillatoria limosa*, *Trichodesmium thiebautii*, *Synechococcus* MA19 and *Nostoc muscorum*.

2.5.4.3 Cyanobacterial consortia for bioremediation purposes

Cyanobacteria has the ability to oxidize oil components and complex organic compounds similar to herbicides and surfactants. For example, *Microcoleus chthonoplastes* and *Phormidium corium* have the ability to degrade n-alkanes. *Agmenellum quadruplicatum* and *Oscillatoria* sp. can oxidize naphthalene to 1-naphthol. *Oscillatoria* can degrade phenanthrene, pristine, dibenzothiophene and n-octadecane. *Microcoleus chthonplastes* has the ability to fix atmospheric nitrogen and can degrade aliphatic heterocyclic organo-sulfur compounds as well as alkylated monocyclic and polycyclic aromatic hydrocarbons. Furthermore, cyanobacteria is used in wastewater treatment such as *Oscillatoria* sp. BDU 30501, *Aphanocapsa* sp. BDU 16, *Halobacterium* US 101, *Phormidium valderianum* BDU 30501 and *Oscillatoria boryana* BDU 92181.

2.5.4.4 Cyanobacterial alternative energy sources

Cyanobacteria can produce hydrogen gas which is an alternative future energy source to substitute fossil fuel resources. Energy sources from cyanobacteria have several advantages including their eco-friendly nature, renewability and efficiency. The culture has the ability to produce hydrogen gas such as Anabaena, *Aphanocapsa*, *Anabaena*, *Oscillatoria*, *Nostoc*, *Chroococcidiopsis*, *Calothrix*, *Cyanothece*, *Microcoleus*, *Microcystis*, *Synechococcus*, *Spirulina* and *Gloeobacter*.

2.5.4.5 Cyanobacterial as biofertilizers

Some cyanobacteria have the ability to fix atmospheric nitrogen and are hence known as nitrogen-fixing cyanobacteria. *Azolla* was used to help the growth of soil micro-organisms and increase the fertility of soils. Nitrogen-fixing cyanobacteria have the potential to dominate desert crusts worldwide.

2.5.4.6 Cyanobacterial as healthy food source

Cyanobacteria are used for healthy foods such as *Spirulina*, *Anabaena*, *Nostoc* and *Arthrospira platensis*. These strains are used as food supplements due to its richness

in nutrients and digestibility. It is composed of 60% protein and beta-carotene, riboflavin, thiamine and vitamin B12.

2.5.4.7 Cyanobacterial emulsifiers

Exopolysaccharides (EPS) can be produced from halophilic cyanobacteria. *Aphanocapsa halophytica*, *Cyanothece* sp. ATCC 51142, *Anabaena* sp. ATCC 33047 and *Synechococcus* sp. are used to produce EPS (Abed *et al*., 2009).

2.5.4.8 Cyanobacterial drugs for cancer

Cyanobacteria has been studied as agent against cancer for natural therapy. The biosynthetic information of chemical structures unique to cyanobacteria will be very valuable in the search for new anticancer agents (Vijayakumar and Menakha, 2015).

2.6 Cancer

Cancer is a disease leading to a high mortality rate in many countries. There are many types of cancer the name of each depending on where the cancer forms, for example, colon cancer, lung cancer, brain cancer, breast cancer, hepatoma cancer, and gastric cancer (Umayaparvathi *et al*., 2014). In 2008, approximately 7.6 million out of 12.7 million cases of cancer patients died from the disease (Samarakoon and Jeon, 2012). Statistics from Khon Kaen regarding cancer pain are comparable to other countries. In Thailand there are ~21,645 cancer cases which did not receive adequate pain care. The prescription of morphine, which is the best pain medicine, has been a significant problem in many of the hospitals (Vatanasapt *et al*., 2002).

Cancer is unlike normal cells with the ability to grow uncontrollably and spread quickly. So, cancers cells can continuously grow to separate without stopping in tissues or organs. The different structures of normal cells and cancer cells are shown in Figure 2.9. Normally, the growth of cancer; can start as a single cell with no control over its normal growth and replication process. Cancer has effect upon other cells and tissues. Cancer is 85% developed from the epithelial cells of the body known as carcinomas. Cancers from glandular tissue including the breast are called adenocarcinomas, cancers

of muscle and bone acquired from mesoderm cells are called sarcomas. Each kind of cancer has distinct characteristics.
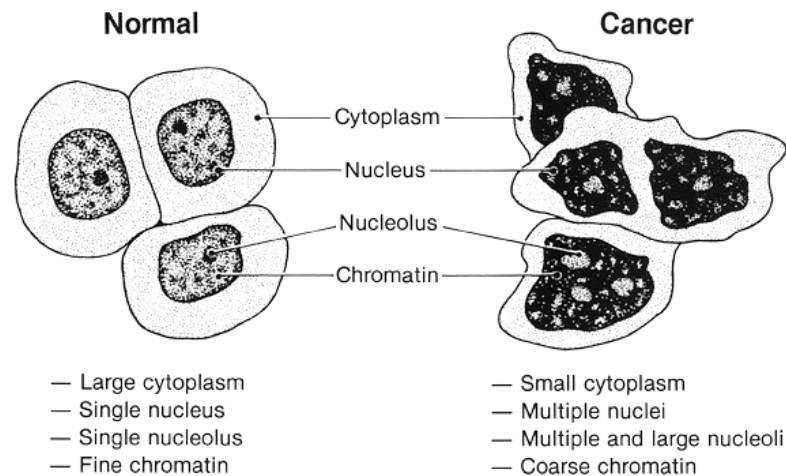
Figure 2.9 Structure of normal and cancer cell

(Pat, 2001)

2.6.1 Causes of cancer

Of the number of cancer cases. 5-10% had genetic causes and 90-95% environmental and other factors, for example, 4-6% alcohol, 10-20% obesity, 15-20% infections, 25-30% smoking and 30-35% diet (Chen *et al*., 2012b; Samarakoon and Jeon, 2012).

2.6.1.1 Endogenous causes

2.6.1.1.1 Oxidative stress

Reactive oxygen species (ROS) created through normal oxidative metabolism have the ability to effect DNA damage. Mechanisms in the body can remove ROS to protect DNA or suppress the effects. Therefore, ROS cause oxidative damage to DNA. The damaged cells has a process to repair through which oxidized bases are released in the urine. Indicators of oxidative DNA damage in the body can be observed from the level of urinary 8-hydrozy-2'-deoxyguanosine and oxidative DNA damage force.

Antioxidants are able to scavenge ROS. Antioxidants such as vitamin C and E are able to donate electrons to free radicals and stop their harmful activity (Ames *et al*., 1993).

    2.6.1.1.2 Inherited germ line mutations

    Approximately 5-10% of cancers are linked to single inherited genes. Inherited alterations means germ line mutations and these are transmitted from the DNA of the sperm or egg. The inherited germ line mutations do not mean it is certain of getting cancer, but that the risk is increased. Mutations in tumour suppressor genes raise the chance of cancer growth: these include retinoblastoma, multiple endocrine neoplasia type 1 and kidney cancer. About 5-10% breast cancer cases are caused by mutations in the BRCA 1 and BRCA 2 genes. Both genes normally create DNA repair proteins. Figure 2.10 illustrates the formation of tumours in cells.
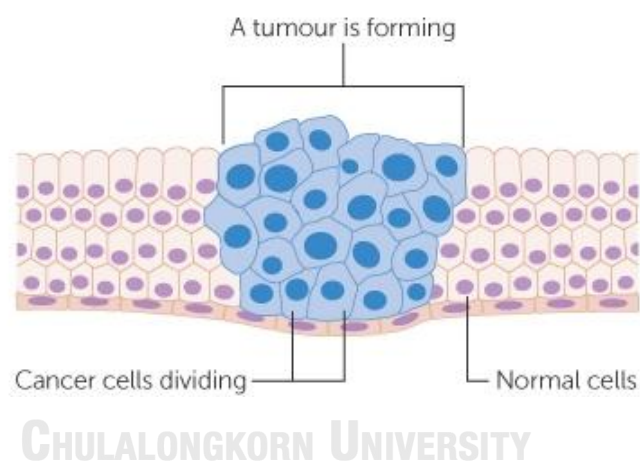
Figure 2.10 Formation of tumour cells from mutation

(Timothy, 2010)

    2.6.1.1.3 Inflammation

    Chronic inflammation is one cause of DNA damage and trigger of cancer. Bioactive chemicals involving cytokines, reactive oxygen and nitrogen species, growth factors, cyclooxygenase and lipoxygenase products cause inflammation of the cells in chronically inflamed tissue. The chronic inflammatory environment affects proliferation and differentiation, preventing apoptosis and activating angiogenesis.

Inflammatory cancer is sensitive to nutritional influences. Dietary constituents lead to the generation of ROS.

2.6.1.1.4 Hormones

Oestrogen is a hormone and can be behind the risk of different cancers in women such as breast cancer, ovarian cancer and endometrial cancer. The combination of oestrogen and progesterone highers the risk of ovarian cancer.

2.6.1.2 Exogenous causes

2.6.1.2.1 Infectious agents

Infectious agents such as bacteria, viruses and parasites cause DNA damage and induce cancer. DNA and RNA viruses are causes of cancer. DNA viruses have viral proteins which can block rumour suppressor genes, while: RNA viruses have oncogenes. Human papilloma virus is the cause of cervical cancer and hepatitis B and C cause liver cancer. These agents do not directly cause cancer but are triggers in the cancer process.

2.6.1.2.2 Radiation

Ionising radiation and UV radiation can cause cancer. Ionising radiation is one factor inducing DNA damage by leading breaks in the DNA strands and interacting with the generated reactive oxygen species and water molecules. Ionising radiation raises the risk of various cancers, including thyroid cancer and breast cancer. UV light has three bands of wavelengths UVA, UVB and UVC but only UVA and UVB can cause cancer. UVA can destroy DNA through the generation of ROS, while UVB can be absorbed by bases in the DNA and hence affecting DNA damage.

2.6.1.2.3 Tobacco

Approximately 80% of cancers in men and 50% in women are because of smoking. Mutagenic carcinogens - arsenic, cadmium, ammonia, benzopyrene and formaldehyde - are all contained in cigarette. Smoking is one factor that can contribute to oxidative stress.

2.6.1.2.4 Carcinogenic agents in food

Moulds, and toxins from the moulds, are known carcinogenics. For example, aflatoxin B is a toxin produced from *Aspergillus* which contaminates grains and peanuts. Aflatoxin B can cause liver cancer. Heterocyclic amines and polycyclic aromatic hydrocarbons are carcinogens formed from cooking meat at high temperatures. Furthermore, polycyclic aromatic hydrocarbons found in industrial and traffic pollution can contaminate foods. N-nitroso compounds can be found in foods with added nitrites and nitrates for preservation including salt, preservatives and drying or smoking. All of these carcinogens are generated by the ingestion of food (Ames *et al*., 1993).

2.6.2 Apoptosis

Apoptosis (from the Greek meaning 'to fall away from') is a programmed cell death that involves the damage of cells (Reed, 2000). Two pathways, the intrinsic pathway (mitochondrial) and the extrinsic pathway (death-receptor) can be initiated (Shrivastava *et al*., 2015). Apoptosis is activated cell death without injury and characterized by cell shrinking, cell blebbing and the compression of the nucleus. Apoptosis is a programmed means of cell death that plays an important role in growth and repair (Favaloro *et al*., 2012). Apoptosis or programmed cell death is characterized by biochemical mechanisms. This process occurs usually during development and as a homeostatic mechanism to sustain cell populations in tissues. Moreover, in many reactions such as immune reactions, cells are damaged by disease and apoptosis can be activated (Elmore, 2007). Figure 2.11 presents the diagram of apoptosis and Figure 2.12 illustrates the apoptosis pathway.
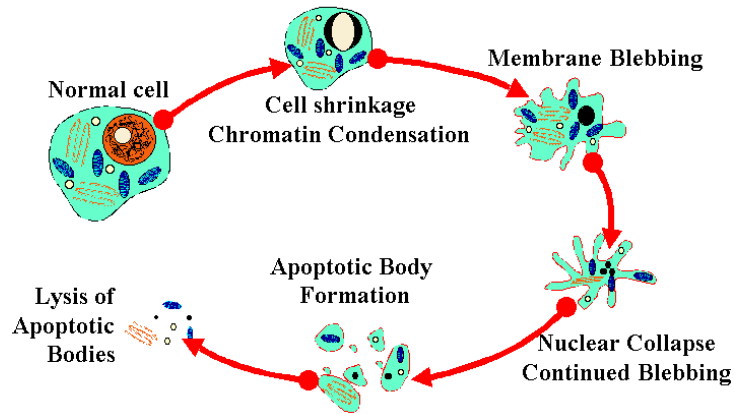
Figure 2.11 Diagram of apoptosis

(Anurag, 2016)
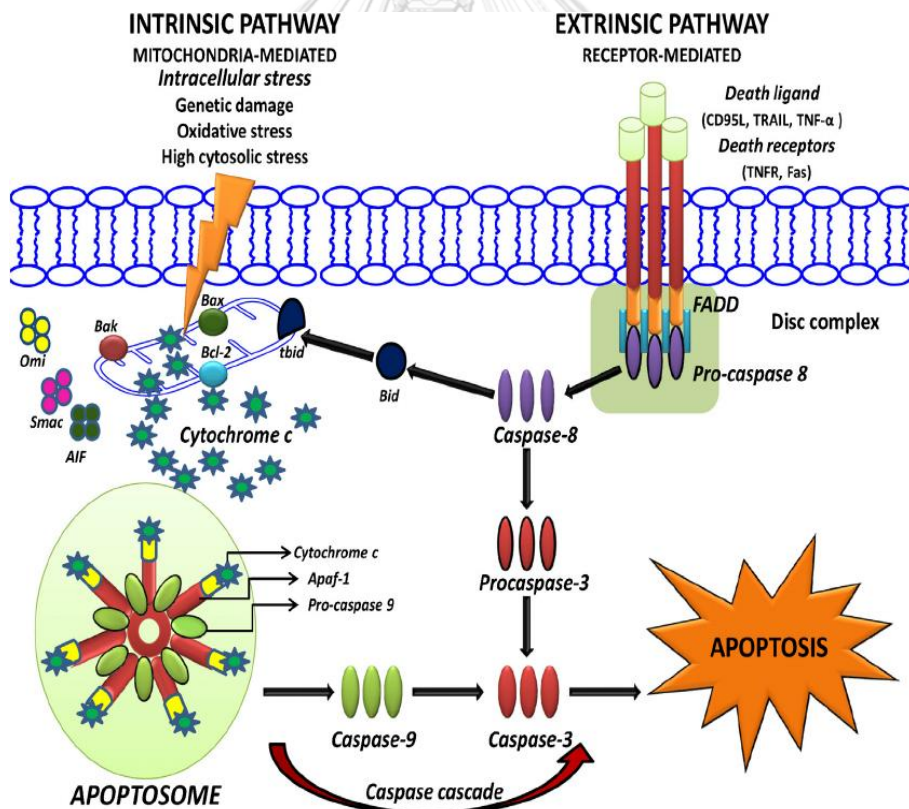
Figure 2.12 Apoptosis pathway

(Beesoo *et al*., 2014)

2.6.2.1 Caspase in apoptosis

The apoptosis pathway is involved in caspase including initiator caspase and executioner caspase. Initiator caspase stimulate executioner caspase which subsequently correlate their activities to destroy key structural proteins and activate other enzymes. Caspase is a cysteine protease which plays an essential role in apoptosis.

2.6.2.1.1 Initiator caspases

Initiator caspases, caspase 8 and caspase 9 are inactive procaspase monomers with both activated by dimerization. Dimerization is the autocatalytic cleavage of caspase monomers to one small subunit and one large subunit leading to the stabilization of the dimer.

2.6.2.1.2 Executioner caspases

Executioner caspases including caspase 3, caspase 6 and caspase 7 are protected by their production because inactive procaspase dimers are cleaved by initiator caspases. A small subunit and large subunit are changed of conformation cause the two active sites of the executioner caspase dimer to produce a functional mature protease. This process leads to caspase activation (McIlwain *et al.*, 2013). Many research suggests that peptides from organisms have the ability to act as anticancer cells as shown in Table 2.3.

Table 2.3 Literature review of anticancer cells from the peptides of different organisms

Source of peptide	Type of cancer cell line	References
Oyster (*Saccostrea cucullata*)	Human colon carcinoma (HT-29) cell lines	(Umayaparvathi *et al*., 2014)
Blood clam (*Tegillarca granosa*)	Prostate cancer cell lines (PC-3)	(Chi *et al*., 2015)
Snow crab	Prostate cancer cell lines (PC-3)	(Doyen *et al*., 2011)
Squid gelatin (*Dosidicus gigas*)	Human breast cancer cell lines (MCF-7)	(Alemán *et al*., 2011)
Mungbean	Hepatoblastoma (HepG2)	(Wongekalak *et al*., 2011)
Chlorella vulgaris	Human breast cancer cell lines(MCF-7), Prostate cancer cell lines (PC-3)	(Sheih *et al*., 2009b)
Purple sweet potato	Human gastric carcinoma SGC7901, human colon cancer SW620	(Wu *et al*., 2015)

2.7 Inflammation

The word inflammation comes from the Latin word "inflammation", meaning to set on fire. Inflammation is a significant process in the immune system in the body to move or repair damaged tissue and to begin the healing process. It is a biological response to harmful stimuli, for example, bacteria, parasites, viruses, irritation and injury causing tissue and cell damage (Ferrero-Miliani *et al*., 2007; Strausbaugh *et al*., 1999). Inflammation can produce or generate pro-inflammatory mediators, including cytokines (IL-1β, IL-6 and IL-12), tumor necrosis factors (TNF-α and TNF-β), interferons (IFN-γ), vasoactive amines (histamine) and eicosanoids (prostaglandins and leukotrienes) (Medzhitov, 2008). Inflammation has signs which indicate damaged tissue such as pain, swelling, heat, and redness (Faro *et al*., 2014). Pain is result from the activation and sensitisation of primary afferent nerve fibers. Swelling is involved in increasing vascular permeability. Heat and redness is involved in increasing blood flow (Strausbaugh *et al*., 1999). Inflammation can lead to diseases, for example, periodontitis, diabetes, obesity, arteriosclerosis, rheumatoid arthritis, pulmonary

diseases, neurologic diseases, cardiovascular diseases and cancer (Aggarwal and Shishodia, 2006).

2.7.1 Types of inflammation

Inflammation can be classified into two types: acute and chronic inflammation. The symptoms of inflammation are shown in Figure 2.13.

2.7.1.1 Acute inflammation

Acute inflammation is the early response of the immune system to manage the stimulant. Acute inflammation can be a rapidly occurring process with the increased movement of plasma and leukocytes into the infected tissue. Acute inflammation can last from a few min to several days (Morgan *et al*., 2008). The nature of acute inflammation is reddening, pain, heat and loss of function (Hortelano, 2009).

2.7.1.2 Chronic inflammation

Chronic inflammation is known as long-term inflammation which can occur for years. Inflammation has three stages: the inflammatory phase, complement phase and resolution phase. Chronic inflammation occurs as the resolution phase. Chronic inflammation is involved with macrophages, monocytes, lymphocytes, cytokines, neutrophils, new vessel proliferation and fibrosis (Yu *et al*., 2009). This inflammation is related to a large number of human diseases such as arthritis, allergy, atherosclerosis and cancer (Medzhitov, 2008).
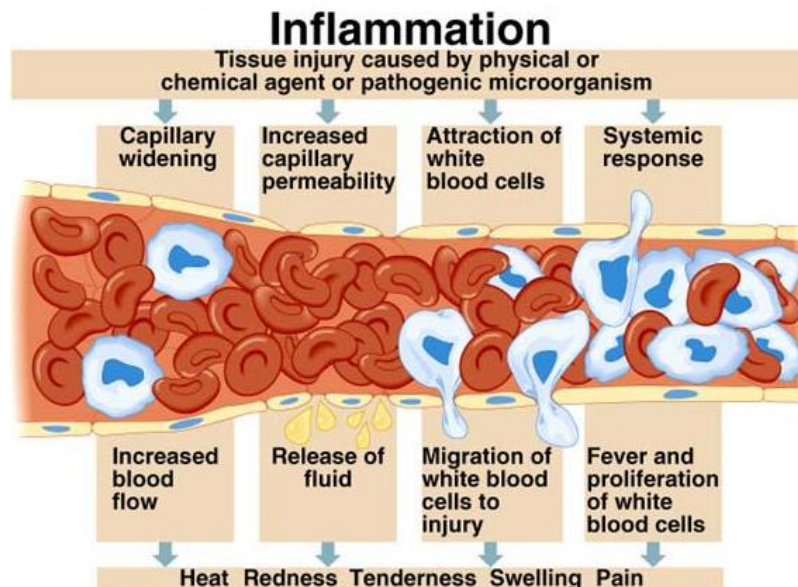
Figure 2.13 The symptoms of inflammation

(Jean, 2012)
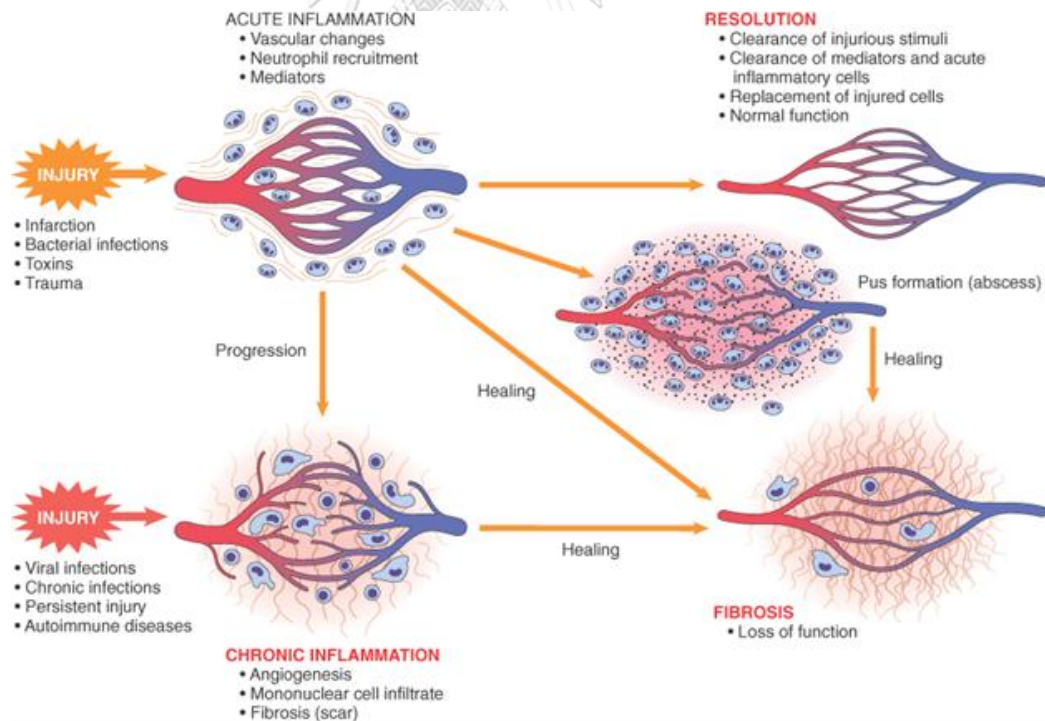
Figure 2.14 Types of inflammation

(Geoffrey, 2011)

2.7.2 Mediators of inflammation

The process of inflammation in the body releases inflammatory mediators. Inflammatory mediators can be triggered by receptors specific to the cells of the inflammation and activated to react to the inflammation. The infection; in the first stages is a resident of macrophages and then mast cells release inflammatory mediators. Mediators of inflammation have seven groups as follows:

1. Vasoactive amines: Mediators in this group include histamine and serotonin, these are generated in an all-or-none manner when platelets and mast cell degranulate.

2. Eicosanoids: Eicosanoids are lipid mediators derived from phospholipids such as prostaglandins and leukotrienes. These mediators are shown in the inner leaflet of cellular membranes. Cyclooxygenases (COX1 and COX2) generate prostaglandins and thromboxanes. COX1 and COX2 enzymes are heme proteins located in the lumenal portion of the endoplasmic reticulum membrane and the nuclear envelope. COX1 can have a predominant action in the endoplasmic reticulum. COX2 operates in the nucleus. COX1 and COX2 are different prostanoid biosynthetic systems which differ in their biological functions. COX1 plays a role in the production of prostaglandin in mammalian tissue and controls normal physiological processes. COX1 is responsible for the housekeeping of prostaglandins synthesis, while; COX2 is an inducible enzyme in control of the production of pro-inflammatory prostaglandins leading to inflammation (Masferrer et al., 1994).

3. Cytokines: Cytokine production is induced by lipopolysaccharide (LPS) that stimulate TLR-4 on the cell surface. TLR-4 is a transmembrane receptor and a binding site of LPS. In the step of the release mediators, activation of nuclear transcription factor kappa-B (NF-κB) occurs after the catching of LPS and TLR-4. NF-κB involved in immune and inflammatory reactions leads to the release of the mediators IL-1, IL-6 and TNF-α. IL-1 and TNF-α can activate inflammatory pathways leading to eicosanoid and nitric oxide (NO) production. Cytokines are produced by macrophages and mast cells (Vandekerckhove et al., 1991).
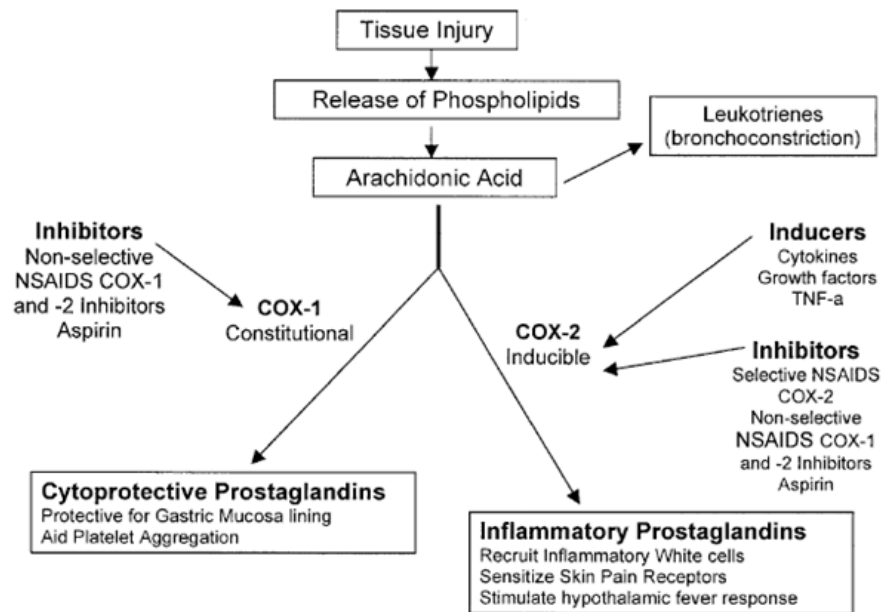
Figure 2.15 Cyclooxygenase (COX) in the inflammation process

(Lee, 2007)
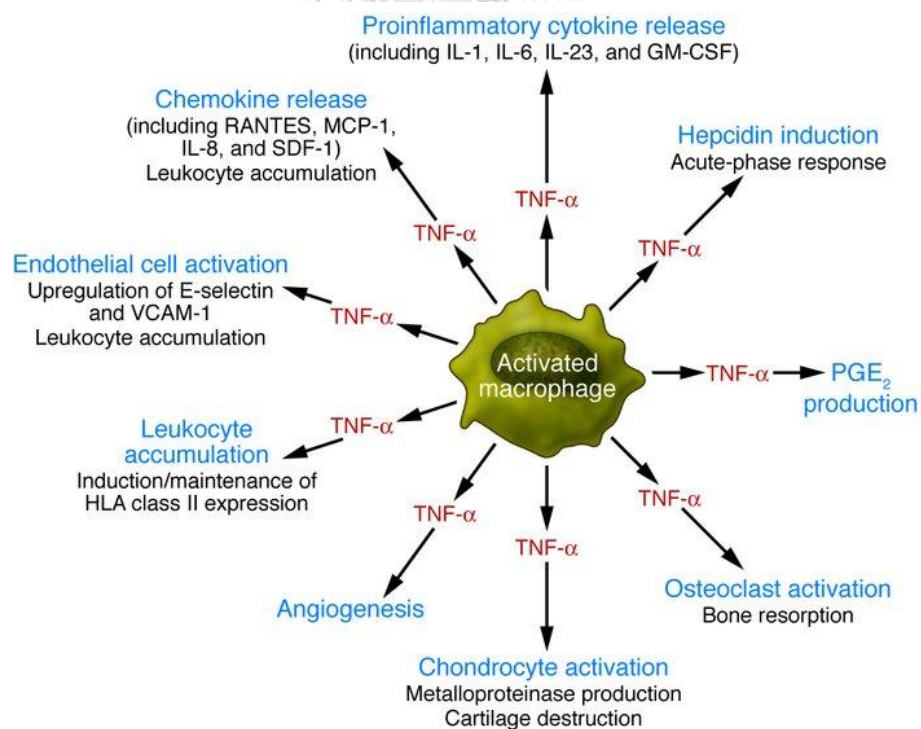
Figure 2.16 TNF- α actions relevant to the inflammation

(Brennan and McInnes, 2008)

4. Chemo-kines: Chemo kines are produced in response to the inducers of inflammation in many cell types. Chemo-kines control chemotaxis towards and leukocyte extravasation.

2.7.3 Nitric oxide synthase pathway

Nitric oxide (NO) is catalyzed in many cell types involved in the inflammation process. This process has important enzymes such as nitric oxide synthase (NOS). NOS have been identified in three isoforms as a nNOS (Type I or NOS-I), iNOS (Type II or NOS-II) and eNOS (Type III or NOS-III). NOSs play necessary roles in the maintenance of homeostasis. eNOS is an enzyme in controlling blood vessel tone, while nNOS is essential in providing neuromodulators and neurotransmitters. iNOS has a function in inflammation. Moreover, iNOS can be expressed by factors, for example, TNF-α, IL-1β, IFN-γ, LPS and oxidative stress (Cedergren *et al*., 2002; Guo *et al*., 2009; Weinberg, 2000).
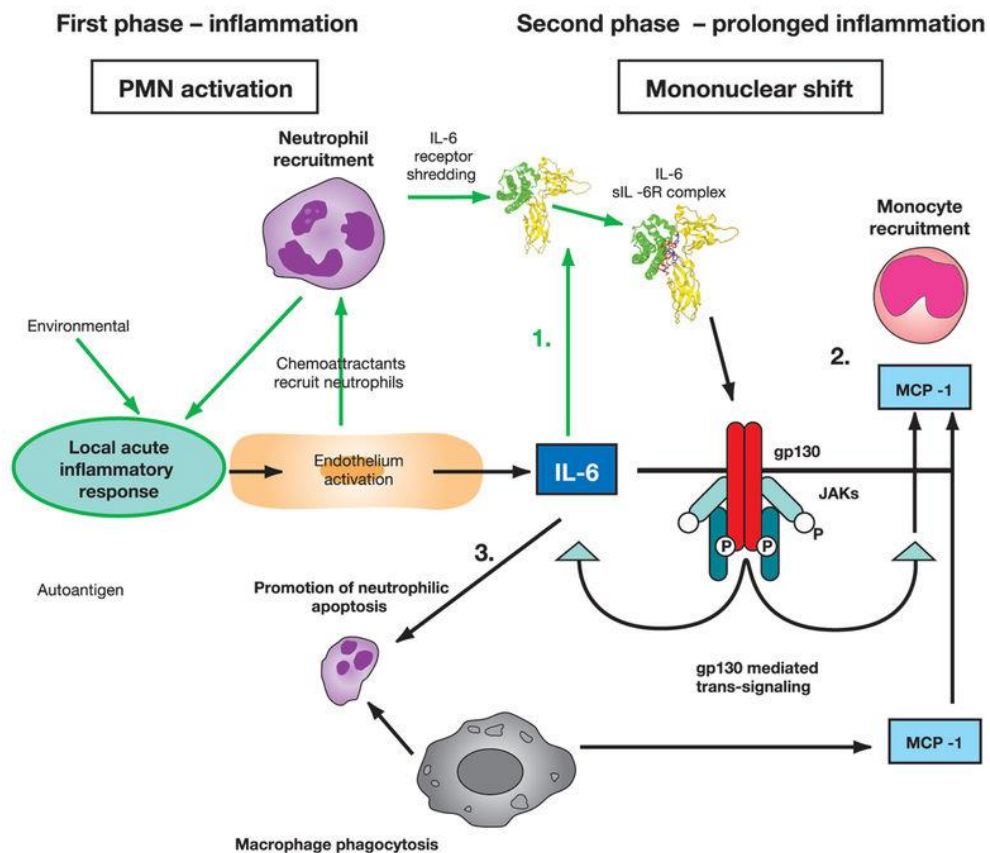
Figure 2.17 IL-6 actions in the chronic inflammation

(Gabay, 2006)
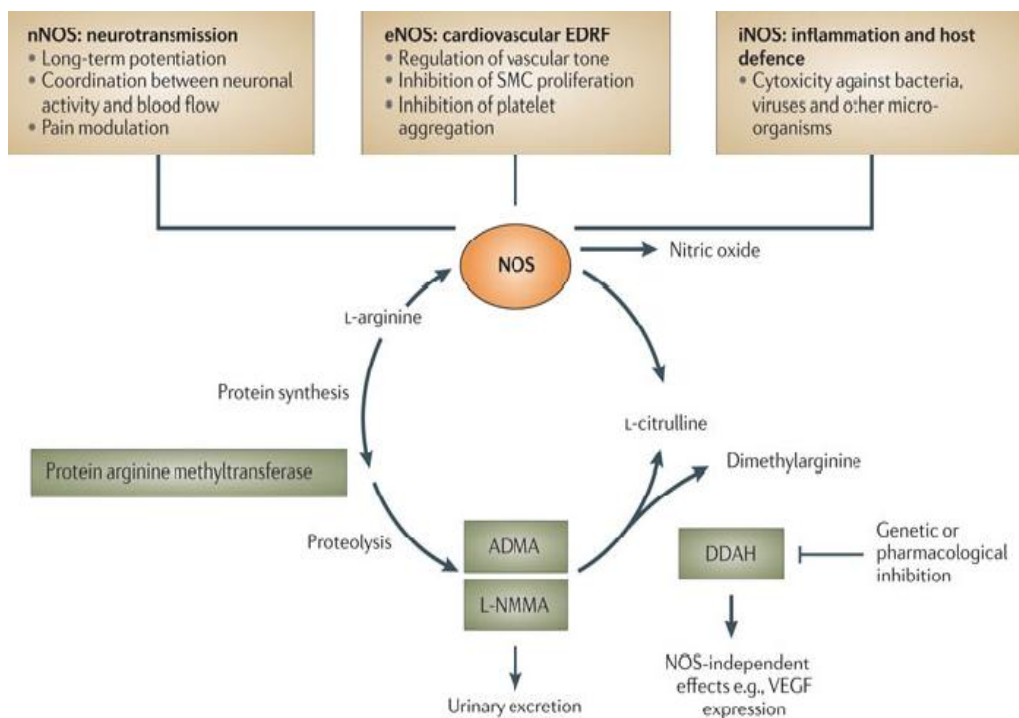
Figure 2.18 Nitric oxide synthase pathway

(Leiper and Nandi, 2011)

Many research has confirmed that the peptide from natural sources has the ability to anti-inflammation as shown in Table 2.4.

Table 2.4 Characteristics of anti-inflammation from natural source

Source of peptide	Type of cytokines	References
Fucoidan (brown seaweed)	iNOS, COX-2, TNF-α, IL-6	(Park *et al*., 2017b)
Rhizomes of Zingiberaceae	iNOS, TNF- α, IL-6	(Chantaranothai *et al*., 2013)
Phyllanthus amarus	iNOS, COX-2, TNF-α, IFN- γ, IL-1, IL-10	(Kiemer *et al*., 2003)
Isatis indigotica	TNF-α	(Xiao *et al*., 2014)
Taraxacum mongolicum	TNF-α, iNOS	(Yang *et al*., 2016)

CHAPTER III
METHODS

3.1 Screening the crude hydrolysates for antioxidant properties by using DPPH, ABTS and NO radical scavenging assays

3.1.1 Growth conditions and preparation of cell biomass

Synechococcus sp. was isolated from Ao Wong Duan, Koh Samet, Thailand. The cultures were grown in BG11 medium combined with Turks Island salt solution for maintenance and cell production (Incharoensakdi and Karnchanatat, 2003). Cultures were incubated at 30 ºC in 250 mL flasks under fluorescent white light (30 µmol photons $m^{-2} s^{-1}$) with shaking. The optical density of cells at 730 nm was evaluated to measure the growth of cells. The centrifugation was used to harvest of cells at 13,000 x g for 15 min, washed twice with 20 mL cold buffer (50 mM potassium phosphate buffer, pH 7.0). The cells were then homogenized in extraction buffer (50 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 0.05% (w/v) polyvinylpyrrolidone-40 (PVP-40). The homogenate was centrifuged at 13,000 x g for 15 min.

3.1.2 Enzymatic hydrolysis of cell biomass

Trypsin from porcine pancreas (Sigma Aldrich, St. Louis, MO, USA) was used for protein hydrolysis of the cell biomass. The concentration of trypsin applied was 50 µg/mL and the reaction was carried out at pH 7.5 for 4 h. The hydrolysis was stopped by heating the reaction at 80 °C for 20 min. The hydrolysate was clarified by centrifugation at 12,000 × g for 20 min at 4 °C, and then lyophilized and stored at –20 °C until use.

3.1.3 Determine antioxidant activities by DPPH, ABTS and NO radical scavenging assays

3.1.3.1 DPPH radical scavenging assays

The DPPH radical scavenging activity assay was determined and modified with the method described by Deng *et al*., (2011). First, 0.004 g of DPPH was dissolved in 100 mL of methanol to prepare a 100 µM DPPH radical solution, and 100 µM DPPH radical solution was then added to each sample in a ratio of 1:4 (80 µL of sample:320 µL of DPPH radical solution) and incubated in the dark at room temperature for 15 min. The solution was then centrifuged at 1,300 rpm for 5 min. Next, 100 µL of each solution was placed into 96-well plates, and the absorbance at 517 nm was measured using a microplate reader. For the positive control, 0.1 mg/mL of ascorbic acid was used.

3.1.3.2 ABTS radical scavenging assays

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

3.1.3.3 NO radical scavenging assays

The NO generated from an aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which are quantified by the Griess Ilosvoy reaction (Govindarajan *et al*., 2003). The reaction mixture was contained 10 mM SNP, phosphate buffered saline pH 7.4 (PBS) and various doses (0-200 µg/mL) of the test solution in a final volume of 3 mL. The reaction was incubate for 150 min. N-(1-Naphthyl) ethylenediamine (NED) 0.1% (w/v) of 1 mL was added in solution reaction. The reaction mixture was incubated for 30 min at 25 ºC. The pink chromophore generated during the diazotination of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at an absorbance wavelength 540 nm against a blank sample. Curcumin was used as the positive control.

3.1.3.4 Percentage inhibition

The percentage of radical scavenging was calculated as follows:

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

where Abs of control is for no sample, Abs of sample is the absorbance of the protein hydrolysate, Abs of background is the color absorbance of the sample and Abs of blank is the absorbance of deionized water. The IC_{50} values was calculated by the GraphPad Prism software version 6. All of the experiment were tested in triplicate and shown as means ± standard error (SE) of the triplicate data.

3.1.4 Determination of protein

The soluble protein concentration was determined by the Bradford method using bovine serum albumin as the standard. The absorbance of the supernatant was measured at 595 nm (Bradford, 1976).

3.2 Purification and identification of peptide from *Synechococcus* sp.

3.2.1 Purification of peptide with ultrafiltration, gel filtration and RP-HPLC technique

3.2.1.1 Ultrafiltration

The peptide solution was fractionated through a range of nominal molecular weight cutoff (MWCO) membranes of 10, 5 and 3 kDa. The protein hydrolysate was stored at -20 °C until use.

3.2.1.2 Gel filtration chromatography

The peptide solution was passed through gel filtration on a Sephadex G-75 column (AKTA^{TM} prime with HiTrap^{TM}, 1.6 cm i.d. × 15 cm; Amersham Biosciences, Uppsala, Sweden) using distilled water as the solvent and at a flow rate of 0.5 mL/min. Each eluate was collected and the absorbance measure at 280 nm.

3.2.1.3 HPLC

The peptide solutions after partial purification with gel filtration were fractionated by RP-HPLC (Shimpak, 250 x 46 mm, Luna 5U; Phenomenex, Torrance, CA, USA) using C-18 column (Shimpak, 250 × 46 mm, Luna 5U; Phenomenex, Torrance, CA, USA). Acetonitrile (0–70%) containing 0.05% trifluoroacetic acid was used to separate species at a flow rate 0.7 mL min^{-1}. The injection volume was 50 μL and the injected sample had a protein concentration of 1.20–2.12 mg protein/mL. Peptides were detected by measuring the absorbance at 215 nm.

3.2.2 Identification of peptide by ion trap mass spectrometer

Peptides isolated from RP-HPLC analysis that showed the highest antioxidant activity were identified by ion trap mass spectrometry coupled with electrospray ionization (Model Amazon SL, Bruker, Germany). The MS/MS data were searched against the SwissProt database with the MASCOT package (www.matrixscience.com).

3.3 Antioxidant activities of the synthetic peptide

Peptides was synthesized by Fmoc solid-phase using an Applied Biosystems Model 433A Synergy peptide synthesizer. The purity of the peptides was verified by an analytical mass spectrometer system (Thermo Mod. Finnigan™ LXQ™) coupled to a Surveyor HPLC. The antioxidant activities of the peptides were determined by DPPH, ABTS and NO radical scavenging assays. The quality of the five synthetic peptides is shown in Table 3.1.

Table 3.1 The quality of the five synthetic peptides

Name	Peptide sequence	Formula	Molecular weight (Da)	Purity
Peptide 1	AILQSYSAGKTK	$C_{56}H_{95}N_{15}O_{18}$	1266.47	99.15%
Peptide 2	ALNKTHLIQTK	$C_{56}H_{99}N_{17}O_{16}$	1266.52	99.10%
Peptide 3	LLVHAPVK	$C_{42}H_{73}N_{11}O_9$	876.12	99.26%
Peptide 4	IPDAHPVK	$C_{40}H_{65}N_{11}O_{11}$	876.03	94.84%
Peptide 5	VVVLRDGAVQQLGTPR	$C_{74}H_{130}N_{24}O_{22}$	1708.01	93.92%

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

3.4.1 Preparation plasmid from *E. coli*

The *E. coli* culture strains containing the pUC19, pBR322 and pKS plasmids were cultivated in LB agar and ampicillin. The culture was incubated overnight at 37°C. Single colonies from the plated *E. coli* containing the pUC19, pBR322 and pKS plasmids were picked, inoculated into LB broth (5 mL) containing ampicillin, incubated for 12–16 h at 30°C with shaking at 200 rpm and harvested by centrifugation.

3.4.2 Plasmid DNA purification

Plasmid DNA purification was performed using the Spinclean Plasmid Miniprep Kit (Norgen Biotek Corp., Canada). The cell pellet was resuspended in 250 μL of the resuspension solution (Sol I) by pipetting up and down until no cell cluster remained, and 250 μL of the lysis solution (Sol II) was added. The tube was mixed completely by inverting the tube 4–6 times until the solution became viscous and slightly clear. The neutralization solution (Sol III; 350 μL) was then added, and the solution was mixed immediately and completely by inverting the tube 4–6 times. The pellet cell debris and chromosomal DNA were separated by centrifugation at 13,000 rpm for 10 min. The supernatant was transferred to a spin column tube and centrifuged for 1 min, and the flow-through was then discarded. The column was then placed back into the same collection tube. The spin column was rinsed with 400 μL of the wash solution I and centrifuged for 2 min, and the flow-through was then discarded. The wash solution (750

μL) was added to the spin column followed by centrifugation for 2 min, and the flow-through was then discarded. The column was then placed into a 1.5 mL microcentrifuge tube, and the elution buffer (50 μL) was added to the center of the spin column membrane to elute the plasmid DNA. The spin column was incubated for 2 min at room temperature and then centrifuged for 2 min.

      3.4.3 DNA damage assay

DNA damage was investigated from the pUC19, pBR322 and pKS plasmids, which existed in a supercoiled form. Oxidative damage can change a supercoiled form to an open-circular form and a linear form. DNA damage was induced by hydroxyl radicals based on the Fenton reaction as described by Sheih et $al.$ (Sheih et $al.$, 2009b). The reactions were composed of 3 μl of DNA (16.5 μg/mL pUC19, 2686 bp; 17.5 μg/mL pBR322, 4361 bp; and 18.8 μg/mL pKS, 2958 bp) and 4 μL of the purified peptides at concentrations of 13.2×10^{-3}, 6.59×10^{-3}, 3.29×10^{-3} and 1.65×10^{-3} μg/mL. The reaction mixtures were incubated for 20 min at room temperature, and 2 mM $FeSO_4$ (3 μL) and 0.06 mM H_2O_2 (4 μL) were then added. The reaction mixtures were incubated at 37°C for 30 min. Finally, electrophoresis was used to visualize the DNA bands.

3.5 Determination of the anti-inflammation activity

      3.5.1 Cell culture of RAW 264.7 cells

The RAW 264.7 murine leukemia macrophage cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 1% (w/v) sodium pyruvate, 1% (w/v) HEPES, 0.4 mg/mL streptomysin sulphate and 100 U/mL penicillin-G at 37 °C in a humidified atmosphere with 5% (v/v) CO_2.

      3.5.2 Pretreatment of RAW 264.7 cells

The RAW 264.7 cells were seeded in 96-well plates at a density of 2×10^4 cells per well and a volume of 100 μL of DMEM supplemented with 10% (v/v) FCS. Cells were incubated overnight in the 5% CO_2 incubator. Subsequently, the medium was replaced with fresh medium containing the peptide sample at various concentrations, fresh DMEM (negative control), or budesonide (positive control), and the cultures

incubated for 1 h. After 1 h incubation, NO production was stimulated with 50 µg/mL lipopolysaccharide (LPS) and the samples incubated for 18–24 h.

3.5.3 MTT assay for the measuring of cell proliferation

RAW 264.7 cell proliferation was measured using the MTT assay with modifications (Chantaranothai *et al*. 2013; Yang *et al*. 2016). Cells were plated at a density of 2×10^4 cells per well in a 96-well plate and 100 µL of a 5 mg/mL MTT solution (in PBS) was added to each well. After incubation at 37 °C and 5% (v/v) CO_2 for 4 h, 100 µL of isopropanol containing 0.04 N HCl was added to dissolve the formazan crystals in the cells. The absorbance of each well was measured at 540 nm using a micro-plate reader. Cell proliferation was reported as the concentration of the sample to suppress cell growth by the IC_{50} values. The IC_{50} values 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 ± standard error of the triplicate data.

3.5.4 Determination of NO production from RAW 264.7 cell

NO production was determined by measuring the nitrite in culture supernatants. Sodium nitrite was used as the standard at concentrations between 0 and 100 µM. 50 µL of the culture supernatant was added to wells of a 96-well plate and 50 µL of sulfanilamide was also added. The samples were incubated at room temperature for 10 min in the dark. 50 µL of the NED solution (Griess reagent) was then added and the sample incubated for 10 min at room temperature in the dark. The absorbance of each well was measured at 540 nm using a micro-plate reader. To eliminate the interaction between the sample and the Griess reagent, NO concentration in the culture medium without cells was also measured, and subtracted from that obtained with cells. The concentration that inhibited LPS-stimulated NO production by 50% (50% inhibitory concentration: IC_{50}) was determined from the dose-response curve. The IC_{50} values were calculated using version 6 of the GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

3.5.5 Total RNA isolation from RAW264.7 cells

RAW 264.7 cells (2×10^5 cell/well) were added to a cell culture plate and allowed to adhere overnight at 37 °C in an incubator containing 5% (v/v) CO_2. The cells were then treated with a peptides sample and 50 μg/mL budesonide for 1 h. Cells were then stimulated with 50 μg/mL LPS. The positive control was treated with LPS and budesonide. The negative control was cells treated with LPS and sterilized water. Cells were harvested after incubation for 12 h. Total cellular RNA was purified using the MasterPure™ DNA and RNA purification kit (Epicentre, USA) following the manufacturer's protocol. The total RNA content was determined by a Nanodrop DS-11FX+ spectrophotometer (DeNovix, Inc., USA). A content of 1 ng total RNA from each condition was subjected to reverse transcription to give single-stranded complementary DNA (cDNA) using the PrimerDesign Ltd *Precision*™ nanoScript 2 Reverse Transcription kit (PrimerDesign Ltd., UK) and following the manufacturer's protocol. The reaction was performed at 42 °C for 20 min and 75 °C for 10 min. The cDNA was stored at –20 °C until used for detection.

3.5.6 Detection *iNOS*, *TNF-α*, *COX-1*, *COX-2* and *IL-6* mRNA by reverse transcription (RT-PCR)

The inducible nitric oxide synthase (*iNOS*), tumor necrosis factor–alpha (*TNF-α*), interleukin–6 (*IL-6*), cyclooxygenase-1 (*COX-1*), cyclooxygenase-2 (*COX-2*) and *β–actin* genes were amplified by PCR. The *β-actin* is a constitutively expressed gene which analyzed as an internal standard (housekeeping gene). The PCR mixture composed of 1 μL cDNA, 9.5 μL ultrapure water (RNase/DNAse free water), 12.5 μL PCR reagent (PCR Biosystem, UK.), 1 μL 10m M forward primer and 1 μL 10 mM reverse primer. The reaction was a final volume at 25 μL. Amplification was performed for 35 cycles by a PCR thermal cycler (Bio-Rad, Laboratories, Inc., USA) with the following programme for *iNOS*, *TNF-α*, *COX-2*, *IL-6* and *β-actin* mRNA : denaturation at 95 °C for 1 min, annealing at 50 °C (except *iNOS* at 65 °C) for 1 min, and extension at 72 °C for 1 min. Amplification was performed for 40 cycles with the following programme for *COX-1* mRNA: denaturation at 95 °C for 1 min, annealing at 62 °C for

1 min, and extension at 72 °C for 1 min. The DNA fragments were obtained and separated on 1.2% (w/v) agarose gel electrophoresis such as 423 base pairs (bp) of *iNOS*, 375 bp of *TNF-α*, 310 bp of *COX-1*, 861 bp of *COX-2*, 417 bp of *IL-6* and 380 bp of *β-actin*. The bands of DNA were stained by ethidium bromide for 10 min and observed under a UV light using a gel documentation system (Bio-Rad, Hercules, USA) with modifications according to Won *et al*. (2006). Table 3.2 shown the primer sequences for each gene. The nucleotide sequences of all genes were searched from GenBank (National Center for Biotechnology Information, NCBI).

Table 3.2 Primer used for RT-PCR analysis

Gene	Primer sequence	Annealing Temperature (°C)	Fragment size (bp)
iNOS	F CCATCATGGACCACCACACA	65	423
	R CCATGCAGACAACCTTGGTG		
TNF-α	F CCTGTAGCCCACGTCGTAGC	50	375
	R TTGACCTCAGCGCTGAGTTG		
COX-1	F AGTGCGGTCCAACCTTATCC	50	316
	R GGTAAAGCCAGGACCCATCTTTC		
COX-2	F GGAGAGACTATCAAGATAGT	50	861
	R ATGGTCAGTAGACTTTTACA		
IL-6	F CATGTTCTCTGGGAAATCGTGG	50	417
	R AACGCACTAGGTTTGCCGAGTA		
β-actin	F ACCAACTGGGACGACATGGAGAA	50	380
	R GTGGTGGTGAAGCTGTAGCC		

3.5.7 Detection *iNOS*, *TNF- α*, *COX-1*, *COX-2* and *IL-6* mRNA by quantitative real time PCR (qRT-PCR)

The gene expression levels of *iNOS*, *TNF-α*, *COX-1*, *COX-2*, *IL-6* and *β-actin* were analyzed by qRT-PCR (Nankar and Pande, 2014). The cDNA was produced using extracted RNA as the DNA template. The real time PCR mixture is compose of 1 μL

cDNA, 1 µL 10 mM forward primer, 1 µL 10 mM reverse primer, 7 µL ultrapure water 7 and 10 µL 2X qPCRBIO SyGreen Mix (PCR Biosystem Ltd, UK). Final volume of real time PCR mixture was 20 µL. The qRT-PCR reactions were amplified by a MyGo Pro® Real time PCR (IT-IS International Ltd, UK) under the following programme : activation step at 95 °C for 2 min, denaturation at 95 °C for 10 s for 40 cycles, annealing at 60 °C for 20 s (except *iNOS*, *COX-1* and *COX-2*, which was performed at 68 °C), extension at 72 °C for 30 s and melting at 55-95 °C for 1 min. Table 3.3 shown the primer sequences of qRT-PCR for each gene. The nucleotide sequences of all genes were searched against the NCBI GenBank.

Table 3.3 Primer used for qRT-PCR analysis

Gene	Primer sequence	Annealing Temperature (°C)	Fragment size (bp)	% GC
iNOS	F CGGCAAACATGACTTCAGGC	68	124	55
	R TAGGTCGATGCACAACTGGG			55
TNF-α	F GGGCAGGTCTACTTTGGAGTCA	60	128	55
	R ACAGACTGGGGGCTCTGAGG			65
COX-1	F AGCTGCTGCTGAGAAGGGAGTT	68	125	55
	R GGTAAAGCCAGGACCCATCTTTC			52
COX-2	F CTGACCCCCAAGGCTCAAAT	68	124	55
	R AAGTCCACTCCATGGCCCAG			60
IL-6	F CTCTCTGCAAGAGACTTCCATCC	60	125	52
	R ACAGGTCTGTTGGGAGTGGTATC			52
β-actin	F GATCAAGATCATTGCTCCTCCTG	68	173	48
	R CGCAGCTCAGTAACAGTCCG			60

β–actin was used as the housekeeping gene and the three qRT-PCR reactions were analyzed by relative quantitation (RQ). The relative gene expression level was determined using the Ct (threshold cycle) value by calculating from the formula as follows (Livak and Schmittgen, 2001):

Relative gene expression = $2^{-\Delta\Delta Ct}$

where $\Delta\Delta Ct$ correlates to the increase in the threshold cycle of the gene. The results derived from the formula are given as: 1 indicates no change, >1 indicates an increase in gene expression and 0–1 indicates a decrease in gene expression. The housekeeping gene threshold cycle value should increase.

3.6 Determination of the antiproliferative activity

3.6.1 Cytotoxicity assay for human malignant cell lines

Antiproliferative activity was determined by the MTT assay. The Wi38 normal cell line and the following human malignant cell lines were used: BT474 (breast), Chago-K1 (lung), Hep-G2 (hepatoma), KATO-III (gastric), and SW620 (colon). All the human malignant cell lines were grown in complete RPMI-1640 medium containing 2.0 mM L-glutamine and 10% (v/v) FCS, and the cells were incubated at 37°C under 5% (v/v) CO_2 conditions. The cells were aspirated, trypsinized, and washed prior to seeding into 96-well plates (200 µL/well) at the following densities: 2.5×10^4 cells/µL for Hep-G2 and SW620; and 1×10^4 cells/µL for Wi38, BT474, Chago-K1 and KATO-III. The cell suspensions were incubated for 1 day. Serial dilutions of F_2 were added into each well, and the cells were incubated for an additional 3 days. MTT solution (5 mg/mL) was added into the 96-well plates (10 µL/well), and the cells were incubated for 4 h. DMSO was added into each well (150 µL), and the cells were measured by a microplate reader at an absorbance of 540 nm.

3.6.2 Apoptosis

Apoptosis was determined by dual staining of Annexin V-FITC and propidium iodide (PI) followed by fluorescence-activated cell sorting (BioLegend Inc., San Diego, CA, USA), following the Annexin V-FITC/PI detection kit protocol. The SW620 cells were seeded in 25 cm^2 culture flasks (1×10^7 cells per flask) in RPMI-1640 complete medium containing 2.0 mM L-glutamine and 10% (v/v) fetal calf serum (FCS), and the cells were incubated at 37°C under 5% (v/v) CO_2 conditions. After incubation overnight, F_2 was added to the cells, and then the cells were incubated for 24, 48 and 72 h at 37 °C under 5% (v/v) CO_2 conditions. The SW620 cells were then harvested using a scraper and were washed with cold phosphate buffer saline (PBS; pH 7.2)

containing 1% fetal calf serum (FCS). The cell pellets were resuspended in Annexin V-binding buffer (100 µL), and 100 µL of the cell suspension was placed into a 1.5 mL microcentrifuge tube followed by the addition of 2.5 µL of Annexin V-FITC and 5 µL of propidium iodide solution. The cell suspension was vortexed and incubated in the dark for 15 min at room temperature, and 200 µL of Annexin V-binding buffer was then added. Apoptosis was immediately detected by flow cytometry (BD FACSCalibur, BD Biosciences, Singapore), and the data were analyzed using Flowjo software.

3.6.3 Caspase 3, 8 and 9 activity assay

3.6.3.1 Preparation of SW 620 cell lysates

The SW620 cells were seeded at 1×10^7 cells/flask, and F_2 was added at the concentration representing the IC_{20} value for the treated cells, and the cells were incubated at 37°C in a 5% CO_2 for 24, 48 and 72 h. The control and treated cells were harvested using a scraper and washed with 20 mM cold PBS. The cell pellets were separated by centrifugation at 600×g for 15 min. The cell pellets were then resuspended in 1× lysis buffer (100 µL) and incubated on ice for 15–20 minutes. The lysed cells were centrifuged at 20,000 for 15 min at 4°C, and the supernatants were moved to new tubes. The lysates were immediately examined or stored at -70°C.

3.6.3.2 Caspase 3 activity assay

A colorimetric caspase 3 assay kit was used to determine caspase 3 activity based on the hydrolysis of the Acetyl-Asp-Glu-Val-Asp *p*-nitroaniline (Ac-DEVD-*p*NA) peptide substrate by caspase 3 leading to release of the *p*-nitroaniline (*p*NA) moiety. *p*-Nitroaniline has high absorbance at 405 nm. The cell lysates and the caspase 3 positive control (5 µL) were loaded into 96-well plates, and 85 µL of 1× assay buffer was then added. The reaction was initiated by loading 10 µL of the caspase 3 substrate into each well and mixing gently. The reaction solution was incubated at 37°C for 70 to 90 min. The activity of caspase 3 was measured by the absorbance at 405 nm. The results were analyzed using a *p*-nitroaniline calibration curve.

3.6.3.3 Caspase 8 activity assay

Caspase 8 assay kit, Colorimetric was used for determine caspase 8 activity. The assay is based A colorimetric caspase 8 assay kit was used to determine caspase 8 activity based on the hydrolysis of the Acetyl-Ile-Glu-Thr-Asp p-nitroaniline (Ac-IETD-pNA) peptide substrate by caspase 8 leading to the release of a p-nitroaniline (pNA) moiety. The cell lysates and the caspase 8 positive control (10 µL) were loaded into 96-well plates, and 80 µL of 1× assay buffer was then added. The reaction was initiated by loading 10 µL of the caspase 8 colorimetric substrate. The activity of caspase 8 was measured by the absorbance at 405 nm.

3.6.3.4 Caspase 9 activity assay

A caspase 9 colorimetric activity assay kit was used to determine caspase 9 activity based on the detection of the p-nitroaniline (pNA) chromophore after cleavage from the labeled LEHD-pNA substrate. The cell lysates and the caspase 9 positive control (10 µL) were loaded into 96-well plates, and 20 µL of 5× assay buffer was then added. The reaction was initiated by adding 10 µL of caspase 9 substrate to each well, and the reaction solution was incubated for 1–2 h at 37°C. The activity of caspase 9 was measured by the absorbance at 405 nm.

Caspase 3, 8, and 9 activity was calculated as follows:

$$\text{Activity (µmol } p\text{NA / min / mL)} = \frac{\mu\text{mol } p\text{NA x d}}{\text{t x V}}$$

where v is the volume of sample in ml; d is the dilution factor; and t is the reaction time in minutes

3.7 Statistical analysis

Each determinations was performed in triplicate and shown as means ± standard error (SE) of the triplicate data. All data was performed by software Statistical Package for Social Sciences (SPSS) version 15.0 by one-way analysis of variance at $P \leq 0.05$ and t-test at $P \leq 0.05$. IC_{50} value was calculated by GraphPad Prism Version 6.01.

CHAPTER IV

Results and discussion

4.1 Screening crude protein hydrolysate for antioxidant activity and its amino acid composition

The age of unicellular cyanobacterium *Synechococcus* sp. played a role in the preliminary conditions for the screening of antioxidant activity using DPPH, ABTS and NO radical scavenging activities as targets. The screening test results are shown in Table 4.1. The age of cyanobacterium had the most important effect on antioxidant activity. The highest DPPH, ABTS and NO radical scavenging activity were 151.16$\pm$12.10 μg protein/mL, 56.90$\pm$0.80 μg protein/mL and 195.27$\pm$2.75 μg protein/mL, respectively at 21 days. The results at 21 days were significantly different ($p \leq 0.05$) compared to the results at 7 and 14 days. Moreover, at 21 days was received yield of cell and antioxidant activity. At 21 days, *Synechococcus* sp. existed in a stationary phase, which was the optimum age for the highest antioxidative DPPH, ABTS and NO radical scavenging activity in crude protein hydrolysate. However, the crude protein hydrolysates from *Synechococcus* sp. at 21 days had the highest antioxidant activity because microalgae produce secondary metabolites during the stationary phase (Abed *et al*., 2009; Falaise *et al*., 2016; Vijayakumar and Menakha, 2015).

The amino acid compositions of the *Synechococcus* sp. cells at 21 days are shown in Table 4.2. The data of the amino acid composition of *Synechococcus* sp. indicated that this strain had important amino acids, which effect the antioxidant activity as a hydrophobic, aromatic and imidazole amino acid, such as leucine (Leu), methionine (Met), valine (Val), cysteine (Cys), proline (Pro) phenylalanine (Phe), tryrosine (Tyr), tryptophan (Trp) and histidine (His) (Mendis *et al*., 2005; Ren *et al*., 2008). The dominate amino acids of this strain were Leu, Phe and Val, which accounted for 0.17%, 0.16% and 0.12%, respectively. Previous studies have shown that protein hydrolysates from organisms, including oyster (Umayaparvathi *et al*., 2014), smooth hound (Bougatef *et al*., 2010), purple sweet potato (Wu *et al*., 2015) and *Chlorella*

vulgaris (Morris *et al*., 2007), have antioxidant activities. Therefore, peptides derived from *Synechococcus* sp. may have antioxidant activities.

Table 4.1 Antioxidant activities of crude protein hydrolysates at different ages of *Synechococcus* sp.

Antioxidant activity	Age of *Synechococcus* sp. (days)		
(IC$_{50}$, µg protein/mL)	7	14	21
DPPH	183.90 $\pm$ 16.00 [b]	132.27 $\pm$ 3.53 [a]	151.16 $\pm$ 12.10 [a]
ABTS	86.00 $\pm$ 3.00 [b]	82.60 $\pm$ 5.00 [b]	56.90 $\pm$ 0.80 [a]
NO	212.80 $\pm$ 6.05 [b]	182.33 $\pm$ 4.63 [a]	195.27 $\pm$ 2.75 [a]

All the data are the mean $\pm$ standard error of the triplicates. Different letters indicate significant differences among the groups according to Duncan's test ($p \leq 0.05$). [a-b] Values with the same letters indicate no significant difference for each group of fraction samples according to Duncan's test ($p \leq 0.05$).

Table 4.2 Amino acid composition of *Synechococcus* sp. cells

Amino acid Profile	Composition (%)
Alanine (Ala)	0.17
Arginine (Arg)	0.07
Glycine (Gly)	0.11
Aspartic acid (Asp)	0.18
Valine (Val)	0.12
Cystine (Cys)	0.01
Glutamic acid (Glu)	0.19
Leucine (Leu)	0.17
Isoleucine (Ile)	0.10
Histidine (His)	0.03
Threonine (Thr)	0.11
Proline (Pro)	0.08
Lysine (Lys)	0.08
Methionine (Met)	0.04
Serine (Ser)	0.09
Phenylalanine (Phe)	0.16
Tyrosine (Tyr)	0.12

4.2 DPPH, ABTS and NO radical scavenging activities after purification of crude protein hydrolysate by ultrafiltration

Synechococcus sp. cells were grown for 21 days and hydrolyzed with trypsin to yield a crude protein hydrolysate, and fractionated by ultrafiltration with molecular weight cut off (MWCO) membranes of 10, 5 and 3 kDa. The four fractions were MW > 10 kDa, MW 10-5 kDa, MW 5-3 kDa and MW <3 kDa. The results of NO radical scavenging activities, ABTS radical scavenging activities and DPPH radical scavenging activities of all the fractions are shown in Table 4.3. The DPPH radical scavenging activities (IC_{50}) of all the fractions were higher than those of the control (ascorbic acid). Moreover, the ABTS radical scavenging activity IC_{50} value of the control was the highest. The ABTS radical scavenging ability of MW<3 kDa fraction had the best efficiency. The DPPH, ABTS and NO radical scavenging activities of MW<3 kDa were an IC_{50} of 13.63$\pm$0.15 µg protein/mL, IC_{50} 11.54$\pm$0.3 µg protein/mL and IC_{50} 34.51$\pm$9.8 µg protein/mL, respectively. Ascorbic acid was used as the positive control for both DPPH and ABTS radical scavenging activity assays, with IC_{50} values of 1.43$\pm$0.26 µg protein/mL and 127.00$\pm$4.29 µg protein/mL, respectively. Curcumin was used as the positive control for NO radical scavenging and gave an IC_{50} value of 164.77$\pm$16.02 µg/mL. The results clearly indicated that the lowest molecular weight peptides displayed the highest antioxidant activity when compared with the higher molecular weight peptides. The efficiency of the isolated antioxidant peptides has been shown to arise from both the molecular weight and amino acid sequence (Wang *et al*., 2013). Moreover, peptides less than 6 kDa were shown to have efficient antioxidant activity after isolation by ultrafiltration (Jiang *et al*., 2014b). The higher activity of these peptides when compared with peptides of higher molecular mass partly arises from their ability to cross internal barriers to exert their biological effect (Ahn *et al*., 2015). Importantly, the activity of the isolated short bioactive peptides herein will also be dependent on their amino acid composition.

Table 4.3 DPPH, ABTS and NO radical scavenging activities of *Synechococcus* sp. hydrolyzed by trypsin to yield a crude protein and its subsequent ultrafiltration through different MWCO membranes

Fraction samples	Radical scavenging activity (IC$_{50}$; μg protein/mL)		
	DPPH	ABTS	NO
Control	1.43 ± 0.26 [a]	127.00 ± 4.29 [d]	164.77 ± 16.02 [b]
Crude hydrolysate	151.16 ± 12.1 [e]	56.9 ± 0.80 [c]	195.27 ± 2.70 [b]
MW > 10 kDa	74.72 ± 8.66 [d]	73.3 ± 1.90 [c]	119.70 ± 3.86 [b]
MW 5–10 kDa	55.63 ± 5.05 [d]	48.26 ± 0.80 [c]	59.43 ± 0.36 [a]
MW 3–5 kDa	17.69 ± 0.86 [c]	18.89 ± 0.10 [b]	35.52 ± 2.44 [a]
MW < 3 kDa	13.63 ± 0.15 [b]	11.54 ± 0.30 [a]	34.51 ± 9.80 [a]

4.3 DPPH, ABTS and NO radical scavenging activities of MW < 3 kDa after purification by gel filtration chromatography (Sephadex G-75)

The peptides were purified by a gel filtration chromatographic method. The MW < 3 kDa fraction was fractionated into two sub-fractions (F$_1$ and F$_2$) by a Sephadex G-75 gel filtration column as shown in Figure 4.1. The DPPH and NO radical scavenging activity of F$_1$ could not be measured, but the IC$_{50}$ value of the ABTS radical scavenging activity of F$_1$ was 35.25±0.7 μg protein/mL. F$_2$ exhibited antioxidant activity of NO and ABTS at IC$_{50}$ 7.68±0.64 μg protein/mL and 9.74±0.2 μg protein/mL, respectively as shown in Table 4.4. F$_2$ showed NO radical scavenging activity which it is activity for screening of anti-inflammation activity. Thus, F$_2$ was selected for further purification by reversed-phase HPLC according to Umayaparavathi and colleague (2014) and Bougatef and colleague (2010).
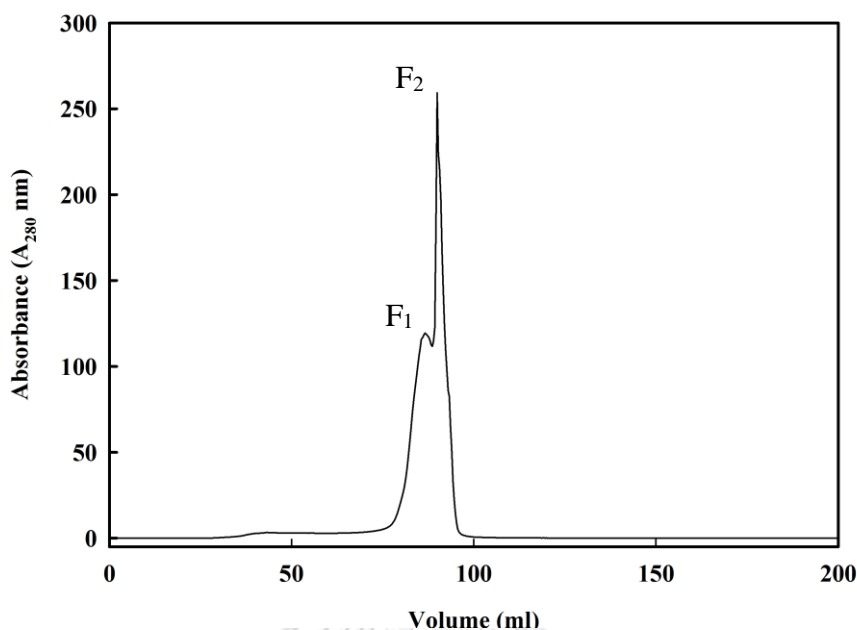
Figure 4.1 Gel filtration chromatography of the MW < 3 kDa on a Sephadex G-75

Table 4.4 Antioxidant activity of gel filtration chromatography fractions as F_1 and F_2

Fraction	IC_{50} (μg protein/mL)		
	NO radical scavenging activity	**ABTS radical scavenging activity**	**DPPH radical scavenging activity**
F_1	>0.06 μg protein/mL	35.25 ± 0.7	>0.04 μg protein/mL
F_2	7.68 ± 0.64	9.74 ± 0.2	>0.01 μg protein/mL

All the data are given as mean $\pm$ standard error of the triplicate data

4.4 DPPH, ABTS and NO radical scavenging activities after purification by HPLC

The F_2 fraction was obtained from gel filtration chromatography. The F_2 fraction was purified by RP-HPLC on a C-18 column. Figure 4.2 shows the chromatogram of the purified peptides, which had many peaks according to the period of time used for selected antioxidant activity of the purified peptides. The fractionate from this step was divided into four fractions as follows: 0–10 min (F_{2-1}), 10–20 min (F_{2-2}), 20–30 min (F_{2-3}) and 30–40 min (F_{2-4}). Table 4.5 shows the DPPH, ABTS and NO radical scavenging activities. From the results, the 30–40 min sub-fraction showed the highest

ABTS and NO radical scavenging activity and this fraction was selected for further analysis by mass spectrometry.
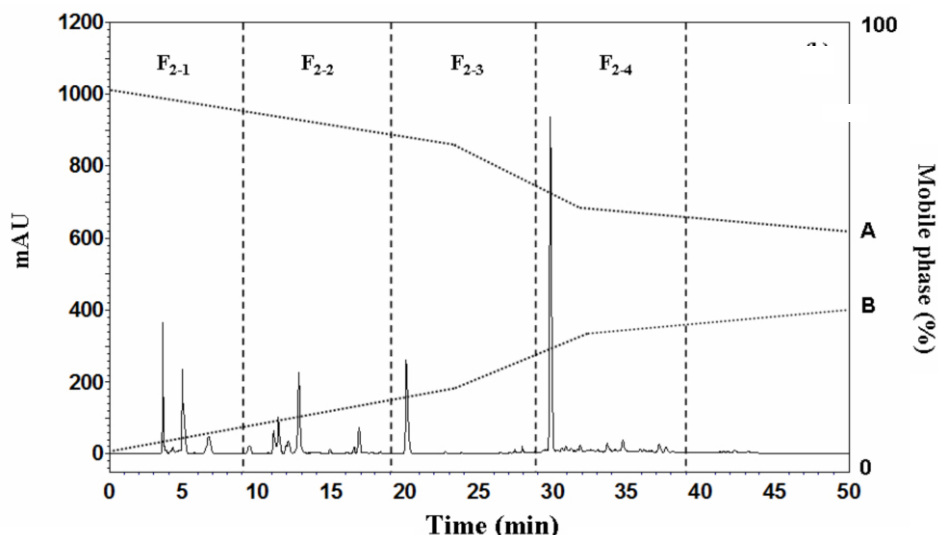
Figure 4.2 RP-HPLC chromatogram of F_2 sub-fraction

Table 4.5 Antioxidant activity of RP-HPLC chromatography of F_2 sub-fraction

Sample	Percentage inhibition of DPPH radical scavenging activity (%)	Percentage inhibition of PABTS radical scavenging activity (%)	Percentage inhibition of NO radical scavenging activity (%)
0 – 10 minute (F_{2-1})	55.28 ± 3.10^a	2.08 ± 0.69^b	56.03 ± 1.01^b
10 – 20 minute (F_{2-2})	47.01 ± 3.77^b	0.91 ± 0.61^b	39.58 ± 0.25^d
20 – 30 minute (F_{2-3})	42.42 ± 1.54^{bc}	1.93 ± 0.56^b	45.38 ± 0.56^c
30 – 40 minute (F_{2-4})	38.28 ± 1.48^c	3.55 ± 0.61^a	77.50 ± 0.55^a

All the data are given as mean $\pm$ standard error of the triplicate data. Different letters indicate significant differences among the groups in Duncan test ($p \leq 0.05$). [a-b] Values with the same letters indicate no significant different for each group of fraction samples in Duncan test ($p \leq 0.05$).

4.5 Identification of peptides by iron trap mass spectrometry and the antioxidant activities of synthetic peptide

The peptides present in the F_{2-4} were identified by iron trap mass spectroscopy. The raw MS/MS data were searched against SwissPlot database using the Mascot software. Database interrogation was: maximum missed cleavages tolerated = 1; variable modifications carbamidomethyl (C) and oxidation (M); MS/MS error tolerance of ± 0.6 Da; peptide charge as 1^+, 2^+ and 3^+; the instrument selected as ESI-iron trap; and the top 5 hits were presented. Table 4.6 shows the amino acid sequence identified from the F_{2-4}. Five purified peptides with amino acid sequences Ala-Iso-Leu-Glu-Ser-Tyr-Ser-Ala-Gly-Lys-Thr-Lys (AILQSYSAGKTK; 1,265.69 Da), Ala-Leu- Asp-Lys-Thr-His-Leu-Iso-Glu-Thr-Lys (ALNKTHLIQTK; 1,265.74 Da), Leu-Leu-Val-His-Ala-Pro-Leu-Lys (LLVHAPVK; 875.55 Da), Iso-Pro-Asp-Ala-His-Pro-Val-Lys (IPDAHPVK; 875.48 Da) and Val-Val-Val-Leu-Arg-Asp-Gly-Ala-Val-Glu-Glu-Leu-Gly-Thr-Pro-Arg (VVVLRDGAVQQLGTPR; 1,706.97 Da) were searched using BLAST database of NCBI.

In this study, aromatic amino acids and amino acids with antioxidant activity showed in the peptide chains were detected. Many researches claimed that aromatic amino acids and hydrophobic amino acids such as Ala, Val, Iso, Leu, Met, Phe, Tyr, Trp have been found to have antioxidant activity (Bougatef *et al.*, 2010; Ren *et al.*, 2008; Zhang *et al.*, 2014). Moreover, histidine is considered to have antioxidant activity because the imidazole group of histidine can donate a proton to another molecule (Li *et al.*, 2007). Saiga *et al.* claimed that a peptide composed of glycine, phenylalanine, proline and leucine was active against lipid peroxidation (Saiga *et al.*, 2003).

Table 4.6 Peptide sequence of protein hydrolysate was identified by iron trap mass spectrometer

Sequence	Organism	Mass	Query cover (%)	Identity (%)	Accession
1. AILQSYSAGKTK	hypothetical protein [*Synechococcus* sp. RCC307]	1265.69	91	73	WP_0508155 35.1
	conserved hypothetical protein [*Synechococcus* sp. RCC307]	1265.69	91	73	CAK27712.1
	elongation factor G [*Synechococcus* sp. NKBG042902]	1265.69	91	73	WP_0300066 66.1
	elongation factor G [*Synechococcus* sp. PCC 7002]	1265.69	91	73	WP_0114330 98.1
	hypothetical protein [*Synechococcus* sp. JA-2-3B'a(2-13)]	1265.69	50	100	WP_0114330 98.1
2. ALNKTHLIQTK	dithiobiotin synthetase [*Synechococcus* sp. PCC 7335]	1265.74	54	100	WP_0064553 63.1
	glycosyl transferase, group 2 family protein [*Synechococcus* sp. PCC 7335]	1265.74	54	100	WP_0064564 69.1
	DNA-binding response regulator [*Synechococcus* sp. RCC307]	1265.74	81	67	WP_0119368 24.1

Sequence	Organism	Mass	Query cover (%)	Identity (%)	Accession
	glycosyl transferase [*Synechococcus* sp. KORDI-100]	1265.74	81	78	WP_0385460 81.1
	type I restriction-modification enzyme S subunit [*Synechococcus* sp. NKBG15041c]	1265.74	63	86	WP_0245469 88.1
3. LLVHAPVK	permease [*Synechococcus* sp. PCC 6312]	875.55	100	100	WP_0151238 56.1
	LPS export ABC transporter periplasmic protein LptC [*Synechococcus* sp. GFB01]	875.55	87	86	WP_0480166 59.1
	hypothetical protein Synpcc7942_0104 [*Synechococcus elongatus* PCC 7942]	875.55	100	88	ABB56136.1
	hypothetical protein [*Synechococcus elongatus*]	875.55	100	88	WP_0112437 12.1
	hypothetical protein [*Synechococcus* sp.]	875.55	100	88	WP_0397555 00.1
4. IPDAHPVK	catalase/hydroperoxidase HPI(I) [*Synechococcus* sp. WH 5701]	875.48	100	78	WP_0061701 70.1
	UDP-glucose 4-epimerase [*Synechococcus* sp. NKBG042902	875.48	75	83	WP_0300074 71.1

Sequence	Organism	Mass	Query cover (%)	Identity (%)	Accession
	UDP-glucose 4-epimerase [*Synechococcus* sp. PCC 7002]	875.48	75	83	WP_0123073 27.1
	hydroperoxidase [*Synechococcus* sp. KORDI-100]	875.48	100	78	WP_0385442 54.1
	hydroperoxidase [*Synechococcus* sp. CC9616]	875.48	100	78	WP_0289517 78.1
5.VVVLRDGAVQQLGTPR	sugar ABC transporter ATP-binding protein [*Synechococcus* sp. PCC 6312]	1706.97	93	67	WP_0151243 78.1
	sugar ABC transporter ATP-binding protein [*Synechococcus* sp. KORDI-52]	1706.97	87	57	WP_0385547 38.1
	sugar ABC transporter ATP-binding protein [*Synechococcus* sp. KORDI-49]	1706.97	87	64	WP_0436910 40.1
	alpha/beta hydrolase superfamily [*Synechococcus* sp. WH 7803]	1706.97	81	77	CAK22581.1
	alpha/beta hydrolase [*Synechococcus* sp. WH 7803]	1706.97	81	77	WP_0414283 25.1

Since fraction F$_{2-4}$ exhibited DPPH, ABTS and NO scavenging activities, peptides of fraction F$_{2-4}$ from de novo sequencing were synthesized and evaluated for antioxidant activity. The antioxidant activity of the five synthesized antioxidant peptides is summarized in Table 4.7. Sadat *et al*. reported that peptides from α-lactalbumin containing either Tyr or Trp at one of the extremities of the sequence displayed the most efficient ABTS radical scavenging activity (Sadat *et al*., 2011). In our study, the ABTS radical scavenging activity of AILQSYSAGKTK peptide would be likely attributed to Tyr residue in the sequence.

Several studies have demonstrated that aromatic amino acids and hydrophobic amino acids, such as Phe, Try, Typ, His, Cys, and Met showed the highest antioxidant activity. Generally, aromatic amino acids in peptides have very good radical scavenging activity due to their special structure which allows the scavenging of unpaired electrons or radicals by donating protons, while the imidazole group in His has proton-donation ability (Bougatef *et al*., 2010; Ren *et al*., 2008; Shangguan *et al*., 2014). 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 (Kannan *et al*., 2008).

Table 4.7 Peptide synthesis derived from the RP-HPLC of 30–40 min sub-fraction (F$_{2-4}$) and their antioxidant activities

Peptide sequence	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)	NO radical scavenging activity (%)
AILQSYSAGKTK	16.61 ± 0.07 [b]	46.90 ± 0.10 [a]	20.89 ± 1.50 [a]
ALNKTHLIQTK	30.85 ± 0.62 [a]	4.23 ± 0.38 [c]	17.73 ± 3.84 [b]
LLVHAPVK	6.31 ± 0.37 [c]	3.78 ± 1.11 [c]	20.55 ± 0.25 [a]
IPDAHPVK	7.74 ± 0.65 [c]	1.76 ± 0.97 [d]	20.67 ± 0.57 [a]
VVVLRDGAVQQLGTPR	16.21 ± 0.05 [b]	11.60 ± 1.93 [b]	10.38 ± 1.10 [c]

All the data are given as the mean ± standard error of the triplicates. [a-d] Values with the same letters indicate no significant difference for each group of fraction samples according to Duncan's test ($p < 0.05$).

4.6 Protective effect of the F$_2$ on oxidation-induced DNA damage

Plasmid DNA, namely, pBR322, pKS and pUC19, were used to test oxidation-induced DNA damage to verify the protective effect of the F$_2$ fraction. DNA damage caused by hydroxyl radicals through the Fenton reaction changes supercoiled DNA into linear and open circular DNA. Figure 4.3 (a) shows the capability of F$_2$ to protect pBR322 plasmid DNA. Supercoiled DNA was completely induced to linear DNA as a result of the hydroxyl radical damage (Lanes 4–5). F$_2$ at concentrations of 13.2×10^{-3} µg protein/mL and 6.59×10^{-3} µg protein/mL (Lanes 4–5) protected oxidation-induced DNA damage. Moreover, this result was similar to that for pKS plasmid DNA (Figure 4.3 (b)), which revealed that the supercoiled DNA of pKS was decreased and was induced to linear DNA.

The protective effect of the F$_2$ fraction on pUC19 plasmid DNA is shown in Figure 4.3 (c). The F$_2$ fraction at concentrations of 13.2×10^{-3} µg protein/mL, 6.59×10^{-3} µg protein/mL and 3.29×10^{-3} µg protein/mL (Lanes 4–6) protected against oxidation-induced DNA damage. The supercoiled DNA form was completely converted to the linear DNA form. Sheih *et al*. reported that the purified peptide from algae protein waste hydrolysate protects DNA from the oxidation-induced DNA damage of PET-28a DNA. The supercoiled DNA was clearly converted to open circular DNA after hydroxyl radical damage through the Fenton reaction (Fe^{2+} + H_2O_2). Increasing concentrations of peptide from 10.6 to 84.9 µM exhibited increasing protective capacity (Sheih *et al*., 2009a). Moreover, Zhang *et al*. reported that peptides from sweet potato protein hydrolysates protect plasmid pBR322 from oxidative damage. The protective effect of the peptide increased with increasing peptide concentrations of 1, 2.5, 5 and 10 mg/mL with protective effects of 28.52%, 36.99%, 50.11% and 61.33%, respectively (Shangguan *et al*., 2014). These data demonstrated that F$_2$ exhibits OH-scavenging activity and Fe^{2+}-chelating ability.
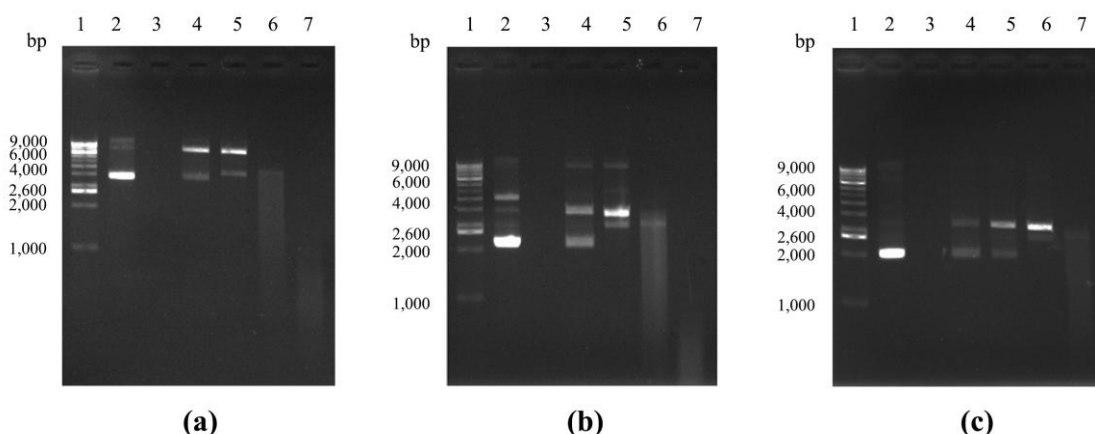
Figure 4.3 Protective effect of the F_2 fraction on hydroxyl radical-induced oxidation of (a) pBR322, (b) pKS, and (c) pUC19 plasmid DNA Lane 1: DNA Ladder, Lane 2: (a) pBR322 plasmid DNA 4,361 bp (DNA = 17.5 µg/mL); (b) pKS plasmid DNA 2,958 bp (DNA = 18.8 µg/mL); (c) pUC19 plasmid DNA 2,686 bp (DNA = 16.5 µg/mL), Lane 3: plasmid DNA with $FeSO_4$ and H_2O_2 treatments (as DNA damage control), Lanes 4-7: plasmid DNA with $FeSO_4$ and H_2O_2 treatments in the presence of the F_2 fraction at concentrations of 13.2×10^{-3} µg protein/mL, 6.59×10^{-3} µg protein/mL, 3.29×10^{-3} µg protein/mL and 1.65×10^{-3} µg protein/mL, respectively

4.7 Determination of the anti-inflammation activity

4.7.1 Anti-inflammatory effect of F_2 fraction in LPS induced RAW264.7 macrophage cells

The anti-inflammatory effect of the F_2 was measured by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and using induced RAW264.7 macrophage cells. The MTT assay is a highly sensitive, quantitative, rapid and reliable colorimetric assay that is used to determine of cytotoxicity activity of peptides derived from sources including algae protein waste, baicalin and deflated walnut (Chen *et al*., 2012a; Gu *et al*., 2015; Sheih *et al*., 2009b). MTT is reduced to purple formazan in living cells. MTT is a yellow water-soluble tetrazolium dye. Viable cells can convert MTT into a purple colored formazan product with mitochondrial enzymes which measured at wavelength 540 nm. Death cell has lose the ability to convert MTT into formazan. Therefore, this assay is a measuring mitochondrial activity with occur only the viable cells (Riss *et al*., 2016). Using this assay we measured the

F_2 at concentrations ranging from 3.75 µg protein/mL to 120 µg protein/mL. F_2 exhibited no cytotoxicity activity toward RAW264.7 macrophage cells as shown in Figure 4.4. Furthermore, the F_2 was used in an assay examining the production of nitric oxide by RAW264.7 macrophage cells treated with LPS. This fraction exhibited activity against NO production with an IC_{50} of 2.45 ± 0.21 µg protein/mL, while budesonide showed an IC_{50} of 3.16 ± 0.06 µg protein/mL. The IC_{50} value of F_2 indicated that F_2 displayed anti-inflammatory activity similar to that of budesonide. A similar observation has been reported for peptides derived from soybean, in which the peptides isolated were able to inhibit significantly NO production in RAW264.7 macrophage cells (Hernández-Ledesma *et al*., 2009).
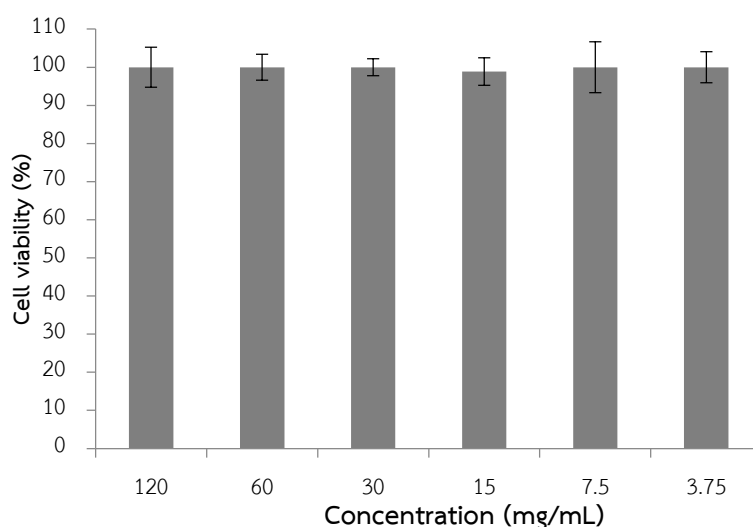
Figure 4.4 Effect of F_2 to cell viability of macrophage RAW 264.7 cells

4.7.2 Gene expression of *iNOS*, *TNF-α*, *COX-1*, *COX-2* and *IL-6* mRNA by reverse transcription (RT-PCR)

The F_2 exhibited an anti-inflammatory effect because this fraction exhibited NO radical scavenging activity when incubated with RAW264.7 macrophage cells. Gene expression was determined by RT-PCR to further understand factors responsible for the observed anti-inflammatory effect of the F_2. Five genes were considered: *iNOS*, *TNF-*

α, *COX-1*, *COX-2* and *IL-6*. The *β-actin* gene was used as the housekeeping control gene. The results of the RT-PCR analysis are presented in Figure 4.5. The F_2 did not decrease expression of *TNF-α*, *COX-1*, *COX-2* and *IL-6* when compare with cells treated with budesonide. However, the concentration of budesonide for treated cells was more than the concentration of the F_2. The F_2 activity towards modulating gene expression in response inflammation may not be investigated by this approach and thus RT-PCR cannot be used to determination the impact of F_2 on gene expression. Nevertheless, it have been reported that the chemical mediator TNF-α is not only a mediator of inflammation but also a chemical mediator to other processes in cells such as cell proliferation via NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) or MAPK (mitogen activated protein kinase) pathways and induction to cell death. Hence, gene expression of *TNF-α* is not only an indicator involved in inflammation (Kaden *et al*., 2005; Wajant *et al*., 2003). In terms of gene expression of macrophage cells exposed to budesonide (50 μg/mL), the results found that budesonide suppressed *iNOS*, *TNF-α*, *COX-2* and *IL-6* expression. In addition, macrophage cells treated with LPS clearly exhibited gene expression of *iNOS*, *TNF-α*, *COX-1*, *COX-2* and *IL-6*. During inflammation, cells or tissue damage cause release of pro-inflammatory cytokines, such as iNOS, TNF-α, COX-2 and IL-6 (Won *et al*., 2006; Zong *et al*., 2015). Lunasin is a peptide from soybean that has been exhibited to modulate gene expression levels, including *TNF-α* and *IL-6* (Hernández-Ledesma *et al*., 2009). Moreover, extracts from *Phyllanthus amarus* have been exhibited to have anti-inflammatory effects when incubated with RAW264.7 macrophage cells. These extracts were found to reduce the expression of *iNOS*, *TNF-α* and *COX-2* (Kiemer *et al*., 2003a). Plant extracts exhibiting anti-inflammatory activity were also exhibited to reduce the expression of *IL-6*, *iNOS*, *COX-2* and *TNF-α* (Mueller *et al*., 2010). These results suggest that F_2 treatment is effective in suppressing the production of pro-inflammatory cytokines at their transcriptional level in LPS-induced RAW264.7 cells. Different mechanisms have been proposed for the inhibition of LPS-induced effects on macrophage cell lines by proteins and peptides. These mechanisms include binding to lipids. For example, LPS-binding proteins have been shown to interfere with the interaction between LPS. Other factors, such as the amino acid sequence of the peptides can affect peptide internalization by cells, which may change their inhibitory effect.
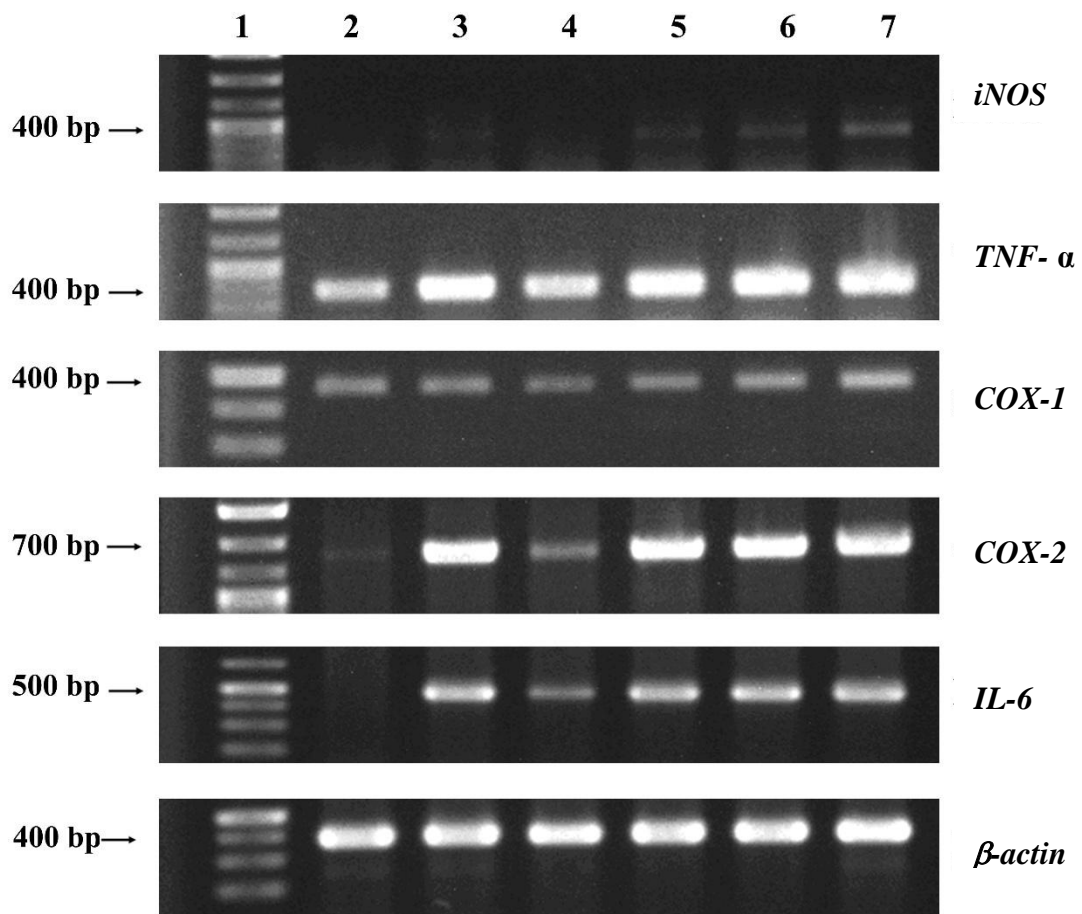
Figure 4.5 Effect of the F_2 on the induce gene expression by RT-PCR of *iNOS*, *TNF-α*, *COX-1*, *COX-2* and *IL-6* from RAW264.7 macrophage cells Lane 1: DNA Ladder 10000 bp, Lane 2: no addition, Lane 3: LPS, Lane: 4 LPS+budesonide (positive control), Lane 5: LPS+F_2 (7.5 µg protein/mL), Lane 6: LPS+F_2 (3.75 µg protein/mL), Lane7: LPS+F_2 (1.88 µg protein/mL)

4.7.3 Gene expression of *iNOS*, *TNF-α*, *COX-1*, *COX-2* and *IL-6* mRNA by quantitative real time PCR (qRT-PCR)

Quantitative real time PCR detection was applied to measure the change in the level of gene expression cause by the anti-inflammatory effect of the F_2 sub-fraction. The qRT-PCR is a very appropriate for quantitative gene expression analysis. During the process, DNA is amplified and can be monitored in real time, and this technique can provide an estimate of the number of gene copies. In particular, this technique is expected to detect gene expression that cannot easily be investigate by RT-PCR, where RT-PCR shows an amplified band on the gel after the PCR cycles.

The results of qRT-PCR analysis are shown in Figure 4.6. Expression levels of the pro-inflammatory cytokines in RAW264.7 macrophage cells were enriched by LPS. Real time PCR data of *iNOS* expression showed a significant reduction for the LPS+budesonide and LPS+ F_2 (7.5, 3.75 and 1.88 µg protein/mL) treated cells with RQ values of 3.53 ± 0.35, 5.05 ± 1.10, 5.91 ± 2.39 and 5.08 ± 1.04 fold, respectively. In contrast, the real time PCR data of *iNOS* treated with only LPS exhibited high expression with a value of 55.55 ± 5.16 fold. Similarly, for expression of *TNF-α* and *COX-2* the F_2 peptide sub-fraction caused a significant reduction in the level of gene expression. The RQ values of *TNF-α* following treatment with LPS, LPS+budesonide and LPS+ F_2 (7.5, 3.75 and 1.88 µg protein/mL) were 11.68 ± 1.30, 0.73 ± 0.2, 2.12 ± 1.01, 1.71 ± 0.19 and 1.91 ± 0.25, respectively. The expression level of *COX-2* was 135 ± 32.85, 2.20 ± 0.21, 17.62 ± 7.27, 19.16 ± 4.68 and 17.93 ± 3.61 fold for LPS, LPS+budesonide and LPS+ F_2 (7.5, 3.75 and 1.88 µg protein/mL), respectively. The level of *IL-6* gene expression was 91.65 ± 5.68, 2.26 ± 0.48, 9.25 ± 0.58, 12.25 ± 2.32 and 6.83 ± 0.57 fold for cells treated with LPS, LPS+budesonide and LPS+ F_2 (7.5, 3.75 and 1.88 µg protein/mL), respectively. However, RAW264.7 macrophage cells treated with F_2 exhibited had significant downregulation of the expression of the *IL-6* gene. The RQ values of *IL-6* for cells treated with LPS, LPS+budesonide and LPS+ F_2 (7.5, 3.75 and 1.88 µg protein/mL) such as 14.74 ± 3.31, 0.09 ± 0.00, 0.1 ± 0.01, 0.15 ± 0.02 and 0.11 ± 0.03, respectively.

This result indicated that F_2 exhibited anti-inflammation activity. Moreover, the data from qRT-PCR corroborated the result from RT-PCR analysis. This is the first

time that a peptide derived from cyanobacteria *Synechococcus* sp. reduced *iNOS*, *TNF-α*, *COX-1*, *COX-2* and *IL-6* gene expression. Many research have been reported relation between the peptides or the properties of peptides and extracts to pro-inflammatory cytokines expression. Apolipoprotein-derived peptides shown up-regulation of the *IL-8* and *COX-2*. The anti-inflammatory properties of peptide depend on physicochemical properties, including physiological pH and type and number of aromatic amino acids present in the peptide (Nankar and Pande, 2014). The release TNF-α involved with induction of COX-2 and PGE2 synthesis (Tsatsanis *et al*., 2007). The extracts from *Taraxacum mongolicum* Hand.-Mazz. (TMHM) have efficiency to anti-inflammation via down-regulate *TNF-α*, *IL-6*, *IL-1β* (Yang *et al*., 2016). Fucoidan shown significantly inhibited the level of *iNOS*, *TNF-α*, *IL-6* and *COX-2* expression in RAW 264.7 macrophage cells (Park *et al*., 2017b). Flavonoids such as isorhamnetine, quercetin and kaempferol suppressed LPS-induced *iNOS* expression and NO production in macrophage cells (Hämäläinen *et al*., 2007).
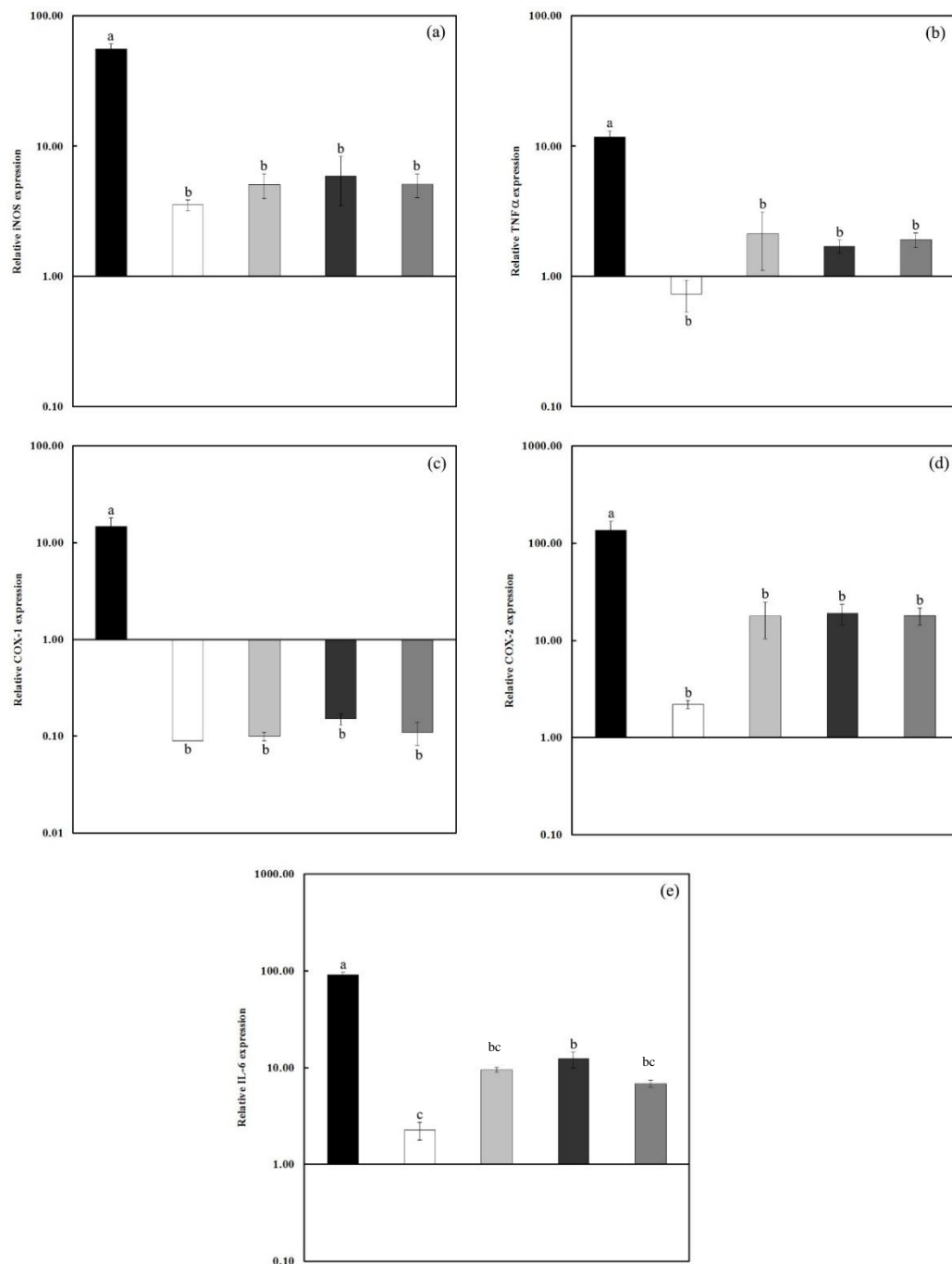
Figure 4.6 Effect of the F_2 on gene expression levels of (a) *iNOS*, (b) *TNF-α*, (c) *COX-1*, (d) *COX-2* and (e) *IL-6* in RAW264.7 macrophage cells Black: LPS; White: LPS + budesonide (positive control); Light gray: LPS + F_2 (7.5 µg protein/mL); Dark gray: LPS + F_2 (3.75 µg protein/mL); Gray: LPS + F_2 (1.88 µg protein/mL). [a-b] Values with the same letters indicate no significant different for each group of fraction samples in Duncan test ($p \leq 0.05$)

4.8 Determination of the antiproliferative activity

    4.8.1 Cytotoxicity assay for human malignant cell lines

    To investigate the cytotoxicity of the F_2 fraction, human normal Wi38 cells were compared to human malignant cell lines, namely, BT474 (breast), Chago-K1 (lung), Hep-G2 (hepatoma), KATO-III (gastric) and SW620 (colon), using an MTT assay. The cells were treated with F_2 at different concentrations for 72 h. The result shows that F_2 exhibited cytotoxic activity at various concentrations in the cell lines as follows: 538.33 ± 3.84 μg protein/mL (Wi38), 155.13 ± 6.17 μg protein/mL (BT474), 164.70 ± 10.70 μg protein/mL (Chago-K1), 166.83 ± 19.63 μg protein/mL (Hep-G2), 270.05 ± 26.46 μg protein/mL (KATO-III) and 106.58 ± 21.46 μg protein/mL (SW620). The results are shown in Figure 4.7. These results suggested that the F_2 fraction has antiproliferative activity. Importantly, F_2 did not have cytotoxic effects in the normal Wi38 cells. Moreover, several studies have reported that several purified peptides exhibit antiproliferative activity in cancer cell lines (Shrivastava *et al.*, 2015; Umayaparvathi *et al.*, 2014) as well as antihypertensive, antiangiogenic and antiobesity effects (Kannan *et al.*, 2008). Because the IC_{50} value of SW620 was the lowest among all the tested cancer cell lines, we used SW620 cells for the determination of apoptosis in subsequent experiments.
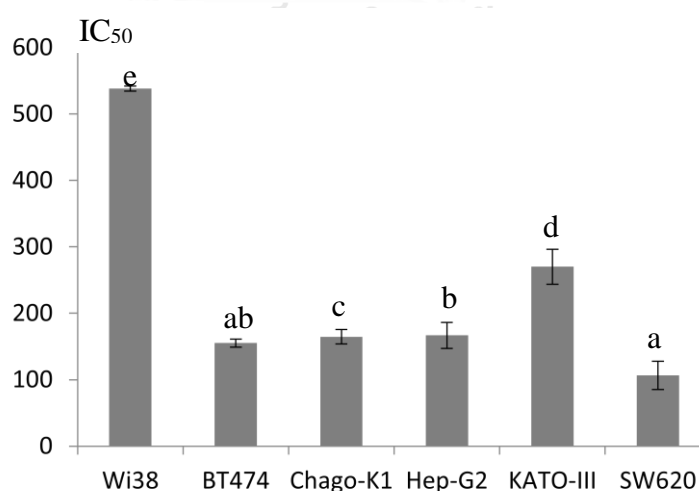
Figure 4.7 Cytotoxicity effects of purified peptide F_2 on five cancer cell lines. All the data are given as mean $\pm$ standard error of the triplicate data. [a-e] Values with the same letters indicate no significant different for each group of fraction samples in Duncan test ($p \leq 0.05$)

4.8.2 Apoptosis

Morphology of the colon cancer cell line SW620 for testing apoptosis is shown in Figure 4.8. This epithelial cell line is received from human.

(A) (B)
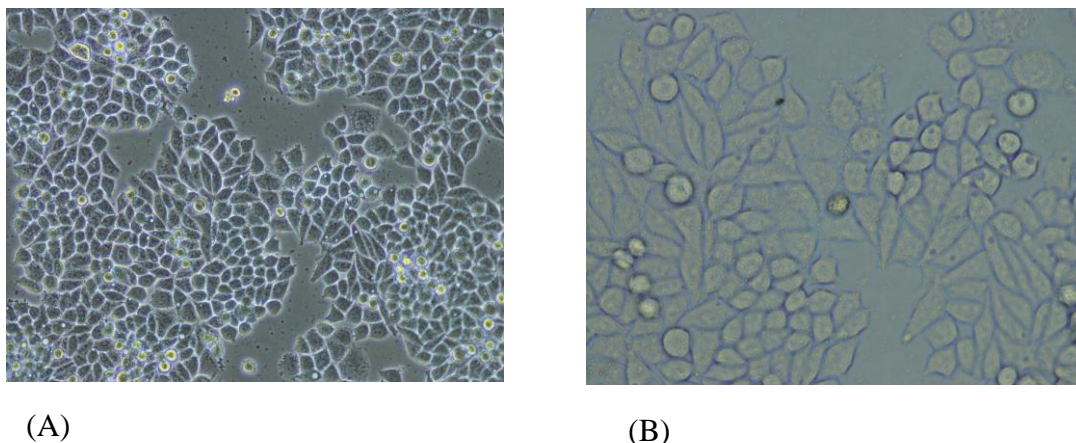
Figure 4.8 Morphology of SW620 (colon) cancer cell lines (A) Cell as observed under microscope with objective x20 (B) Cell as observed under microscope with objective x40

Apoptosis or programmed cell death results from cell damage. Cell apoptosis was measured by flow cytometry using an Annexin V-FITC/PI detection kit. Annexin V labels phosphatidylserine externalization on the outer plasma membranes, which occurs in early apoptotic cells, and PI staining of DNA indicates cell membrane deficiency and quantitates cellular DNA content. Apoptosis and necrosis were monitored using quadrant statistics for the various states as follows: viable (Annexin-/PI-), early apoptotic (Annexin+/PI-), late apoptotic (Annexin+/PI+) and necrotic (Annexin-/PI+). The SW620 cells were treated with the F_2 fraction (IC_{50} value of 23.06 µg protein/mL) to detect apoptosis, as shown in Figure 4.9, 4.10 and 4.11 The SW620 cells treated with 23.06 µg protein/mL peptide showed increasing percentages of early apoptotic cells over time, with values of 31.99%, 45.65% and 52.31% at 24, 48 and 72 h, respectively. Moreover, the percentages of late apoptosis remained constant over time, with values of 46.99%, 48.46% and 46.59% at 24, 48 and 72 h, respectively. This research was used doxorubicin with concentration of 0.5 µg/mL because it is an optimum concentration to test for appear early apoptosis and late apoptosis. The concentration of doxorubicin with more than 0.5 µg/mL can induce cell line to all

necrosis which can not to see early apoptosis and late apoptosis. The concentration of doxorubicin with less than 0.5 µg/mL was not clear to see early apoptosis, late apoptosis and necrosis.

Early apoptosis and total apoptosis were investigated, as shown in Figure 4.12 and Figure 4.13. The percentage of early apoptosis after F_2 treatment for 48 h was similar to that at 72 h but was higher than that at 24 h. The percentage of total apoptosis was likely affected the most by early apoptosis due to intrinsic inducers of apoptosis, even in control cells. This result indicated that the control showed apoptotic cells because apoptosis was induced by intrinsic inducers. The cells were under stress conditions, such as heat, chemotherapeutic agents, oxidative stress, irradiation and nutrient deficiency, which serve as stimuli to induce the apoptotic process (Degterev *et al.*, 2003). Apoptotic cell death occurs through two pathways, namely, an intrinsic pathway and an extrinsic pathway. The intrinsic pathway or mitochondrial pathway is initiated by the upregulation of wild-type p53 and involves the transcriptional or post-transcriptional regulation of Bcl-2 proteins, and cytochrome c is released from mitochondrial intermembrane spaces, thereby activating executioner caspases. The extrinsic pathway or cell surface death receptor pathway is activated by death receptor ligation, adaptor recruitment, procaspase-8 recruitment, caspase-8 activation and activation of executioner caspases. Additionally, the intrinsic and extrinsic pathways are linked via Bid cleavage (Chipuk and Green, 2005; Shangguan *et al.*, 2014). In addition, necrosis is another cell death process. Apoptosis eliminates cells during development and homeostasis in tissue. Furthermore, apoptosis is important for the disposal of cancer cells that are damaged. Apoptosis is a necessary process in cancer cells (Evan and Vousden, 2001; Muppidi *et al.*, 2004; Norbury and Hickson, 2001).

This study suggested that the F_2 fraction from *Synechococcus* sp. cells induces the apoptotic pathway in SW620 colon cancer cells. Several studies have concluded that purified peptides induce apoptosis as measured by flow cytometry (Sui *et al.*, 2016). The anticancer effect of *Angelica dahurica* extract has been determined in HT-29 colon cancer cell lines according to flow cytometry analysis of apoptotic cells, with increasing concentrations of *A. dahurica* extract resulting in increasing percentages of early apoptosis and total apoptosis after treatment for 48 h (Zheng *et al.*, 2016). Furthermore, protein hydrolysate from clam muscle blood exhibits anticancer effects in

PC-3 prostate cancer cell lines, with 3 mg/mL showing more activity than 2 and 2.5 mg/mL after a 24 h treatment, and flow cytometry analysis of these cells indicates early apoptosis and late apoptosis percentages of 20.28% and 21.77%, respectively (Chi *et al.*, 2015).
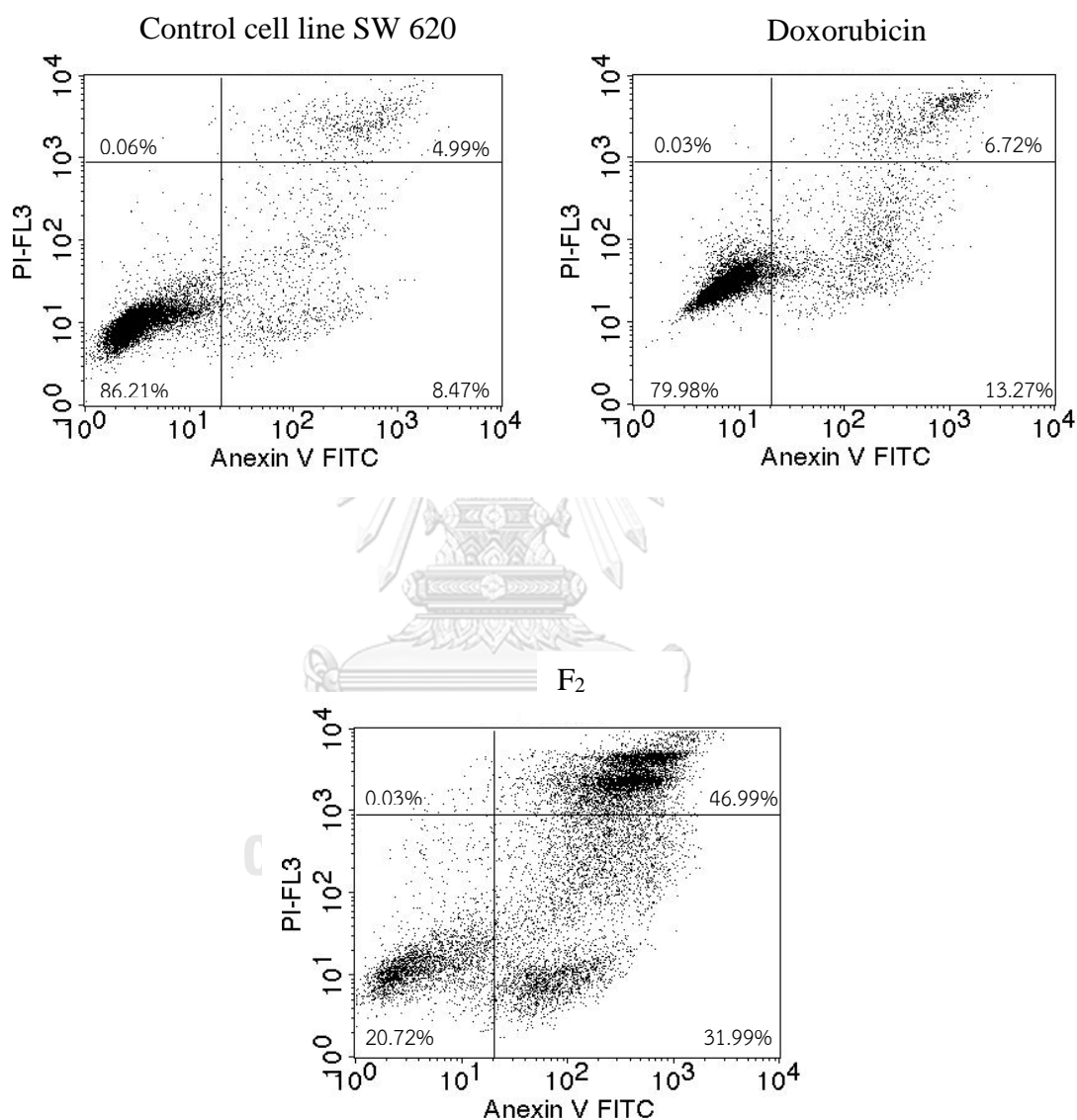
Figure 4.9 The apoptosis rate of SW 620 cells was induced by purified peptide F_2 (IC_{50} 23.06 µg protein/mL) at 24 h and analyzed by flow cytometric analysis (upper right quadrant refers to late apoptotic cells, upper left quadrant refers to necrotic cells, lower left quadrant refers to viable cells and lower right quadrant refers to early apoptotic cells)
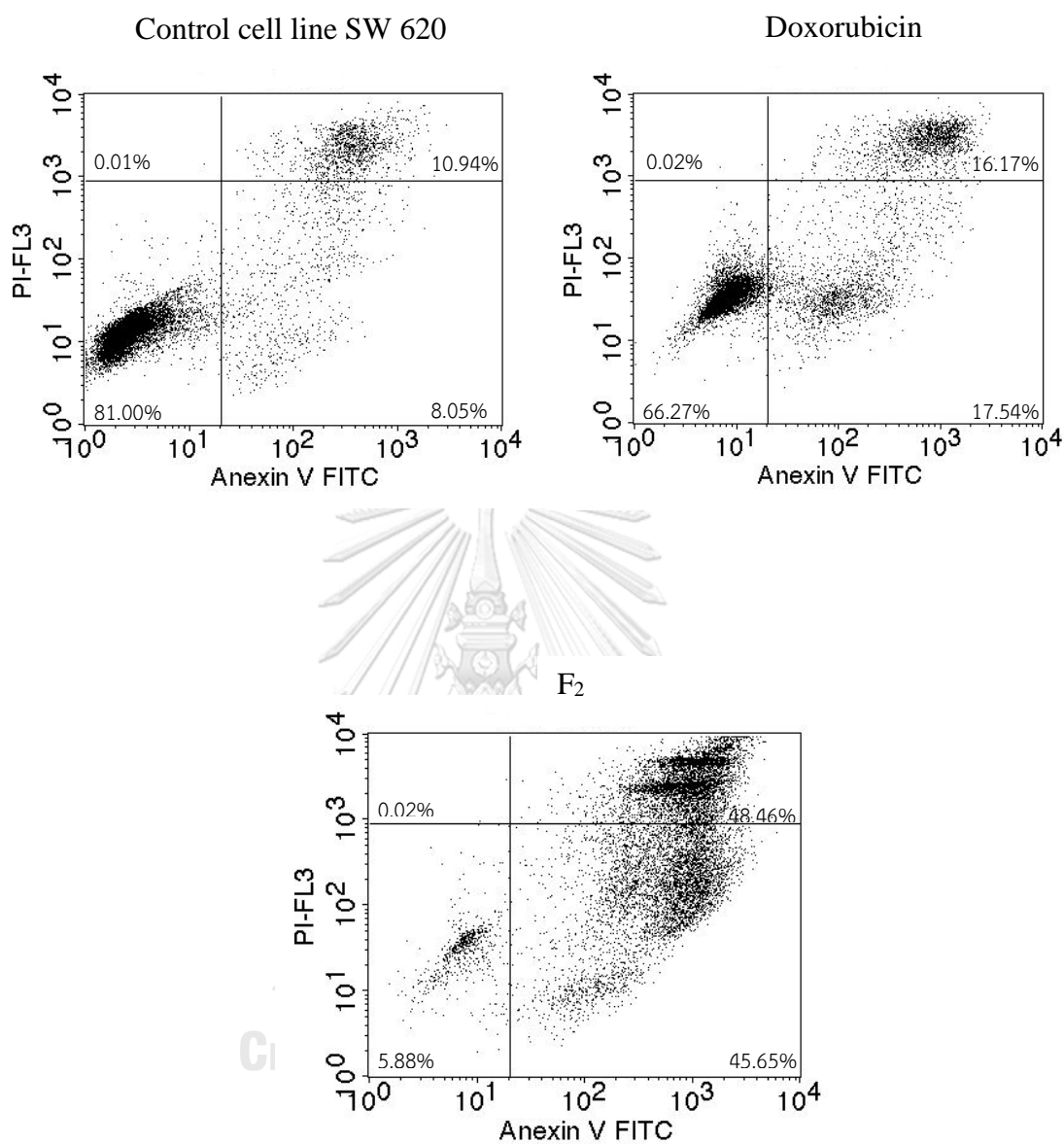
Figure 4.10 The apoptosis rate of SW 620 cells was induced by purified peptide F_2 (IC_{50} 23.06 μg protein/mL) at 48 h and analyzed by flow cytometric analysis (upper right quadrant refers to late apoptotic cells, upper left quadrant refers to necrotic cells, lower left quadrant refers to viable cells and lower right quadrant refers to early apoptotic cells)
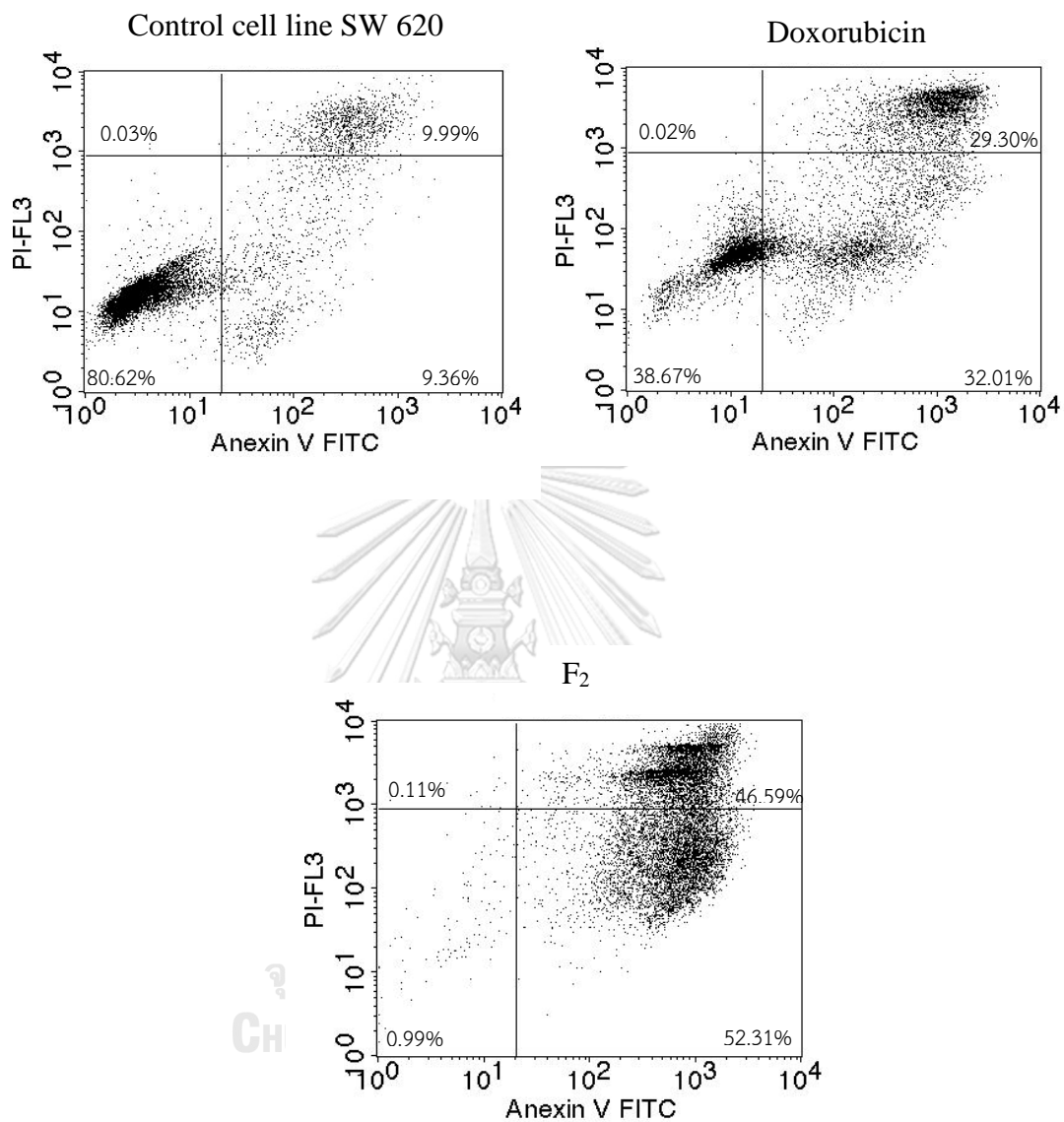
Figure 4.11 The apoptosis rate of SW 620 cells was induced by purified peptide F_2 (IC_{50} 23.06 µg protein/mL) at 72 h and analyzed by flow cytometric analysis (upper right quadrant refers to late apoptotic cells, upper left quadrant refers to necrotic cells, lower left quadrant refers to viable cells and lower right quadrant refers to early apoptotic cells)
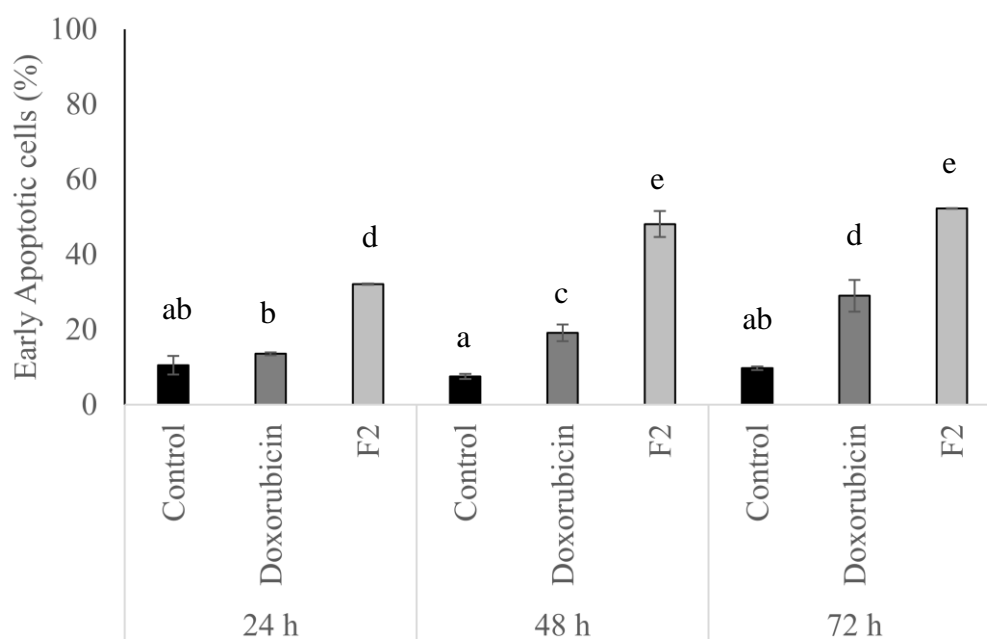
Figure 4.12 The percentage of the early apoptotic SW620 cells was analyzed by flow cytometry. Data are the mean ± standard error of the triplicates. [a-e] Values with the same letters indicate no significant difference for each group of fraction samples according to Duncan's test ($p < 0.05$)
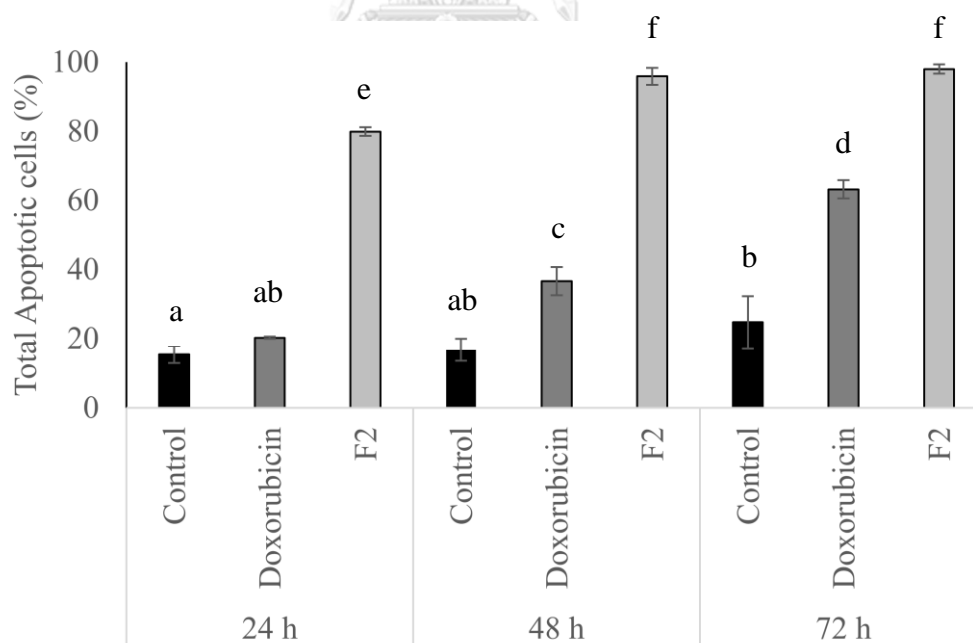
Figure 4.13 The percentage of the total apoptotic SW620 cells was analyzed by flow cytometry. Data are the mean ± standard error of the triplicates. [a-e] Values with the same letters indicate no significant difference for each group of fraction samples according to Duncan's test ($p < 0.05$)

Doxorubicin is an anthracycline which react by blocking topoisomerase II activity. This agent can act of disrupt DNA replication and transcription. Doxorubicin can transport into cell membrane by passive diffusion (Speelmans *et al*., 1996; Thirumaran *et al*., 2007; Thorn *et al*., 2011). The transport of peptides into cells has been reported. Peptides can transport into cells through an endocytic meachanism by directly penetrating the cell membrane as shown in Figure 4.14. The mechanism of transportation has depend on concentration, type and the cargo of peptide (Ramsey and Flynn, 2015).
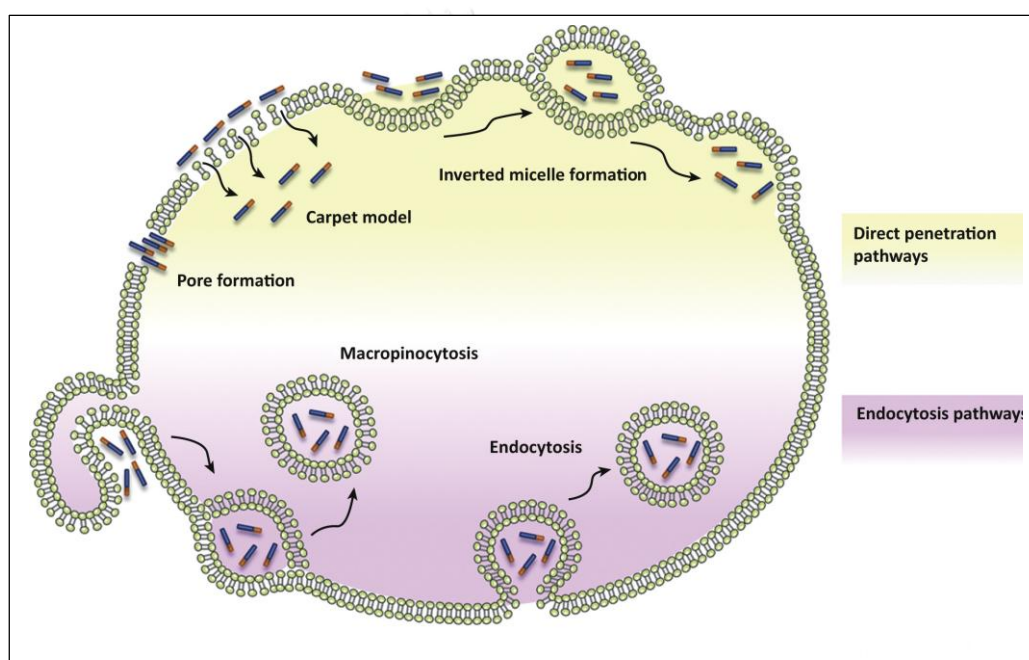
Figure 4.14 The transport of peptide into the cell membrane

(Guidotti *et al*., 2017)

4.8.3 Caspase 3, caspase 8 and caspase 9 activity

The F_2 fraction at an IC_{50} value of 23.06 µg protein/mL was used to treat the SW620 cells for 24, 48 and 72 h to investigate caspase 3, 8 and 9 activities. Caspase 3, 8 and 9 activities were determined with colorimetric assays (Table 4.8). F_2 induced the highest activities of caspases 3, 8 and 9 at 72 h, implying that F_2 induced apoptosis via caspases 3, 8 and 9. Moreover, caspase 3, 8 and 9 activities progressively increased from 24 h to 72 h. Thus, these findings suggested that F_2 can activate both the intrinsic

and extrinsic pathways of apoptosis. The intrinsic pathway is implicated by caspase 9 activity, which was detected at every time points, with the highest activity exhibited at 72 h. Stress conditions activate caspase 9 in cancer cell lines (Degterev et al., 2003). F_2 induces caspase 9 via the mitochondrial pathway (Shangguan et al., 2014). The extrinsic pathway is implicated by caspase 3 and 8 activities. The results demonstrated that F_2 induced caspase 3 and 8 activities, suggesting that the death receptor-mediated apoptotic pathway is involved in this process (Park et al., 2017a). Caspases 3 and 8 are important enzymes that control programmed cell death (Wu et al., 2016). Caspase 3 is a central caspase, and it plays a key role in the apoptosis pathway and is used to investigate apoptosis (Ichikawa et al., 2012). Apoptotic pathways involving caspases 3, 8 and 9 in mammalian cells indicate the involvement of both extrinsic and intrinsic apoptotic pathways. Caspases 3 and 8 are key enzymes in the extrinsic pathway, but caspase 9 is an important enzyme in the intrinsic pathway (Shrivastava et al., 2015; Zheng et al., 2016).

This study suggests that F_2 can activate apoptotic pathways, both the intrinsic pathway and the extrinsic pathway. The intrinsic pathway was involved with caspase 9, which was detected in all of the groups, particularly at 72 h, showing the highest activity of caspase 9. This result may be due to stress condition effects that activate caspase 9. Moreover, F_2 has the ability to induce caspase 9 via the mitochondrial pathway. Notably, the activities of caspases 3 and 8 were found to involve the extrinsic pathway. Chen et al. reported that baicalin (200 µM) induces apoptosis in SW620 human colorectal carcinoma cells as indicated by increased activities of caspases 3, 8 and 9 as well as suppressed SW620 cell growth (Chen et al., 2012a). Colon cancer cell (HCT-116) apoptosis is induced by the Dae-Hwang-Mok-Dan-Tang (DHMDT) extract (increasing concentrations of 0 to 1 mg/mL), which is a traditional Korean medicine, via both the intrinsic and extrinsic pathways as measured by colorimetric caspase 3, 8 and 9 activity assays (Park et al., 2017a). The activation of caspases 3, 8 and 9 in the apoptotic process in MG-63 human osteosarcoma cells treated with ginsenoside Rf for 24 h was investigated, and they showed that ginsenoside Rf increases the activities of caspases 3 and 9 (Shangguan et al., 2014). Moreover, it have been report that caspases are a family of cysteine proteases involved in apoptosis via activation by cleavage at the post translational level. Apoptosis is induced by both intrinsic and extrinsic

pathways. Caspases 3 and 8 are activated via the extrinsic pathway which caspase 8 will be activated and it is now able to directly activate caspase 3 to apoptotic cell. Caspase 8 can cleave Bid protein. Truncated Bid protein (tBid) translocates to mitochondria and induce cytochrome c for promote cell death. In the intrinsic pathway, cytochrome c is released and forms the apoptosome, which causes the cleavage of caspase 9 and the activation of caspase 3 send to the has a degradation. Intrinsic and extrinsic pathway have connection at Bid protein as shown in Figure 2.12. Our findings indicated that the purified F_2 peptide activates apoptosis via both the intrinsic and extrinsic pathways (Jin *et al.*, 2015).

Table 4.8 Caspase 3, caspase 8 and caspase 9 activities in SW 620 cells

Treatment	Activity (µmol pNA/min/mL)		
	Caspase 3	**Caspase 8**	**Caspase 9**
Control	$0.10 \times 10^5 \pm 0.07 \times 10^{5\,c}$	$0.60 \times 10^5 \pm 0.22 \times 10^{5\,c}$	$14.21 \times 10^5 \pm 4.34 \times 10^{5\,d}$
F_2 fraction for 24 h	$0.30 \times 10^5 \pm 0.19 \times 10^{5\,c}$	$0.70 \times 10^5 \pm 0.44 \times 10^{5\,c}$	$18.00 \times 10^5 \pm 1.64 \times 10^{5\,c}$
F_2 fraction for 48 h	$1.00 \times 10^5 \pm 0.07 \times 10^{5\,b}$	$5.00 \times 10^5 \pm 0.68 \times 10^{5\,b}$	$105.16 \times 10^5 \pm 9.14 \times 10^{5\,b}$
F_2 fraction for 72 h	$1.60 \times 10^5 \pm 0.07 \times 10^{5\,a}$	$6.20 \times 10^5 \pm 0.66 \times 10^{5\,a}$	$161.05 \times 10^5 \pm 17.29 \times 10^{5\,a}$

All the data are given as the mean ± standard error of the triplicates. Different letters indicate significant differences among the groups according to Duncan's test ($p \leq 0.05$). [a-d] Values with the same letters indicate no significant difference for each group of fraction samples according to Duncan's test ($p \leq 0.05$).

CHAPTER V
CONCLUSION

The results of the present study showed that *Synechococcus* sp. cells cultured for 21 days and hydrolyzed with trypsin had antioxidant anti-inflammation and anticancer properties. The purification by ultrafiltration shown that the MW < 3 kDa fraction had the highest ABTS, DPPH and NO radical scavenging activities when compare with all the fractions. The MW < 3 kDa fraction was further separated by gel filtration into two sub-fractions (F_1 and F_2). The F_2 fraction had the strongest ABTS and NO radical scavenging activity. The F_2 fraction was purified by reversed-phase HPLC to yield four fractions. The 30 – 40 min sub-fraction (F_{2-4}) had the highest ABTS and NO radical scavenging activity. The F_{2-4} fraction was selected for further analysis by mass spectrometry. Five isolated peptides with amino acid sequence namely, AILQSYSAGKTK, ALNKTHLIQTK, LLVHAPVK, IPDAHPVK and VVVLRDGAVQQLGTPR were identified. The F_{2-4} fraction had higher DPPH and NO radical scavenging activity compared to the synthetic peptide while the synthetic peptide of AILQSYSAGKTK had the highest ABTS radical scavenging activity. The protective abilities of the F_2 fraction in DNA damage were tested. The increased concentration of the F_2 fraction was enhanced ability of protection on oxidation-induced DNA damage when test with pBR322, pKS and pUC19 plasmid DNA. Moreover, our findings indicated that F_2 fraction exhibited anti-inflammatory properties because gene expression levels of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophage cells were reduced. Furthermore, F_2 fraction at an IC_{50} value of 23.06 µg protein/mL induces the apoptotic pathway in SW620 colon cancer cells after treatment for 24, 48 and 72 h and the apoptotic pathway in SW620 cells involved caspases 3, 8 and 9. This is a new research with involve application of *Synechococcus* sp. cells in term of antioxidant. The advantage of cell cyanobacteria is can easy to grow in a medium. Hence, cultivation of cell cyanobacteria give the mass of cells more than cultivation of other organisms such as sponge and plant and this research is a development to use *Synechococcus* sp. cells for value added. In addition, these findings demonstrated that these peptides might be used for new natural antioxidant agents and antitumor drugs in the future. Further studies have to be made on determination of the

F_2 fraction to test the ability of anti-inflammation in vivo and the production these peptide can be use genetic engineering which produce in a large scale by fermenter.

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APPENDIX

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APPENDIX A
MEDIUM AND REAGENTS PREPARATION

1. BG11 Turks Island salt solution medium

1.1 Stock A (10x)

KCl	6.66 g
$MgCl_2.6H_2O$	55 g
$CaCl_2.2H_2O$	14.66 g

Adjusted volume to 1 L with deionized water

1.2 Stock B (10x)

$MgSO_4.7H_2O$	74.8 g

Adjusted volume to 1 L with deionized water

1.3 Stock BG11 (100x)

$NaNO_3$	149.5 g
$MgSO_4.7H_2O$	7.48 g
$CaCl_2.2H_2O$	3.6 g
Citric acid	0.6 g
Na_2EDTA	0.104 g

Adjusted volume to 1 L with deionized water

1.4 Trace element (1000x)

H_3BO_3	2.86 g
$MnCl_2.4H_2O$	1.81 g
$ZnSO_4.7H_2O$	0.22 g
$NaMoO_4.2H_2O$	0.39 g
$CuSO_4.5H_2O$	0.079 g
$Co(NO_3)_2.6H_2O$	0.0494 g
Na_2CO_3	20 g
KH_2PO_4	30.5 g
$(NH_4)_5[Fe(C_6H_4O_7)_2]$	6 g

Adjusted volume to 1 L with deionized water

Mixed 10 mL of BG 11, 1 mL of trace element, 100 mL of stock A, 100 mL of stock B and 29.22 g of NaCl after that adjusted pH to 7.6 and adjusted volume to 1 L with deionized water then autoclave at 121 ºC, 15 min.

2. 20 mM Phosphate buffer

KH_2PO_4	2.72 g
K_2HPO_4	3.48 g

Adjusted volume to 1 L with deionized water and adjusted pH to 7.2 with 1M KOH

3. Bradford solution and protocal

3.1 Bradford stock solution

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

3.2 Bradford working buffer

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

4. DPPH solution

0.1 M DPPH	0.004 mg
Methanol	100 mL

5. ABTS solution

5.1 7 mM ABTS (solution A)

ABTS	0.096 g

Dissolve in 25 mL deionized water.

5.2 2.45 mM potassium persulphate (solution B)

Potassium persulphate	0.016 g

Dissolve in 25 mL deionized water

5.3 ABTS solution

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

6. Solution for nitric oxide radical scavenging assay

   6.1 10 mM sodium nitroprusside (SNP) in PBS pH 7.2

     Sodium nitroprusside   0.29 g

     Dissolve in 100 mL Phosphate buffer pH 7.2

   6.2 0.33% (w/v) sulfanilamide in 20% acetic acid

     Sulfanilamide   0.33 g

     Dissolve in 100 mL 20% acetic acid (20 mL acetic in 80 mL distilled water).

   6.3 0.1% (w/v) N-(1-Napthyl) ethylenediamine dihydrochloride (NED)

     NED   0.1 g

     Dissolve in 100mL deionized water.

7. Mobile phase in RP-HPLC analysis

   7.1 Eluent A: 0.1% trifluoroacetic acid (TFA), 1000 mL

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

   7.2 Eluent B: 70 % acetonitrile containing 0.05% TFA, 1000 mL

     Adding 300 mL 0.05% TFA in double deionized water into 700 mL 70% acetonitrile and mixing followed by filtration using PTFE membrane.

8. MTT solution (5mg/mL)

     MTT   5 mg

     Dissolve in 1 mL deionized water.

9. LB Broth for *E. coli*

     Peptone   1 g

     Yeast extract   0.5 g

     NaCl   1 g

     Dissolve to 100 mL with deionized water then sterilization at 121°C, 15 min.

10. LB agar for *E. coli*

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

Dissolve to 100 mL with deionized water then sterilization at 121°C, 15 min.

11. DNA damage

11.1 2mM FeSO₄

$FeSO_4 \cdot 7H_2O$	0.0278 g

Dissolve in 50 mL deionized water.

11.2 30% H₂O₂

Protocal: 1. Pipet DNA plasmid into PCR tube at 3 μl.

2. Add 4 μl of protein and incubate for 20 min at room temperature.

3. Add 3 μl of 2mM FeSO₄

4. Add 3 μl of 30% H2O2 and mix solution then incubate at 37°C for 30 min

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

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APPENDIX B

STANDARD CURVE

Standard curve for determine the protein concentration by Bradford method

y = 1.5118x
R² = 0.9956
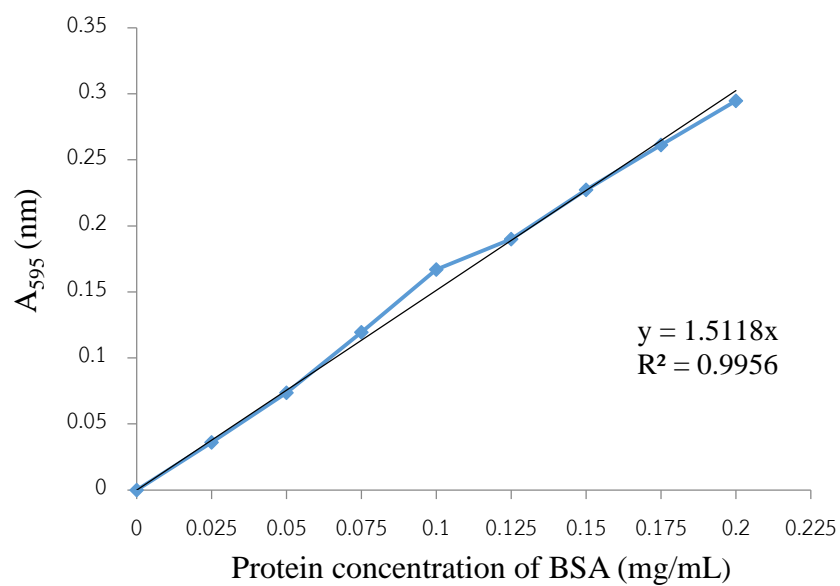
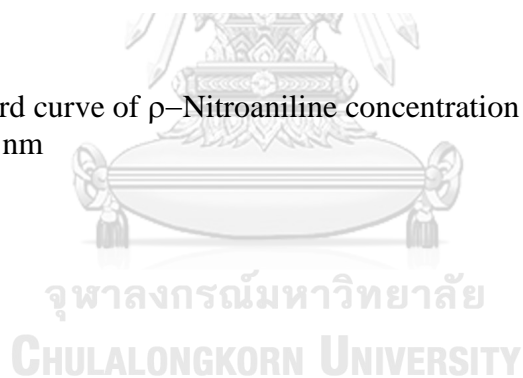**Figure B 1** Standard curve of BSA concentration at the absorbance of 595 nm

Standard curve for determine the ρ–Nitroaniline concentration for caspase 3 and caspase 8

$y = 88.112x$
$R^2 = 0.9997$
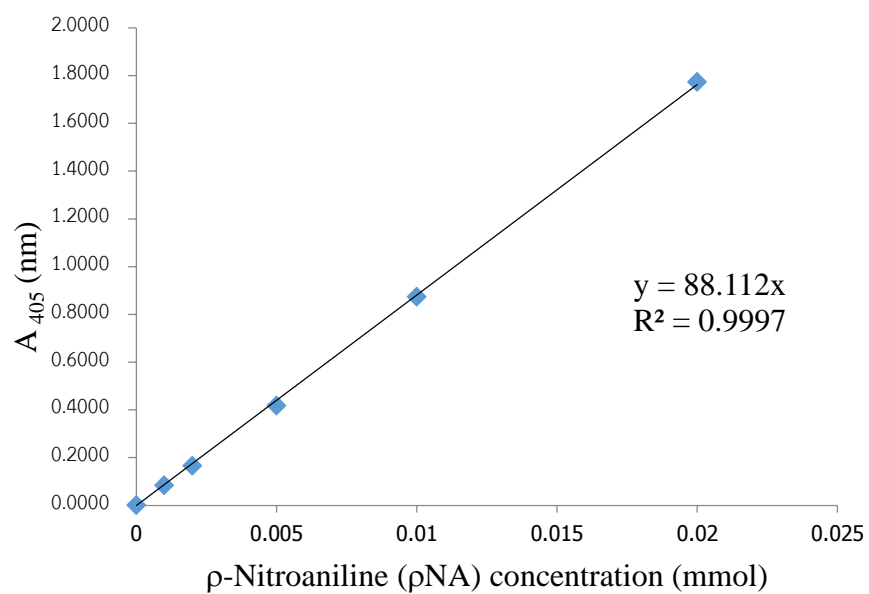
Figure B 2 Standard curve of ρ–Nitroaniline concentration for caspase 3 and 8 at the absorbance of 405 nm

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Standard curve for determine the ρ–Nitroaniline concentration for caspase 9

$$y = 2.9321x$$
$$R^2 = 0.9891$$
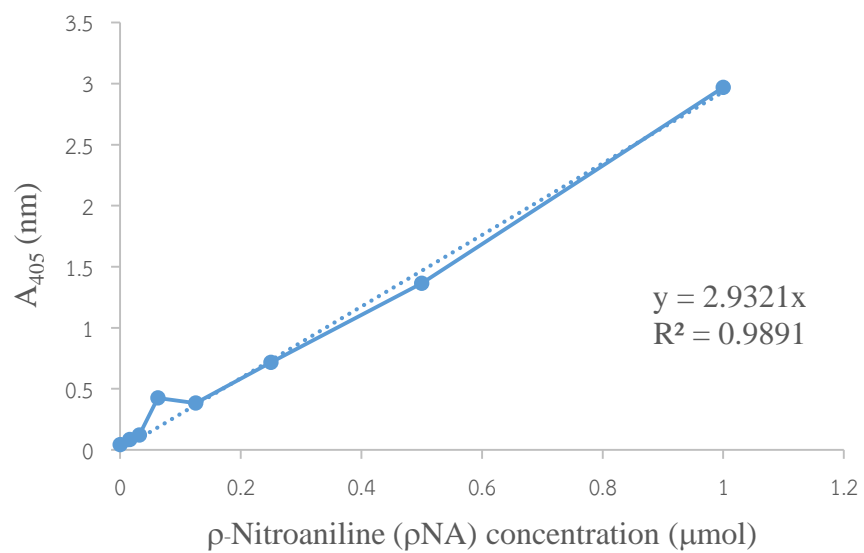
Figure B 3 Standard curve of ρ-Nitroaniline concentration for caspase 9 at the absorbance of 405 nm

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	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	v

APPENDIX D

MASS SPECTRUM ANALYSIS

MS/MS fragmentation of AILQSYSAGKTK

Found in gi[514355063 in NCBInr,
hydroxymethylpyrimidine/phosphomethylpyrimidine kinase [*Leptospira wolffii*]
Match to query 179: 1264.862472 from(422.628100.3+) intensity(36120.0000)
index(108)
Title: Cmpd 109. +MSn(422.6281). 24.8 min
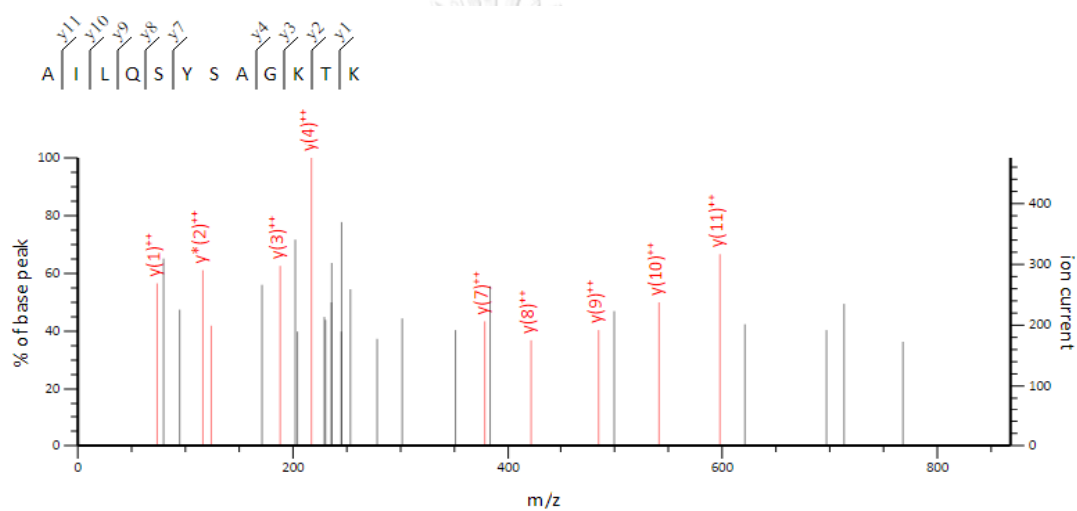
MS/MS fragmentation of ALNKTHLIQTK
Found in gi[654614087 in NCBInr, hypothetical protein [*Solobacterium mooreo*]
Match to query 179: 1264.862472 from(422.628100.3+) intensity(36120.0000)
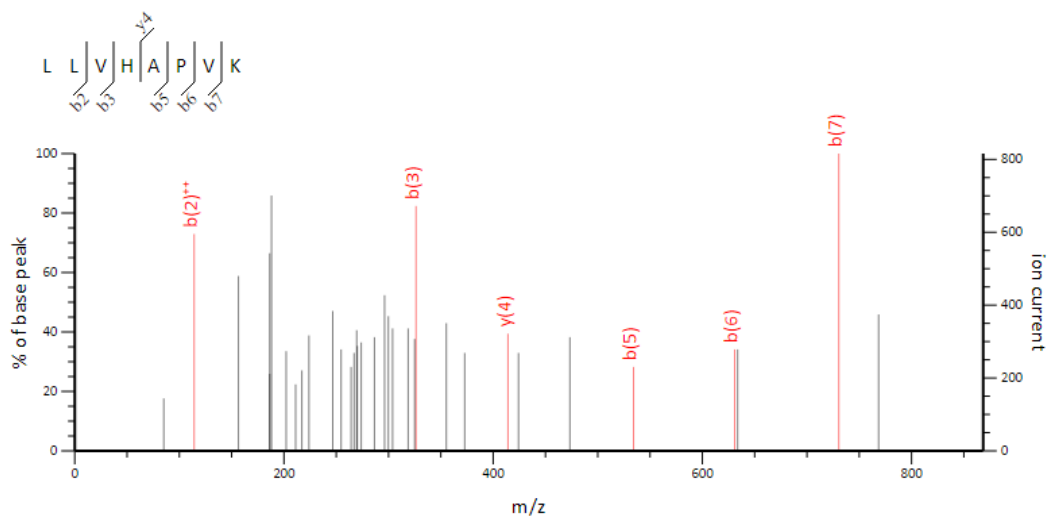index(108)
Title: Cmpd 109. +MSn(422.6281). 24.8 min
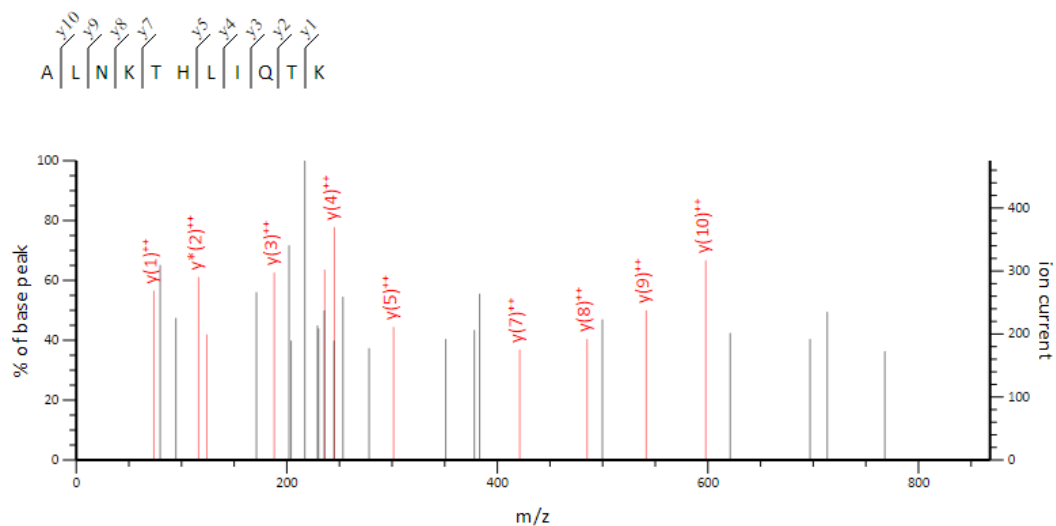
MS/MS fragmentation of LLVHAPVK
Found in gi[504936754 in NCBInr, permease [*Synechococcus* sp. PCC 6312]
Match to query 83: 874.571348 from(438.292950.2+) intensity(2728.0000)
index(136)
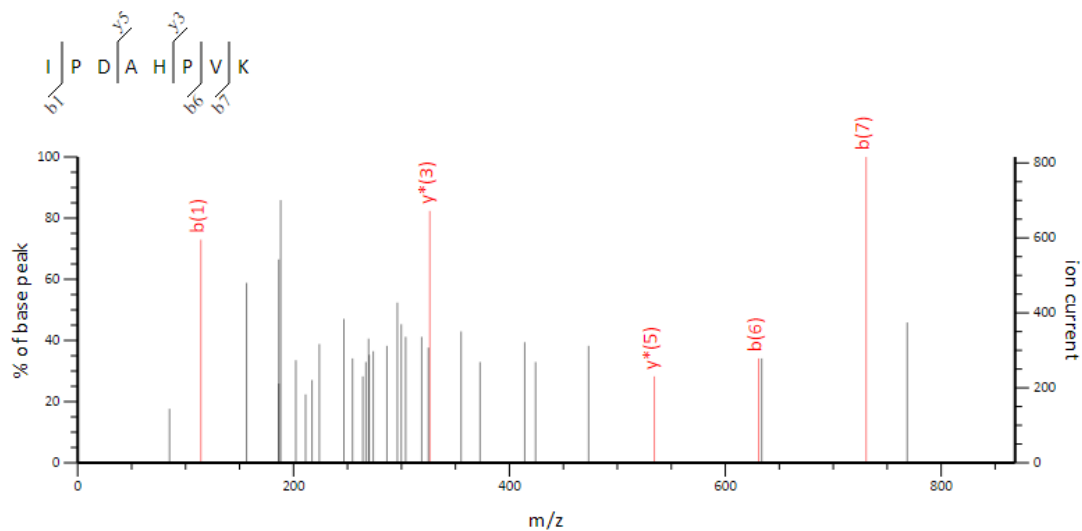Title: Cmpd 137. +MSn(438.2929). 26.8 min

MS/MS fragmentation of IPDAHPVK

Found in gi[701253099 in NCBInr, DNA (cytosine-5-)-methyltransferase family protein [*Burkholderia pseudomallei* MSHR 3965]

Match to query 83: 874.571348 from(438.292950.2+) intensity(2728.0000) index(136)
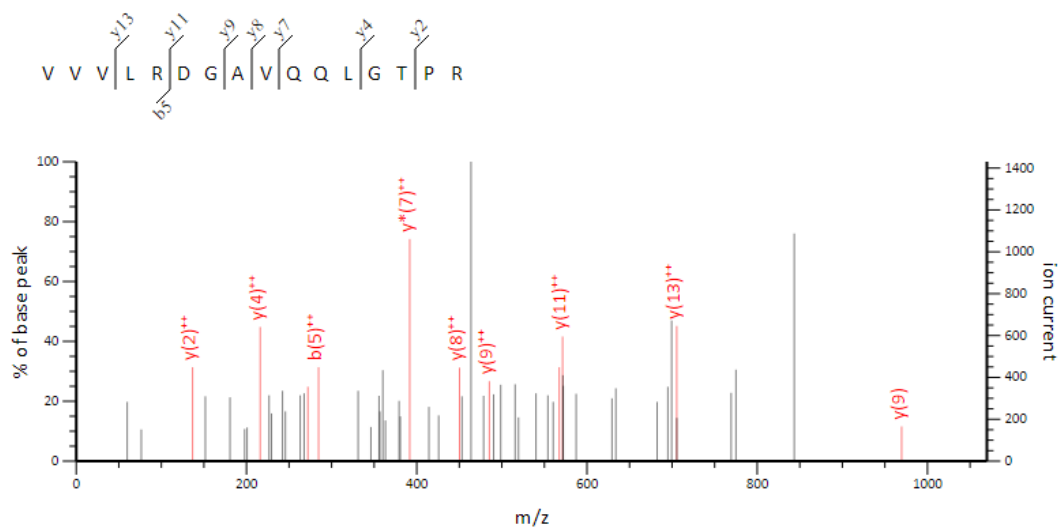
Title: Cmpd 137. +MSn(438.2929). 26.8 min

MS/MS fragmentation of VVVLRDGAVQQLGTPR
Found in gi[868626300 in NCBInr, spermidine/putrescine import ATP-binding
protein PotA [*Marinobacter subterrani*]
Match to query 238: 1706.135412 from(569.719080.3+) intensity(10432.0000)
index(74)
Title: Cmpd 75. +MSn(569.7191). 22.1 min

VITA

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Academic presentations;

1. Suttisuwan, R., Phunpruch, S., Thongchul, N., Sangtanoo, P. and Kanchanatat, A. 2016. Purification and Identification of antioxidant peptides from trypsin hydrolysates of microalgae Synechococcus sp. VDW protein. The 5th International Biochemistry and Molecular Biology Conference: Biochemistry for a sustainable future, 26-27th May 2016, B.P. Samila Beach Hotel, Songkhla, Thailand.