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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

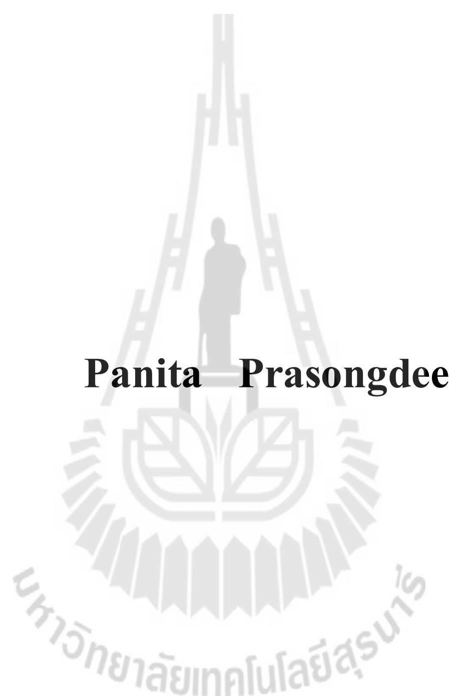
สาขาวิชาเทคโนโลยีอาหาร

มหาวิทยาลัยเทคโนโลยีสุรนารี

ปีการศึกษา 2558

EXTRACTION AND BIOACTIVITIES OF THAI BASIL

(*Ocimum basilicum* L.)



Panita Prasongdee

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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Academic Year 2015

EXTRACTION AND BIOACTIVITIES OF THAI BASIL

(*Ocimum basilicum* L.)

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ปณิตา ประสงค์ดี : การสกัดและฤทธิ์ทางชีวภาพของโหระพาไทย (EXTRACTION AND BIOACTIVITIES OF THAI BASIL (*Ocimum basilicum* L.)) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.รัชฎาพร อุ่นศิวิไลย์, 110 หน้า.

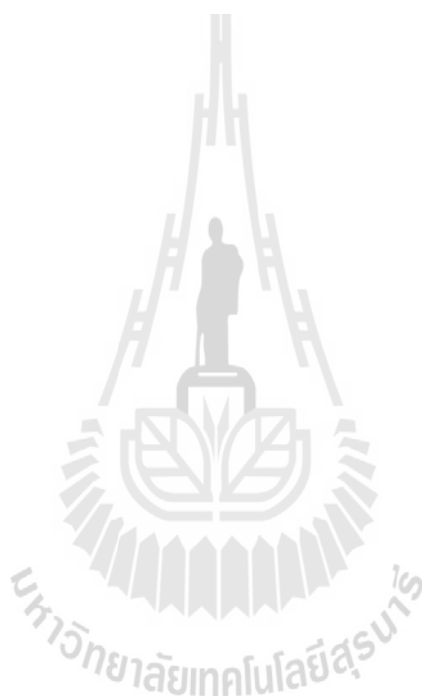
โหระพาไทย (*Ocimum basilicum*) สายพันธุ์ *thyrsiflorum* และ Jumbo 4320 ได้ถูกนำมาสกัดด้วยน้ำ เอทานอล และเอทิลอะซิเตท จากการตรวจสอบสารพฤกษเคมีของสารสกัดโหระพาไทยทั้งสองสายพันธุ์ พบว่า สารสกัดโหระพาไทยมีสารพฤกษเคมีคือ คลอโรฟิลล์ แคโรทีนอยด์ และสารประกอบฟีนอลิก ผลการวิเคราะห์โดยวิธี HPLC พบว่า สารสกัดเอทานอล และสารสกัดเอทิลอะซิเตท มีส่วนประกอบหลัก คือ คลอโรฟิลล์เอ คลอโรฟิลล์บี ฟีโอไฟตินเอ ฟีโอไฟตินบี และลูทีน สารสกัดเอทานอลสายพันธุ์ *thyrsiflorum* มีปริมาณคลอโรฟิลล์ และลูทีนสูงสุด กรดฟีนอลิก และฟลาโวนอยด์ที่พบคือ กรดกาลิค คาเทชิน อะพีจีนิน กรดคาเฟอิก กรดคูมาริค และกรดซินแนปปิก จากการวิเคราะห์ปริมาณสาร ฟีนอลิกทั้งหมดโดยวิธี Folin-Ciocalteu พบว่า สารสกัดน้ำสายพันธุ์ Jumbo 4320 มีปริมาณฟีนอลิกทั้งหมดสูงสุดที่ 4,596.19±3.07 ไมโครกรัมกาลิก/กรัมวัตถุดิบ ตามด้วยสารสกัดเอทานอล และสารสกัดเอทิลอะซิเตท ตามลำดับ จากการศึกษาปริมาณสารฟลาโวนอยด์ทั้งหมด พบว่า สารสกัดเอทานอลสายพันธุ์ Jumbo 4320 มีปริมาณฟลาโวนอยด์ทั้งหมดสูงสุดที่ 5,571.16±14.27 ไมโครกรัมคาเทชิน/กรัมวัตถุดิบ ตามด้วยสารสกัดเอทิลอะซิเตท และสารสกัดน้ำ ตามลำดับ

ในส่วนของคุณสมบัติการเป็นสารต้านอนุมูลอิสระของสารสกัดโหระพาไทยซึ่งทำการวิเคราะห์ด้วยวิธี DPPH FRAP และ DCFH-DA พบว่า สารสกัดน้ำสายพันธุ์ Jumbo 4320 แสดงฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH สูงสุดที่ค่า IC_{50} 48.52±1.15 มิลลิกรัมวัตถุดิบ/มิลลิลิตร ตามด้วยสารสกัดเอทานอล และสารสกัดเอทิลอะซิเตท โดยสารต้านอนุมูลอิสระอ้างอิงมาตรฐาน BHT และกรดแอสคอร์บิก มีค่า IC_{50} 0.18 และ 0.06 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ อย่างไรก็ตาม สารสกัดเอทานอลสายพันธุ์ *thyrsiflorum* แสดงฤทธิ์ต้านอนุมูลอิสระด้วยวิธี FRAP สูงสุดที่ค่า 18.64±0.13 ไมโครโมล Fe^{2+}/กรัมวัตถุดิบ ตามด้วยสารสกัดน้ำ และสารสกัดเอทิลอะซิเตท สารสกัดน้ำสายพันธุ์ Jumbo 4320 แสดงฤทธิ์ต้านอนุมูลอิสระสูงสุดด้วยวิธี DCFH-DA ที่ความเข้มสัมพัทธ์ของสารเรืองแสง (relative fluorescence intensity) 80.62±0.00%

การวิเคราะห์คุณสมบัติต้านจุลินทรีย์ โดยวิธี agar disk diffusion พบว่า สารสกัดโหระพาเกือบทั้งหมดไม่มีผลต้านเชื้อจุลินทรีย์ก่อโรค มีเพียงเชื้อ *Bacillus cereus* ที่ถูกยับยั้งโดยสารสกัดเอทิลอะซิเตทสายพันธุ์ *thyrsiflorum* และสารสกัดน้ำสายพันธุ์ Jumbo 4320

การวิเคราะห์คุณสมบัติต้านลิ่มเลือดของสารสกัดโหระพาไทย โดยวิธี Prothrombin time พบว่า สารสกัดเอทิลอะซิเตท สายพันธุ์ *thyrsiflorum* แสดงการยืดระยะเวลาการแข็งตัวเป็นลิ่มของเลือดสูงสุดที่ 78.3±17.56 วินาที

กล่าวโดยสรุป สารสกัดโหระพาทั้งสองสายพันธุ์มีศักยภาพในการพัฒนาให้เป็นส่วนประกอบของอาหารเพื่อสุขภาพได้



สาขาวิชา เทคโนโลยีอาหาร ลายมือชื่อนักศึกษา________________________

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา__________________

PANITA PRASONGDEE : EXTRACTION AND BIOACTIVITIES OF THAI BASIL (*Ocimum basilicum* L.). THESIS ADVISOR : ASST. PROF. RATCHADAPORN OONSIVILAI, Ph.D., 110 PP.

THAI BASIL/PHYTOCHEMICAL/ANTIOXIDANT ACTIVITY/ANTIMICROBIAL ACTIVITY/ANTITHROMBOTIC ACTIVITY

Thai basils *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 were extracted by 3 solvents, namely water, ethanol and ethyl acetate. Phytochemical analyses of both species of Thai basil extracts revealed the presence of chlorophylls, carotenoids and phenolic compounds. HPLC analysis showed that ethanol and ethyl acetate extracts contained primarily chlorophyll *a*, chlorophyll *b*, pheophytin *a*, pheophytin *b* and lutein. *O. basilicum* var. *thyrsiflorum* ethanol extract showed the highest chlorophylls and lutein contents. The contents of the phenolic acids and flavonoids were found to be gallic acid, catechin, apigenin, caffeic acid, coumaric acid and sinapic acid. Results of total phenolic contents, evaluated by Folin-Ciocalteu method, revealed that *O. basilicum* cv. Jumbo 4320 water extract showed the highest total phenolic contents at 4,596.19±3.07 µg gallic acid equivalent/g of raw material (RM) followed by ethanol and ethyl acetate extracts. The study of total flavonoid contents revealed that *O. basilicum* cv. Jumbo 4320 ethanol extract showed the highest total flavonoid content at 5,571.16±14.27 µg catechin equivalent/g of RM followed by ethyl acetate and water extracts.

Antioxidant activities of Thai basil extracts were evaluated by DPPH, FRAP and DCFH-DA assays. *O. basilicum* cv. Jumbo 4320 water extract showed the highest

antioxidant activity by DPPH assay at IC_{50} 48.52±1.15 mg of RM/ml followed by ethanol and ethyl acetate extracts, whereas the antioxidant controls, BHT and ascorbic acid, showed IC_{50} at 0.18 and 0.06 mg/ml, respectively. However, *O.basilicum* var. *thyrsiflorum* ethanol extract showed the highest antioxidant activity by FRAP assay at 18.64±0.13 µmol Fe^{2+}/g RM followed by water and ethyl acetate extracts. *O. basilicum* cv. Jumbo 4320 water extract showed the highest cellular antioxidant activity by DCFH-DA assay at relative fluorescence intensity 80.62±0.00%.

The antimicrobial activity was evaluated by the agar disk diffusion method. Almost all extracts had no effect on tested pathogenic strains. Only *Bacillus cereus* was inhibited by *O. basilicum* var. *thyrsiflorum* ethyl acetate extract and *O. basilicum* cv. Jumbo 4320 water extract.

Antithrombotic activity of Thai basils extracts was evaluated by prothrombin time assay. *O. basilicum* var. *thyrsiflorum* ethyl acetate extract showed the highest prolonged prothrombin time at 78.3±17.56 seconds.

In conclusion, both species of Thai basils extracts showed the potential for healthy food ingredients.

มหาวิทยาลัยเทคโนโลยีสุรนารี

School of Food Technology Student's Signature__________________

Academic Year 2015 Advisor's Signature__________________

ACKNOWLEDGEMENTS

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Finally, I most gratefully acknowledge my family for their support and faith in me. They have always been the best of inspiration for me in my life. I dedicate this thesis to them.

Panita Prasongdee

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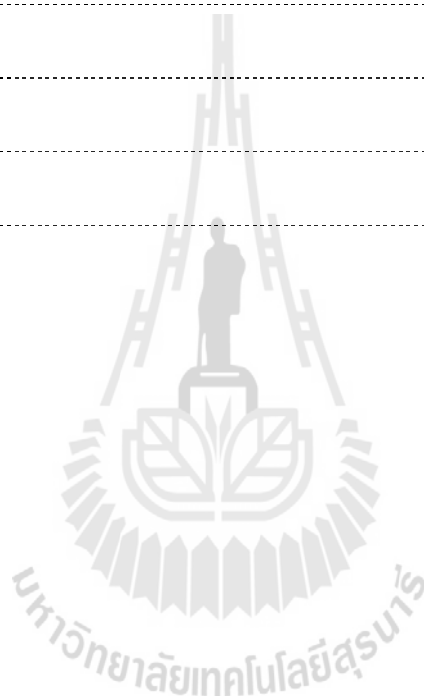

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1B Hemacytometer 107

LIST OF ABBREVIATIONS

ºC	=	Degree of Celsius
ABAP	=	2,2’-Azobis (2-amidinopropane) dihydrochloride
ADP	=	Adenosine 5’-diphosphate
ANOVA	=	analysis of variance
AOAC	=	Association of Official Chemists
ATCC	=	American Type Culture Collection
ATP	=	Adenosine triphosphate
BHT	=	Butylated hydroxytoluene
cm	=	Centimetre
cv.	=	Cultivars
DCF	=	2’, 7’-dichlorofluorescin
DCFH-DA	=	2’, 7’-dichlorofluorescin diacetate
DI	=	*Deionized*
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DPPH	=	2, 2-diphenyl-1-picrylhydrazyl
DMEM	=	Dulbecco’s modified eagle’s medium
EC_{50}	=	50% Effective concentration
EtOH	=	Ethanol
FRAP	=	Ferric reducing antioxidant power

LIST OF ABBREVIATIONS (Continued)

GAE	=	Gallic acid equivalent
GPx	=	Glutathione peroxidase 1
HBSS	=	Hanks' Balanced Salt Solution
HepG2	=	Human hepatomacarcinoma
HPLC	=	High Performance Liquid Chromatography
IC_{50}	=	50% Inhibitory concentration
IU	=	International unit
J	=	*Joule*
kg	=	Kilogram
l	=	Liter
MeOH	=	Methanol
mg	=	Milligram
µg	=	Microgram
MIC	=	Minimal inhibitory concentration
min	=	Minute
mM	=	Millimolar
mm	=	Millimetre
µm	=	Micrometre
mmol	=	Millimole
ml	=	Milliliter
µl	=	Microliter
LC_{50}	=	50% Lethal concentration

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF ABBREVIATIONS (Continued)

MHA	=	Mueller Hinton agar
MTT	=	3-(4, 5-dimethylthiazol-2-yl)2, 5-diphenyltetrazolium bromide
N	=	*Normal*
NADH	=	*Nicotinamide adenine dinucleotide*
nm	=	*Nanometre*
PBS	=	Phosphate buffered saline
PDA	=	Photodiode array
pH	=	Potential of Hydrogenion
p.o.	=	Per *oral*
PTFE	=	Polytetrafluoroethylene
ppm	=	Part per million
RBC	=	Red blood cells
RM	=	*Raw* material
ROS	=	Reactive oxygen species
Rpm	=	Round per minute
SRBC	=	*Sheep* red blood cells
USDA	=	United States Department of Agriculture
UV-vis	=	Ultra violet -visible
var.	=	Variety
WBC	=	White blood cells
Wt.	=	Weight

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF ABBREVIATIONS

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ANOVA = analysis of variance

AOAC = Association of Official Chemists

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ATP = Adenosine triphosphate

BHT = Butylated hydroxytoluene

cm = Centimetre

cv. = Cultivars

DCF = 2’, 7’-dichlorofluorescin

DCFH-DA = 2’, 7’-dichlorofluorescin diacetate

DI = *Deionized*

DMSO = Dimethyl sulfoxide

DNA = Deoxyribonucleic acid

DPPH = 2, 2-diphenyl-1-picrylhydrazyl

DMEM = Dulbecco’s modified eagle’s medium

EC_{50} = 50% Effective concentration

EtOH = Ethanol

FRAP = Ferric reducing antioxidant power

LIST OF ABBREVIATIONS (Continued)

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LIST OF ABBREVIATIONS (Continued)

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PBS	=	Phosphate buffered saline
PDA	=	Photodiode array
pH	=	Potential of Hydrogenion
p.o.	=	Per *oral*
PTFE	=	Polytetrafluoroethylene
ppm	=	Part per million
RBC	=	Red blood cells
RM	=	*Raw* material
ROS	=	Reactive oxygen species
Rpm	=	Round per minute
SRBC	=	*Sheep* red blood cells
USDA	=	United States Department of Agriculture
UV-vis	=	Ultra violet -visible
var.	=	Variety
WBC	=	White blood cells
Wt.	=	Weight

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER I

INTRODUCTION

1.1 Introduction

Medicinal plants have been used for centuries and have become part of complementary medicine worldwide because of their potential health benefits. Some of their metabolites have been successfully used directly in the treatment and prevention of infectious diseases and cancer, or indirectly by stimulating the immune system. Now a day large number of drugs in use are derived from plants, such as Aswagandha from *Withania somnifera*, Atrophine from *Atropa belladonna*, Ephedrine from *Ephedra vulgaris*, morphine from *Papaver somniferum* etc. Plants play an essential role in the health care needs for the treatment of diseases and to improve the immunological response against much pathology. The immune system is designed to protect the host from invading pathogens and to eliminate disease. Immunomodulatory agents are used to either suppress or stimulate the immune responsiveness of an organism. Plant extracts are potentially curative. Some of these extracts can boost the humoral and cell mediated immunity against bacteria, fungi, protozoa, viruses and cancer (Jeba et al., 2011).

In the last three decades, especially in the developed countries of Europe and America, scientists have shown increasing interest in plant research. It is estimated that today about 60% of the total world population in treatment relies on herbs and natural products that are thus recognized as an important source of drugs (Harvey, 2000).

Phytochemistry studies a large variety of organic substances that have been discovered, and which accumulate in plants. Moreover, phytochemistry is also defining the structure of these compounds, their biosynthesis, metabolism, natural distribution and biological activities (Harborne, 1998). An important place among them is occupied by aromatic plants, whose aroma is associated with the presence of essential oils, complex mixtures of volatile compounds, dominated by mono- and sesquiterpenes. In addition to essential oils, aromatic plants are characterized by the presence of plant phenolic compounds, primarily coumarins and phenylpropanoids, that have been shown to possess multiple pharmacological activities. Investigations of these secondary biomolecules intensified when some commercial synthetic antioxidants were found to exhibit toxic, mutagenic and carcinogenic effects. It was also found that excessive production of oxygen radicals in the body initiates the oxidation and degradation of polyunsaturated fatty acids. It is known that free radicals attack the highly unsaturated fatty acid membrane systems and induce lipid peroxidation, which is a key process in many pathological conditions and one of the reactions that cause oxidative stress. Particularly vulnerable are the biological membrane lipids in the spinal cord and brain, because they contain high levels of polyunsaturated fatty acids. Furthermore, the brain contains significant amounts of transitional pro-oxidant metals and consumes a lot of oxygen. These features facilitate the formation of oxygen radicals involved in the processes of aging, Alzheimer’s and Parkinson’s disease, arthritis, myocardial infarction, ischemic heart damage, arteriosclerosis and cancer. Phenolic antioxidants “stop” free oxygen radicals and free radicals formed from the substrate by donating hydrogen atoms or electrons. Many plant species and aromatic plants have been tested because of their antioxidant and antiradical activity (Kaurinovic et al., 2011).

Thai food is currently attracting considerable interest because of its harmonious blend of flavors, aromas and health benefits. Thai vegetables commonly used for flavor and/or condiment have been reported to possess antitumor properties and antimutagenicity (Tangkanakul et al., 2009). The antioxidant property of culinary herbs and spices extensively used in Thai cooking such as lemon grass, kaffir lime, mints, and sweet basil have been documented (Tachakittirungrod et al., 2007). Especially, the genus *Ocimum* (sweet basil, holy basil, hairy basil and wild basil) is known for its antioxidant activity (Jayasinghe et al., 2003; Javanmardi et al., 2003; Chanwitheesuk et al., 2005). Galangal is another common ingredient applied in both Thai chili paste and Malaysian dishes which has demonstrated high antioxidant activity and total phenolic contents. In addition, tumeric and tomato have been reported to have total phenolic contents and antioxidant activity. Thai foods are not only rich in plant species which contain a variety of phytochemicals, but they also provided nutrient contents (Tangkanakul et al., 2009).

These health promoting culinary herbs and spices are commonly used in Central Thai foods. Traditional Thai foods from other regions of the country contain various kinds of indigenous vegetables other than herbs and spices. Thai local vegetables exhibited their health benefits such as antioxidant, antitumor and antimutagen properties (Tangkanakul et al., 2009). After cooking, vegetables present in assayed foods still provide considerable antioxidant capability (Tangkanakul et al., 2006).

Ocimum basilicum Linn. commonly known as basil or sweet basil (family: Lamiaceae) is a perennial herb, native to Asia, Africa, South America, and the Mediterranean but widely cultivated in many countries (Grayer et al., 1996). It grows to a height of 50-70 cm, leaves are oval and slightly toothed and flowers are white in

color. It is mostly used in cosmetics, liqueurs, medicines and perfumes. The leaves and its oil have insecticidal property (Jeba et al., 2011). Commercial sweet basil leaves contain per 100 g fresh material approximately: water 87 g, protein 3 g, fat 1 g, carbohydrates 5 g, fiber 2 g and ash 2 g. The seed is rich in protein and fat, but contains little carbohydrates. Sweet basil is a popular savory herb. Both fresh and dried leaves are used to impart a fragrant, warm and sweet flavor with pungent and clove-like notes to dishes and drinks. The leaves complement many soups and salads and vegetable dishes and have a special affinity with tomatoes, e.g. in tomato paste, pasta sauces and even in a bloody Mary. Lamb is also often flavored with sweet basil leaves. In Italian cooking the leaves are used in pizzas, pasta, chicken and cheese dishes and in the famous pesto. In France the leaves are particularly popular in omelettes and soups. Sweet basil leaves should be added towards the end of cooking to best retain their flavor. The leaves are a source of essential oil and oleoresin mainly applied in industry to flavor baked goods, sauces, pickles, vinegar and meat products and to modify the flavor of chartreuse liqueurs. The oil is also used in cosmetics, dental and oral products and occasionally in perfumes (Aguilar et al., 1999).

In medicine, this is an important medicinal plant and culinary herb. It has been used traditionally for treatment of anxiety, headaches, cough, cold, fevers, migraines, diabetes, nerve pain, cardiovascular diseases, as anticonvulsant and anti-inflammatory, digestive disorders, insect bites, menstrual cramps, sinusitis, and in a variety of neurodegenerative disorders (Bora et al., 2011). Furthermore, the leaves and flowering tops of the plant are perceived as galactogogue, stomachic, carminative and antispasmodic in folk medicine (Sajjadi, 2006). In Vietnam sweet basil is used to treat fever and malaria. The mucilaginous seed enters into the preparation of non-alcoholic

drinks and in medicine. The essential oil is used to repel bugs and flies. Purple-leaved selections are popular ornamentals, but many are as aromatic as green-leaved ones and may be used similarly (Aguilar et al., 1999).

Sweet basil is well known for its essential oil which is responsible for the aroma of plant and the flavor of condiments. The plant also contains acylated and glycosylated anthocyanins; cinnamic acid ester, triterpenoids and steroidal glycosides (Bora et al., 2011); phenolic acids such as rosamarinic acid, lithospermic acid B, vanillic acid, p-coumaric acid, hydroxybenzoic acid, syringic acid, ferulic acid, protocatechuic acid, caffeic acid and gentisic acid, chicoric acid (Bais et al., 2002; Lee et al., 2010); flavonoids and tannins (Grayer et al., 1996). The high economic value of sweet basil oil is due to the presence of phenyl propanoids, like eugenol, chavicol and their derivatives or terpenoids like monoterpen alchole linalool, methyl cinnamate, and limonene (Taie et al., 2010).

Pharmacological reports revealed that various *Ocimum basilicum* extracts have anticandidal, antibacterial, antifungal effects, antioxidant activities (Bora et al., 2011) anti-giardial and antiviral (Chiang et al., 2005). The plant has also shown hypolipidemic (Amrani et al., 2006), anti-inflammatory (Selvakkumar et al., 2007), anti-platelet aggregation, antithrombotic, bronchodilatory, antiulcerogenic and anticarcinogenic (Beric et al., 2008). It can reduce LDL cholesterol level and increase HDL cholesterol level in blood and prevent diseases related to cardiovascular system (Tohti et al., 2006; Harnafi et al., 2008; Wannissorn et al., 2005). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can be play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Many of these phytochemicals possess significant antioxidant

capacities that may be associated with lower incidence and lower mortality rates of cancer in several human populations (Asami et al., 2003).

Extracts from seeds, roots, flowers, stems and leafs of sweet basil were applied to various medical. Recent studies found that sweet basil extracts have antibacterial properties such as *Staphylococcu aureus* and *Escherichia coli*, antifungal such as *Aspergillus niger* and *Rhizopus solani* and antiviral (some strains) (Nguefack et al., 2004; Opalchenova et al., 2003; Orafidiya et al., 2001; Maisuthisakul et al., 2008). However, the phytochemicals and biological activities of various species of sweet basil remain unknown. Therefore, this present study aims at comparing phytochemical and bioactivities of Thai basil extracts from two species, *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320, the biological properties such as antioxidant, antimicrobial and antithrombotic activity of these two plants would be investigated and compared.

1.2 Research objectives

1. To study bioactivities of Thai basil extracts from two species, *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320.

2. To study phytochemical of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320.

3. To study *in vitro* antioxidant activity of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320.

4. To study *in vitro* antimicrobial activity of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320.

5. To study *in vitro* antithrombotic activity of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320.

1.3 Research hypothesis

Thai basil extracts of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 contain phytochemicals as bioactive compounds and show antioxidant, antimicrobial, and antithrombotic activities.

1.4 Scope and limitation of the study

1. Thai basil extracts were prepared from two species *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320. The extraction was by three solvents, hot water, ethanol and ethyl acetate.

2. Phytochemical profile of the performed Thai basil extracts was evaluated by total phenolic and flavonoid content.

3. The antioxidant activity of Thai basil extracts was performed *in vitro* and was limited to DPPH, FRAP and DCFH-DA assays.

4. *In vitro* antimicrobial activity of Thai basil extracts was performed by agar disk diffusion method.

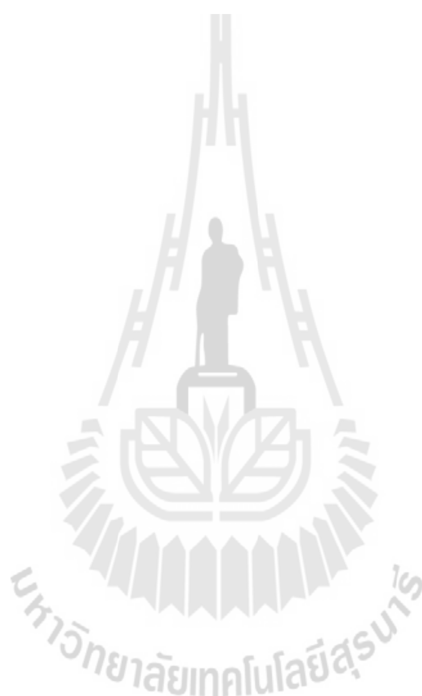
5. *In vitro* antithrombotic activity of Thai basil extracts was evaluated by prothrombin time.

1.5 Expected results

1. To know the phytochemical profiling of Thai basil extracts of two species,

Ocimum basilicum var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320, extracted by 3 solvents namely, hot water, ethanol and ethyl acetate.

2. More understanding about variation of antioxidant, antimicrobial, and antithrombotic activities of two species of Thai basil extracts.



CHAPTER II

LITERATURE REVIEW

2.1 Sweet basil (*Ocimum basilicum* L.)

Basil belongs to the genus *Ocimum* and is a member of the mint family (Lamiaceae). Basil cultivars are cultivated varieties of basil. They are used in a variety of ways: as culinary herbs, landscape plants, healing herbs, teas, and worship implements. The genus *Ocimum* is particularly diverse, and includes annuals, non-woody perennials and shrubs native to Africa and other tropical and subtropical regions of the Old and New World (Meyers, 2003). Although it is estimated that there are 50 to 150 species of basil (Simon et al., 1990), most, but not all, culinary basils are cultivars of *Ocimum basilicum*, or sweet basil (Paton et al., 1999).

Some are cultivars of other basil species, and others are hybrids. It is particularly challenging to determine which species a basil belongs to. This is because basil cross-breeds easily, and drawing boundaries between species is particularly difficult (Paton et al., 1999). Physically, basils are characterized by square, branching stems, opposite leaves, brown or black seeds (also called nutlets) and flower spikes, but flower color and the size, shape, and texture of the leaves vary by species. Leaf textures range from smooth and shiny to curled and hairy, and flowers are white to lavender/purple. Leaf color can also vary, from green to blue/purple, and plants can grow to from 1 to 10 feet in height, depending on the species. Most people are familiar with sweet basil, *Ocimum basilicum*, the common culinary basil, but the world of basils offers a wide

array of plants with a great diversity of flavors, scents, and uses. There are many species and cultivars of basil. Some of the more popular basils include sweet, specialty fragrant (cinnamon, lemon and Thai/anise), purple-leaved, bush, and miniature or dwarf (Meyers, 2003).

Table 2.1 Classification for *Ocimum basilicum* L.

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Lamiales
Family	Lamiaceae
Genus	*Ocimum* L.
Species	*Ocimum basilicum* L.

Source: http://plants.usda.gov/java/

มหาวิทยาลัยเทคโนโลยีสุรนารี

Nowadays, medicinal and aromatic plants occupy a prominent economic position because of the continuous and increasing demand for their products. Sweet basil is one of the most important plants in this concern. Sweet basil has been used as a medicinal and aromatic plant for centuries.it is used in many fields of human activity, especially in the food industry and in gastronomy. Sweet basil is also important for the pharmaceutical industry and is still used in traditional medicines in many parts of the world. It is appreciated for its essential oil, which gives it a unique flavor. The oil is

extensively employed in several countries for flavoring of food stuffs, confectionery goods, and condiments and in toiletry products. It also finds a prominent place in the flavoring of foods, and in perfumes industry. Various uses are attributed to different parts of the plant in indigenous system of medicine and homoeopathy. It is also recognized as a febrifuge and ant malarial plant. The levels of essential oil and other compounds vary between the different sweet basil species and cultivars, and are also influenced by growing conditions. According to, the variation in essential oil content and composition of *Ocimum basilicum* coming from different countries might be attributed to different environmental conditions, as well as genetic factors, different chemotypes and differences in the nutritional status of the plants. Most of the essential oil is concentrated in the leaves, and the amounts obtained from branches and stems is not economically significant. (Said-Al Ahl et al., 2015; Svecova et al., 2010).

Figure 2.1 *Ocimum basilicum* L.

Source: http://www.thaiseed.co.th

Sweet basil is characterized as having an aroma like French tarragon, a sweet licorice scent, attributed to the aroma compound methyl chavicol or estragole (Tucker et al., 2009). Sweet basil is an annual, 20-60 cm long, it has green leaves, stems, and green flower spikes with white flowers (Klimankova et al., 2008; Simon et al., 1999). Two other popular cultivars of sweet basil include 'Genovese', which has a lavender-like floral aroma attributed to linalool, and is typically used to make pesto; and Thai basil 'Siam Queen' which has a stronger licorice scent than sweet basil, and a higher percentage of methyl chavicol, and is used in salads, sauces, and Thai cooking (Tucker et al., 2009). 'Siam Queen' grows to about 35 cm in height, 44 cm in width, with green-purple leaves, green stems, and flower spikes with pink to purple flowers (Simon et al., 1999).

2.2 Bioactive compounds

The distinctive scents and flavors of the many basil species and cultivars are due to the composition of essential oils found in the leaves and other parts of the plant. Most basils contain methyl chavicol, eugenol and linalool. The amount of each of these chemical constituents varies depending on the species or variety. Methyl chavicol provides a sweet flavor that has been compared to anise and French tarragon, linalool produces a floral scent, and eugenol is reminiscent of cloves. Sweet basils tend to have a higher percentage of methyl chavicol while spicy basils get their "spice" from large amounts of eugenol. Other scents come from thymol (thyme), trans-methyl cinnamate (cinnamon), camphor, citral (lemon), and geranial (rose) (Meyers, 2003).

Sweet basil is cultivated worldwide: in particular because it is a rich source of natural compounds, such as monoterpenes, sesquiterpenes, phenylpropanoids, anthocyanins

and phenolic acids (Hussain et al., 2008). Sweet basil's antioxidant, antimutagenic, antitumorigenic, antiviral, and antibacterial properties likely arise from a variety of components including linalool, 1,8-cineole, estragole, eugenol (Muller et al., 1994; Chiang et al., 2005; Makri et al., 2007), camphor, methyl cinnamate, chlorophyll, vitamin C and carotenoids, a wide range of phenolic compounds displaying various antioxidant activities, depending on the basil species and cultivars (Dumbrava et al., 2012).

Table 2.2 Phenolic composition of basil (*Ocimum basilicum*) leaves and stems from two unnamed cultivars of sweet and Thai basil.

Components	Sweet basil		Thai basil	
	Leaves	Stems	Leaves	Stems
Total anthocyanins (mg of cyanidin-glucoside/100 g tissue)	nd	nd	2.00	13.00
Total phenolics (mg gallic acid/100 g tissue)	523.00	244.00	605.00	231.00
Polyphenolics (mg/100 g tissue)				
- caftaric acid	16.50	nd	1.93	nd
- chicoric acid	51.80	nd	88.50	0.30
- rosmarinic acid	112.00	31.90	128.00	40.30
- other minor compounds	28.30	3.98	17.60	9.41
Total polyphenolics (phenolics other than anthocyanins)	208.00	35.90	236.00	50.00

nd means not detected.

Source: Lee et al. (2009)

2.2.1 Phenolic compounds

Plant metabolites can roughly be divided into two categories: primary and secondary metabolites. The primary metabolites participate in the nutrition and essential metabolic processes such as growth, development, or reproduction, and these compounds include, for example, carbohydrates, lipids, and proteins. Secondary metabolites often function in important ecological roles between a plant and its environment, such as being pigments in plant leaves and flowers, or as defensive compounds against herbivores and pathogens. The secondary plant compounds can be divided into three main categories: alkaloids, terpenoids, and phenolics (Bernards, 2010). Phenolic compounds are further divided into subgroups, such as flavonoids, tannins, and phenolic acids. Many phenolics contain several phenolic rings with multiple hydroxyl groups, and thus the term polyphenolic is often used. Quideau et al. (2011) proposed a revised definition of a polyphenolic compound that also explains their biosynthetic origin: "*The term "polyphenol" should be used to define plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen based functional group in their most basic structural expression*". This definition excludes terpenoids, alkaloids, and compounds with only one benzene ring (e.g., vanillin and salicylic acid) outside of polyphenolic compounds.

In the last few years, great attention has been paid to the bioactive compounds due to their ability to promote benefits for human health, such as the reduction in the incidence of somedegenerative diseases like cancer and diabetes (Conforti et al., 2009; Kim et al., 2009), reduction in risk factors of cardiovascular diseases, antioxidant, anti-mutagenic, anti-allergenic, anti-inflammatory, and anti-microbial effects among

others. Due to these countless beneficial characteristics for human health, researches have been intensified aiming to find fruits, vegetables, plants, agricultural and agro-industrial residues as sources of bioactive phenolic compounds (Martins et al., 2011).

Bioactive compounds are extra nutritional constituents that naturally occur in small quantities in plant and food products. Most common bioactive compounds include secondary metabolites such as antibiotics, mycotoxins, alkaloids, food grade pigments, plant growth factors, and phenolic compounds (Martins et al., 2011). Phenolic compounds are synthesized in plants partly as a response to ecological and physiological pressures such as pathogen and insect attack, UV radiation and wounding (Khoddami et al., 2013). The basic structural feature of phenolic compounds is an aromatic ring bearing one or more hydroxyl groups (Figure 2.2; Chirinos et al., 2009). Plant phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule (Khoddami et al., 2013).

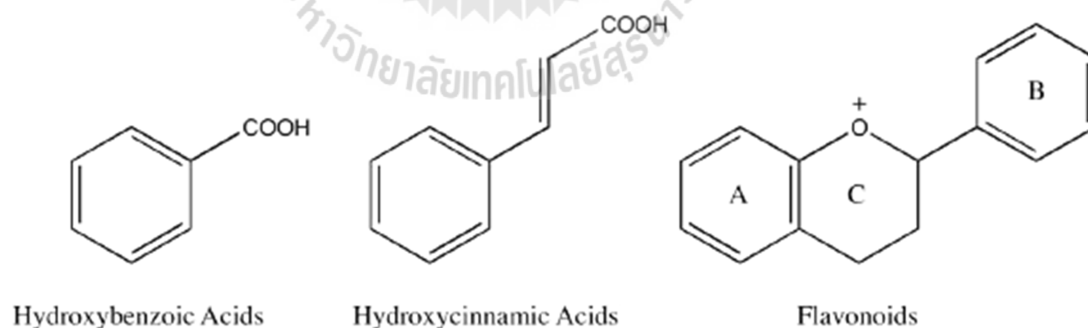


Figure 2.2 Basic structures of phenolic acids and flavonoids.

Source: Chirinos et al., 2009

Phenolic compounds comprise flavonoids, phenolic acids, and tannins, among others. Flavonoids constitute the largest group of plant phenolics, accounting for over

half of the eight thousand naturally occurring phenolic compounds. Flavonoids are based on a C6–C3–C6 backbone. Flavonoids are divided into three main classes: flavonoids, isoflavonoids, and neoflavonoids (Figure 2.3; Waterman et al., 1994). Variations in substitution patterns to ring C in the structure of these compounds result in the major flavonoid classes, i.e., flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanidins (Martins et al., 2011).

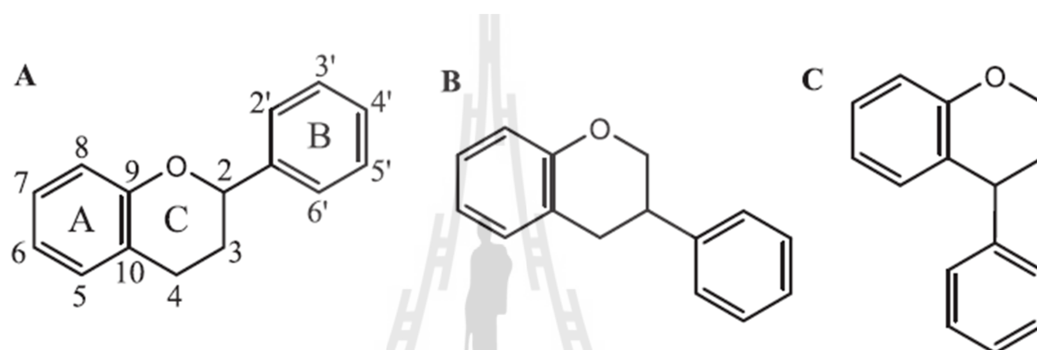


Figure 2.3 The main classes of flavonoids: flavonoids (A), isoflavonoids (B), and neoflavonoids (C). The labeling of flavonoid rings, and the numbering of atoms is presented.

Source: Waterman et al., 1994

Similarly to the flavonoids, phenolic acids constitute also an important class of phenolic compounds with bioactive functions, usually found in plant and food products. Phenolic acids can be divided in two subgroups according to their structure: the hydroxybenzoic and the hydroxycinnamic acids. The most commonly found hydroxybenzoic acids include gallic, phydroxybenzoic, protocatechuic, vanillic and syringic acids, while among the hydroxycinnamic acids, caffeic, ferulic, p-coumaric and sinapic acids can be pointed out (Martins et al., 2011). Phenolic compounds, also referred as polyphenols, are considered to be natural antioxidants and represent an

important group of bioactive compounds in foods (Duenas et al., 2005). These compounds are present in all plant foods but their type and levels vary enormously depending on the plant, genetic factors and environmental conditions (Kris-Etherton et al., 2002).

Anthocyanins belong to flavonoids which represent a large class of plant secondary metabolites. This group of flavonoids are water soluble pigments which occur in most species in the plant kingdom. Anthocyanins are accumulated in cell vacuoles, especially in flowers, fruits and vegetables, but they are also found in leaves, stems, seeds and other tissues (Szymanowska et al., 2015). Anti-. Anthocyanins functions within plants include UV protection, attraction of animals for pollination and seed dispersal, defense against pathogens and pests, and protecting DNA and the photosynthetic apparatus from high radiation fluxes. Additionally, they can serve both as antioxidants and in the acquisition of tolerance to many different kinds of environmental stressors, such as cold or frost, heavy metal contamination, desiccation and wounding (Hatier et al., 2009). Antioxidant and antiinflammatory activities of anthocyanin are manifested via its ability to neutralize free radicals as well as inhibition of lipoxygenase and cyclooxygenase, enzymes metabolizing arachidonic acid (Bowen-Forbes et al., 2010). Phenolic compounds, especially anthocyanins, are known from their antioxidant, anti-inflammatory and anticancer properties (Bowen-Forbes et al., 2010). Anthocyanins responsible for the various attractive colours in plants are also becoming important alternatives to many synthetic colourants due to increased public concerns over the safety of artificial food colours (Szymanowska et al., 2015).

2.2.2 Chlorophyll

Leaf is one essential part of plants, since it plays several important roles such as food production (photosynthesis), food storage, water transport, gas exchanging (respiration), and protection of vegetative and floral buds. In addition to the plants themselves, leaves are also necessary to other living organisms, because the photosynthesis process generates oxygen which is released to an environment. Plant leaves are also a major source of food for human and other animals. In green leaf plants, quality of leaves can be simply determined by chlorophyll content in the leaves, because chlorophyll is responsible for the characteristic green color of several vegetables and also fruit. However, this green pigment can degrade to other forms of molecule with color changes such as phaeophytin and phaeophorbide, olive-brown compounds, and the degradation products can be further metabolized to colorless compounds. There are several factors affecting chlorophyll loss such as normal aging, lack of light, temperature changes, nutrient deficiency, and viral infection etc (Arunrangsi et al., 2013).

Chlorophyll is a specifically pigment of green plants, which plays a key role in photosynthesis (the most important biochemical process on earth). In plants there are several types of chlorophyll, denoted by letters of the alphabet (a, b, c, d). The most important in photosynthesis is chlorophyll “a”, which is darker. Chlorophyll has effects on the human body. External acts as deodorant and skin tonic, internally, stimulates respiration, helps in cleansing waste and help combat anemia (Dumbrava et al., 2012). Chlorophyll is often referred to as the green blood of plants due to the identical molecular structure with hemoglobin with only difference in centre atom (iron or magnesium). This similarity makes chlorophyll so important to our health, it

improve digestive, immune and detoxification systems of human body (Kopsell et al., 2005).

There are a few different forms of chlorophyll. Chlorophyll *a*, blue-green in solution, is the primary photosynthetic pigment in green plants for the transfer of light energy to a chemical acceptor. Light that is absorbed provides the energy for photosynthesis. A green leaf absorbs blue light (mostly at 430 nm) and red light (mostly at 660 nm). It reflects the green wavelengths, appearing green to human eye. Chlorophyll *a*, alone, is found in blue-green and some red algae. Accessory pigments in photosynthesis transfer light energy to chlorophyll *a*. One of these is chlorophyll *b*, greenish-yellow in solution, found in higher plants and green algae with chlorophyll *a*. Chlorophyll *c* is also an accessory pigment found with chlorophyll *a* in brown algae and diatoms. Chlorophyll *d*, together with chlorophyll *a*, is in some red algae. All forms of chlorophyll are oil-soluble. Chlorophyll forms, except *c*, have a 'head' and a long 'tail'. The head consists of a porphyrin ring or tetrapyrrole nucleus, from which extends a tail made up of a 20-carbon grouping called the phytol. The tail of form *c* is short and links to porphyrin ring from C_{17}. In chlorophyll, the porphyrin is very similar in structure to the heme group found in hemoglobin, except that in heme the central atom is iron, whereas in chlorophyll it is magnesium (Inanc, 2011).

Chlorophyll is decomposed by heat and an olive-green color is produced. The time of heating and the temperature affect on the extent of decomposition, for example, the temperature is high in a pressure cooker and acidity is not decreased because the volatile acids are retained, thus the change is rapid. The usage of alkaline compounds such as alkaline water reduces acidity of medium. However, if it is used at excess amounts, chlorophyll reacts with base. Figure 2.4 and 2.5 show simple

reactions of chlorophyll with acid and base. Reaction of chlorophyll a with acid removes the magnesium ion replacing it with two hydrogen atoms giving an olive-brown solid, phaeophytin *a*. Hydrolysis of this (reverse of esterification) splits off phytol and gives phaeophorbide *a*. Similar compounds are obtained if chlorophyll *b* is used. If chlorophyll is reacted with a base, it forms a series of phyllins, magnesium porphyrin compounds. Treatment of phyllins with acid gives porphyrins (Inanc, 2011).

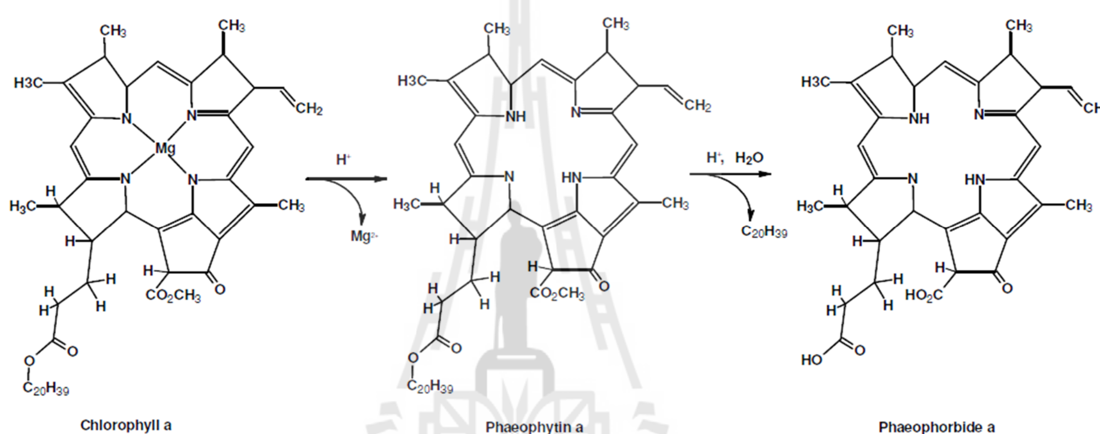


Figure 2.4 Reaction of Chlorophyll a with acid.

Source: Inanc, 2011

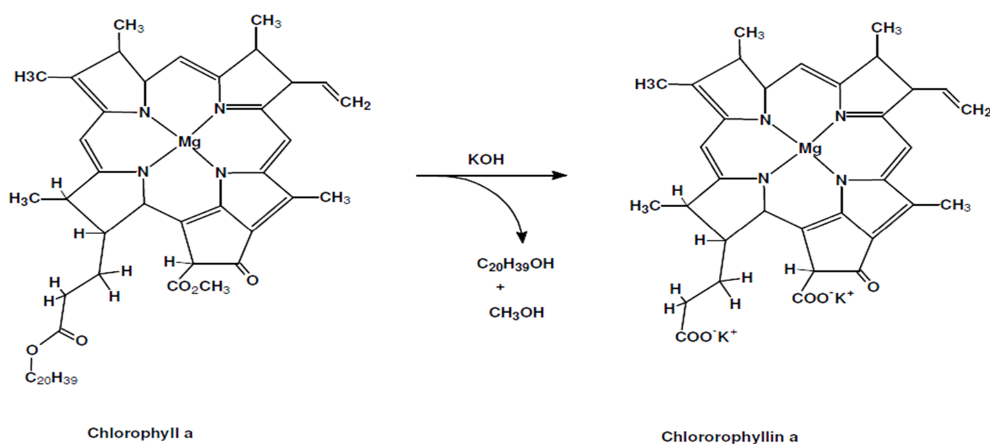


Figure 2.5 Reaction of Chlorophyll a with base.

Source: Inanc, 2011

2.3 Nutrition

Sweet basil is low in calories, has almost no fat, and is a good source of vitamin A. Fresh basil leaves (2.5 grams) has less than 1 calorie, 96.6 IU of vitamin A (Meyers, 2003). One hundred grams of fresh sweet basil leaves contain 26 mg of vitamin C, 3.95 mg of carotene, 0.08 mg of thiamin, 0.31 mg of riboflavin, and 1.1 mg of niacin. Its potassium content is 30 times that of sodium. One hundred grams of fresh sweet basil leaves contain 250 mg of calcium, 37 mg of phosphorus, 5.5 mg of iron, and 11 mg of magnesium (Dumbrava et al., 2012). Basil seeds, in particular, are high in dietary fiber. Basil also includes flavonoids and antioxidants. Sweet basil (*Ocimum basilicum*) is on the USDA's GRAS (Generally Recognized as Safe) list at 2-680 ppm (parts-per-million) for the leaf and 0.01-50 ppm for the oil, but some suggest that it may be hazardous in excessively large doses due to possible carcinogens. Note that the amount of essential oil that is considered GRAS is very small and basil essential oil should not be taken internally (Meyers, 2003).

2.4 Biochemical properties of sweet basil (*Ocimum basilicum*)

2.4.1 Antioxidant

Antioxidants are compounds or systems that delay autoxidation by inhibiting formation of free radicals or by interrupting propagation of the free radical by one (or more) of several mechanisms: (1) scavenging species that initiate peroxidation, (2) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, (3) quenching $*O_2^-$ preventing formation of peroxides, (4) breaking the autoxidative chain reaction, and/or (5) reducing localized O_2 concentrations. Highly reactive free radicals and oxygen species are present in

biological systems from a wide variety of sources (Asimi et al., 2013). Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Jayasinghe et al., 2003). Usually synthetic antioxidants such as butylhydroxyanisole (BHA) or butylhydroxytoluene (BHT) are used to decelerate these processes (Asimi et al., 2013). Synthetic phenolic antioxidants (butylated hydroxyanisole [BHA], butylated hydroxytoluene [BHT], and propyl gallate) effectively inhibit oxidation; chelating agents, such as ethylene diamine tetra acetic acid (EDTA), can bind metals reducing their contribution to the process. Some vitamins (ascorbic acid and α-tocopherol), many herbs and spices (rosemary, thyme, oregano, sage, sweet basil, pepper, clove, cinnamon, and nutmeg), and plant extracts (tea and grapeseed) contain antioxidant components as well (Hinneburg et al., 2006). Additionally, it is still unclear whether prolonged consumption can lead to health risks. Natural antioxidants are considered safe and have consumer acceptant as compared to synthetic antioxidants for example, herbs and spices (Asimi et al., 2013).

The major antioxidant capacities of plants such as fruits and vegetables are vitamins C and E and phenolic compounds, especially flavonoids. Phenolic compounds possess different biological activities, but most important are antioxidant activities. Phenolics are able to scavenge reactive oxygen species due to their electron donating properties. Their antioxidant effectiveness in food depends on not only the number and location of hydroxyl groups but also on factors such as physical location, interaction with other food components, and environmental conditions (Re et al., 1999; Velioglu et al., 1998).

Literature surveys have revealed that sweet basil possess a high level of antioxidant

activity, which is attributed to the large class of polyphenols and flavonoids like quercetin, kaempferol and myricetin, tannins like catechin, pigment such as anthocyanins found in purple basil, and essential oil such as eugenol and methyl chavicol (Bora et al., 2011).

Maisuthisakula et al. (2008) reported that sweet basil extracted by ethanol showed total phenolic content at 50.5 mg GAE/g and it showed antioxidant activity by DPPH assay at EC_{50} value of 1.8 μg/ml.

Sweet basil can lower oxidative damage in animal models. Feeding mice 200 and 400 mg/kg body weight with a hydroalcoholic extract of sweet basil leaves for 15 days markedly increased GPx (1.22-1.4 fold), glutathione (GSH) reductase (1.16-1.28 fold), catalase (1.56-1.58 fold), and superoxide dismutase (1.1-1.4 fold). The change in activity in one or more of these enzymes may explain the decrease in lipid peroxidation caused by sweet basil in studies by Dasgupta et al. (2004). (Kaefer et al., 2011)

Dragan et al. (2007) examined the effects of balsamic vinegar-enriched extracts from several herbs (rosemary, sage, and basil) in soups and salads on oxidative stress and quality of life measures in women with stage IIIB and IV breast cancer. While there was a decrease in oxidative stress, the complexity of the dietary intervention made it impossible to determine the components that led to improvements.

2.4.1.1 Measurement of antioxidant activity

Methods of assessing antioxidant behavior fall into two broad categories reflecting the focus on activity in foods or bioactivity in humans. In the case of food systems, the need is to assess the efficacy of an antioxidant(s) in providing protection

for the food against oxidative spoilage. A subcategory involves measurement of activity in foods, particularly fruits, vegetables and beverages, but with a view to predicting dietary burden and *in vivo* activity. Oxidative stress in humans arises from an imbalance in the antioxidant status (reactive oxygen species *versus* defence and repair mechanisms). Among the endogenous defences are enzymes such as superoxide dismutase, catalase and glutathione peroxidase, plus vitamin E, uric acid and serum albumins. Besides these defences, consumption of dietary antioxidants is also important. An important distinction from food-based systems is the absence of a single, definable substrate in many instances *in vivo*. Antioxidant activity cannot be measured directly but rather by the effects of the antioxidant in controlling the extent of oxidation. Methods show extreme diversity. Some methods involve a distinct oxidation step followed by measurement of the outcome as, for example, oxidation of linoleic acid followed by determination of diene conjugation. In other instances, there is no clear distinction between the various steps in the procedure. The features of an oxidation are a substrate, an oxidant and an initiator, intermediates and final products and measurement of any one of these can be used to assess antioxidant activity (Antolovich et al., 2002).

2.4.1.1.1 2,2-diphenyl-1-picrylhydrazyl assay (DPPH assay)

DPPH is a stable radical with a deep purple color whose reaction with other radicals, reducing agents, or compounds capable of hydrogen atom transfer leads to loss of color at 515 nm. DPPH• (Figure 2.6) reacts with both electron and hydrogen donors, though more slowly, and steric accessibility to the radical site is a clear issue. No antioxidant assay is simpler or less expensive to run than the DPPH assay, which accounts for its popularity and extensive use. Needed only are the reagent, some

cuvettes, and a UV-vis spectrophotometer, the latter of which are found in even the most rudimentary laboratories. DPPH crystals are dissolved in MeOH or EtOH. Antioxidant activity is reported either as IC_{50} (the antioxidant concentration required to reduce the DPPH absorbance by half) or % loss or original absorbance (Apak et al., 2013). DPPH method is widely used to determine antiradical/antioxidant activity of purified phenolic compounds as well as natural plant extracts (Shalaby et al., 2013).

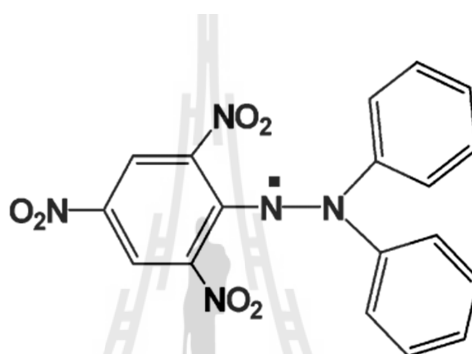


Figure 2.6 Structure of 2,2-diphenyl-1-picrylhydrazyl (DPPH•).

Source: Prior et al., 2005

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2.4.1.1.2 Ferric reducing antioxidant power (FRAP assay)

FRAP assay was originally developed by Benzie and Strain to measure reducing power in plasma, but the assay subsequently has also been adapted and used for the assay of antioxidants in botanicals. The reaction measures reduction of ferric 2,4,6-tripyridyl-s-triazine (TPTZ) to a colored product (Figure 2.7; Prior et al., 2005).

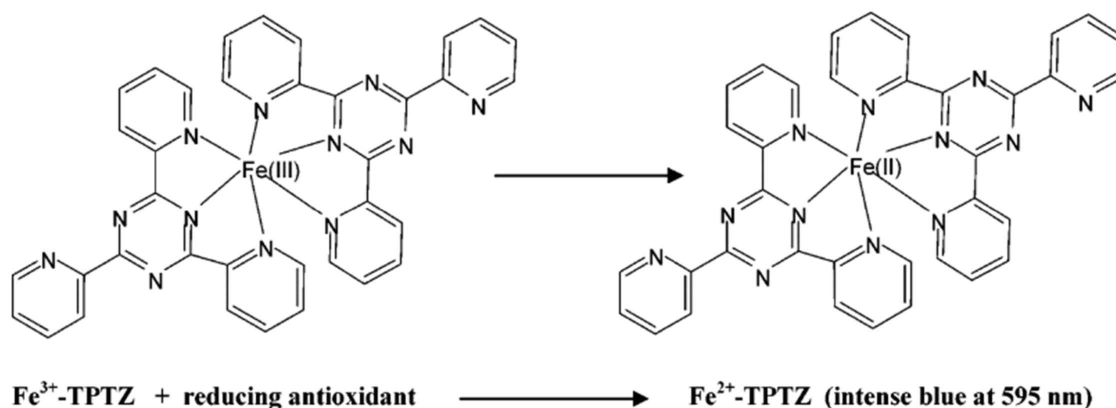

Figure 2.7 Reaction for FRAP assay.

Source: Prior et al., 2005

FRAP assay is based on the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$, to the intensely blue coloured Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic medium. Results are obtained as absorbance increases at 593 nm and can be expressed as micromolar Fe^{2+} equivalents or relative to an antioxidant standard (Antolovich et al., 2002).

Advantages/Disadvantages of the FRAP assay. The FRAP assays evolve from assays that rely on the hypothesis that the redox reactions proceed so rapidly that all reactions is complete within 4 min, but in fact this is not always true. FRAP results can vary tremendously depending on the time scale of analysis. Fast-reacting phenols that bind the iron or break down to compounds with lower or different reactivity are best analyzed with short reaction times, for example, 4 min. However, some polyphenols react more slowly and require longer reaction time for detection, for example, 30 min. The order of reactivity of a series of antioxidants can vary tremendously and even invert, depending on the analysis time. The FRAP assay does not measure thiol antioxidants,

such as glutathione. FRAP actually measures only the reducing capability based upon the ferric ion, which is not relevant to antioxidant activity mechanistically and physiologically. However, in contrast to other tests of total antioxidant power, the FRAP assay is simple, speedy, inexpensive, and robust and does not require specialized equipment. The FRAP assay can be performed using automated, semiautomatic, or manual methods (Prior et al., 2005).

2.4.1.1.3 Cellular Antioxidant Activity (CAA) assay

The measurement of antioxidant activity is an important screening method to compare the oxidation/reduction potentials of fruits and vegetables and their phytochemicals in various systems. Many chemistry methods are currently in wide use, including the oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), Trolox equivalent antioxidant capacity (TEAC), total oxyradical scavenging capacity (TOSC), and the peroxyl radical scavenging capacity (PSC) assays, the latter of which was recently developed by our laboratory. The ferric reducing/antioxidant power (FRAP) assay and the DPPH free radical method measure the ability of antioxidants to reduce ferric iron and 2,2- diphenylpicrylhydrazyl, respectively (Wolfe et al., 2007). Despite wide usage of these chemical antioxidant activity assays, their ability to predict *in vivo* activity is questioned for a number of reasons. Some are performed at nonphysiological pH and temperature, and none of them take into account the bioavailability, uptake, and metabolism of the antioxidant compounds (Liu et al., 2005). The protocols often do not include the appropriate biological substrates to be protected, relevant types of oxidants encountered, or the partitioning of compounds between the water and lipid phases and the influence of interfacial behavior (Frankel et al., 2000). Biological systems are much more complex

than the simple chemical mixtures employed, and antioxidant compounds may operate via multiple mechanisms (Wolfe et al., 2007). The different efficacies of compounds in the various assays attest to the functional variation. The best measures are from animal models and human studies; however, these are expensive and time-consuming and not suitable for initial antioxidant screening of foods and dietary supplements (Liu et al., 2005). Cell culture models provide an approach that is cost-effective, relatively fast, and address some issues of uptake, distribution, and metabolism (Wolfe et al., 2007).

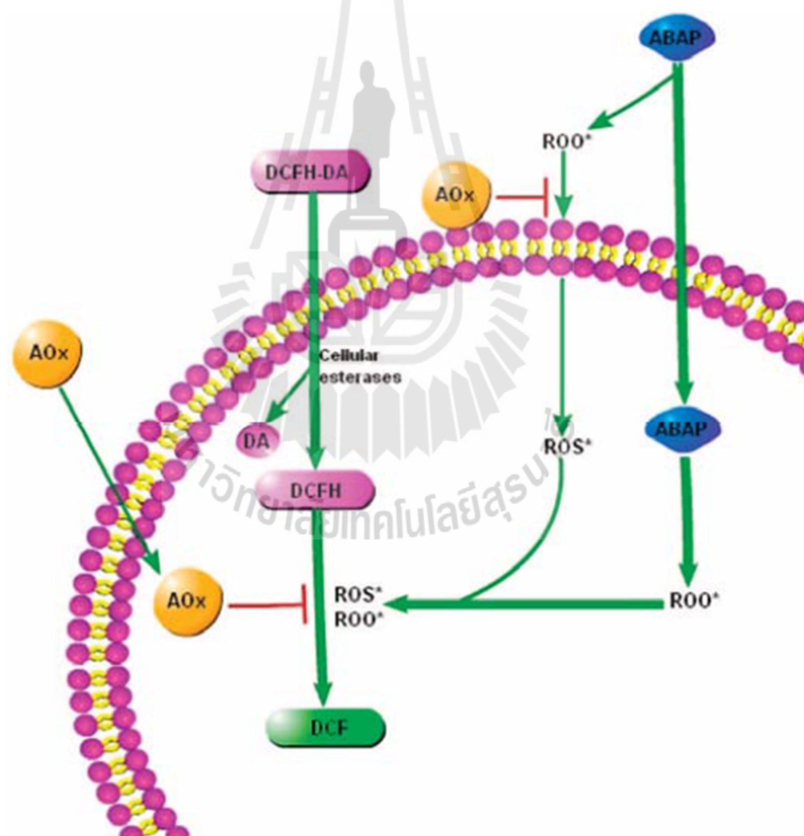

Figure 2.8 Method and proposed principle of the cellular antioxidant activity (CAA) assay.

Source: Wolfe et al., 2007

Method and proposed principle of the cellular antioxidant activity (CAA) assay (Figure 2.8). Cells were pretreated with antioxidant compounds or extracts and DCFH-DA. The antioxidants bound to the cell membrane and/or passed through the membrane to enter the cell. DCFH-DA diffused into the cell where cellular esterases cleaved the diacetate moiety to form the more polar DCFH, which was trapped within the cell. Cells were treated with ABAP, which was able to diffuse into cells. ABAP spontaneously decomposed to form peroxyl radicals. These peroxyl radicals attacked the cell membrane to produce more radicals and oxidized the intracellular DCFH to the fluorescent DCF. Antioxidants prevented oxidation of DCFH and membrane lipids and reduced the formation of DCF (Wolfe et al., 2007).

2.4.2 Antiplatelet aggregation

The mechanism of blood coagulation constitutes a complex and dynamic interaction of platelets, plasma, and blood vessel endothelium. Blood coagulation is an important part of the hemostatic process. It is usually initiated through damage to the vessel wall and subsequent activation of protease enzymes and ends with the transformation of soluble fibrinogen into insoluble fibrin. Natural anticoagulant mechanisms limit and localize hemostatic plug (thrombus) formation at sites of blood vessel injury, and disorders of coagulation can lead to an increased risk of hemorrhage and/or clotting (thrombosis; Alesci et al., 2008). The model generally used to describe the mechanism of coagulation is the cascade system, which is separated into 3 areas. The intrinsic system, commonly measured by the activated partial thromboplastin test, is activated by surface contact. The extrinsic system, commonly measured by the prothrombin test, is activated by vascular injury. The common pathway leading to clot formation is activated by the intrinsic and/or extrinsic pathways (Casella et al., 2009).

Although modern coagulation diagnostics is becoming increasingly complex, screening tests such as prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen are still important for the basic assessment of hemostasis. Measurement of PT, aPTT, and fibrinogen concentration is performed using citrated plasma, and they are the most commonly employed laboratory tests in patients with a suspected coagulopathy. Prothrombin time is a laboratory screening test used to detect disorders involving the activity of the factors I, II, V, VII, and X of the extrinsic and common pathways. Activated partial thromboplastin time is an assay used to screen for abnormalities of the intrinsic and common clotting systems and to monitor the anticoagulant effect of circulating heparin. It measures the activities of factors I, II, V, VIII, IX–XI, and XII of the intrinsic and common pathways. Fibrinogen is a protein that originates in the liver and is converted to fibrin during the blood-clotting process. Evaluation of fibrinogen abnormalities aids in the diagnosis of suspected clotting or bleeding disorders (Casella et al., 2009).

Anticoagulants can be classified according to chemical structure as indandiones (e.g. pindone, diphacinone) or coumarins (e.g. warfarin, brodifacoum), and also as first- or second-generation compounds, according to the timing of their development. All of these compounds act by interfering with the normal synthesis of vitamin K-dependent blood-clotting factors in the live. Vitamin K (hydroquinone form) is a co-factor for a carboxylase enzyme in the production of proteins such as blood clotting factors II, VII, IX, and X. In this carboxylation cycle, vitamin K is oxidised (VKO) and is then unavailable until recycled back to vitamin K hydroquinone by the enzyme vitamin K epoxide reductase (VKOR). It is this enzyme that is inhibited by the action of coumarin anticoagulants, which bind strongly to the enzyme, leaving it unavailable

for VKO. Recycling of vitamin K is required to maintain normal levels of the different blood clotting factors, which mediate the coagulation response to injury. When anticoagulants inhibit the recycling of Vitamin K to the extent that levels of blood clotting factors are depleted, fatal haemorrhaging may result from the inability of blood to clot normally (Bailey et al., 2005).

Bora et al. (2011) studied the effect of ethyl acetate extract of *Ocimum basilicum* leaves on ischemia and reperfusion-induced cerebral damage, and motor dysfunctions in mice. Global cerebral ischemia was induced by bilateral carotid artery occlusion for 15 min followed by reperfusion for 24 hours. Cerebral infarct size was measured using triphenyltetrazolium chloride staining. The concentration of thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) content was determined by colorimetric assay. Short-term memory was evaluated using elevated plus-maze. Inclined beam walking was employed to assess motor coordination. Bilateral carotid artery occlusion followed by reperfusion produced significant increase in cerebral infarct size and lipid peroxidation (TBARS), and reduced GSH content, and impaired short-term memory and motor coordination. Pre-treatment with standardized ethyl acetate extract of *Ocimum basilicum* (100 and 200 mg/kg, p.o) markedly reduced cerebral infarct size and lipid peroxidation, restored GSH content, and attenuated impairment in short-term memory and motor coordination.

Tohti et al. (2006) studied the effects of aqueous extract of *Ocimum basilicum* L. on platelet aggregation and experimental thrombus. Platelet aggregations induced by ADP and thrombin, and thrombus weight in an arteriovenous thrombosis (AVT) model were tested. *Ocimum basilicum* L. possesses an inhibitory effect on platelet aggregation induced by ADP and thrombin that is dose-dependent and results in an

anti-thrombotic effect *in vivo* which develops progressively over 7 days and disappears over 3-7 days.

Amrani et al. (2009) studied the endothelium-dependant vasorelaxant and anti-platelet aggregation activities of an aqueous extract from *Ocimum basilicum*. The results show that *Ocimum basilicum* extract exerts a significant vasorelaxant effect. The extract inhibits ADP-induced platelet aggregation and reduced thrombin-induced platelet activation. The use of *Ocimum basilicum* as medicinal plant could be beneficial for cardiovascular system.

2.4.3 Anticarcinogenic

Dasgupta et al. (2004) studied the effects of doses of 200 and 400 mg/kg body weight of hydroalcoholic extract (80% ethanol, 20% water) of the fresh leaves of *Ocimum basilicum* on xenobiotic metabolizing phase I and phase II enzymes, antioxidant enzymes, glutathione content, lactate dehydrogenase and lipid peroxidation in the liver of 8-9 weeks old Swiss albino mice. The anticarcinogenic potential of sweet basil leaf extract was studied, using the model of benzo(a)pyrene-induced forestomach and 7,12 dimethyl benz(a)anthracene (DMBA)-initiated skin papillomagenesis. It found that sweet basil leaf, as deduced from the results, augmented mainly the phase II enzyme activity that is associated with detoxification of xenobiotics, while inhibiting the phase I enzyme activity. There was an induction in antioxidant level that correlates with the significant reduction of lipid peroxidation and lactate dehydrogenase formation. Moreover, sweet basil leaf extract was highly effective in inhibiting carcinogen-induced tumor incidence in both the tumor models at peri-initiational level.

The anticancer properties of sweet basil in preclinical studies are mixed. In studies

with Sprague-Dawley rats fed with an AIN-76 diet with or without high concentrations of sweet basil (6.25% and 12.5%), there was no clear indication of a decrease in 9,10-dimethyl-1,2-benzathracene (DMBA)-induced mammary cancer. It is unclear whether the quantity of the procarcinogen examined, the simultaneous induction of both phase I and II enzymes, or some other factors accounted for the lack of protection by adding sweet basil to the animals' diet. Nevertheless, there is evidence that sweet basil can decrease DMBA-induced carcinogenesis. Providing Swiss mice with a diet containing 150 or 300 mg/kg body weight of sweet basil extract decreased DMBA-induced skin tumors (12.5% reduction and 18.75% reduction for lower and higher doses, respectively), and lowered the tumor burden per mouse. Compared to the average number of tumors per mouse in the controls, the tumor burden was approximately 2.4 times lower ($p<0.01$) in the low-dose sweet basil group and 4.6 times lower ($p<0.001$) in the high-dose sweet basil group. It is unclear whether differences in the response between mice and rats reflect the species, the cancer site, or the dietary or procarcinogen exposures (Kaefer et al., 2011).

DNA methyltransferase (MGMT) is a critical repair protein in the cellular defense against alkylation damage. MGMT is highly expressed in human cancers and in tumors resistant to many anticancer alkylating agents. Niture et al. (2006) examined the ability of several medicinal plants to upregulate O^6-methylguanine adducts. Both ethanol and aqueous extracts of sweet basil increased MGMT protein levels in HT29 human colon carcinoma cell lines 1.25-fold compared to controls after 72-hours incubation. Compared to the control, sweet basil increased glutathione-S-transferase (GST) protein activity 1.33-fold after 12 hours of incubation; after 24 hours, GST activity increased 1.68-fold compared to the control, which declined to 1.47-fold after

72 hours incubation. Because MGMT is one of the body's first lines of defense against alkylation DNA damage, a small increase (two- to threefold) in this enzyme may protect against mutagenic lesions.

The anticancer properties of sweet basil may also relate to its ability to influence viral infections. Individuals with hepatitis B are recognized to be at increased risk for hepatocellular carcinoma (Kaefer et al., 2011). Chiang et al. (2005) evaluated the antiviral activities of sweet basil extract and selected sweet basil constituents in a human skin basal cell carcinoma cell line (BCC-1/ KMC) and a cell line derived from hepatoblastoma HepG2 cells against several viruses, including hepatitis B. Impressively, Chiang et al. (2005) found that the aqueous extract of sweet basil, along with apigenin and ursolic acid, displayed greater anti-hepatitis B activity than two commercially available drugs, glycyrrhizin and lamivudine (3TC). Overall, these studies raise intriguing questions about the merits of using commercially available spices to retard viruses and potentially cancer. Undeniably, much more information is needed to clarify the amounts and durations needed to bring about a desired viral response and the mechanism by which a response occurs.

Jeba et al. (2011) studied immunomodulatory effect of *Ocimum basilicum*. Low and high dose of *Ocimum basilicum* was administrated in wister albino rat. Antibody titre was estimated by SRBC titre method. RBC, WBC, Haemoglobin count was recorded. The biochemical parameters also estimated in treated and control animals. It enhanced the antibody titre value. It improves the RBC and haemoglobin count. Biochemical results were good when compared to control.

2.4.4 Anti-inflammatory

Selvakkumar et al. (2007) investigated the anti-inflammatory activity of crude

extracts of *Ocimum basilicum* using peripheral blood mononuclear cells (PBMC) of healthy individuals. *Ocimum basilicum* crude methanolic extract showed a good inhibitory effect on the proliferative response of PBMC in mitogenic lymphocyte proliferation assays. Furthermore, gene expression studies on lipopolysaccharide (LPS) induced production of proinflammatory cytokines like Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β) and IL-2 showed down regulation of the markers. It also suppressed the induction of inducible nitric oxide synthase (iNOS) and the subsequent production of nitric oxide (NO) in LPS-stimulated RAW 264.7 macrophages in a timedependent manner. The results showed that *Ocimum basilicum* crude methanolic extract inhibits the key proinflammatory cytokines and mediators, which accounts for its anti-inflammatory effect.

Gomez-Flores et al. (2008) investigated the effects of methanol and aqueous extracts of *Ocimum basilicum* on *in vitro* rat lymphocyte proliferation. Methanol extracts of *Ocimum basilicum* stimulated up to 80% lymphoproliferation, whereas aqueous extracts induced up to 83% lymphoproliferation, as compared with untreated controls. Methanol and aqueous vehicles did not affect lymphocyte viability nor proliferation activity. The observed immunostimulatory effect may be of benefit in increasing the pool of lymphocytes in immunodeficiency patients.

2.4.5 Antimicrobial

The essential oil of sweet basil possesses antimicrobial properties. Moghaddam et al. (2009) investigated the effect of sweet basil on *Helicobacter pylori* and found that methanol, butanol, and n-hexane fractions of sweet basil demonstrated antagonistic activity against the bacteria (MIC=39-117 μg/disk). While not as potent as amoxicillin, its effectiveness raises possibilities of using individual or multiple

spices as potent antimicrobials, especially in areas where commercial antibiotics are in limited supply.

Sienkiewicz et al. (2013) studied the activities of sweet basil (*Ocimum basilicum* L.) essential oils against multidrug-resistant clinical strains of *Escherichia coli*. The antibacterial activity of the oils was tested against standard strain *Escherichia coli* ATCC 25922 as well as 60 other clinical strains of *Escherichia coli*. The inhibition of microbial growth by essential oils was determined by agar dilution. Susceptibility testing to antibiotics was carried out using disc diffusion. The results showed that tested essential oils are active against all of the clinical strains from *Escherichia coli* including extended-spectrum β-lactamase positive bacteria (extended-spectrum β-lactamases are enzymes produced by gram-negative bacilli that mediate resistance to penicillin, cephalosporins and monobactams.

The literature reports that basil oil, which contains mainly estragole and linalool, also possesses antibacterial agents which are effective against a variety of gram-positive and gram-negative bacteria. According to Sakovic et al. (2010), *Ocimum basilicum* essential oil possesses antibacterial activity against the human pathogenic bacteria *Bacillus subtilis*, *Enterobacter cloacae*, *Escherichia coli* O157:H7, *Micrococcus flavus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *S. typhimurium*, *Staphylococcus epidermidis* and *S. aureus*.

Sweet basil oil obtained from *Ocimum basilicum,* containing mainly estragole (86.4%), inhibited the growth all strains isolated from various clinical materials. Among them were bacteria isolated from urine, which were also extended-spectrum beta-lactamase positive (Sienkiewicz et al., 2013).

Lopez et al. (2005) studied the oils from *Ocimum basilicum*, it has an antibacteria

l potential against the gram-positive bacteria *Staphylococcus aureus*, *Enterococcus faecalis* and *Listeria monocytogenes* and against gram-negative bacteria *Escherichia coli*, *Yersinia enterocolitica*, *Salmonella choleraesuis* and *Pseudomonas aeruginosa* as foodborne bacterial strains.

Antimicrobial activity is understood as the ability of some agents to eliminate microorganisms (aiming at different metabolic or structural targets, as nucleic acid synthesis disruption or peptidoglycan synthesis inhibition) or by inhibiting their growth. The highest antibacterial activity is demonstrated by phenolic compounds such as carvacrol, thymol and eugenol. Another effective group of active compounds are alcohols: terpinen-4-ol, γ-terpineol, geraniol, cytronellol, menthol and linalol. Many of them are synthesized by plants from the *Lamiaceae* family (Sienkiewicz et al., 2013).

2.4.5.1 Mechanisms of antimicrobial activity

The mechanism of action of essential oils and their components as antimicrobials has not been fully elucidated. This is complicated by the fact that there are a large number of chemical compounds present in essential oils and often they are all needed for antibacterial activity and the essential oils does not seem to have a specific cellular target. Thus the antimicrobial mechanism of essential oils may not be attributable to one specific mechanism, but rather there may be several targets in the cell. Most of the focus on antimicrobial mechanisms for essential oils has been on the cell membrane and targets interconnected with the membrane. For bioactivity, the essential oils pass through the cell wall and cytoplasmic membrane, disrupt the structure of different layers of polysaccharides, fatty acids and phospholipids and permeabilize them (Li, 2011), including degradation of the cell wall, damage to the

cytoplasmic membrane, damage to membrane proteins, leakage of cell contents, coagulation of cytoplasm, and depletion of proton motive force (Rattanachaikunsopon et al., 2010). Several major factors will be discussed individually including cell membrane integrity, leakage of ions and other contents and internal pH. For cell membrane integrity; potential explanations for this mechanism is that the hydrophobic essential oils components disrupt in the lipids of the bacterial cell membrane thus disturbing the structures of the membrane and rendering them more permeable, It is also associated with loss of ions and reduction of membrane potential, collapse of the proton pump and depletion of the ATP pool. For leakage of ions and other cell components; because the cytoplasmic membrane is rendered more permeable by essential oils components, enzymes such as ATPases, which are known to be located in the cytoplasmic membrane, may be disrupted, and further leakage of ions and other cell contents may occur. Bacteria can tolerate a small amount of leakage from their bacterial cytoplasm without loss of viability, but extensive loss of cell contents or the loss of critical molecules and ions will lead to death (Li, 2011).

Microbial cells are negatively affected by plant-derived substances via various mechanisms of actions that attack the phospholipid bilayer of the cell membrane and disrupt enzyme systems. They have an influence on enzyme activity associated with energy production or they can cause denaturation of proteins, modify cell wall permeability, causing the loss of macromolecules. Therefore, it is difficult for microorganisms to create resistance against these (Dostalova et al., 2014).

2.4.5.2 Molecules involved in antimicrobial activity

2.4.5.2.1 Phenols and polyphenols

This group comprises a large number of different molecules like

simple phenols, phenolic acids, quinones, flavonoids, flavones, flavonols, tannins and coumarins. They have in common the fact of participating in the aromatic characteristics of plants and are very common. Phenols like catechol and epicatechin or cinnamic acid (phenolic acid) participate in membrane disruption. Because phenols are very common, the antibacterial activity of plants and herbs is very often related with this phenomenon. The antibacterial activity of quinones, flavonoids, flavones and flavonols seems to be similar. Some examples are hyperacin, chrysin, and abyssinone. Their mechanism of inhibition includes ability to bind to adhesins and specially, enzymatic inhibition. Tannins are one of the most commom chemical groups present in plants and vegetable material. It is easily found in seeds, bark, and other parts of the plant. In terms of organoleptic characteristics, tannins are responsible for astringency. An example of tannins with antimicrobial activity is ellagitannin, and the mechanism of inhibition is related with their ability to bind to proteins, blocking some metabolic pathways (Silverio et al., 2012).

2.4.5.2.2 Terpenoids

Terpenoids are one of the most common constituents in plants, herbs and spices. As plant metabolites, terpenoids play a role in growth and development but also in the process of resistance against environmental stresses. Their inhibition ability is based on membrane disruption, a mechanism widely refered in scientific literature, but yet not fully understood. Diterpene metabolites like totarol and abietic acid are active against gram positive bacteria. In fact, diterpenes is one of the largest groups of plantderived natural products with anti-staphylococcal activity. This is justified by their ability to cross the bacterial cytoplasmatic membrane (due to their amphipathic character).

2.4.5.2.3 Essential oils

Essential oils do not constitute a separate molecular group. In fact, many of the molecules present in essential oils are terpenoids. However, they are treated separately because scientific literature studies them intensively and they are commercially traded as medicinal products, perfume ingredients or food additives. Essential oils are aromatic oily liquids extracted from plants. As said, essential oils are mixtures, but among the most common molecules present in some essential oils we find carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde, cinnamic acid, camphor or linalool. Some essential oils constituents like carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde and cinnamic acid have been shown to have antibacterial activity against.

2.4.5.2.4 Alkaloids

In terms of antimicrobial properties, molecules like berberine and piperine seem have the ability to intercalate into cell wall or DNA (deoxyrribonucleic acid)

2.4.5.2.5 Lectins and polypeptides

Lectins and polypeptides, whose main characteristic is the ability to form disulfide bridges, are targeted to virus. These molecules are able to block viral fusion or adsorption, probably through competition for cellular binding spots (Silverio et al., 2012).

2.4.6 Antimutagenic

Several studies provide evidence that sweet basil is an antimutagenic spice. Stajkovic et al. (2007) studied the antimutagenic effects of sweet basil on mutagenicity in *Salmonella typhimurium* TA98, TA100, and TA102 cells in the presence or absence

of liver microsomal activation. The essential oil of sweet basil, at concentrations ranging from 0.5 μl/plate to 2.0 μl/plate, inhibited mutations from ultraviolet irradiation (dose=6 J/m^2) by 22-76%. Mutations caused by 4-nitroquinoline-N-oxide (0.15 μg/plate) were decreased by 23-52%, and those from 2-nitropropane (14.9 mg/plate) by 8-30%. These findings are consistent with studies by Jeurissen et al. (2008), who demonstrated that 50 μg/ml sweet basil largely blocked DNA adduct formation caused by 1′-hydroxyestragole in the human hepatoma (HepG2) cell line, possibly by promoting phase II enzymes and thereby conjugation and elimination of this carcinogen. These findings likely explain the ability of sweet basil to decrease the mutagenicity of aflatoxin B1 (AFB1) and benzo(a)pyrene (B(a)P) (Stajkovic et al. 2007). The mutagenicity of AFB1 was inhibited by>30% by the presence of 1-2 mg/plate of a hexane-based sweet basil extract and 0.5-1 mg/plate of chloroform- and methanol-based sweet basil extracts. Because B(a)P mutagenicity was only inhibited by chloroform- and methanol-based sweet basil extracts at doses of 2-5 mg/plate, multiple constituents might be responsible for sweet basil's antimutagenic activities.

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2.5 Cytotoxicity

2.5.1 MTT assay

MTT is a standard calorimetric laboratory test used to measure the color change its used for the analysis of cell proliferation and cell cytotoxicity of cell against several potential toxic and medical agents. MTT which is a yellow color solution turns in to insoluble formazan (Figure 2.9).

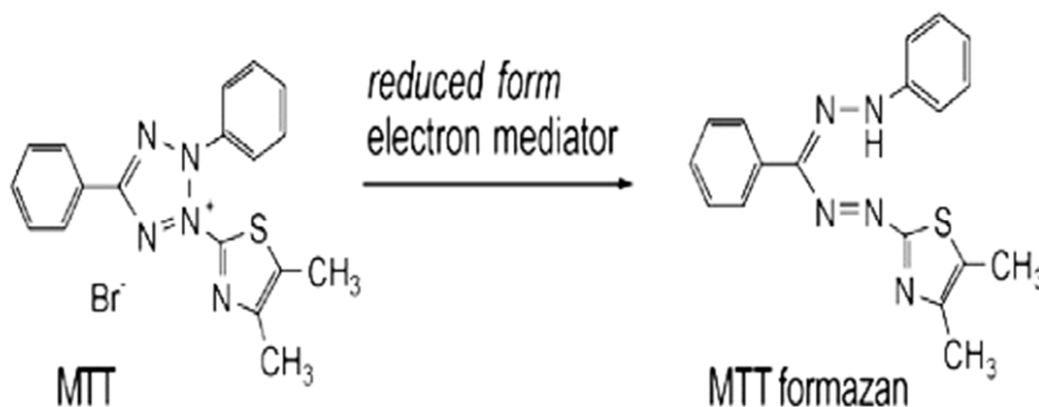

Figure 2.9 Principle of MTT assay.

Source: Talupula, 2011

In the method, MTT is reduced to a purple formazan by NADH. However, MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Therefore prior to measuring the absorbance, an organic solvent is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error. This method is easy-to-use, safe, has a high reproducibility, and is widely used in both cell viability and cytotoxicity tests. The use of the MTT method does have limitations influenced by: (1) the physiological state of cells and (2) variance in mitochondrial dehydrogenase activity in different cell types. Nevertheless, the MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves. The MTT method of cell determination is most useful when cultures are prepared in multiwell plates. For best results, cell numbers should be determined during log growth stage (Talupula, 2011).

2.6 Carcinogenicity of sweet basil (*Ocimum basilicum*)

It should be noted that there are concerns about excess sweet basil exposure. Estragole, a suspect procarcinogen/mutagenic found in sweet basil, raises questions about the balance between benefits and risks with the use of this and other spices (Muller et al. 1994). Now, the majority of evidence points to the antimutagenic effects of sweet basil outweighing the potential adverse effects associated with estragole-induced cell damage (Jeurissen et al. 2008).

Estragole (Figure 2.10) occurs naturally in a variety of traditional foods and spices such as basil, anise, fennel, bay leaves, and tarragon. Estragole is a natural organic compound that is used as an additive, flavoring agent, or fragrance in a variety of food, cleaning, and cosmetic products; as an herbal medicine; as an antimicrobial agent against acid-tolerant food microflora; and to produce synthetic anise oil (Bristol, 2011).

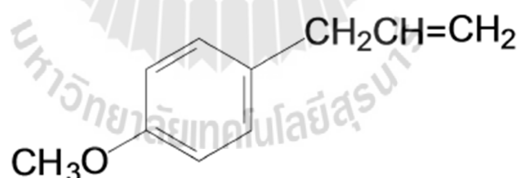

Figure 2.10 Structure of estragole.

Source: Bristol, 2011

Estragole or its metabolites administered to adult or newborn mice of different strains, through different routes of administration, produced malignant liver tumors. Carcinogenicity of estragole has not been adequately studied in the rat. One subcutaneous injection study of derivatives of estragole in adult male rats did not

observe any treatment-related increase in tumors. Regarding other relevant data, estragole produced genotoxic effects in *Salmonella typhimurium*, yeast, and mammalian cells. Several DNA adducts have been characterized. Further strong supporting evidence of carcinogenicity comes from comparisons of compounds structurally similar to estragole (e.g., safrole, methyleugenol) which produce liver tumors and tumors at other sites in rodents (McDonald, 1999).

The mode of action for estragole carcinogenicity (Figure 2.11) has been well characterized and proceeds through a genotoxic mechanism. Estragole is metabolized by the liver to 1'-hydroxyestragole and several epoxide compounds. 1'-Hydroxyestragole is further conjugated with sulfate to form a sulfuric acid ester compound that readily binds to DNA and is responsible for most, if not all, of estragole's hepatocellular carcinogenicity in mice. Metabolism of estragole through this pathway appears to be quantitatively consistent among humans and rodents (McDonald, 1999).

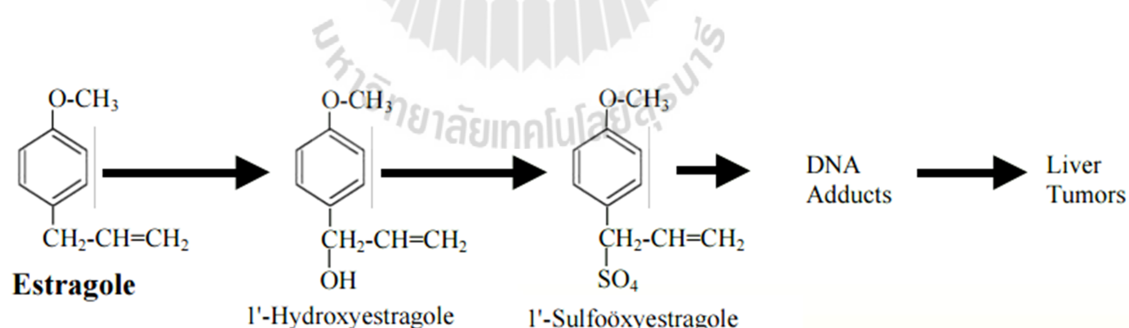

Figure 2.11 Carcinogenic mode of action of estragole.

Source: McDonald, 1999

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw materials

Thai basil leaves were prepared from *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 cultivated in Nakhon Ratchasima province, Thailand. Thai basil leaves were rinsed, dried at 50°C for 24 hours, after which they were ground by Ultra Centrifugal Mill Model ZM-1000 (Retch, Haan, Germany), sieved by mesh size 0.2 mm and stored in vacuum package at -20ºC until use.

3.1.2 Chemicals

1,1-diphenyl-2-picryhydrazyl free radical (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), ferric, chloride-6-hydrate, ferrous, sulphate 7-hydrate, acetate buffer pH 4.6, Folin-Ciocalteu phenol reagent, anhydrous sodium carbonate (Na_2CO_3), sodium nitrite *($NaNO_2$)*, *aluminum chloride* (*$AlCl_3$)* and *sodium hydroxide* (*NaOH*) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Mueller Hinton agar (MHA) was from Difco (Detroit, MI, USA). Solvents including 95% ethanol and ethyl acetate were purchased from Mallinckrodt-Baker (Phillipsburg, NJ, USA) for extraction. Solvents including acetone, acetonitrile, ethanol, ethyl acetate and methanol (Mallinckrodt-Baker, Phillipsburg, NJ, USA) were of certified HPLC and ACS grades for HPLC. The following standards were obtained: chlorophyll *a*, chlorophyll *b*, lutein, gallic acid, catechin, caffeic acid, coumaric, ferulic acid, sinapic acid and

apigenin (Sigma Aldrich, St. Louis, MO, USA). Pheophytin *a* and *b* standards were synthesized from chlorophyll *a* and *b* as described by Oonsivilai et al. (2007). Briefly, 1 mg of chlorophyll *a* or chlorophyll *b* was dissolved in 10 ml of acetone. Then 400 µl of 0.1 N HCl was added into 5 ml of chlorophyll *a* or *b* solution. Complete conversion to pheophytins was followed by HPLC analysis as described below. Pheophytins were extracted with 5 ml of petroleum ether, dried under a stream of nitrogen and kept at -80°C until use. Prior to HPLC calibration, each standard was dissolved in appropriate solvent and filtered through a 0.45 µm PTFE membrane.

3.2 Proximate analysis

Dry Thai basil powders were analyzed for chemical composition. Moisture analysis was done by AOAC method 925.10. Ash and sand quantification were done using AOAC method 900.02A and 900.02D, respectively. Protein and fat quantification were conducted by AOAC method 928.08 and 963.15, respectively. Crude fiber determination was done by AOAC method 978.10. (AOAC, 2005)

3.2.1 Moisture

Moisture was determined by oven drying method. One g of sample was placed into a pre-weighed and dried moisture can. The moisture can was allowed in an oven at 105°C for 6-12 hours or until a constant weight was obtained. Then, the moisture can was placed in the desiccator to cool. After cooling it was weighed again. The percent moisture was calculated by following formula:

$$\text{Moisture percentage} = \frac{(A-B)\times 100}{C}$$

A = weight of moisture can + weight of sample before oven

B = weight of moisture can+ weight of sample after oven

C = weight of sample

3.2.2 Ash

For the determination of ash, empty porcelain crucible was placed in a muffle furnace at 600°C for an hour, cooled in a desiccator, and then weight of empty porcelain crucible was noted. One gram of sample was taken in porcelain crucible in a furnace at 550°C for 3 hours. After ashing, the samples were removed from the furnace, cooled to room temperature in a desiccator and then reweighed. Percent ash was calculated by following formula:

$$\%Ash = \frac{\text{Difference in Wt.of Ash(g)} \times 100}{\text{Wt.of sample(g)}}$$

3.2.3 Sand

Twenty-five ml of 10% hydrochloric acid was added to the ash that has been burned off with watch glass. Boil sample gently for 5 min, then filtered while hot through ashless filter paper and washed with water several times. Take filter paper in the same cup glaze, then burned in a muffle furnace at 550°C for 12-18 hours or until a constant weight of the sample. After that bring the sample out of the furnace, cooled at room temperature in a desiccator and weighed. Percent sand was calculated by following formula:

$$\%Sand = \frac{\text{Wt.of sand(g)} \times 100}{\text{Wt.of sample(g)}}$$

3.2.4 Crude protein

Crude protein was determined by Kjeldahl method and was based on the measurement the nitrogen content of the feed and multiplying it by a factor of 6.25. This factor is based on the fact that most protein contains 16% nitrogen. One g of sample was taken in kjeldahl flask and 15 ml of concentrated H_2SO_4 and 5 g of

digestion mixture that is K_2SO_4:$CuSO_4$ (10:1) was added. The flask was swirled in order to mix the contents thoroughly then placed on the heater to start digestion untill the mixture become clear (blue green in color). The digest was cooled and 15-20 ml of distilled water was added. Distillation of the digested sample was performed in Markham distillation apparatus. Fifty ml of 40% sodium hydroxide was added into the kjeldahl flask, distilled until the volume is 150 ml. Distillation was continued for 7-10 min. Ammonia produced was collected as ammonium hydroxide in a conical flask containing 25 ml of 4% boric acid solution with a few drops of modified methyl red indicator. The distillate was then titrated against standard 0.1 N HCl solution till the appearance of pink color. A blank was also run through all steps as above. Percent crude protein content of the sample was calculated by using the following formula:

$$\%\text{Crude protein} = \text{N} \times \frac{\text{Corrected acid vol.(ml)}}{\text{Wt.of sample(g)}} \times 14 \times 100 \times \text{Factor}$$

N = Normality of HCl

Corrected acid vol. = Sample titration reading-Blank titration reading

3.2.5 Crude fat

Dry extraction method for fat determination was indicated. It consisted of extracting dry sample with some organic solvent, since all the fat materials such as fats, phospholipids, sterols, fatty acids, carotenoids, pigments, chlorophyll etc. are extracted together. Therefore, the results are frequently referred to as crude fat.

Crude fat of dry Thai basil powders was determined by Soxhlet extraction using petroleum ether on a SoxtecTM 2050 auto fat extraction system. Briefly, one g of the sample was wrapped in filter paper and placed in fat free thimble then 80 ml of petroleum ether were added. The thimble was moved to the Soxtec System. Extraction was performed for 3 hours. The solvent was evaporated off and

the extraction was removed and placed into an oven at 105°C for 2 hours. The drying cup was taken from the oven and cooled down in a desiccator. The percent of crude fat was determined by using the following formula:

$$\%\text{Fat} = \frac{\text{Wt.of fat(g)}}{\text{Wt.of sample(g)}} \times 100$$

Wt.of fat =Wt.of can after extraction(g)-Wt.of can before extraction(g)

3.2.6 Crude fiber

One g of the fat-free sample was transferred into a flask/beaker. One hundred and fifty ml of preheated 1.25% H_2SO_4 solution was added and the solution was gently boiled for about 30 min. The buckner flask funnel fitted with whatman filter was pre-heated by pouring hot water into the funnel. The boiled acid sample mixture was filtered hot through the funnel under sufficient suction. The residue was washed several times with boiling water and transferred back into the beaker. Then, 150 ml of preheated 1.25% NaOH solution was added and the solution was gently boiled for about 30 min. Filter under suction and wash thoroughly with hot water several times and wash with 10 ml of 95% ethanol. The residue was transferred into a crucible and it was dried at 105˚C for 3 hours or until a constant weight was obtained, the residue (including crucible) was placed in the desiccator to cool. After cooling it was weighed. The residue with crucible was placed in muffle furnace at 500˚C for 2 hours. After that, it was cooled to room temperature in a desiccator and reweighed. Calculations were done by using the formula:

$$\%\text{Crude fiber} = \frac{\text{Wt.of fiber(g)}}{\text{Wt.of sample(g)}} \times 100$$

Wt.of fiber =Dry Wt.of residue before burned-Wt.of residue after burned

3.2.7 Energy

The energy was determined by using the following formula:

Energy (calories/100g) =[(%available carbbohydrate×4)+(%protein×4)+(%fat×9)]

1 calories = 4.2 Joules

%Available carbohydrate =100-[%moisture+%Ash+%Fat+%Protein+%Fiber]

3.3 Preparation of Thai basil Extracts

Thai basil powder about 5.0 g was extracted with 50 ml portions of boiling water, ethanol and ethyl acetate by shaking for 18 hours and then centrifuged at 2800 rpm (Thermo Scientific, Braunschweig, Germany) for 15 min, filtered and the volumes were adjusted to 50 ml with the same solvent. Aliquaots of 2 ml were added in test tubes (VWR, Wilmington, NC). The solvent was removed with rotary evaporator (Buchi R-114, *Flawil*, Switzerland) for ethanol and ethyl acetate, and for water with freeze dryer (GEA, LYOVAC GT2-S, Dusseldorf, Germany). The samples were kept at -20°C until use (modified method of Bora et al., 2011). The quantification of the extraction based on assumption of complete extraction of active ingredient from raw materials, used in all experiment.

3.4 Phytochemical profiling

3.4.1 Chlorophyll and carotenoid profiling of Thai basil extracts

Chlorophyll and carotenoid of Thai basil extracts were determined by HPLC according to the method described by Oonsivilai et al. (2007). Briefly, separation was determined using a Grace-Vydac 201TP54 reversed-phase (4.6 mm×250 mm) polymeric C18 column with guard column containing the same

stationary phase (Grace Vydac, Apple Valley, MN). A gradient elution profile was used based on a binary mobile phase system consisting of methanol:water: ammonium acetate (73:25:2, v/v/v) in reservoir A and ethyl acetate in reservoir B. A flow rate of 1.0 ml/min was applied with initial setting at 100% (A) with a linear gradient to 50:50 (A/B) over 10 min. The gradient was held for 10 min followed by a 5 min linear gradient back to 100% (A) and equilibration at the initial condition for 5 min for a total run time of 30 min. Detection and tentative identification of chlorophyll a and b were accomplished by comparison of retention times and on line-electronic absorption spectra with that of authentic standards collected between 250 and 600 nm. Quantification were made by the calibration curves of the respectively standards. The calibration curves of chlorophylls a and b were linear over the range of 0.1-5 ppm (correlation coefficient = 0.99)

3.4.2 Phenolics profiling of Thai basil extracts

Phenolic compounds were determined as described by Oonsivilai et al. (2007) with modification. A Waters NovaPak C18 (3.8 mm×150 mm) reversed phase column (Milford, MA) with a guard column also packed with Waters NovaPak C18. Phenolic compounds were separated by gradient elution with a flow rate of 1.0 ml/min at 35°C using a binary mobile phase of water/acetic acid (98:2, v/v) in reservoir A and acetonitrile in reservoir B. Initial solvent proportion of 99:1 A/B with a linear gradient to 70:30 A/B over 20 min was used, followed by a 5 min linear gradient back to 99:1 A/B and 5 min equilibration at the initial condition for a total chromatographic run time of 30 min. Detection and tentative identification of phenolic compounds was accomplished using in-line PDA data between 200 and 500 nm. Calibrations plots for

quantification were constructed from injection of authentic standards of gallic acid, catechin, caffeic acid, coumaric, ferulic acid, sinapic acid and apigenin (5-5ppm).

3.4.3 Total phenolics

Total phenolics were determined by the Folin Ciocalteu procedure described by Oonsivilai et al. (2007) and gallic acid was used as a standard. Aliquot (20 µl) of samples was added to test tube, after that 1.58 ml DI water was added followed by 100 µl Folin-Ciocalteu reagent, then the mixture was thoroughly mixed and further incubated for 5 min at room temperature. Following incubation, 300 µl of the Na_2CO_3 (20% w/v) solution was added and the mixture was allowed to stand at room temperature in the dark for 2 hours. Absorbance was measured at 765 nm using spectrophotometer (Biochrom Libra S22, Cambridge, UK). Gallic acid (50, 100, 250, 500 and 750 ppm (mg/l)) was used as standard. Results were expressed as gallic acid equivalents.

3.4.4 Total flavonoid contents

Total flavonoids contents of the samples were determined by a modified colorimetric method described by Juan et al. (2010) and used catechin as a standard. Aliquot (250 µl) of samples was added to test tube. After that 1.25 ml DI water was added followed by 5% $NaNO_2$ 75 µl, then mixture was incubated for 6 min. Following incubation, 150 µl of the 10% $AlCl_3$ solution was added followed by 1 M NaOH 0.5 ml, 275 µl DI water, respectively. The mixture was thoroughly mixed and incubated for 5 min. Absorbance was measured at 510 nm using spectrophotometer (Biochrom Libra S22, Cambridge, UK). Catechin (0-350 ppm (mg/l)) was used as standard. Results were expressed as catechin equivalents.

3.5 Antioxidant activity

3.5.1 DPPH assay

Antioxidant activity by DPPH assay of Thai basil extracts (hot water, ethanol and ethyl acetate), BHT (Acros Organics, NJ, USA) and ascorbic acid (Carlo Erba Reagents, Peypin, France) were determined using spectrophotometer (Biochrom Libra S22, Cambridge, UK) according to the method described by Oonsivilai et al. (2008). Briefly, 0.1 mM solution of DPPH in methanol was prepared. An aliquot (100 μl) of samples at the varied concentration was mixed with 1.9 ml of methanolic DPPH solution and incubated for 15 min. The decrease in absorbance at 515 nm was determined by using spectrophotometer (Biochrom Libra S22, Cambridge, UK). The percentage of scavenging was calculated as the ratio of the absorption of the sample relative to the control DPPH solution, without the extracts. The BHT and ascorbic acid in methanol solution were used as positive controls. The inhibition percentage of the DPPH radical was calculated according to the formula:

$$\text{Inhibition\%} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right) \text{x}100$$

A_{blank} = Absorbance of blank

A_{sample} = Absorbance of sample

3.5.2 FRAP assay

Antioxidant activity by FRAP assay of Thai basil extracts (hot water, ethanol and ethyl acetate) and BHT were determined using spectrophotometer (Biochrom Libra S22, Cambridge, UK) according to the method described by Oonsivilai et al. (2008). Briefly, the working FRAP reagent was prepared by acetate buffer (pH 3.6), 10 mmol TPTZ solution in 40 mmol HCl and 20 mmol ferrous chloride

solution in the proportion of 10:1:1 (v/v). Freshly prepared FRAP reagent was warmed to 37°C in a water bath prior to use. The extract of 50 µl was added to 1.5 ml of the FRAP reagent and incubated for 4 min. Absorbance was measured at 593 nm using spectrophotometer (Biochrom Libra S22, Cambridge, UK). All solutions were used on the day of preparation. The BHT was used as positive controls and standard curve was prepared using different concentrations of ferric sulphate solution (0.2-1 mM). The results were expressed as µmol equivalents of ferric per g dry weight of plant materials.

3.5.3 Cellular antioxidant activity

3.5.3.1 Cell culture

HepG2 (human liver hepatocarcinoma ATCC Cat. No. HB-8065). HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% 2mM L-glutamine and, 1% penicillin-streptomycin (Gibco, Gaithersburg, MD, USA). Cells were incubated at 37°C in a fully humidified incubator with 5% CO_2:95% air atmosphere.

3.5.3.2 Cytotoxicity of extracts

Cells were seeded into a 96 well plate at a density of $0.5x10^4$ cells/well and incubated in a humidified incubator containing 5% CO_2 and 95% air at 37°C for 24 hours. Thai basil dried extracts were dissolved and diluted to various extracts concentrations in culture medium containing 0.1% DMSO (Amresco, Solon, OH, USA). The extracts were added to the wells and incubated for 24 hours. After 24 hours, the extracts were removed and the cells were washed with PBS (Gibco, Gaithersburg, MD, USA). Add 100 µl of culture media containing 0.5 mg/ml MTT (Invitrogen, Carlsbad, CA, USA) into each well and incubated for 4 hours. After 4

hours of incubation, the media containing MTT was gently removed, washed gently in PBS and 100 µl DMSO was added to each well to solubilize the crystallized formazan product. Absorbance was monitored at 570 nm using a microplate reader (Bio-Rad Benmark Plus, CA, USA). Two controls were set up, one with medium as reagent control and the second with the 0.1% DMSO as solvent control. A dose-response curve was derived from 5 concentrations in the test range of 50-500 µg/ml. Results of toxic compounds were expressed as the lethal concentration 50% (LC_{50}) of the cells compared with controls.

3.5.3.3 Cellular antioxidant activity (CAA) by DCFH-DA assay

Cellular antioxidant activity of Thai basil extracts were determined by a modified method of Wolfe et al. (2007). HepG2 cells were seeded at a density of 6×10^4 per well on a 96-well plate in 100 µl of growth medium/well. Twenty-four hours after seeding, the growth medium was removed and the wells were washed with PBS. Wells were treated for 1 hours with 100 µl of Thai basil (25 µg/ml-500 µg/ml) extracts and then the extracts were removed and washed with PBS. One hundred µl 25 µM DCFH-DA (Sigma Aldrich, St. Louis, MO, USA) was added into the wells and wells were treated for 30 min. When a PBS wash was utilized, wells were then washed with 100 µl of PBS. Then 600 µM ABAP (Sigma Aldrich, St. Louis, MO, USA) in 100 µl of HBSS (Gibco, Gaithersburg, MD, USA) was applied to the cells, and the 96-well plate was placed into a Spectramax Gemini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 37°C. Emission at 538 nm was measured with excitation at 485 nm every 5 min for 1 hours. Each plate included control and blank wells: control wells contained cells treated with DCFH-DA and

oxidant; blank wells contained cells treated with dye and HBSS without oxidant. Vitamin E (Abcam, Cambridge, MA, USA) was used to positive control.

3.6 Antimicrobial activity

3.6.1 Microbial strains

The samples were evaluated against a panel of microorganisms including the bacterial strains *Bacillus cereus* (TISTR687), *Bacillus subtilis* (TISTR008), *Enterobacter aerogenes* (bcc6719), *Escherichia coli* (TISTR3436), *Pseudomonas aeruginosa* (TISTR781) and *Staphylococcus aureus* (TISTR1466). Bacterial strains were cultured overnight at 37°C in MHA.

3.6.2 Disk diffusion method

The antimicrobial activities of the extracts were determined by agar disk diffusion method of Lorian V. (1996). Briefly, microorganisms were prepared for the test by increasing the number of bacteria in broth and measurement turbidity of bacteria to 10^8 colony-forming units (cfu)/ml (0.5 McFarland scale). Spread 100 µl a suspension of bacteria on MHA. The filter disks (6 mm in diameter) were placed on the agar plates and treated with 10 µl of extracts. Disk without samples were used as a negative control (DMSO 75% with solvent). Chloramphenicol (Sigma Aldrich, St. Louis, MO, USA) was used as positive references. The plates were incubated at 37°C for 24 hours. Antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zones in millimeters (including disk diameter of 6 mm) for the test organisms and comparing to the controls. The measurements of inhibition zones were carried for three sample replications, and values are the average of three replicates.

3.7 Antithrombotic activity

The antithrombotic activity of the extracts was determined using prothrombin time (PT) method by Rizzo et al. (2008) with modification. Reconstitute tissue thromboplastin from rabbit (Sigma Aldrich, St. Louis, MO, USA) according to instructions. The tissue thromboplastin-calcium chloride reagent (PT reagent) was incubated at 37°C. Pipet 100 µl of citrated (3.8% sodium citrate) rabbit plasma (NLAC, Mahidol University, Thailand) containing various concentrations of Thai basil (100 µg/ml-500 µg/ml) extract into a 12x75 mm test tube and incubated for 1 min at 37°C. Two hundred µl of PT reagent was added into the tube. Start the stopwatch simultaneously. The time from the plasma-reagent mixing to clot formation (visually detected) was defined as the prothrombin time. Warfarin sodium (Sriprasit Pharma, Bangkok, Thailand) was used as positive references.

3.8 Statistical analysis

Results were expressed as mean ± standard deviation. Analyses of variance (ANOVA) were conducted and differences among samples means were analyzed by Duncan's multiple range test ($p<0.05$) by using SPSS

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Proximate composition

The proximate composition of dried *Ocimum basilicum* var. *Thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 powder are presented in Table 4.1. It was found that *Ocimum basilicum* var. *Thyrsiflorum* powder showed significantly higher moisture, crude fat and carbohydrate than *Ocimum basilicum* cv. Jumbo 4320 powder ($p<0.05$). While the *Ocimum basilicum* cv. Jumbo 4320 powder showed significantly higher ash and crude protein than *Ocimum basilicum* var. *Thyrsiflorum* powder ($p<0.05$).

Table 4.1 Proximate composition of dried Thai basil powder.

Chemical composition	Percentage (% dry weight)	
	Ocimum basilicum* var. *Thyrsiflorum	***Ocimum basilicum* cv. Jumbo 4320**
Moisture	5.10 ± 0.11^{b}	4.07 ± 0.04^{a}
Ash	13.89 ± 0.03^{a}	14.60 ± 0.04^{b}
Crude protein	25.92 ± 0.13^{a}	28.72 ± 0.16^{b}
Crude fat	3.26 ± 0.04^{b}	2.55 ± 0.34^{a}
Crude fiber	9.28 ± 0.21	9.34 ± 0.32
Carbohydrate	42.54 ± 0.30^{b}	40.72 ± 0.29^{a}
Energy (cal/100 g)	303.19 ± 1.12	300.70 ± 2.88

All values are means of triplicate determinations ± standard deviation.

^{a,b} Data in the same row with different superscripts are significantly different ($p<0.05$).

The vegetables, being a good source of protein, may serve as a food supplement for malnourished communities. Their high crude protein provides complementary-supplementary amino acids with animal protein or cereal protein that characterizes human foods especially in the tropical regions (Agunbiade et al., 2015).

4.2 Phytochemical profiling

4.2.1 Chlorophylls and carotenoid profile of Thai basil extracts

Results from a reverse phase HPLC suggested that chlorophylls and carotenoid (lutein) were achieved in Thai basil ethanol and ethyl acetate extracts but not in hot water extracts. Lutein and major chlorophyll derivatives including, chlorophyll *a*, chlorophyll *b*, pheophytin *a* and *b* were identified as evaluated by UV-vis spectra and co-chromatography with authentic standards.

Chlorophylls and lutein of *Ocimum basilicum* var. *Thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 crude extracts are presented in Table 4.2. For comparison between the solvents extraction, it was found that the chlorophyll *a*, chlorophyll *b*, pheophytin *a*, pheophytin *b*, total chlorophylls and lutein contents of ethanol and ethyl acetate extracts are significantly different ($p<0.05$). The ethanol extraction showed higher values of chlorophyll *b*, pheophytin *a*, pheophytin *b*, total chlorophylls and lutein contents than ethyl acetate extraction. Only chlorophyll *a* content of ethyl acetate extract was higher than ethanol extract. When compared between the species, it was found that the *Ocimum basilicum* var. *Thyrsiflorum* crude extract showed significantly higher chlorophyll *a*, chlorophyll *b*, total chlorophyll and lutein contents than *Ocimum basilicum* cv. Jumbo 4320 crude extract ($p<0.05$), while *Ocimum basilicum* cv. Jumbo 4320 crude extract showed significantly higher pheophytin *a* and

pheophytin *b* contents than *Ocimum basilicum* var. *Thyrsiflorum* crude extract ($p<0.05$).

Ocimum basilicum var. *Thyrsiflorum* extracted by ethyl acetate showed the highest chlorophyll *a* contents at the value of 4,188.29±6.96 µg/g RM. *Ocimum basilicum* var. *Thyrsiflorum* extracted by ethanol showed the highest chlorophyll *b*, total chlorophylls and lutein contents at the value of 1,355.73±0.86 µg/g RM, 7,557.84±6.25 µg/g RM and 405.24±0.23 µg/g RM, respectively. *Ocimum basilicum* cv. Jumbo 4320 extracted by ethanol showed the highest pheophytin *a* and pheophytin *b* at the values of 4,127.16±1.95 µg/g RM and 124.58±0.37 µg/g RM, respectively.

These results indicated that chlorophyll derivatives are the predominant lipophilic phytochemicals presented in Thai basil ethanol and ethyl acetate extracts and efficiently extracted by the conditions utilized in these preparations. Moreover, the presence of significant amounts of chlorophylls degradation products (pheophytin *a* and *b*) is likely a result of post-harvest handling of leaves prior to extraction. Hot air drying and milling of the Thai basil leaves during raw material preparation, similar to the method generally used by local people, would further expose leaf material to conditions favorable for degradation of chlorophylls. The extreme sensitivity of chlorophylls to heat is known to generate metal free pheophytin derivative (Oonsivilai et al., 2007).

4.2.2 Phenolics profiling of Thai basil extracts

HPLC separation of the phenolic Thai basil extracts was recognized to contain several phenolic acids and flavonoid including gallic acid, catechin, apigenin, caffeic acid, coumaric acid, ferulic acid and sinapic acid. The separation profile from *Ocimum basilicum* var. *Thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 crude extracts are

presented in Table 4.3. Gallic acid, catechin, apigenin, caffeic acid, coumaric acid, ferulic acid and sinapic acid were identified by co-chromatography with authentic standards. Gallic acid of *Ocimum basilicum* var. *Thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 crude extracts were quantified to be at 248.60±7.61, 186.98±0.66, 189.55±1.14 µg/g RM and 227.70±2.39, 195.78±0.93, 190.14±0.56 µg/g RM in water, ethanol, and ethyl acetate extracts, respectively. Catechin of *Ocimum basilicum* var. *Thyrsiflorum* crude extracts was quantified to be at 111.49±7.65 and 131.94±5.10 µg/g RM in water and ethanol extracts, respectively. For *Ocimum basilicum* cv. Jumbo 4320 crude extracts was quantified to be at 201.05±9.18 µg/g RM in water. Catechin in ethanol extract of *Ocimum basilicum* var. Jumbo 4320 and ethyl acetate extract of both species was not detectable. Apigenin could be detected only in water extract of *Ocimum basilicum* cv. Jumbo 4320. Caffeic acid of *Ocimum basilicum* var. *Thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 crude extracts were quantified to be at 19.52±0.71, 32.83±2.32 µg/g RM and 63.76±0.79, 32.94±2.50 µg/g RM in water and ethanol extracts, respectively. Caffeic acid in ethyl acetate extract of both species was not detected. Coumaric acid of *Ocimum basilicum* var. *Thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 crude extracts were quantified to be at 178.81±0.54, 175.00±0.55 µg/g RM and 178.65±0.36, 188.69±2.14 µg/g RM in water and ethanol extracts, respectively. Coumaric acid in ethyl acetate extracts of both species were not detectable. Ferulic acid was also undetectable in both species for all solvents extraction. Sinapic acid at 107.53±0.96 µg/g RM could be detected in *Ocimum basilicum* var. *Thyrsiflorum* ethanol extract. Sinapic acid could also be found in water and ethanol extracts of *Ocimum basilicum* cv. Jumbo 4320 at the values of 104.88±1.08 and 111.42±1.01 µg/g RM, respectively.

In agreement with our results, other investigators also reported the presence of phenolic acids such as p-coumaric acid, caffeic acid, sinapic acid (Bais et al., 2002; Lee et al., 2010; Javanmardi et al., 2003); flavonoids and tannins (Grayer et al., 1996) in sweet basil. However, variation of plant cultivars and environmental conditions of growth could contribute to differences in phenolic profiling.

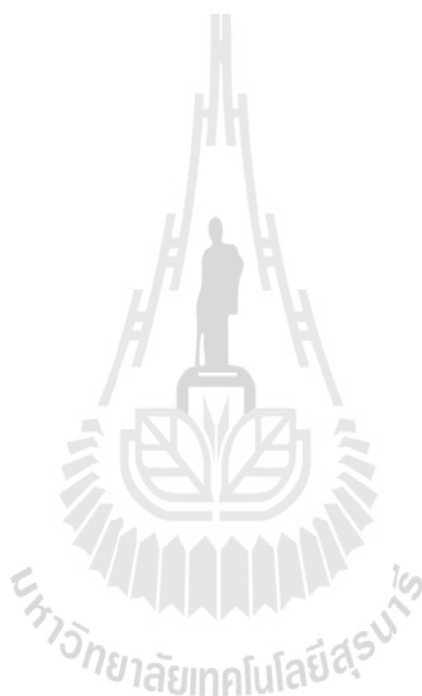

Table 4.2 Phytochemical profile of Thai basil crude extracts.

Phytochemical Contents (µg/g RM)	*Ocimum basilicum* var. *thyrsiflorum*			*Ocimum basilicum* cv. Jumbo 4320		
	Solvent			Solvent		
	Water	Ethanol	Ethyl acetate	Water	Ethanol	Ethyl acetate
Chlorophyll *a*	ND	2,191.18 ± 3.61^{b,e}	4,188.29 ± 6.96^{d,f}	ND	1,547.30 ± 2.69^{a,e}	3,369.72 ± 6.58^{c,f}
Chlorophyll *b*	ND	1,355.73 ± 0.86^{b,f}	1,280.29 ± 0.79^{d,e}	ND	1,130.35 ± 0.10^{a,f}	1,089.77 ± 1.92^{c,e}
Pheophytin *a*	ND	3,916.84 ± 1.06^{a,f}	1,159.26 ± 2.75^{c,e}	ND	4,127.16 ± 1.95^{b,f}	1,707.61 ± 1.87^{d,e}
Pheophytin *b*	ND	94.09 ± 0.72^{a,f}	20.34 ± 0.61^{c,e}	ND	124.58 ± 0.37^{b,f}	70.47 ± 2.36^{d,e}
Lutein	ND	405.24 ± 0.23^{b,f}	374.31 ± 0.62^{d,e}	ND	360.51 ± 0.30^{a,f}	306.01 ± 0.68^{c,e}
Total chlorophylls	ND	7,557.84 ± 6.25^{b,f}	6,648.18 ± 11.11^{d,e}	ND	6,929.39 ± 5.11^{a,f}	6,237.57 ± 12.73^{c,e}

Note: Each value is mean ± SD (n=3), ND Means not detected

^{a,b} Data within the same row with different superscripts are significantly different ($p<0.05$) for ethanol extraction between species.

^{c,d} Data within the same row with different superscripts are significantly different ($p<0.05$) for ethyl acetate extraction between species.

^{e,f} Data within the same row with different superscripts are significantly different ($p<0.05$) for different solvent extraction within the same species.

Table 4.3 Phenolics profile of Thai basil crude extracts.

Phenolics Contents (µg/g RM)	*Ocimum basilicum* var. *thyrsiflorum*			*Ocimum basilicum* cv. Jumbo 4320		
	Solvent			Solvent		
	Water	**Ethanol**	**Ethyl acetate**	**Water**	**Ethanol**	**Ethyl acetate**
Gallic acid	248.60 ± 7.61^{b,f}	186.98 ± 0.66^{c,e}	189.55 ± 1.14^{e}	227.70 ± 2.39^{a,g}	195.78 ± 0.93^{d,f}	190.14 ± 0.56^{e}
Catechin	111.49 ± 7.65^{a,e}	131.94 ± 5.10^{f}	ND	201.05 ± 9.18^{b}	ND	ND
Apigenin	ND	ND	ND	29.56 ± 0.65	ND	ND
Caffeic acid	19.52 ± 0.71^{a,e}	32.83 ± 2.32^{f}	ND	63.76 ± 0.79^{b,f}	32.94 ± 2.50^{e}	ND
Coumaric acid	178.81 ± 0.54^{f}	175.00 ± 0.55^{c,e}	ND	178.65 ± 0.36^{e}	188.69 ± 2.14^{d,f}	ND
Ferulic acid	ND	ND	ND	ND	ND	ND
Sinapic acid	ND	107.53 ± 0.96^{c}	ND	104.88 ± 1.08^{e}	111.42 ± 1.01^{d,f}	ND

Note: Each value is mean ± SD (n=3), ND Means not detected.

^{a,b} Data within the same row with different superscripts are significantly different ($p<0.05$) for water extraction between species.

^{c,d} Data within the same row with different superscripts are significantly different ($p<0.05$) for ethanol extraction between species.

^{e,f,g} Data within the same row with different superscripts are significantly different ($p<0.05$) for different solvent extraction within the same species.

4.2.3 Total phenolic

Total phenolic contents of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 extracted by water, ethanol and ethyl acetate were investigated by Folin-Ciocalteu method. The total phenolic contents in all extracts are presented in Figure 4.1.

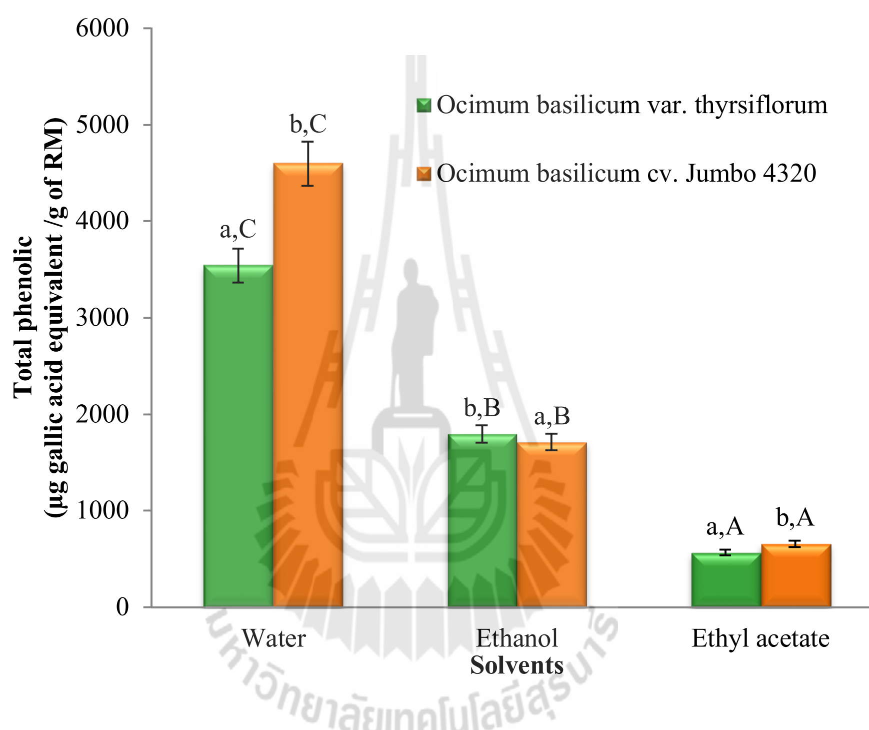

Figure 4.1 Total phenolic contents of Thai basil crude extracts.

Data are mean ± SD (n=3)

[a,b] Different letters over the error bars denote that the means differed significantly ($p<0.05$) on species.

[A,B,C] Different letters over the error bars denote that the means differed significantly ($p<0.05$) on solvents.

The total phenolic contents in all solvents extracts are significantly different ($p<0.05$). Also, the total phenolic contents of water extract showed the highest value

followed by ethanol and ethyl acetate extracts, respectively. Furthermore, total phenolic contents of both species are also significantly different ($p<0.05$). *Ocimum basilicum* cv. Jumbo 4320 extracted by water showed the highest total phenolic contents at 4,596.19±3.07 µg gallic acid equivalent/g of RM. Phenolic compounds are often extracted in higher amounts in more polar solvents such as aqueous methanol/ethanol (Quy et al., 2014). This result supported similar findings.

4.2.4 Total flavonoid contents

The total flavonoid contents in all extracts are presented in Figure 4.2. The total flavonoid contents of all solvents extracts are significantly different ($p<0.05$) with ethanol extracts showed the highest value followed by ethyl acetate and water extracts, respectively. When compared between two species studied, the *Ocimum basilicum* cv. Jumbo 4320 extract showed higher values of flavonoid contents than *Ocimum basilicum* var. *thyrsiflorum* with significantly different ($p<0.05$). Both species of Thai basils ethyl acetate extract is not significant different ($p<0.05$). *Ocimum basilicum* cv. Jumbo 4320 extracted by ethanol showed the highest total flavonoid contents at 5,571.16±14.27 µg catechin equivalent/g of RM.

The phenolic and flavonoids compounds such as cinnamic acid, caffeic acid, sinapic acid, ferulic acid and rosemarinic acid in *Ocimum basilicum* have also been reported (Grayer *et al*., 1996). The chemical composition of sweet basil varies significantly depending upon the cultivar, season, environmental and genetic factors, chemotype and origin of the plants (Sajjadi, 2006). Polar solvents are frequently used for recovering polyphenols from plant matrices. The most suitable solvents are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate. Ethanol has

been known as a good solvent for polyphenol extraction and is safe for human consumption (Quy et al., 2014).

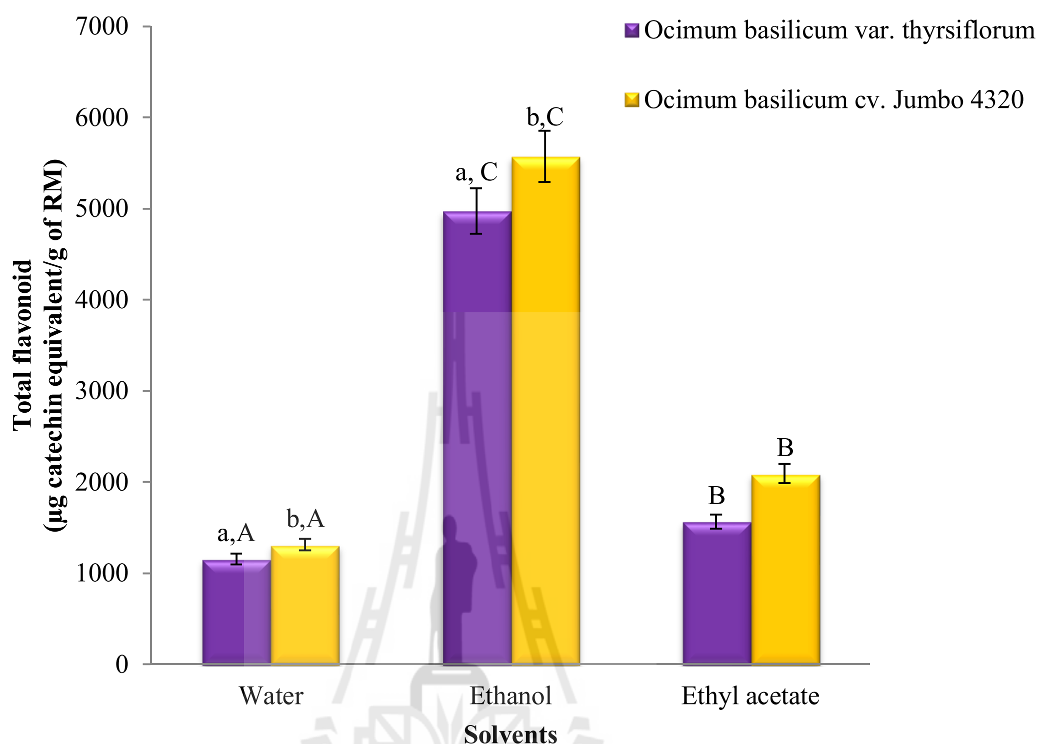

Figure 4.2 Total flavonoid contents of Thai basil crude extracts.

Data are mean ± SD (n=3)

[a,b] Different letters over the error bars denote that the means differed significantly ($p<0.05$) on species.

[A,B,C] Different letters over the error bars denote that the means differed significantly ($p<0.05$) on solvents.

Most of the antioxidant substances in plants are phenolic compounds. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which could be play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Asami et al., 2003).

The choice of extraction method is an important factor for extracting antioxidant phytochemicals. Lipophilicity or hydrophilicity affects the solubility of a phytochemical in the extracting solvent and, conversely, polarity of a solvent also has an impact on the extraction efficiency. Many different extractions methods are available for extracting antioxidant phytochemicals, but most popular procedures are based on solvent extraction using water, organic solvent or liquefied gas, or combinations of these under different temperature and pressure, although other methods such as physical press, filtration, stream distillation and solid absorption have been used. Polar antioxidants such as phenolic acids and glycosides of many flavonoids are generally extracted using water, alcohol or a mixture of water and alcohols. (Oonsivilai et al., 2008).

4.3 Antioxidant activity

4.3.1 DPPH assay

The DPPH radical scavenging activity of Thai basil crude extracts are presented in Table 4.4. Extracts obtained from different extraction solvents, differed significantly in scavenging DPPH radicals ($p<0.05$). When compared between two studied species, the antioxidant activities of Thai basils extracted by water and ethanol are significantly different ($p<0.05$). Whereas there was no different in ethyl acetate extraction. *Ocimum basilicum* cv. Jumbo 4320 water extract show the highest scavenging capacity against DPPH radicals at IC_{50} value of 48.52±1.15 mg RM/ml. The antioxidant activity by DPPH assay of all Thai basil crude extract was less than BHT and ascorbic acid, the commercial standards.

Table 4.4 DPPH radical scavenging activity of Thai basil crude extracts.

Solvents	DPPH IC$_{50}$ (mg RM/ml)	
	Ocimum basilicum* var. *thyrsiflorum	***Ocimum basilicum* cv. Jumbo 4320**
Water	$105.62 \pm 3.77^{b,D}$	$48.52 \pm 1.15^{a,B}$
Ethanol	$53.88 \pm 0.74^{a,B}$	$60.54 \pm 0.52^{b,C}$
Ethyl acetate	72.48 ± 5.57^{C}	82.09 ± 3.18^{D}
BHT	0.18 ± 0.00^{A}	0.18 ± 0.00^{A}
Ascorbic acid	0.06 ± 0.00^{A}	0.06 ± 0.00^{A}

Note: Each value is mean ± SD (n=3)

^{a,b} Data within the same row with different superscripts are significantly different ($p<0.05$).

^{A,B,C,D} Data within the same column with different superscripts are significantly different ($p<0.05$).

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4.3.2 FRAP assay

The antioxidant potential of the *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 extracts were estimated for their power to reduce the TPTZ-Fe(III) complex to TPTZ-Fe(II) complex in the FRAP assay, which is simple, fast and reproducible (Wong et al., 2006). The results are expressed as mmol ferrous equivalents/g of sample (Oonsivilai et al., 2008).

The antioxidant activities in each extract are presented in Table 4.5. For the comparison among extraction solvents, the antioxidant activities in the extracts are no

significantly different. When compared between two species, the antioxidant activities of both species are significantly different ($p<0.05$) in all extraction solvents. The antioxidant activity by FRAP assay of all Thai basil crude extract was less than BHT, the commercial standards.

Table 4.5 Ferric reducing antioxidant power of Thai basil crude extracts.

Solvents	**FRAP (μmol Fe^{2+}/g RM)**	
	Ocimum basilicum* var. *thyrsiflorum	***Ocimum basilicum* cv. Jumbo 4320**
Water	$13.86 \pm 0.07^{b,A}$	$8.99 \pm 0.02^{a,A}$
Ethanol	$18.64 \pm 0.13^{b,A}$	$15.30 \pm 0.11^{a,A}$
Ethyl acetate	$1.16 \pm 0.02^{a,A}$	$1.61 \pm 0.04^{b,A}$
BHT	$2,615.95 \pm 0.04^{B}$	$2,615.95 \pm 0.04^{B}$

Note: Each value is mean ± SD (n=3)

^{a,b} Data within the same row with different superscripts are significantly different ($p<0.05$).

^{A,B} Data within the same column with different superscripts are significantly different ($p<0.05$).

Wong et al. (2006) categorized medicinal plants based on their antioxidant activities in the FRAP assay as extremely high (>500 µmol Fe(II)/g, high (100-500 µmol Fe(II)/g, medium (10-100 µmol Fe(II)/g), and low (<10 µmol Fe(II)/g). Under this criteria, the *Ocimum basilicum* var. *thyrsiflorum* water and ethanol extracts and

Ocimum basilicum cv. Jumbo 4320 ethanol extract could be classified to exhibit medium antioxidant activity. The others are classified to possess low antioxidant activity. *Ocimum basilicum* var. *thyrsiflorum* extracted by ethanol showed the highest antioxidant activity by FRAP assay at the value of 18.64±0.13 µmol Fe^{2+}/g RM.

4.3.3 Cellular antioxidant activity

4.3.3.1 Cytotoxicity

Biological systems are much more complex than the simple chemical mixtures employed, and antioxidant compounds may operate via multiple mechanisms (Wolfe et al., 2007). The different efficacies of compounds in the various assays attest to the functional variation. The best measures are from animal models and human studies; however, these are expensive and time-consuming and not suitable for initial antioxidant screening of foods and dietary supplements (Liu et al., 2005). Cell culture models provide an approach that is cost-effective, relatively fast, and address some issues of uptake, distribution, and metabolism (Wolfe et al., 2007).

The cytotoxicities of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 extracts were evaluated in HepG2 cell line with the MTT assay. When cells were treated for 24 h with concentration range from 50 to 500 µg/ml of extract, the relative cell survival progressively decreased in a dose-dependent manner. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaved the tetrazolium ring, converting the MTT to an insoluble purple formazan. Absorbance was monitored at 570 nm. Results of toxic compounds were expressed as the LC_{50} of the cells compared with controls. The LC_{50} value for *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 extract are shown in Table 4.6. The results showed that all Thai basil crude extract exhibited extremely high value of

LC_{50} (>200 μg/ml) against cell types indicating non-toxic activity to the cells (Okonogi et al., 2007).

Table 4.6 Cytotoxicity of Thai basil crude extracts.

Solvents	Cytotoxicity LC_{50} (μg/ml)	
	Ocimum basilicum* var. *thyrsiflorum	***Ocimum basilicum* cv. Jumbo 4320**
Water	$966.50 \pm 39.07^{b,B}$	$567.49 \pm 4.17^{a,A}$
Ethanol	688.85 ± 31.37^{A}	632.19 ± 20.92^{B}
Ethyl acetate	624.36 ± 30.54^{A}	640.82 ± 11.96^{B}

Note: Each value is mean ± SD (n=5)

^{a,b} Data within the same row with different superscripts are significantly different ($p<0.05$) on species.

^{A,B} Data within the same column with different superscripts are significantly different ($p<0.05$) on solvents.

4.3.3.2 Cellular antioxidant activity (CAA)

Numerous *in vitro* solution-based chemical assay systems, such as the thiobarbituric acid (TBA) colorimetric method, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and oxygen radical absorbing capacity (ORAC) were reported for the evaluation of antioxidants. However, it is important to know how to feedback the results of *in vitro* assays into an evaluation of *in vivo* activity. Fluorescent technology has made it possible to evaluate antioxidants in live cells using specific probes such as 2′,7′-

dichlorofluorescin diacetate (DCFH-DA). A cell-based method with fluorescent technology is useful for the direct examination of the ability of natural products, e.g. polyphenols and flavonoids, to inhibit ROS in live human cells (Takamatsu et al., 2003).

The antioxidant activity of Thai basil extracted by water, ethanol and ethyl acetate was evaluated using DCFH-DA-cell-based assay. HepG2 cells were treated for 1 hour with various concentrations of Thai basil and were incubated for 1 hour in the presence of 600 µM 2,2'-Azobis (2-amidinopropane) dihydrochloride (ABAP). The cellular antioxidant activities in each extract are presented in Figure 4.3. The result shows the dose-response curve of Thai basil depend on concentration of the extract. When the concentrations of Thai basil increase, it can decreases ABAP-induced DCFH oxidation. The comparison among the extraction solvents, it was found that the cellular antioxidant activities by DCFH-DA assay are significantly different ($p<0.05$) in both solvents and species that used to study. *Ocimum basilicum* cv. Jumbo 4320 extracted by water show the highest cellular antioxidant activity by DCFH-DA assay at relative fluorescence intensity 80.62±0.00%. This result suggested that Thai basil had a cellular radical scavenging effect. The cellular antioxidant activity by DCFH-DA assay of all Thai basil crude extract was less than vitamin E, the commercial standards.

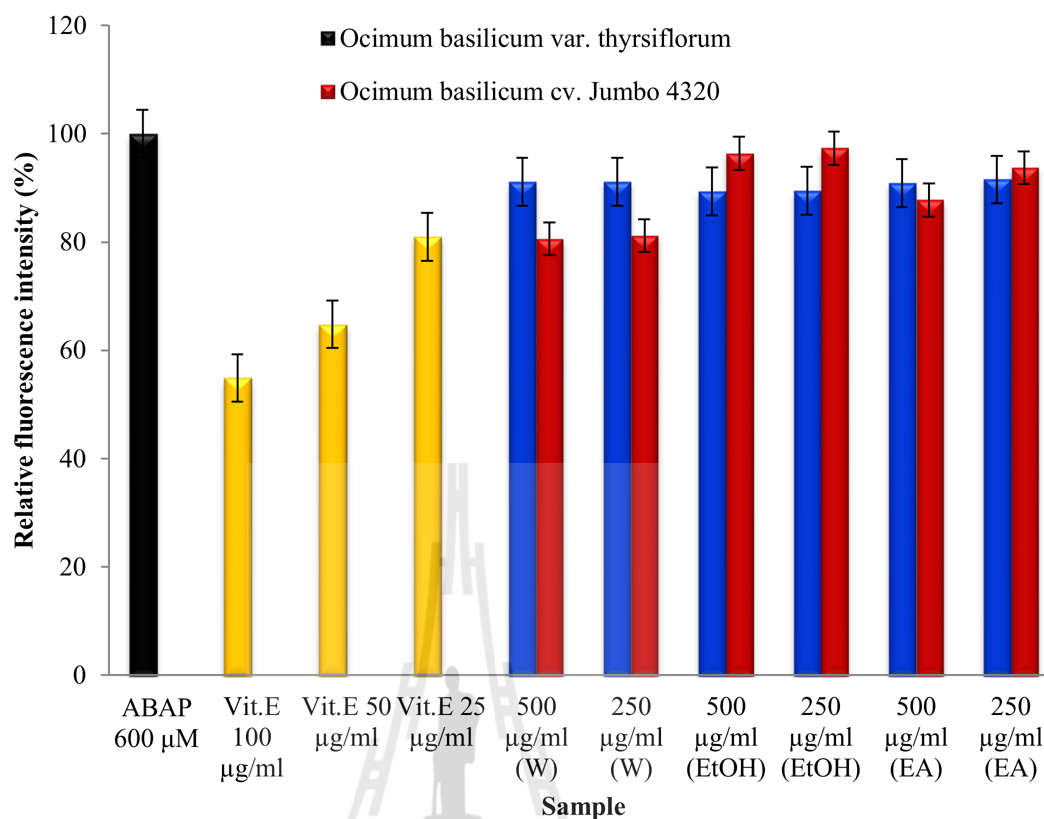

Figure 4.3 Cellular antioxidant activity (CAA) by DCFH-DA assay of Thai basil crude extracts (W = basil extracted by water, EtOH = basil extracted by ethanol and EA = basil extracted by ethyl acetate).

Data are mean ± SD (n=5), all sample are significantly different ($p<0.05$).

Takamatsu et al. (2003) found that the antioxidant efficacy depends to a great extent on the ability to penetrate the cell membranes. Vitamin C, a hydrophilic antioxidant, exhibited the strongest activity. On the contrary, carotene, a lipophilic compound and a well-known antioxidant, did not inhibit intercellular DCF-production, presumably because of a cell passage problem. Vitamin E, is also a lipophilic compound and showed a weak scavenging effect in HL-60 cells.

For all evaluation methods (DPPH, FRAP and CAA), all extracts of Thai basils in this study showed the antioxidant activities but antioxidant activities are differently depending on components in each extract. The different in antioxidant activities among Thai basil extracts could be due to multiple factors including concentration of the extracts and qualitative profile of extracts (Table 4.3 and 4.4). Water extract had the primarily components as phenolic compounds, whereas ethanol extract had main components as phenolic compounds, chlorophyll derivatives and lutein. The ethyl acetate extract which showed less antioxidant activity was composed of chlorophylls, chlorophyll derivatives and luteins. This could be explained that chlorophylls and chlorophylls derivative exhibited low antioxidant activity, also the amount of luteins that presented in ethyl acetate extract was very low resulting in low antioxidant activity.

Most of the antioxidant substances in plants are phenolic compounds. Phenolic substances serve as oxidation terminators by scavenging radicals to form resonance stabilized radicals (Maisuthisakul et al., 2008). Sweet basil is also known to have strong antioxidant activity, since its leaves contain phenolic compounds, especially rosmarinic acid, chicoric acid, caftaric acid (all derivatives of caffeic acid in order from the most abundant to the least), and cinnamic acid, caffeic acid, sinapic acid, and ferulic acid (Boggia et al., 2015).

The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agent, hydrogen donors and single oxygen quenchers. They may also have a metal chelating activity (Rice-Evans et al., 1995). Sweet basil is attributed to the large class of polyphenols and flavonoids like quercetin, kaempferol and myricetin; tannins like catechin, pigment such as anthocyanins found

in purple basil, and essential oil such as eugenol and methyl chavicol (Bora et al., 2011). The presence of electron-donating and electron-withdrawing substituents in the ring structure of phenolics as well as the number and arrangement of the hydroxyl groups determines their antioxidant potential. Additionally, a huge number of phenolic compounds which have been reported in the literature, show differences in possible biochemical modification (glycosylation, acetylation, manolynation, esterification to organic acids etc.). Differences in the structure of phenolic compounds also determine their solubility in solvents of different polarity. Therefore type of extraction solvent as well as the isolation procedures may have a significant impact on the yield of extraction polyphenols from plant materials (Zlotek et al., 2015). Different solvents used in extraction resulted in differences in compositions and antioxidant activities of the extracts; an extract possessing a phenolic compound that contains a higher number of hydroxyl groups has a higher antioxidant activity. The presence of various antioxidant compounds with different chemical characteristics and polarities may or may not be soluble in a particular solvent. Polar solvents are frequently used for recovering polyphenols from plant matrices. Ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption (Quy et al., 2014).

The results from the antioxidant assays show that all extracts could act as radical scavengers to a certain extent. These results suggested that Thai basil extract could be natural sources for antioxidant in food but the amount required to produce antioxidant activity similar to standard use antioxidants could be large. The leaves of Thai basil are available at a low cost and thus provide an economic source of potential natural antioxidants for use as food supplements or food additives.

4.4 Antimicrobial activity

4.4.1 Disk diffusion method

The antimicrobial activity of the extracts of *Ocimum basilicum* leaves was tested by agar disk diffusion method. The diameter of the clear zone indicated the inhibition activity. The antimicrobial activities in each extract are presented in Table 4.7. All the extracts (water, ethanol and ethyl acetate) of Thai basil leaves had no effect on test pathogenic strains except that *Bacillus cereus* was susceptible to *Ocimum basilicum* var. *thyrsiflorum* ethyl acetate extract and *Ocimum basilicum* cv. Jumbo 4320 water extract. The antimicrobial activity of Thai basil crude extract was less than chloramphenicol, the reference standards.

Both species of Thai basil showed no antimicrobial effect against most test strains. This finding is in accordance to Shafique et al. (2010) who reported that ethanolic extracts of sweet basil were inactive against *Bacillus subtilis*, *Enterobacter* species, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains. Chaisawadi et al. (2005) reported that extracts of sweet basil were inactive against *Bacillus cereus* and *Staphylococcus aureus* strains. Balamurugan (2013) also reported that water extracts of sweet basil were inactive against *Bacillus subtilis* and *Escherichia coli* strains. In addition, Adiguzel et al. (2005) reported that ethanol extracts of sweet basil were inactive against *Bacillus cereus*, *Bacillus subtilis*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa* strains.

Table 4.7 Antimicrobial activity of Thai basil crude extracts.

Type strains	Inhibition zone (mm)						
	Ocimum basilicum var. *thyrsiflorum* (800 mg RM/ml)			*Ocimum basilicum* cv. Jumbo 4320 (800 mg RM/ml)			Chloramphenicol (16.67 mg/ml)
	Water	Ethanol	Ethyl acetate	Water	Ethanol	Ethyl acetate	
Bacillus cereus	-	-	7.00 ± 0.00	6.5 ± 0.00	-	-	30.4 ± 1.33^{b}
Bacillus subtilis	-	-	-	-	-	-	13.3 ± 0.98^{a}
Enterobacter aerogenes	-	-	-	-	-	-	32.0 ± 0.99^{c}
Escherichia coli	-	-	-	-	-	-	12.6 ± 1.03^{a}
Pseudomonas aeruginosa	-	-	-	-	-	-	32.1 ± 1.03^{c}
Staphylococcus aureus	-	-	-	-	-	-	31.5 ± 0.00^{c}

Note: Each value is mean ± SD (n=3)

- No inhibition zone

^{a,b,c} Data within the same column with different superscripts are significantly different ($p<0.05$).

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4.5 Antithrombotic activity

4.5.1 Prothrombin time assay

The antithrombotic activities of Thai basil extracted by water, ethanol and ethyl acetate were evaluated using prothrombin time assay. The prothrombin time of each extract are presented in Table 4.8. The result showed that the prothrombin time of Thai basil depend on concentration of the extracts. When the concentrations of Thai basil increased, the prothrombin time was prolonged. Plasma containing extract showed prolonged prothrombin time in the antithrombotic activity. From species comparison, it was found that the *Ocimum basilicum* var. *thyrsiflorum* water extract (100 μg/ml) showed more significantly prolonged prothrombin time than *Ocimum basilicum* cv. Jumbo 4320 water extract ($p<0.05$). The prothrombin time of other extracts between two species are not significantly different ($p<0.05$). For comparison of extraction solvents, it was found that the antithrombotic activity, assessed by prothrombin time of Thai basil extracted by all solvents are significantly different ($p<0.05$) at 100 μg/ml. However, for other concentrations of water and ethanol extracts, the prolonged prothrombin time were not significantly different ($p<0.05$), but they were significantly different ($p<0.05$) in Thai basils ethyl acetate extract. *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 extracted by ethyl acetate at 500 μg RM/ml showed the highest prolonged prothrombin time at 78.3±17.56 and 77.3±3.21 second, respectively. The antithrombotic activity of all Thai basil crude extract was less than warfarin sodium, the reference standards.

The process of coagulation occurs via a cascade of sequential reactions and thrombin, the final enzyme in the coagulation cascade, converts soluble fibrinogen into insoluble fibrin. Clotting arises from a complex interaction of various mechanisms.

Platelets initiate clotting by breaking and converting the blood prothrombin into thrombin which is one of the basic substances to form the clot (Kishore, 2013).

The prothrombin time pathways do not directly reflect *in vivo* hemostasis; nevertheless, they represent useful laboratory tests by which the function of factors can be measured. The prothrombin time represents the *in vitro* analysis of coagulation factors in the extrinsic (Factor VII) and common pathways (Factors II, V, X and fibrinogen). The prothrombin time is functional determination of the extrinsic (tissue factor) pathway of coagulation and is extremely sensitive to the vitamin-K dependent clotting factors (factors II, VII, and X). Thromboplastin is a tissue extract containing tissue factor and a phospholipid. Prothrombin time measures the time taken by citrated platelet poor plasma to clot in presence of optimum concentration of tissue thromboplastin and calcium (Gadag et al., 2010).

Anticoagulants can act by interfering with the normal synthesis of vitamin K-dependent blood-clotting factors (Bailey et al., 2005). Substances in these extract may interfere with vitamin K metabolism and therefore prevent synthesis of those vitamin K-dependent coagulation factors.

Table 4.8 Prothrombin time of Thai basil crude extracts on rabbit plasma.

Solvents	Prothrombin Time (second)					
	***Ocimum basilicum* var. *thyrsiflorum* (µg RM/ml)**			***Ocimum basilicum* cv. Jumbo 4320 (µg RM/ml)**		
	100	250	500	100	250	500
Water	$34.7 \pm 0.58^{b,A}$	43.0 ± 0.00^{B}	45.3 ± 0.58^{AB}	$31.0 \pm 1.00^{a,B}$	42.0 ± 1.00^{B}	46.0 ± 1.00^{B}
Ethanol	38.7 ± 1.15^{B}	47.7 ± 0.58^{C}	53.7 ± 3.21^{B}	37.0 ± 1.00^{C}	43.7 ± 4.93^{B}	46.3 ± 0.58^{B}
Ethyl acetate	57.3 ± 2.52^{C}	69.3 ± 4.04^{D}	78.3 ± 17.56^{C}	54.3 ± 1.15^{D}	69.7 ± 1.53^{C}	77.3 ± 3.21^{C}
Warfarin (6 mg/ml)	144.3 ± 0.58^{D}	144.3 ± 0.58^{E}	144.3 ± 0.58^{D}	144.3 ± 0.58^{E}	144.3 ± 0.58^{D}	144.3 ± 0.58^{D}
Control	33.3 ± 1.53^{A}	33.3 ± 1.53^{A}	33.3 ± 1.53^{A}	33.3 ± 1.53^{A}	33.3 ± 1.53^{A}	33.3 ± 1.53^{A}

Note: Each value is mean ± SD (n=4)

^{a,b} Data within the same row with different superscripts are significantly different ($p<0.05$) for different species within the same concentration of sample.

^{A,B,C,D,E} Data within the same column with different superscripts are significantly different ($p<0.05$).

This result correspond with Tohti et al. (2006) who reported that sweet basil has been shown to decrease the occurrence of platelet aggregation and experimental thrombus in rats. The results showed that sweet basil aqueous extract when given orally to rats for 15 days inhibits both ADP and thrombin induced platelet aggregation, and reduce thrombus weight in an *in vivo* model of arterio-venous shunt thrombosis. Both these effects are dose-dependent. Amrani et al. (2009) reported that the endothelium-dependant vasorelaxant and anti-platelet aggregation activities of an aqueous extract from *Ocimum basilicum*. The results show that *Ocimum basilicum* extract exerts a significant vasorelaxant effect. The extract inhibits ADP-induced platelet aggregation and reduced thrombin-induced platelet activation. The use of *Ocimum basilicum* as medicinal plant could be beneficial for cardiovascular system.

Recently, the polyphenol-rich diets have been shown to play a positive role in vascular functioning including platelet aggregation in humans. In fact, recent studies on platelet function demonstrated that the phenolic compounds isolated from medicinal plants are responsible for their beneficial effect (Amrani et al., 2009). Some flavonoids inhibit blood platelet aggregation and provide antioxidant protection for low density lipoprotein (Frankel et al., 1993). Tognolini et al. (2006), demonstrating that the substances with high content in phenol such as thyme oil, oregano oil and *Ocimum basilicum* oil play a high anti-platelet activity towards arachidonic acid and moderate activity towards ADP. *Ocimum basilicum* showed a moderate inhibition against platelet aggregation with 12.3% content of eugenol. Such result is in accordance with previous reports regarding the efficacy of eugenol-containing essential oils in inhibiting Thromboxane A_2 formation and calcium mobilization in platelets (Tognolini et al., 2006).

CHAPTER V

CONCLUSIONS

Thai basils in species of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 were extracted by 3 solvents namely water, ethanol and ethyl acetate. Thai basils extracts were analyzed for chlorophylls and carotenoids profiling as well as phenolic profiling. Main chemical compositions of Thai basils ethanol and ethyl acetate extracts contained chlorophylls derivatives and carotenoids. *Ocimum basilicum* var. *thyrsiflorum* ethanol extract showed the highest chlorophylls and carotenoids contents. *Ocimum basilicum* cv. Jumbo 4320 water extract showed the highest total phenolic contents including some phenolic acids and flavonoid as gallic acid, catechin, apigenin, caffeic acid, coumaric acid and sinapic acid.

Total phenolic and total flavonoid contents of Thai basils extracts were evaluated using Folin-Ciocalteu and aluminum chloride colorimetric methods, respectively. *Ocimum basilicum* cv. Jumbo 4320 extracted by water showed the highest total phenolic contents and *Ocimum basilicum* cv. Jumbo 4320 extracted by ethanol showed the highest total flavonoid contents.

Antioxidant activities of Thai basil extracts were evaluated using DPPH, FRAP and DCFH-DA assay. *Ocimum basilicum* cv. Jumbo 4320 extracted by water show the highest antioxidant activity by DPPH assay. *Ocimum basilicum* var. *thyrsiflorum* extracted by ethanol showed the highest antioxidant activity by FRAP assay. *Ocimum basilicum* cv. Jumbo 4320 extracted by water show the highest cellular antioxidant

activity by DCFH-DA assay. The ethyl acetate extract showed the lowest antioxidant activity. Total phenolic and flavonoid contents and antioxidant activities of *Ocimum basilicum* are significantly different ($p<0.05$) between species and solvent extraction. Total phenolic and flavonoid contents of the extracts showed the relationship with antioxidant activities. At high total phenolic and flavonoid contents of *Ocimum basilicum* crude extracts showed strong antioxidant activities.

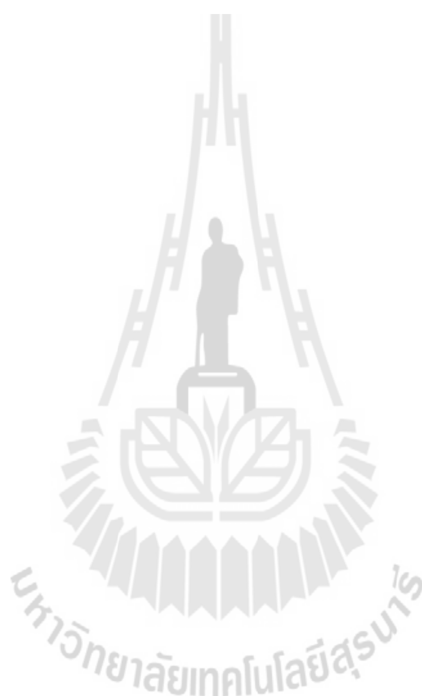
Thai basils extracts were investigated for their cytotoxicities in HepG2 cell lines. All Thai basils crude extracts exhibited extremely high value of LC_{50} >200 μg/ml, against cell types indicating all crude extracts are relatively non-toxic to the cells.

Antimicrobial activities of Thai basils crude extracts were evaluated using agar disk diffusion method. The comparison of antimicrobial activities between the extraction solvents and two species of Thai basils, almost all extracts have no antimicrobial activity against test pathogenic strains.

Antithrombotic activities of Thai basils crude extracts were evaluated using prothrombin time assay. *Ocimum basilicum* var. *thyrsiflorum* extracted by ethyl acetate showed the highest prolonged prothrombin time.

Ocimum basilicum cv. Jumbo 4320 extracted by water might be suitable for application in healthy food products because of its highest total phenolic contents and antioxidant activity evaluated by DPPH and DCFH-DA assay, and its antimicrobial activity against *Bacillus cereus*. Ethanol is the best solvent to extract flavonoids from Thai basils and Thai basils ethanol extract showed the highest antioxidant activity by FRAP assay. Thai basils ethyl acetate extract of both species showed the equal

($p>0.05$) highest prolonged prothrombin time. So, both species of Thai basils extracts possessed the potential for application as healthy food ingredients.



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Appendix A

Chemical and cell culture media preparations

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Chemical and cell culture media preparations

1. 20% (w/v) Na_2CO_3 (Sodium carbonate)

Na_2CO_3	20.0	g
Add distilled water and adjust the volume up to	100.0	ml

2. 5 mg/ml Standard gallic acid

Gallic acid	125.0	mg
95% Ethanol	2.5	ml
Add distilled water and adjust the volume up to	25.0	ml

3. 5% $NaNO_2$ (Sodium nitrite)

$NaNO_2$	1.25	g
Add distilled water and adjust the volume up to	25.0	ml

4. 10% $AlCl_3$ (Aluminium chloride)

$AlCl_3$	2.5	g
Add distilled water and adjust the volume up to	25.0	ml

5. 1 M NaOH (Sodium hydroxide)

NaOH	2.0	g
Add distilled water and adjust the volume up to	50.0	ml

6. 5 mg/ml Standard catechin

Catechin	125.0	mg
95% Ethanol	2.5	ml
Add distilled water and adjust the volume up to	25.0	ml

7. 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl)

DPPH	3.9432	mg
Add methanol and adjust the volume up to	100.0	ml

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8. 1 mg/ml Standard BHT (Butylated hydroxytoluene)

BHT	50	mg
Add methanol and adjust the volume up to	50.0	ml

9. 1 mg/ml Standard ascorbic acid

Ascorbic acid	50	mg
Add methanol and adjust the volume up to	50.0	ml

10. 300 mM Acetate buffer pH 3.6

Sodium acetate	4.0824	g
Glacial acetic acid	1.6	ml
Add distilled water and adjust the volume up to	100.0	ml

11. 40 mM HCl (Hydrochloric acid)

HCl (37% w/w)	333.0	µl
Add distilled water and adjust the volume up to	100.0	ml

12. 10 mM TPTZ (2,4,6-tripyridyl-s-triazine)

TPTZ	31.23	mg
Add 40 mM HCl and adjust the volume up to	10.0	ml

13. 20 mM Ferric (III) chloride

Ferric chloride	54.06	mg
Add distilled water and adjust the volume up to	10.0	ml

14. 1 mM Standard ferrous (II) sulphate

Ferrous sulphate	27.80	mg
Add distilled water and adjust the volume up to	100.0	ml

15. 5 mg/ml MTT solution

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MTT	125.0	mg
Add PBS and adjust the volume up to	25.0	ml

16. Complete cell culture media

DMEM	40.0	ml
FBS	4.5	ml
L-glutamine	500	µl
Penicillin-streptomycin	500	µl

17. 20 mM Stock solution of DCFH-DA

DCFH-DA	48.729	mg
Add DMSO and adjust the volume up to	5.0	ml

18. 25 µM DCFH-DA

20 mM Stock solution of DCFH-DA	15.0	µl
Add HBSS and adjust the volume up to	12.0	ml

19. 600 mM Stock solution ABAP

ABAP	813.57	mg
Add HBSS and adjust the volume up to	5.0	ml

20. 600 µM ABAP

600 mM Stock solution ABAP	12.0	µl
Add HBSS and adjust the volume up to	12.0	ml

21. MHA

MHA	38.0	g
Add distilled water and adjust the volume up to	1000.0	ml

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22. 16.67 mg/ml Chloramphenicol

Chloramphenicol	50.0	mg
Add 95% ethanol and adjust the volume up to	3.0	ml

23. 10 mM $CaCl_2$ (Calcium chloride)

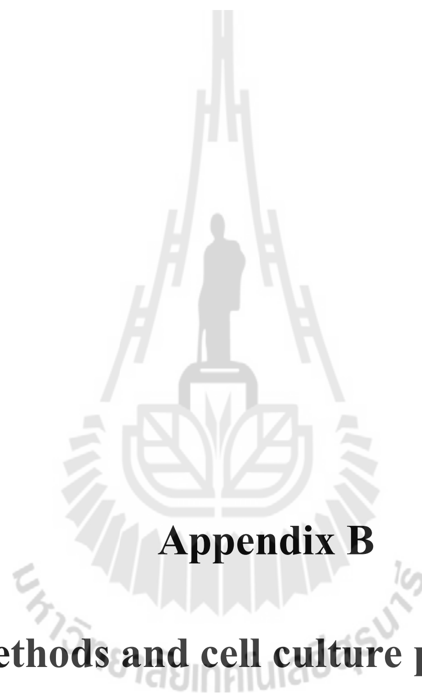
$CaCl_2$	55.495	mg
Add distilled water and adjust the volume up to	50.0	ml

24. Thromboplastin

Thromboplastin	1	vial
10 M $CaCl_2$	4.0	ml

25. Warfarin sodium

Warfarin sodium	3	mg
Citrated rabbit plasma	500	µl



Appendix B

Methods and cell culture protocols

Methods and cell culture protocols

Counting Cells in a hemacytometer

1. Clean the chamber and cover slip with alcohol. Dry and fix the coverslip in position.
2. Harvest the cells. Add 10 µl of the cells to the hemacytometer. Do not overfill.
3. Place the chamber in the inverted microscope under a 10X objective. Use phase contrast to distinguish the cells.
4. Count the cells in the large, central gridded square (1 mm^2). The gridded square is circled in the graphic below. Multiply by 10^4 to estimate the number of cells per ml. Prepare duplicate samples and average the count.

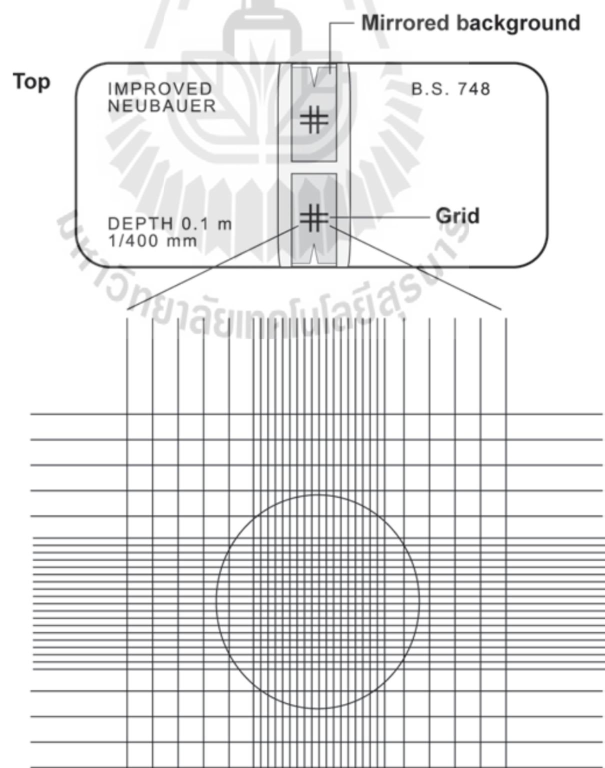


Figure 1B Hemacytometer

Source: http://www.thermofisher.com

Trypan blue exclusion

1. The cell suspension was adjusted to the appropriate concentration for counting cell about 100 µl and prepared 100 µl of 0.4% trypan blue, mixed and incubated for 2 minutes at room temperature.
2. Added cell with 1 drop of trypan blue into hemocytometer.
3. Load a hemacytometer and examine immediately under a microscope.
4. Count the number of blue staining cells and non-staining cells (total cells).
5. The percent viable cell was calculated by following formula:

   % viable cells = (Number of blue cells x 100)/ Number of total cells

Thawing Frozen Cells

1. Quickly thaw the cells (<1 minute) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial.
2. Transfer the vial it into a laminar flow hood. Before opening, wipe the outside of the vial with 70% ethanol.
3. Transfer the desired amount of pre-warmed complete growth medium appropriate for your cell line dropwise into the centrifuge tube containing the thawed cells.
4. Centrifuge the cell suspension at approximately 200×g for 3-5 minutes.
5. After the centrifugation, check the clarity of supernatant and visibility of a complete pellet. Aseptically decant the supernatant without disturbing the cell pellet.
6. Gently resuspend the cells in complete growth medium, and transfer them into the appropriate culture vessel and into the recommended culture environment (37°C, 5% CO_2).

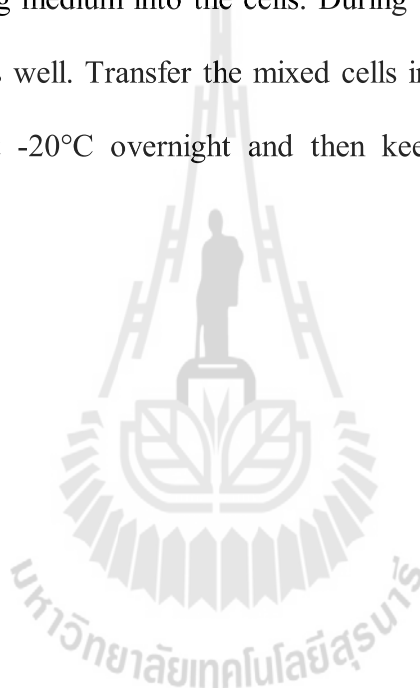
Freezing Cells

1. Stock batches: $1.5x10^6$-$2.0x10^6$ cells/1 ml in Cryovial.
2. Prepare freezing medium by following ratio:

   30%DMEM (The remain of 30% DMEM was mixed with cells and soak in ice)

   30%FBS

   10%DMSO (DMSO was prepared by filtering with 0.2 µm filter size)
3. Gently drop freezing medium into the cells. During this time, gently shake the tube to make it mixed as well. Transfer the mixed cells into Cryovial (soak in ice), then cells was stored at -20°C overnight and then keep at -80°C or keep in liquid nitrogen.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BIOGRAPHY

Panita Prasongdee was born on January 24th, 1989 in Nakhon Ratchasima Province, Thailand. She studied for high school diploma at Huaithalaeng Pittayakom School (2001-2006) in Nakhon Ratchasima Province. In 2010, she received her Bachelor's Degree in Science (Food Technology) from Suranaree University of Technology. In 2011, she started to study for her Master's degree in Food Technology at Suranaree University of Technology, Nakhon Ratchasima, Thailand. During graduate study, she was a research assistant in the project "Extraction, bioactivities, and functional properties of Thai radish extracts" and "Bioactive properties of Thai basil extract". The results from her research were also presented.