องค์ประกอบทางเคมีและฤทธิ์กำจัดอนุมูลอิสระของเกสรผึ้งของผึ้งพันธุ์ *Apis mellifera* จากจังหวัดน่าน ประเทศไทย

นายอธิป จันทฤดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย
บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
เป็นแฟ้มข้อมูลของนิสิตเจ้าของวิทยานิพนธ์ที่ส่งผ่านทางบัณฑิตวิทยาลัย
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CHEMICAL CONSTITUENTS AND FREE RADICAL SCAVENGING ACTIVITY OF BEE POLLEN OF *Apis mellifera* FROM NAN PROVINCE, THAILAND

Mr. Atip Chantarudee

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2011

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Thesis Title	CHEMICAL CONSTITUENTS AND FREE RADICAL SCAVENGING ACTIVITY OF BEE POLLEN OF *Apis mellifera* FROM NAN PROVINCE, THAILAND
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เกสรผึ้งเป็นหนึ่งในผลิตภัณฑ์ของผึ้งซึ่งได้จากการเก็บสะสมของผึ้งงานในสกุลเอพิสและชันโรงในบางสปีชีส์ เกสรผึ้งเป็นของผสมระหว่างเรณูจากพืช น้ำหวาน สิ่งคัดหลั่งจากตัวผึ้ง เกสรผึ้งอุดมไปด้วยสารอาหารต่างๆ เช่น น้ำตาล โปรตีน ไขมัน วิตามินและฟลาโวนอยด์ เป็นต้น มีรายงานว่าเกสรผึ้งมีฤทธิ์ทางชีวภาพที่หลากหลาย เช่น ฤทธิ์ต้านการแบ่งตัวของเซลล์มะเร็ง ฤทธิ์ต้านอาการแพ้ ฤทธิ์ต้านการเกิดเส้นเลือดฝอยไปเลี้ยงเซลล์มะเร็ง และฤทธิ์ในการกำจัดอนุมูลอิสระ เป็นต้น ในงานวิจัยนี้ มีจุดมุ่งหมายเพื่อตรวจหาองค์ประกอบทางเคมีและตรวจวัดฤทธิ์กำจัดอนุมูลอิสระของสารสกัดจากเกสรผึ้ง ทำการเก็บเกสรผึ้งของผึ้งพันธุ์ *A. mellifera* จากตำบลเจดีย์ชัย อำเภอปัว จังหวัดน่าน ประเทศไทย ในช่วงเดือนมิถุนายน 2553 ในช่วงเวลาดังกล่าว พบว่าพืชหลักที่ทำการเพาะปลูกรอบๆ ฟาร์มผึ้งคือข้าวโพด จากนั้นจึงนำเกสรผึ้งมาสกัดด้วยเอทานอลและสกัดแยกสารตามขั้วโดยใช้ตัวทำละลายต่างๆ ได้แก่ เมทานอล ไดคลอโรมีเทน เฮกเซน แล้วนำไปทดสอบฤทธิ์ในการกำจัดอนุมูลอิสระโดยวิธีดีพีพีเอช โดยวัดค่าการดูดกลืนแสงที่ความยาวคลื่น 517 นาโนเมตร ทำการคำนวณเปอร์เซ็นต์ของฤทธิ์ในการกำจัดอนุมูลอิสระและค่าความเข้มข้นที่สัมฤทธิ์ผลที่ 50 เปอร์เซนต์ (อีซี 50) พบว่าสารสกัดหยาบไดคลอโรมีเทนที่ได้มาจากสารสกัดหยาบเอทานอลมีฤทธิ์ในการกำจัดอนุมูลอิสระดีสุดซึ่งมีค่าอีซี 50 เท่ากับ 7.47 ± 0.12 ไมโครกรัมต่อมิลลิลิตร จึงนำไปทำให้บริสุทธิ์ขึ้นโดยใช้ควิกคอลัมน์โครมาโทกราฟี ไซส์เอ็กซ์คลูชันโครมาโทกราฟีและแอดซอร์ปชันโครมาโทกราฟี สังเกตความบริสุทธิ์ของแต่ละลำดับส่วนที่เก็บได้ในแต่ละขั้นตอนโดยทินเลเยอร์โครมาโทกราฟี (ทีแอลซี) และการวิเคราะห์โครงสร้างทางเคมีของสารในลำดับส่วนที่ออกฤทธิ์ดีที่สุดโดยใช้นิวเคลียร์แมกเนติกเรโซแนนซ์ (เอ็นเอ็มอาร์) ได้สารบริสุทธิ์ สองตัวคือไฮโดรควิโนน ซึ่งมีปริมาณน้อยเป็นสารในกลุ่มสารประกอบฟีนอลิก และเอพิเจนิน ซึ่งมีปริมาณมากเป็นสารในกลุ่มฟลาโวน นอกจากนี้ได้ทำการวัดสมบัติต่างๆ ในเกสรผึ้ง ได้แก่ ปริมาณทั้งหมดของน้ำตาลโมเลกุลคู่ น้ำตาลรีดิวซิง ฟีนอลิกและฟลาโวนอยด์ จึงสามารถสรุปได้ว่าเกสรผึ้งจากจังหวัดน่าน ประเทศไทย ไม่เพียงแต่มีคุณค่าทางโภชนาการ แต่ยังมีฤทธิ์ในการกำจัดอนุมูลอิสระที่ดีอีกด้วย

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต..

ปีการศึกษา 2554 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก..................

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม ....

5272613123: MAJOR BIOTECHNOLOGY
KEYWORDS: *Apis mellifera*/ BEE POLLEN/ FLAVONOID/ FREE RADICAL SCAVENGING ACTIVITY/ NAN PROVINCE

ATIP CHANTARUDEE: CHEMICAL CONSTITUENTS AND FREE RADICAL SCAVENGING ACTIVITY OF BEE POLLEN OF *Apis mellifera* FROM NAN PROVINCE, THAILAND. ADVISOR: ASSOC. PROF. CHANPEN CHANCHAO, Ph.D., CO-ADVISOR: ASST. PROF. PREECHA PHUWAPRAISIRISAN, Ph.D., 104 pp.

Bee pollen is one of honeybee products. It is collected by foragers of *Apis* spp. and some species of stingless bees. It is the mixture of natural flower pollens, nectar, and secretion of bees. It is rich in nutrients such as sugars, proteins, lipids, vitamins, flavonoids, etc. It was reported that bee pollens provided various bioactivities such as antiproliferative, anti-allergenic, anti-angiogenic, and free radical scavenging activities, etc. In this research, it was aimed to investigate chemical constituents and to determine the free radical scavenging activity of bee pollen extracts. Bee pollens were directly collected from *A. mellifera* colonies in Chedeechai subdistrict, Pua district, Nan province, Thailand, in June, 2010. During that period, the majority of cultivated crops surrounding an apiary were corn. Crude bee pollen was extracted by EtOH and was partitioned by different solvents (hexane, CH_2Cl_2, and MeOH). Later, they were tested for free radical scavenging activity by DPPH assay. The absorbance was measured at 517 nm. The percentage of scavenging activity and effective concentration at 50% (EC_{50}) were estimated. Crude CH_2Cl_2 extract originated from EtOH extract providing the most active free radical scavenging activity has the EC_{50} value of 7.47 ± 0.12 μg/ml. This crude was purified by quick column, size exclusion chromatography, and adsorption chromatography. The purity of all fractions in each step was observed by thin layer chromatography (TLC). Finally, chemical constituents in the most active fraction was analysed by nuclear magnetic resonance (NMR). Two compound structures were revealed. The first compound, a minor compound, was hydroquinone belonging in a phenolic compound group. The second compound, a major compound, was apigenin belonging to a flavone group. The properties of bee pollen were tested in term of total disaccharide sugar, reducing sugar, total phenolics, and total flavonoid contents. It could be concluded that not only bee pollens from Nan province in Thailand were nutritional, but it also had the free radical scavenging activity.

Field of Study: Biotechnology .... Student's Signature ....

Academic Year: 2011 .... Advisor's Signature ....

Co-advisor's Signature ....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and great appreciation to Associate Professor Dr. Chanpen Chanchao, my advisor, for her kindness, meaningful guidance, invaluable suggestions, and encouragement throughout this study.

My deep appreciation is expressed to Assistant Professor Dr. Preecha Phuwapraisirisan, my co-advisor, for his valuable suggestions and comments.

Furthermore, I would like to express my gratitude to Associate Professor Dr. Kumthorn Thirakhupt, Associate Professor Dr. Nattaya Ngamrojanavanich, Assistant Professor Dr. Chumpol Khunwasi, and Dr. Ubolsree Leartsakulpanich for serving as thesis committee, for their valuable comments, and also for useful suggestions.

I am particularly indebted to Assistant Professor Dr. Preecha Phuwapraisirisan, Assistant Professor Dr. Jessada Denduangboripant, and Assistant Professor Dr. Orawan Sattayalai for laboratory facilities.

I am grateful to Mr. Sawang Piyapichart for bee pollen collection in my research.

I would also like to extend my thanks to members of Central Molecular Laboratory, Department of Biology, members of Natural Product Research Unit, Department of Chemistry, and all of my friends in Program of Biotechnology, Faculty of Science, Chulalongkorn University for their help, suggestions, and kind friendship.

I wish to acknowledge the Science for Locale Project under the Chulalongkorn University Academic Development Plan (2008-2012) and the 90th Anniversary of Chulalongkorn University Fund(Ratchadaphiseksomphot Endowment Fund) for financial supports.

Finally, I would like to express my infinite appreciation to my family members for their unlimited love, encouragement, and continuous support throughout my life.

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LIST OF ABBREVIATIONS

ABS	absorbance
α	alpha
β	beta
J	coupling constant
δ	chemical shift
δ_H	chemical shift of proton
CC	column chromatography
CC, SiO_2	column chromatography using silica gel as absorbent
CAP	crude ethyl acetate extract of bee pollen
CEE	crude ethanol extract of bee pollen
CWE	crude water extract of bee pollen
CME	crude methanol extract of bee pollen
CDE	crude CH_2Cl_2 extract of bee pollen
CHE	crude hexane extract of bee pollen
CME (CEE)	crude methanol extract originated from CEE
CDE (CEE)	crude CH_2Cl_2 extract originated from CEE
CHE (CEE)	crude hexane extract originated from CEE
CME (CWE)	crude hexane extract originated from CWE
CDE (CWE)	crude CH_2Cl_2 extract originated from CWE
CHE (CWE)	crude hexane extract originated from CWE
CDE1-CDE7	fraction 1-7 originated from CDE
CDE5-1 to CDE5-6	fraction 1-6 originated from CDE5

CEC	crude ethanol extract of corn pollen
CMC	crude methanol extract of corn pollen
CDC	crude CH_2Cl_2 extract of corn pollen
CHC	crude hexane extract of corn pollen
cm	centimeter
°C	degree Celsius
CD_3OD	deuterated methanol
CH_2Cl_2	methylene chloride or dichloromethane
COSY	two- dimensional ^1H correlation spectroscopy
DMSO-d_6	deuterated dimethyl sulfoxide
DNSA	3, 5-Dinitrosalicylic acid
DPPH	1, 1-Diphenyl-2-picrylhydrazyl or 2, 2- diphenyl-1-picryl hydrazyl
d	doublet for NMR spectra
EC_{50}	efficient concentration at 50%
EtOH	ethanol
EtOAc	ethyl acetate
γ	gamma
GC-MS	gas chromatography-mass spectrometry
g	gram
H^+	proton
HMBC	^1H- detected heteronuclear multiple bond correlation
^1H NMR	proton nuclear magnetic resonance
HO·	hydroxyl radical

H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HPLC/DAD	high performance liquid chromatography/ diode array detector
H_2SO_4	sulfuric acid
HSQC	^{1}H-detected heteronuclear single quantum correlation
Hz	hertz
h	hour
m.p.	melting point
MeOH	methanol
µg	microgram
µl	microliter
mg	milligram
ml	milliliter
mM	millimolar
min	minute
M	molar
M.W.	molecular weight
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
nm	nanometer
$O^{\cdot}{}_2^-$	superoxide anion
ppm	part per million
/	per

%	percentage
ROO·	peroxide radical
:	ratio
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	room temperature (25°C)
rpm	revolution per minute
SEM	scanning electron microscope
s	singlet for NMR spectra
sp.	species
spp.	more than one species
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultra-violet
VCC	vacuum column chromatography
v/v	volume by volume
w/w	weight by weight
w/v	weight by volume

CHAPTER I
INTRODUCTION

Bee pollen is one of economic bee products which are honey, propolis, bee pollen, royal jelly, bee venom, and wax. It contains various nutrients such as protein, lipid, carbohydrate, sugar, minerals, and vitamins which are useful for human health. Bee pollen is originally processed by bees. When foragers collect nectar from flowers of plant as food sources, they will also collect flower pollen. The collected pollen will be mixed with nectar, enzyme, wax, and bee secretion. Then, a small pellet will be formed and stuck in a pollen basket at hind legs. After the foragers return to their hives, the collected bee pollen will be stored in a hive and used as food.

Not only bee pollen has a variety of nutrients as mentioned above, but it also contains phenolic compounds such as flavanoid, flavone, etc. Thus, it has long been used for traditional medicine in order to relieve a symptom of many diseases such as diabetes, sinus, asthma, allergy, flu, and rheumatism, etc. In addition, bee pollen is applied to many cosmetics and supplementary foods. Although many species of bees can collect bee pollen, it seems to be that bee pollen from European honeybee or *Apis mellifera* is the most popular for consumers. *A. mellifera* is native to Europe and African. Since it can be well managed in an apiary and it can produce high quantity of honey, it is imported to many countries including Thailand.

Until now, people still suffer with many diseases. It also includes a disease resistant to present treatment. Thus, it is necessary to search for a new source of effective agent which will be developed to be a drug in the future. A natural product that can provide an interesting bioactivity has been a target for this purpose. Bee pollen was also reported to provide many bioactivities such as antiproliferative activity, anti-allergic activity, anti-angiogenesis, and free radical scavenging activity.

Free radical scavenging activity is my interest because free radical can lead to many diseases such as cancer, atherosclerosis, cerebral-cardiac ischemia, Parkinson's disease, and aging. As known, free radical consists of O_2 at a center of a molecule, so

called reactive oxygen species (ROS). The molecule in this group may consist of $^3O_2$, O^-_2, H_2O_2, OH^-, and $1O_2$. Normally, this ROS is originated from active metabolism in a mammalian cell. It is stimulated by an antioxidant producing enzyme influenced by an external factor. Therefore, ROS is likely to be a connection among signals inside the cells involving in stress responsiveness, cell proliferation, aging, and cancer.

Since an interesting bioactivity of natural products depends on external factors such as biogeography, climates, seasons, period of harvesting, etc., it is very important to find an active compound in that natural product. This will be fruitful in controlling the standard of the product. Thus, the objective of this research is to search for a chemical constituent in *A. mellifera's* bee pollen that can provide a free radical scavenging activity. Bee pollen from Nan province, Thailand is my interest since there are many bee farms. Furthermore, the geography and climate of this province is unique. This may lead to typical food plants of *A. mellifera.* At last, it may bring to the finding of a new free radical scavenging agent from bee pollen from this area. The outcome of this research not only will be useful in medical treatment, but it will also increase the income of bee farmers.

CHAPTER II
LITERATURE REVIEWS

2.1 Biology of European honeybee (*Apis mellifera*)

A. mellifera is native to Europe and Africa. It is worldwide imported into many countries since it can produce high quantity of economic honeybee products (bee pollen, honey, wax, propolis, royal jelly, and bee venom) as in Figure 2.1. As known, bee products are useful both in food and medical purposes (Wongsiri, 1989; Oldroyd and Wongsiri, 2006). Within a colony of *A. mellifera*, there are around 40,000-50,000 bees. In each year, bees in a hive can produce honey more than 20-100 kg. Due to diligently foraging and harmless behaviors, *A. mellifera* is mostly chosen in a bee farm. In addition, it can be well managed in a hive box. This leads to the convenience in product harvesting. *A. mellifera* was brought into Thailand since the 18th century (Wongsiri, 1989). It can adapt itself well in Thailand although there are 4 native species of *A. dorsata, A. cerana, A. florea,* and *A. andriniformis* in the country. Considering a body size, *A. mellifera* is larger than *A. cerana, A. florea,* and *A. andreniformis* but is smaller than *A. dorata*.

(A)

(B)

(C)

(D)

Figure 2.1 European honeybee (*A. mellifera)* and its products. A worker of *A. mellifera* performs all duties, except fertilization, within a hive (A). Bee products are economically important such as royal jelly (B), propolis (C), and bee pollen (D).

The taxonomy of *A. mellifera* is shown below (Wongsiri, 1989):

Kingdom Animalia

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Super-family Apoidea

Family Apidae

Subfamily Apinae

Genus *Apis*

Species *A. mellifera*

2.1.1 Morphology of honeybee

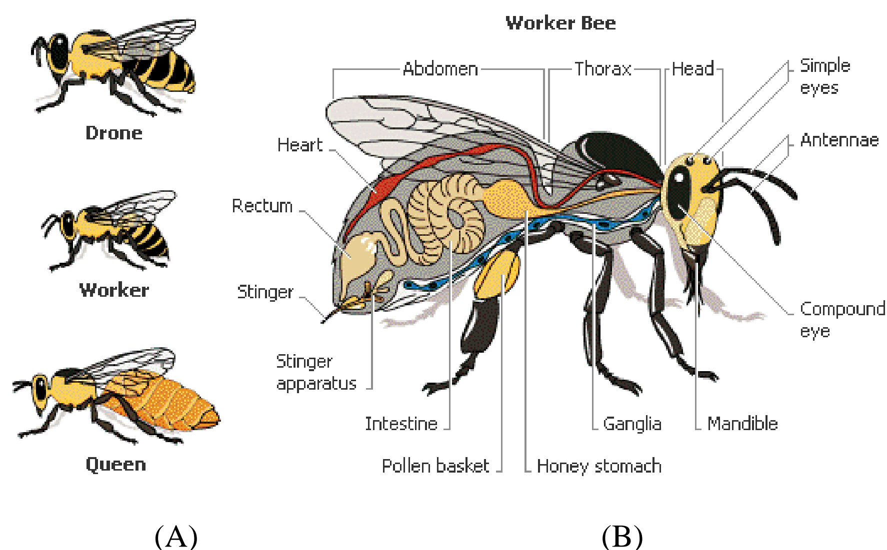
A body of honeybee can be divided into three parts: head, thorax, and abdomen (Figure 2.2). A head is composed of antennae, eyes, and mouth. Antennae are used as a receptor and a sense organ. Eyes in a pair provided the vision ability in a long distance and a wide area. The flower color that bees see is almost similar to what human sees, except the red color which a bee sees in black instead. The size of eyes can be used to distinguish the gender of bees since a drone's eyes are larger than a worker's and a queen's eyes. A bee uses proboscis to suck nectar. Within a mouth, it consists of many small parts such as a pair of strong jaws, teeth, and a trunk for absorbing nectar. A mouth of a drone and a queen is short because it is not in function. They are fed by workers.

Thorax is a portion of muscle consisting of legs and wings. A worker has three pairs of legs and a special organ called a pollen basket for collecting flower pollen. A drone and a queen do not have this organ because they do not have to forage food. There are 2 pairs of wings which front pairs of wings are larger than back pairs

of wings. Each wing is connected to each other with a small hook called Hamulai (Wongsiri, 1989; Oldroyd and Wongsiri, 2006).

Abdomen of female and abdomen of male is diferent in segment number. Female's abdomen consists of 6 segments while male's abdomen consists of 7 segments. Each segment has a pair of breathing organs. Total of 10 pairs of breathing holes are located on the side of thorax and abdomen. Three pairs are located at the thorax while seven pairs are located at the abdomen. The last segment of a female bee is used for spawning. At the end of an abdomen, there is a sting in a female bee, a modified form of the last segment of an abdomen but not in a male bee (Wongsiri, 1989; Oldroyd and Wongsiri, 2006). It looks like a sharp needle.

About breathing hold, it could be turned on and shut down all the time because a bee breathes in and out through these holes which are connected to the respiratory system including air bags. In addition, a bee has large air sacs within its body in order to support the body while flying. A bee's body has a lot of hair which consist of receptor and sensory nerves. For example, face hair is used for sensing, moving, and detecting the wind direction. A bee tends to fly upwind to the location of food sources. Hair on thorax and abdomen of a bee is sensitive to the gravity force which is necessary while flying. In addition, hair can detect the movement of an enemy and the position of food from plants (Wongsiri, 1989; Oldroyd and Wongsiri, 2006).



(A) (B)

Figure 2.2 The social castes and characteristics of *A. mellifera*. Within a colony, there are 3 social castes which are drones, workers, and a queen. A bee body consists of 3 parts which are head, thorax, and abdomen (B).

2.1.2 Life cycle and Social

Bees are eusocial insects. Obviously, within a hive, bees are classified into 3 castes of a queen (female), workers (female), and drones (male) (Figure. 2.2)

A queen is diploid (2n=32). Although the chromosome number is the same as the chromosome number of a worker, its body is larger than a worker's body. That is due to the food they uptake. A queen is fed by royal jelly through the entire life. In contrast, a worker's larva is fed by royal jelly for only 3 days. After that, it will fed by pollen and honey. An average life span of a queen is around 1-2 years. It acts as a leader of a colony and performs the fertilization.

A drone is haploid (n=16) and developed by parthenogenesis. Its body is smaller than a queen's body. In addition, it is stingless. It has a short tongue and can not forage. Drones get food from the feeding by a worker only. The only function in their life is to perform fertilization.

A worker is also diploid (2n=32). It is the smallest in a hive but performs all duties within a hive. Workers performing different duties depend on age. A young

worker (a nurse bee) will feed their broods by synthesizing and secreting a royal jelly (bee milk) whereas an old worker (a forager) will go to forage nectar and pollen from plants (Wongsiri, 1989; Oldroyd and Wongsiri, 2006).

2.2 Bee pollen

Bee pollen is collected by honeybees and stingless bees in some genus. It is a combination of flower pollens with nectar or honey, enzymes, wax, and bee secretion. Also, it is packed as a small pellet and kept at a forager's hind legs which is called "pollen basket" (Figure 2.3). Bee pollen is highly nutritional since it is rich in sugar, protein, lipid, vitamins, enzymes, and flavonoids, etc. Other than that, it has long been used in traditional medicine to treat some diseases such as diabetes, sinus, asthma, allergy, flu, and rheumatism, etc (Campos *et al*., 1997; Silva et al., 2006; Wei *et al*., 2008).

(A)

(B)

Figure 2.3 Bees collecting pollen with the help of pollen basket. The shape of pollen basket of *A. mellifera* is shown in (A). Providing the better attach of bee pollen to a pollen basket, a lot of hair at a pollen basket is presented (B).

There were many researches reporting the bee pollen compositions. For example, Saric *et al*. (2009) reported that, by chemical analysis, bee pollen from

Croatia consisted of 12.05% water, 6.92% (w/w) fat, 58.61% (w/w) carbohydrates, 1.83% (w/w) cellulose, 18.42% (w/w) protein(Kjeldahlu, 1960), and (w/w) 1.72% ashes.

Considering minerals in bee pollen, it consisted of 0.060 mg/kg Lead (Pb), 99.90 mg/kg Iron (Fe), 7.330 mg/kg Copper (Cu), 0.013 mg/kg Mercury (Hg), 74.70 mg/kg Zinc (Zn), 31.50 mg/kg Manganese (Mn), 0.379 mg/kg Chromium (Cr), 997.0 mg/kg Calcium (Ca), 0.060 mg/kg Cadmium (Cd), 564 mg/kg Magnesium (Mg), 0.999 mg/kg Selenium (Se), > 0.005 mg/kg Molybdenum (Mo), 4.006 mg/kg Potassium (K), 192.0 mg/kg Sodium (Na), 654.0 mg/kg Chlorine (Cl), 3.084 mg/kg Phosphorus (P), and < 0.01 mg/kg Arsenic (As) (Gorsuch, 1970; Saric *et al*., 2009).

Furthermore, bee pollen also consists of vitamins which are 12.80 mg/100g vitamin E, 11.90 mg/100g α-carotene, 11.91 mg/100g *β*-caratene, 3.44 mg/100g vitamin C, 1.10 mg/100g vitamin B_1, 0.44 mg/100g vitamin B_2, and 0.30 mg/100g vitamin B_6 (Song *et al*., 2000; Saric *et al*., 2009).

Types and chemical compositions of bee pollen depend on plants as food sources of bees. In addition, it also depends on geographies, harvesting period, seasons, and other external factors which can affect the bioactivities, properties, and the appearance of bee pollen such as color, taste, and smell. Chemical constituents of bee pollen from European honeybee and stingless bees in some genus of *Melipona* sp. and plant species are shown in Table 2.1.

Table 2.1 Botanical origins of bee pollen.

Reference	Area	Plant type	Compound
Compos *et al.* (1997)	Portugal	*Salix atrocinerea*	kaempferol-3-neohesperidoside
		Erica australia	quercetin-3-rhamnoside
		Raphanus raphanistrum	myricetin-3-galactoside
			kaempferol-3-sophoroside
		Eucalyptus globules	quercetin-3-sophoroside
			Tricetin
			myricetin
			luteolin

Table2.1 (continued)

Reference	Area	Plant type	Compound
Compos *et al*. (1997)	Portugal	*Eucalyptus globules*	3-*O*-methylquercetin
		Ranunculus sardous	7-*O*-methylherbacetin-3-diglycoside
			8-*O*-methylherbacetin-3-diglycoside
			7-*O*-methylherbacetin-3-sophoroside
			quercetin-3-diglycoside
			herbacetin glycoside
	New Zealand	*Salix atrocinerea*	kaempferol-3-neohesperidoside
		Raphanus raphanistrum	myricetin-3-galactoside
			kaempferol-3-sophoroside
		Ranunculus sardous	7-*O*-methylherbacetin-3-diglycoside
			8-*O*-methylherbacetin-3-diglycoside
			7-*O*-methylherbacetin-3-sophoroside
			quercetin-3-digtycoside
			herbacetin glycoside
		Taraxacum sp.	isorhamnetin-3-sophoroside-diglycoside
Silva *et al*. (2006)	Brazil	Family Miosaceae and Fabaceae	*β*-sitosterol
			tricetin
			selagin
			8-methoxy herbacetin
			D-mannitol
			naringenin
			isorhamnetin
Abarca *et al*. (2007)	Mexico	*Prosopis juliflora* (mesquite tree)	apigenin derivative-7-*O*-R
			luteolin derivative
			flavonol glycoside
			quercetin-3-glycoside

Table2.1 (continued)

Reference	Area	Plant type	Compound
Abarca *et al.* (2007)	Mexico	*Prosopis juliflora* (mesquite tree)	genistein glycoside (dihidroquercetin)
			isorhamnetin-3-*O*-R
			chalcone
			cinnamic acid derivative
Leblanc *et al.* (2009)	Sonoran desert, USA	Mesquite tree	naringenin
			4′, 5-dihydroxy-7-methoxy flavanone
			7, 8, 2′, 4′-tetrahydroxy isoflavone
			benzene acetic acid, α-OXO, methylester
			anthraquinone derivative
			5-methoxy-7-methyl-1,2-naphthoquinone
			7-hydroxy-1-indanone
			1-*p*-tolyl-anthraquinone
		Yucca	naringenin
			2-methyl-5-hydroxybenzofuran
			4′,5-dihydroxy-7-methoxy flavanone
			anthraquinone derivative
			5-methoxy-7-methyl-1,2-naphthoquinone
			1, 2, 3, 4-tetrahydro-2-(2-hydroxy-3- phenoxypropyl)-6,7-dimethoxyisoquinoline
			1-(2-methoxy phenyl)-9,10-anthracenedione
		Palm	2,6-dihydroxy-6-methylbenzaldehyde
			2-formyloxy-1-phenylethanone
			4′,5-dihydroxy-7-methoxyflavone
			anthraquinone derivative

Table 2.1 (continued)

Reference	Area	Plant type	Compound
Leblanc *et al*. (2009)	Sonoran desert, USA	Palm	5-methoxy-7-methyl-1, 2-naphthoquinone
			7-hydroxy-1-indanone
			naringenin
			methyl benzoate
		Terpentine bush	naringenin
			benzene acetic acid, α-OXO, methyl ester
			4′,5-dihydroxy-7-methoxy flavanone
			5-methoxy-7-methyl-1,2 naphthoquinone
			1,1-diphenyl-9-methyldeca-3,5-dien-1,9-diol-8-one
			1-*p*-tolyl-anthraquinone
		Mimosa	naringenin
			4′,5-dihydroxy-7-methoxy flavanone
			benzene acetic acid, α-OXO, methyl ester
			5-hydroxy-7-7-methoxy-2-methyl-3- phenyl-4-chromene
			1-*p*-tolyl-anthraquinone
		Chenopot	naringenin
			α-OXO, methyl ester
			4′,5-dihydroxy-7-methoxy flavanone
			5-methoxy-7-methyl-1,2-naphthoquinone
			1-(3-methoxy phenyl)-anthraquinone
			5-hydroxy-7-metoxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one

Table 2.1 (continued)

Reference	Area	Plant type	Compound
Leblanc *et al.* (2009)	Sonoran desert, USA	Chenopot	Anthraquinone derivative
			7-methoxy-6-(3-methyl-2-butenyl)-2*H*-1-benzopyran-2-one
Marghitas *et al.* (2009)	Romania	*Capsella bursa- pastoris* L.	ND
		Helianthus annuus L.	
		Crataegus monogyna J.	
		Pinus sp.	
		Matricaria chamomilla L.	
		Carduus sp.	
		Taraxacum officinale Web.	
		Onobrychis viviifolia Scop.	
		Centaurea cyanus L.	
		Knautia arvensis L.	
		Salix sp.	
		Corex sp.	
Saric *et al.* (2009)	Croatia	*Cytus incanus* L. (Cistaceae)	pinocembrin
			quercetin
			kaempferol
			galangin
			isorhamnetin
			chrysin
			caffeic acid
Silva *et al.* (2009)	Brazil	*Scoparia dulcis* L. and *Senna obtusifolia* L.	*p*-hydroxycinnamic acid
			dihydroquercetin
			isorhamnetin
			isorhamnetin-3-*O*-(6″-*O*-*E*-*p*-coumaroyl)-*β*-D-glucopyranoside
			luteolin
			quercetin

Remark: ND refers to no data available.

2.3 Flavonoids

Interestingly, flavonoids perform significant antioxidant and chelating properties. In general, they can be found in several parts of plants such as leaves, seeds, barks, and flowers. Plants will produce these compounds for the protection of UV and herbivores together with the defense for pathogens (Harborne *et al*., 2000; Heim *et al*., 2002). Moreover, anthocyanin, especially found in a flower, will help to attach an insect or a pollinator.

Other than plants, flavanoids are also useful to our health since it is an antioxidant and a chelating agent. Not only it can also inhibit low density lipoprotein (LDL) oxidation, but it also provides the cardioprotective effect, the free radical scavenging activity, and the activity of reactive oxygen species removal (Kondo *et al*., 1996; Mazur *et al*., 1999; Tsimogiannis and Oreopoulou, 2006).

Flavonoids (4,000 compounds) belong to a class of secondary plant phenolic compounds (Grotewold, 2006). They are characterized by a flavan nucleus and are low molecular weight compounds. Considering the chemical structure of flavonoids, it is benzo-γ-pyrone derivative consisting of the phenolic and pyrane ring (Figure 2.4). Within the structure of flavonoid, there might be an attached glycoside, hydroxyl, or methoxyl groups or (a) double bond position (s) inside A and B rings (Rice-Evans *et al*., 1997).

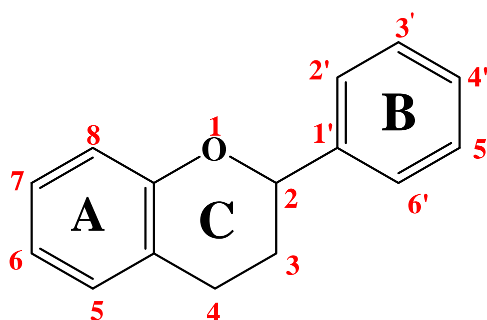


Figure 2.4 The structure of flavonoids. It contains A, B, and C rings as a backbone. The number in a structure showed the position of carbon atom in A, C, and B rings.

Flavonoids can be classified into 6 groups of flavanol, flavone, flavonol, flavanone (dihydroflavone), isoflavone, and anthocyanidin. Each group can be sub-divided. That depends on the attachment and the source. Thus, phenolic compounds in bee pollen can be divided as below:

2.3.1 Flavanone

The flavonoid structure is attached by glycosidic side groups. If disaccharide is attached to the 7th position, it is called flavanone or glycoside flavanone (Figure 2.5). It may be a metabolic product inside a plant cell. Two enzymes are reported to involve in synthesizing it. The first one is chalcone isomerase which can use chalcone as a substrate to produce flavanone. The second one is flavanone 4-redutase which can use (2s)-flavan-4-ol and $NADP^+$ as substrates to produce (2s)-flavanone, NADPH, and H^+. Compounds belonging to the group of flavanone are butin, eriodictyol, hesperetin, hesperidin, homoeriodictyol, isosakuranetin, naringenin, naringin, pinocembrin, poncirin, and sakuranetin. In addition, other compounds those can be found in bee pollen are pinocembrin and naringenin (Rice-Evans *et al*., 1997; Grotewold, 2006; Tsimogiannis and Oreopoulou, 2006).

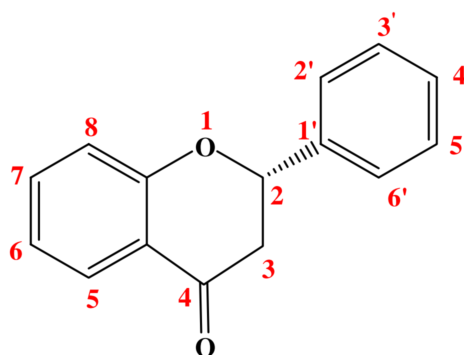


Figure 2.5 The structure of flavanone. It has a ketone group at the 4th position in a C ring.

1) Pinocembrin

The 5, 7-dihydroxyflavanone or pinocembrin is reported to perform many interesting bioactivities such as antioxidant, antimicrobial, and anti-inflammatory activities. Also, it can provide the endothelium relaxation effect. This compound can be found in some bee plants. Thus, it is possible to be found in bee products such as honey, bee pollen (Saric *et al.*, 2009), and propolis with the substitution at the 5th and 7th positions with the –OH group as in Figure 2.6 (Rice-Evans *et al.*, 1997; Heim *et al.*, 2002; Gao *et al.*, 2008; Liu *et al.*, 2008).

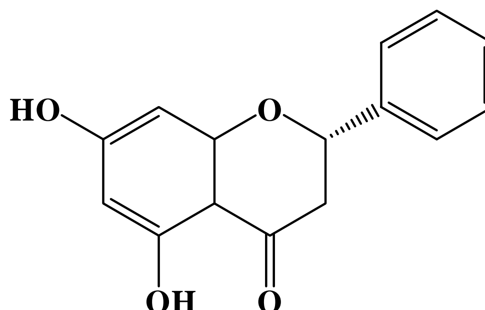


Figure 2.6 The structure of pinocembrin.

2) Naringenin

The 4′, 5, 7- trihydroxyflavanone or naringenin can be isolated from plants, especially in grapefruit and citrus fruit. It is reported to perform many bioactivities such as antioxidant, antiproliferative, free radical scavenging, and anti-inflammatory activities. Generally, it has a substitution at the 4′th, 5th, and 7th positions with the -OH group as in Figure 2.7 (Rice-Evans *et al.*, 1997; Jin *et al.*, 2009).

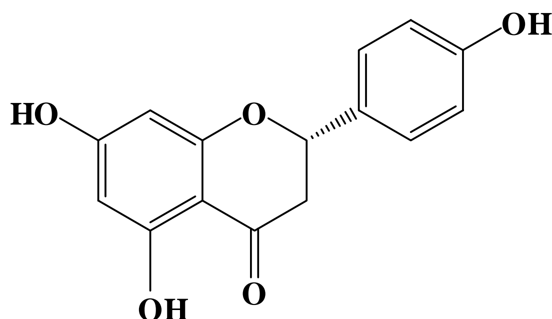


Figure 2.7 The structure of naringenin.

2.3.2 Flavonol

Considering flavonol, there is the –OH group at the 3rd position and the ketone group at the 4th position of the structure (Figure 2.8). If the -OH group is attached to the 3rd position of the structure, it is called 3-hydroxyflavone backbone. If the glycosidic side is attached instead, it is called flavonol glycoside. Compounds in this group are azaleatina, fisetin, galangin, gossypeuin, kaempferide, kaempferol isorhamnetin, morin, myricetin, natsudaidain, pachypodol, quercetin, rhamnazin, and rhamnetin (Rice-Evans *et al.*, 1997; Grotewold, 2006; Tsimogiannis and Oreopoulou, 2006). These compounds are widely found in fruits and vegetables. Some flavonols such as galangin, kaempferol, quercetin, myricetin, and isorhamnetin are also found in bee pollen.

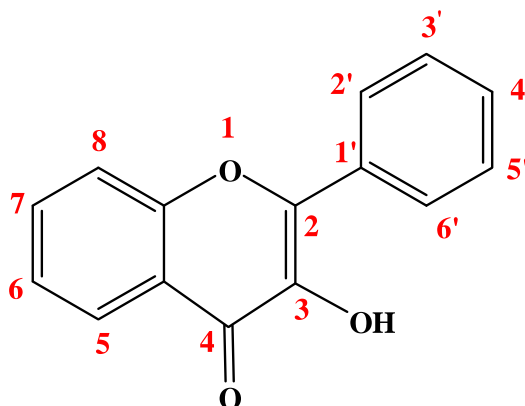


Figure 2.8 The structure of flavonol.

1) Galangin

Galagin is 3, 5, 7-trihydroxyflavone with the substitution at the 3rd, 5th, and 7th position with the -OH group (Figure 2.9). High concentration of this compound can be found in bee pollen of *Alpinia officinarum* (lesser galangal). Moreover, it can be found in galangal rhizome (*Alpinia galanga*) and propolis. Galangin can slow down the growth of breast tumor cells (Ciolino and Yeh, 1999; Tosi *et al.*, 2007; Kaur *et al.*, 2010).

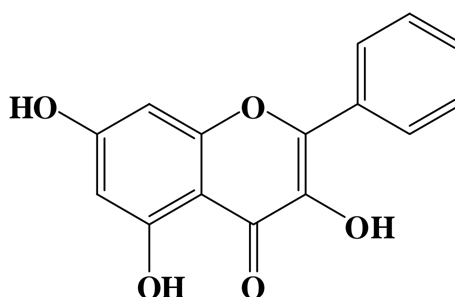


Figure 2.9 The structure of galangin.

2) Kaempferol

Kaempferol is 3, 4′, 5, 7-tetrahydroxyflavone with the substitution at the 3rd, 5th, 7th, and $4^{\prime th}$ positions with the –OH group (Figure 2.10). This compound can be isolated from many plants such as tea, broccoli, delphinium, witch hazal, grapefruit, brussel sprout, and apple, etc (Park *et al.*, 2006; Yoshida *et al.*, 2008). It is a yellow crystal solid with a melting point(m.p.) between 276-278 °C. It was found out that there was a relation between food containing kaempferol and the decreasing risk in cancer and cardiovascular disease. Moreover, kaempferol and glycosides of kaempferol have pharmacological activities like antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, anti-osteoporotic, anti-estrogenic, anxiolytic, analgesic, and anti-allergic activities. It was also reported that keampferol in tea and broccoli could reduce the risk of heart attack (Park *et al.*, 2006; Calderon-Montano *et al.* 2011).

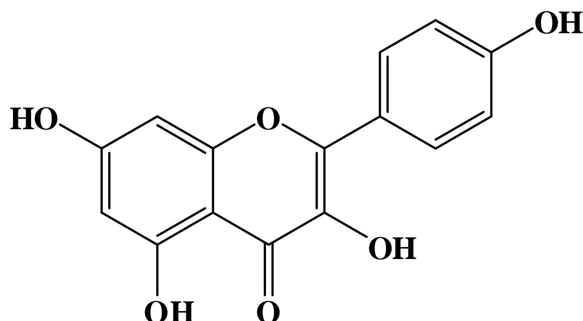


Figure 2.10 The structure of Kaempferol.

3) Quercetin

Quercetin or 3, 3′, 4′, 5, 7- pentahydroxyflavone (Ishisaka *et al*., 2011) was substituted with the –OH group at the 3rd, 5th, 7th, 3′rd, and 4′th positions (Figure 2.11). It is yellow powder and has anti-inflammatory and antioxidant activities. It was reported that the -OH group at the 3′rd and 4′th positions of B ring is very important for a substituent reaction in order to catch free radicals (Rice-Evans *et al*., 1997; Heim *et al*., 2002; David *et al*., 2009). Thus, consuming food which has quercetin could reduce the risk of a cancer (Harwood *et al*., 2007). Citrus fruit, buckwheat, and onions are the sources for quercetin. Moreover, it was found in bee products like bee pollen and honey.

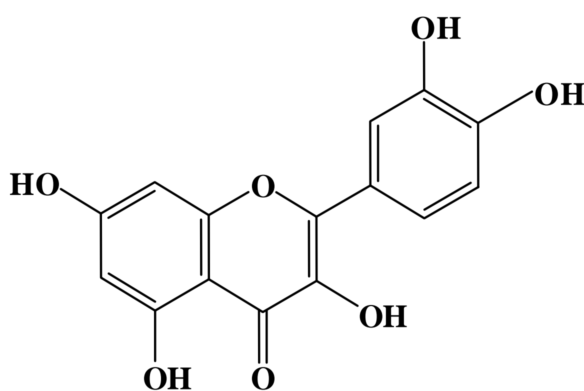


Figure 2.11 The structure of quercetin.

4) Myricetin

Myricetin is 3, 3′, 4′, 5, 5′, 7 hexahydroxyflavone. Its structure is substituted with the –OH group at the 3rd, 5th, 7th, 3′rd, 4′th, and 5′th positions (Figure 2.12). It is commonly found in grape, vegetable, fruit, and herb, etc. Other than that, myricetin in glycoside (exp. myricitrin and myricetin 3-*O*-rutinoside) is mostly found in walnut and red wine (Knekt, *et al.*, 2002; Rice-Evans *et al.*, 1997). This compound has an activity which can relief an anti-allergic symptom (Medeiros *et al.*, 2008) and the antioxidant activity which can reduce prostate cancer.

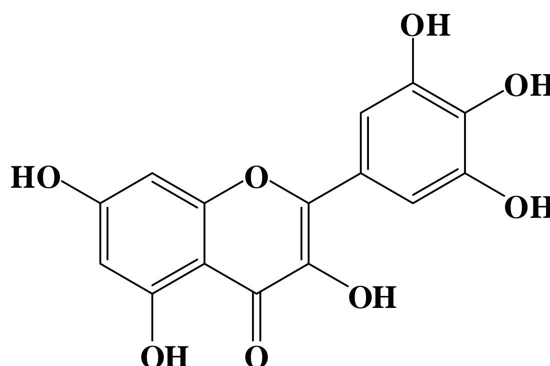


Figure 2.12 The structure of myricetin.

5) Isorhamnetin

Isorhamnetin is 3′-*O*-methyl quercetin. Its structure is substituted with the –OH group at the 3rd, 5th, 7th, and 4′th positions and the methoxyl group (-OCH_3) at the 3′rd position (Figure 2.13). Moreover, isorhamnetin in glycosides can be found in the form of isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside and isorhamnetin-3-*O*-rutinoside-4′-*O*-glucoside (Rice-Evans *et al.*, 1997; Heim *et al.*, 2002). This compound can be found in *Tagetes lucida* and Psychedelic plant which is a local plant in Mexico and Central America (Bohm and Stuessy, 2001). It can inhibit the cell proliferation by inducing apoptosis. In addition, it is used in treatment of heart disease (Ma *et al.*, 2007; Lee *et al.*, 2008).

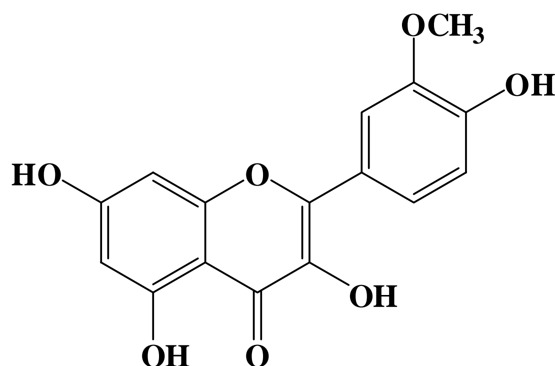


Figure 2.13 The structure of isorhamnetin.

2.3.3 Flavone

Flavone is categorized in a group of flavonoids. It has a structure which is called "2-phenylchromen-4-one (2-phenyl-1-benzopyron-4-one)". Due to Figure 2.14, there is no -OH group at the 3rd position.

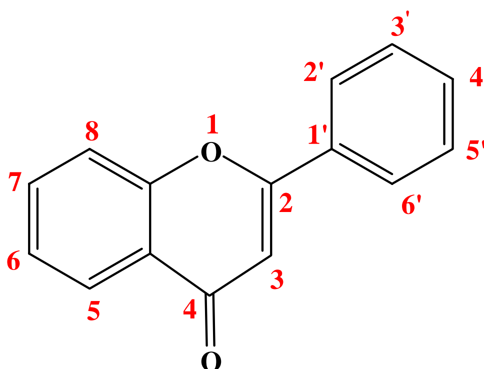


Figure 2.14 The structure of flavone.

Compounds belonging to this group are apigenin (4′, 5, 7-trihydroxyflavone), luteolin (3′, 4′, 5, 7-tetrahydroxyflavone), tangeritin (4′, 5, 6, 7, 8-pentamethoxyflavone), chrysin (5, 7-dihydroxyflavone), baicalein (5, 6, 7-trihydroxyflavone), scutellarein (5, 6, 7, 4′-tetrahydroxyflavone), and wogonin (5, 7-dihydroxy-8-methoxyflavone). Flavones were reported to perform bioactivities against artherosclerosis, osteoporosis, diabetes mellitus, and cancers. The compounds are mainly found in grains and herbs (Rice-Evans *et al*., 1997; Grotewold, 2006). In

addition, some kinds of flavones such as chrysin, luteolin, and apigenin, etc. were found in bee pollen (Table 2.1).

1) Chrysin

Chrysin or 5, 7-dihydroxyflavone is a natural flavones. It is substituted with the -OH group at the 5th and 7th positions (Figure 2.15). This compound was reported to have the anti-inflammatory, anticancer, and antioxidant activities (Rice-Evans *et al.*, 1997; Woo *et al.*, 2004). It could be isolated from many plants. Also, it could be found in bee pollen and propolis.

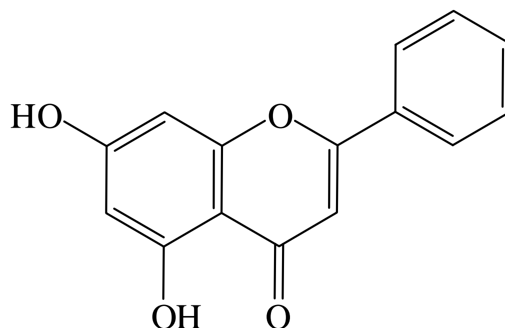


Figure 2.15 The structure of chrysin.

2) Luteolin

Luteolin or 3′, 4′, 5, 7-tetrahydroxyflavone has a yellow crystal. It was substituted with the -OH group at the 5th, 7th, $3'^{rd}$, and $4'^{th}$ positions (Figure 2.16). This compound has the antioxidant, free radical scarvenging, antiproliferative, and anti-inflammatory activities (Kim *et al.*, 2003). It is mostly found in vegetables and herbs such as celery, green pepper, thyme, perillo, chamomile tea, carrot, olive oil, peppermint, rosemary, navel orange, and oregano (Shimoi *et al.*, 1998). It was also reported to be in ragweed pollen (Mahn, 1992).

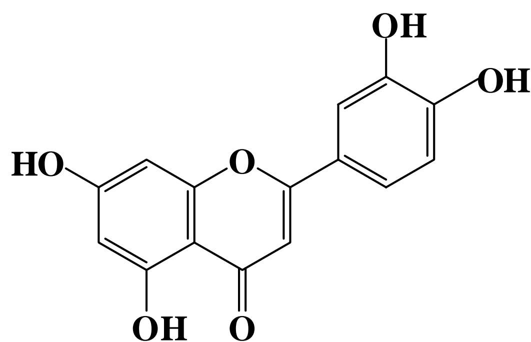


Figure 2.16 The structure of luteolin.

3) Apigenin

Apigenin or 4′, 5, 7 trihydroxyflavone is a yellow powder (Figure 2.17). Apigenin is a aglycone which is attached with several types of chemical compounds such as apiin, apigetrin (apigenin-7-glucoside), and vitexin (apigenin 7-*O*-neohesperidoside), etc. Commonly, apigenin can be found in many types of fruits and vegetables, especially in parsley, onion, orange, tea, chamomile, celery, and cereal. In addition, apigenin glycoside is found in pollen. It has many bioactivities such as anti-inflammatory, anticarcinogenic, and free radical scavenging activities, etc. (Rice-Evans *et al.*, 1997; Heim *et al.*, 2002; Zheng *et al.*, 2005).

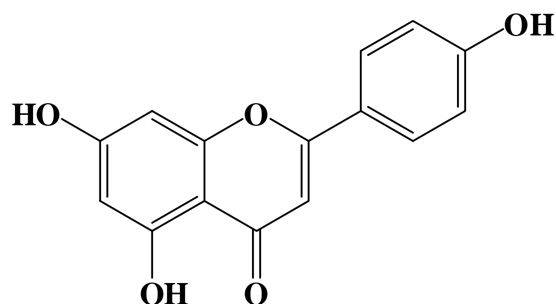


Figure 2.17 The structure of apigenin**.**

2.3.4 Hydroxycinnamic acid

Hydroxycinamic acid is a chemical compound in the phenolic compound group. Compounds belonging to the group of hydroxycinnamic acid are caffeic acid (Figure 2.18), chlorogenic acid, ferulic acid, and *p*-coumaric acid (Rice-Evans *et al.*, 1997). Hydroxycinnamic acid which can be found in fruits and vegetables has the anti-inflammatory and anticancer activities (Kono *et al.*, 1997; Terpinc *et al.*, 2011). In addition, caffeic acid is usually found in bee pollen and propolis.

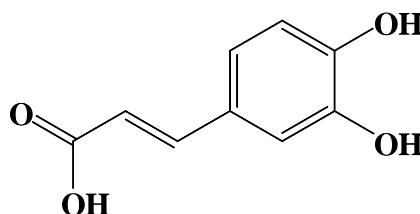


Figure 2.18 The structure of caffeic acid.

2.4 Bioactivities of bee pollen

2.4.1 Antiproliferative activity

Since cancer which is a rapid and uncontrolled division of cells can mainly cause the death nowadays, it is really important to find an alternative way to cure cancer. Rice-Evans *et al.* (1997) and Marghitas *et al.* (2009) found that flavoniod, one of the phenolic compounds, in bee pollen could perform the antiproliferative activity.

Furthermore, in 2009, Saric *et al.* used High Performance Liquid Chromatography (HPLC) to analyse bioactive compounds from *A. mellifera* bee pollen in Croatia. Those analysed bioactive compounds were phenolic compounds such as flavoniod (pinocembin), flavonol (quercetin, kaempferol, galangin, and isohamnetin), flavones (chrysin), and phenylpropanoid (caffeic acid). By doing pollen

analysis, it was found out that dominant pollens in the mentioned bee pollen were from *Cystus incanus* L., one of the native plants in Croatia.

2.4.2 Anti-allergic activity

Basically, there are several types of allergic symptoms which may be caused by protein, immunity reaction, some intruding molecules into a body, etc. Thus, it is still necessary to find a new anti-allergic agent for an alternative treatment. It was reported that the extract of berry, fruit, vegetable, and tea leaf could perform the anti-allergic activity (Park *et al*., 2006; Calderon-Montano *et al*. 2011). In 2008, Medeiros *et al*. could indicate the anti-allergic activity from bee pollen of *A. mellifera* in Brazil. Other than the anti-allergic activity, bee pollen could still present the anti-oxidant and anti-cancer activities. After being analyzed by High Performance Liquid Chromatography/Diode Array Detector (HPLC/DAD), the most bioactive compound against an allergic symptom in bee pollen was myricetin, one of the flavanol derivatives. This compound was tested *in vivo* with mice. They were injected by an allergenic protein and ovalbumin to stimulate them to be allergic. After being treated with myricetin, it was obviously observed that the symptom of paw ederma was relieved, the production of immunoglobulin G_1 protein was inhibited, the migration of pulmonary cells was noticed, and the eosinophil peroxidase activity was reduced (Fathiazad *et al*., 2006).

2.4.3 Anti-angiogenesis activity

In addition, it is really interesting to find anti-oxidant activity from bee pollen. This activity was relatively able to inhibit the angiogenesis of cancer. As known, angiogenesis of cancer is the new construction of capillaries supplying blood nutrients for cell cancers. It was found that an anti-oxidant agent could reduce and prevent some inflammation of the bruise and some symptoms caused by the newborn capillaries such as rheumatoid arthritis (Wang *et al*., 2004; Almaraz-Abarca *et al*., 2007).

Krishnamachari *et al*. (2002) found quercetin which was an anti-oxidant agent in bee pollen. It could decrease a free radical in Reactive Oxygen Species (ROS).

Also, its ability to be substituted in B-ring, C-ring, and olefinic linkage could reduce an angiogenesis. The obtained data in this research coincided to the work of Saric *et al*. (2009) that quercetin was the main compound in bee pollen. In overall, quercetin in bee pollen was both anti-oxidant and anti-allergic agents.

2.4.4 Free radical scavenging activity

Free radical is defined as a molecule, a compound or an ion that has an electron donor surrounded. A molecule of oxygen such as $O^{\cdot}{}_2^{-}$, $HO^{\cdot}$, $ROO^{\cdot}$, and H_2O_2, etc. is in the center of the structure (Wettasinghe and Shahidi, 2000). The most common molecule of free radical is a molecule of reactive oxygen species (ROS). Normally, ROS could be produced by 2 ways. The first one was from the aerobic respiration process, metabolism inside a mammal cell, or was caused by an infection or stress. The second one was from an external factor such as burnt food consuming and ultraviolet or pollution exposure (Noipa *et al*., 2011). In mammalian cells, ROS occurs by metabolism and is stimulated by an oxidant producing enzyme influenced by an external factor. Therefore, ROS is so called a connection among signals inside the cells involving in stress responsiveness, cell proliferation, aging, and cancer (Campos *et al*., 2003).

However, free radical has a very short life span. It can be said that free radical is an unstable but an active reaction molecule. If there is an excess amount of free radical in our body, it can cause our body's cells to die. Moreover, ROS can cause many diseases such as cancer, cataract, age-related and mascular degeneration, antherosclerosis, cardiac ischemia, Parkinson's disease, gastrointestinal disturbance, aging, and rheumatoid arthritis (Ames *et al*., 1993; Luis and Navab, 1993; Diplock, 1994). Also, too much free radical inside our body could affect low density lipoprotein (LDL), protein, and DNA (Heim *et al*., 1993). Thus, an antioxidant agent is very important for our health since it can destroy a free radical system.

Silva *et al*. (2009) showed that chemical constituents in bee pollen of stingless bees, *Melipona subnitida*, in Brazil could present the free radical scavenging activity. The collected bee pollen was from the pollen of *Mimosa gemmalata* (a plant

in family Mimosaceae) and a plant in family of Fabaceae. Seven active compounds were naringenin, isorhamnetin, D-manitol, β-sitosterol, tricetin, selagin, and 8-methoxiherbacetin. The quantity of those active compounds was varied in solvent extracts. The highest to the lowest quantity of active compounds were in EtOAc > EtOH > hexane extracts, respectively (Silva *et al*., 2006; 2009). The reported active chemical constituents were also supported by Saric *et al*. (2009) but the active anti-oxidant agents were from bee pollen of *A. mellifera*.

2.5 The 2, 2-diphenyl-1-picryl hydrazyl (DPPH) assay

The anti-oxidative capacity could be detected by many methods but a DPPH assay which is very simple is always used. This assay is one of the colorimetric methods. DPPH is the stable radical and its electron can delocalize in the structure. Whenever, it attaches to other radicals or electron donor, the radical reactions will stop. DPPH radical is a violet solid compound which could be dissolved easily in organic solvents such as ethanol and methanol (Locatelli *et al*., 2009). The violet color will be fade after it is attached to free radical or hydrogen donor (Figure 2.19).

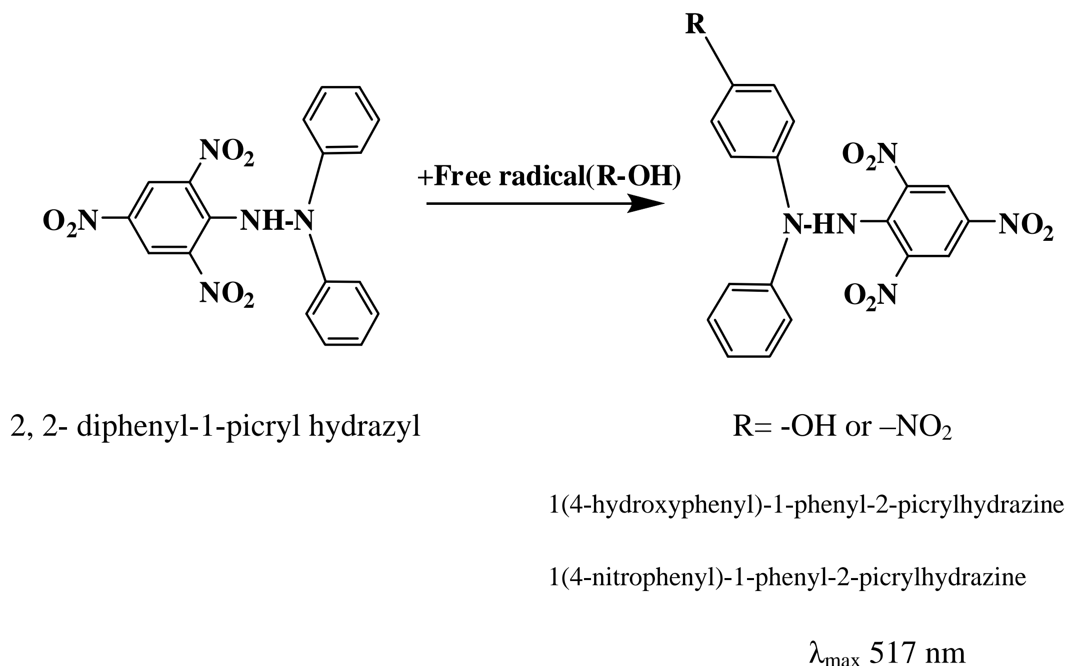


Figure 2.19 The mechanism of DPPH assay. A molecule will become pale yellow after being attached to a hydrogen donor or free radical (Ebada *et al.*, 2008; Chantaranothai, 2010).

From the data mentioned above, it could be considered that bioactive chemical constituents in bee pollen could be an alternative source for the free radical scavenging activity. That led to our interest in studying chemical constituents and their active free radical scavenging activity from bee pollen of *A. mellifera.* In addition, we focused on bee pollen in Nan province according to the unique or typical geography and botanical diversity. New active chemical components may be possible. The benefit of this work may be that new active anti-oxidant compounds could be obtained and may be developed to be an anti-oxidant agent useful in pharmaceutical industry. Finally, this may be benefit to the promotion of bee industry in Thailand which brings the increased income to bee farmers.

CHAPTER III
MATERIALS AND METHODS

3.1 Equipments

- Freezer (-20°C), model: MDF-U332, Sanyo, Japan
- Refrigerator (4°C), model: NR-BT262, Panasonic, Thailand
- 96 well cell culture cluster, Costar®, Corning Inc., USA
- Automatic micropipette, model: P10, P20, P100, P200, and P1000 µl, Gilson, France
- Beaker, model: 50, 250, 600 and 1000 ml, Pyrex®, Germany
- Centrifuge, model: Universal 32 R, Hettich zentrifugen GmbH & Co. Kg., Germany
- Centrifuge/ vortex, model: Combi-spin FVL-2400, Biosan, USA
- Centrifuge tube, model: 15 and 50 ml, Sarstedt, Germany
- Circulating aspirator, model: WJ-20, Sibata, Japan
- Column chromatography, model: 250 ml, Schott Duran, Germany
- Cuvette, model: 1.5 ml semi- microcuvette, Brand, Germany
- Filter paper (catalog# 1003-110), model: no. 3, Whatman®, England
- Flask, model: 50, 500 and 1000 ml, Schott Duran, Germany
- Hot plate stirrer, Schott, Germany
- Measuring cylinder, model: 10, 100, 500 and 1000 ml, Witex, Germany
- Microplate reader, model: Sunrise remote/ touch screen, Tecan, Austria
- Microtube, model: 1.5 ml, Sarstedt, Germany
- Pipette tips (catalog# 10040), model: 100-1000 µl, Sorenson, Bioscience, Inc., USA
- Pipette tips (catalog# 31930), model: 1-200 µl, Sorenson, Bioscience, Inc., USA
- Pipette tips, model: T-300 (0.5-10 µl), Axygen Scientific, USA
- Rota evaporator, model: Buchi rotavapor R-114, Switzerland

- Round bottom flask, model: 50, 100, 500 and 1,000 ml, NK Laboratory, Schott Duran, Germany
- Separatory funnel, model: 500 and 1,000 ml, Bucher®, Germany
- Shaking incubator, model: SI-23MC, Bioer Technology, China
- Spectrophotometer, model: Ultraspec II, LKB Biochrom, England
- Sonicator, Branson, France
- Test tube, model: 3 ml, Pyrex, Mexico
- Ultraviolet light, model: AB-409U electronic money detector, China
- Vacuum column chromatography, model: 500 ml, Schott Duran, Germany
- Vortex mixer, model: MS I Minishaker, IKA-works, Inc., USA
- Water bath, Memmert Schwabach, Germany
- Weighing machine, model: 1600C, Precisa instrument Ltd., Switzerland
- Weighing machine, model: XT220A, Precisa instrument Ltd., Switzerland

3.2 Chemicals

- 1, 1-Diphenyl-2-picrylhydrazyl, $C_{18}H_{12}N_5O_6$, F.W. = 394.32, Sigma-Aldrich, USA
- 3, 5-Dinitrosalicylic acid, $C_7H_4N_2O_7$, M.W. = 228.19, Sigma-Aldrich, USA
- Aluminium chloride hydrate, $AlCl_3 .6H_2O$, M.W. = 241.43, Univar, Ajax Finechem, Australia
- D(+) Glucose monohydrate, $C_6H_{12}O_6.H_2O$, M.W. = 198.7, Merck KGaA Darmstadt, Germany
- Di-sodium hydrogen orthophosphate, Na_2HPO_4, M.W. = 141.96, Univar, Ajax Finechem, Australia
- Ethanol, C_2H_5OH, M.W. = 46.07, Merck KGaA Darmstadt, Germany
- Folin-Ciocalteu's phenol reagent (catalog# 109001), Merck KGaA Darmstadt, Germany
- Gallic acid monohydrate, $C_7H_6O_5.H_2O$, F.W. = 188.14, Sigma-Aldrich, USA
- Hydrochloric acid, HCl, M.W. = 36.46, J.T. Baker, USA
- Hexane, C_6H_{14}, M.W. = 86.18, TSL chemical, Thailand
- Methanol, CH_3OH, M.W. = 32.04, TSL chemical, Thailand

- Methanol-D4 (catalog# 106028), CD_3OD, M.W. = 36.07, Merck KGaA Darmstadt, Germany
- Methylene Chloride, CH_2Cl_2, M.W. = 84.93, TSL chemical, Thailand
- Phenol, C_6H_6OH, M.W. 94.11, Merck KGaA Darmstadt, Germany
- Potassium-sodium tartrate tetrahydrate, $C_4H_4KNaO_6.4H_2O$, M.W. = 282.23, Merck KGaA Darmstadt, Germany
- Quercetin hydrate (catalog# 33,795-1), $C_{15}H_{10}O_7.H_2O$, M.W. = 302.24, Sigma-Aldrich, USA
- Sephadex LH-20, GE Healthcare Bio-sciences AB, Sweden
- Silica gel 60 for column chromatography (0.063- 0.200 mm), SiO_2, M = 60.08 g/mol, Merck KGaA Darmstadt, Germany
- Silica gel 60 for thin layer chromatography, M = 60.08 g/mol, Merck KGaA Darmstadt, Germany
- Sodium carbonate anhydrous, Na_2CO_3, M.W. = 105.99, Univar, Ajax Finechem, Australia
- Sodium dihydrogen orthophosphate, $NaH_2PO_4.H_2O$, M.W. = 156.01, Univar, Ajax Finechem, Australia
- Sodium hydroxide, NaOH, M.W. = 39.99, Univar, Ajax Finechem, Australia
- TLC silica gel 60 F_{254}, Merck KGaA Darmstadt, Germany

3.3 Sample collection

Bee pollen was directly collected from a collecting box in front of an entrance of *A. mellifera* hives (about 80-100 hives). After that, bee pollen from each collecting box was pooled together as shown in Figure 3.1. The collection sites were in Chedeechai subdistrict, Pua district, Nan province, Thailand and the sampling period was on June, 16, 2010. The freshly harvested bee pollen was dried in an oven at 40^oC and was stored at RT (25°C) until used. Since corn (*Zea mays*) was the dominant plant surrounding a sampling site, its pollen was collected, dried, and stored at RT until used.

(A)

(B)

(C)

Figure 3.1 *Apis mellifera***:** (A) a colony of *A. mellifera*; (B) bee pollen in a collecting box at an entrance of a hive; and (C) bee hives for collected sampling in Nan province.

3.4 Pollen morphology by Scanning Electron Microscope (SEM)

Both bee and corn pollens were sent to the Scientific and Technological Research Equipment Centre (STREC) of Chulalongkorn University for morphological analysis.

3.5 Extraction

3.5.1 Ethanol extraction

It was followed by Najafi *et al*. (2007). Bee pollen (70 g) was mixed by 400 ml of 96% (v/v) ethanol. After it was shaken at 100 rpm, 15°C for 18 h, it was centrifuged at 7,000 rpm, 20°C for 15 min. The supernatant was collected. The pellet was re-extracted by 100 ml of 96% (v/v) ethanol. Both supernatant were pooled together. That would bring the total volume to be 500 ml. Then, 300 ml of pooled supernatant was evaporated. The obtained sample from this step was named to be "crude ethanol extract" or "CEE". It was kept at –20°C in the dark until used. Later, it was tested for the free radical scavenging activity by DPPH assay (as mentioned in 3.8.1).

3.5.2 Water extraction

Pooled supernatant (200 ml) from the previous step (as mentioned in 3.5.1) was stirred with 200 ml of 20 mM phosphate buffer (pH 7.0) at 20°C for 20 min. Then, it was centrifuged at 7,000 rpm, 20°C for 15 min. The supernatant was collected and evaporated. The sample from this step was named to be "crude water extract" or "CWE". It was kept at -20°C in the dark until used. Later, it was tested for the free radical scavenging activity by DPPH assay (as mentioned in 3.8.1).

3.6 Partition

It was followed by Bioassay-guide isolation(Umthong *et al*., 2011). CEE and CWE were separately partitioned by three organic solvents with different polarities (MeOH, high polar; CH_2Cl_2, medium polar; and hexane, low polar). At the beginning, each of crude extracts was dissolved in 80% (v/v) MeOH until it was not sticky. The volume of 80% (v/v) MeOH was recorded. The mixture was poured into a separating funnel. Later, it was mixed with the equal volume of hexane. After two phases were clearly separated, the lower phase (hexane part) was removed from the funnel and collected. The left-over upper phase in a separating funnel was re-mixed with the equal volume of hexane. This step was repeated at least 2 times. Later, the hexane parts were pooled together and evaporated. The sample from this step was named to be "crude hexane extract" or "CHE". After that, the left-over upper phase [80% (v/v) MeOH part] was adjusted to be 40% (v/v) MeOH part by adding the equal volume of

deionized water. The volume of 40% (v/v) MeOH part was recorded. Equal volume of CH_2Cl_2 was mixed to re-partition. After two phases were clearly separated, the lower phase (CH_2Cl_2 part) was collected. The left-over upper phase [40% (v/v) MeOH part] was re-extracted by CH_2Cl_2 at least 2 times. The CH_2Cl_2 parts were pooled together and evaporated. The sample from this step was named to be "crude CH_2Cl_2 extract" or "CDE". At last, the 40% (v/v) MeOH part was evaporated. The obtained sample was named to be "crude MeOH extract" or "CME". Finally, all 6 crude extracts were tested for the free radical scavenging activity by DPPH assay (as mentioned in 3.8.1).

3.7 Isolation

The crude extract providing the best free radical scavenging activity was further purified.

3.7.1 Quick column chromatography

A vacuum column chromatography (VCC) at the volume of 500 ml was packed with fine silica gel 60 (Merck's silica gel 60 for thin layer chromatography). It was more tightly packed by using a circulating aspirator. Then, 2 g of the targeted crude extract was dissolved in the appropriate solvent (3 ml) and combined with 10 g of rough silica gel (Merck's silica gel 60 for column chromatography). After being dry, it was sprinkled all over the surface of the packed VCC. After that, pure rough silica gel was sprinkled all over. It was, then, covered with a piece of filter paper and cotton, respectively, in order to protect the crude layer. The elution of the packed chromatography was begun with the lowest polar solvent. Later on, the polarity of the eluting solvents was gradually increasing like the gradient. Since the CDE provided the best free radical scavenging activity, it was firstly eluted by 250 ml of 75% (v/v) CH_2Cl_2: hexane for 3 times. After that, it was followed by eluting with 250 ml of 100% (v/v) CH_2Cl_2 for 2 times, 250 ml of 5% (v/v) MeOH: CH_2Cl_2 for 5 times, 250 ml of 10% (v/v) MeOH: CH_2Cl_2 for 5 times, 250 ml of 20% (v/v) MeOH: CH_2Cl_2 for 16 times, and 250 ml of 100% (v/v) MeOH for 8 times, respectively. In order to make the fast elution, an aspirator was used. Eluted fractions were collected in a flask (500 ml in size). Later, all fractions were evaporated. The pattern of chemical compounds in each fraction was tested by Thin Layer Chromatography or TLC (as mentioned in 3.7.4). Fractions providing the same pattern of chemical compounds were pooled

together. Each pooled fraction was assayed for a free radical scavenging activity by DPPH assay (as mentioned in 3.8.1). Active fractions were further purified by size exclusion (sephadex LH-20) and adsorption chromatographies.

3.7.2 Size exclusion chromatography (sephadex LH-20 chromatography)

Before packing the column, sephadex LH-20 gel (100 g) was immersed in absolute MeOH (500 ml) overnight to make the beads swelling. Cotton was placed at the bottom of the column (250 ml in size). Prepared sephadex LH-20 gel was packed into the column at the appropriate height. After that, the active fraction would be dissolved in absolute MeOH until it was not too viscous. The sample was dropped onto the top of sephadex LH-20 gel carefully by using a pasteur pipette. A valve of the packed column was on in order to let the crude be absorbed into the texture of a gel better. After that, absolute MeOH (500 ml) was added to elute. Fractions (2.5 ml of each) were collected. The pattern of chemical compounds in all fractions was tested by TLC (as mentioned in 3.7.4). Fractions providing the same pattern of chemical compounds were pooled together. Active pooled fractions were determined by spraying the developed TLC plate with 0.2% (w/v) DPPH in MeOH (as mentioned in 3.7.3).

3.7.3 Adsorption chromatography

Before packing the column, silica gel 60 (90 g) was mixed with 2% (v/v) MeOH: CH_2Cl_2 (200 ml). Cotton was placed at the bottom of the column (250 ml in size). The prepared silica gel 60 was packed into the column at the appropriate height. The active pooled fraction from the above (as mentioned in 3.7.2) was mixed with siliga gel 60 (10 g). After it was dry, it was put onto the top of the packed gel in the column. Later, pure silica gel 60 was applied to cover the sample. It was followed by cotton. The elution was begun with 500 ml of 2% (v/v) MeOH: CH_2Cl_2. Fractions (2.5 ml of each) were collected. After that, the purity of all fractions was tested by TLC (as mentioned in 3.7.4). Fractions providing the same pattern of chemical compounds were pooled together. Active pooled fractions were determined by spraying the developed TLC plate with 0.2% (w/v) DPPH in MeOH (as mentioned in 3.7.3).

3.7.4 Thin layer chromatography

Thin Layer Chromatography (TLC) plate as an immobile phase was cut into the size of 5 x 5 cm^2. A solvent front line at the position of 0.5 cm from the bottom edge of the plate was drawn by a pencil. A sample was spotted onto the solvent front line by using a capillary tube. If the sample was too sticky, the capillary tube containing the sample could be dipped into the sample dissolving solvent for 3-4 times in order to make the sample less sticky. While the TLC plate containing samples was left at RT to make it dry, a TLC tank was prepared. A rectangular piece of filter paper was cut and placed inside the TLC tank. The solvent [which depended on the sample dissolving solvent such as 100% (v/v) hexane, 50% (v/v) CH_2Cl_2-hexane, 75% (v/v) CH_2Cl_2-hexane, 100% (v/v) CH_2Cl_2, 5% (v/v) MeOH-CH_2Cl_2, 10% (v/v) MeOH-CH_2Cl_2, 15% (v/v) MeOH-CH_2Cl_2, and 20% (v/v) MeOH-CH_2Cl_2] as a mobile phase was poured into the tank. Then, the chamber was closed for about 5 min in order to make the chamber saturated. The TLC plate containing samples was put into the saturated tank until the mobile solvent reached the top front line of the TLC plate. After the TLC plate was dry, the result of migrating chemical compounds on the plate could be visualized under ultraviolet light at the wavelengths of 254 and 356 nm.

3.8 Free-radical scavenging activity

3.8.1 The 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay

The method was followed by Chen *et al.* (2003). Various concentrations of an interesting sample were prepared with MeOH. For example, it might be in the serial dilution of 0, 25, 50, 75, 100, 200, 300, and 400 µg/ml (for CEE and CWE) or in the serial dilution of 0, 10, 50, 100, and 400 µg/ml (for the rest of samples). The diluted sample (50 µl) was mixed with 50 µl of 0.15 mM DPPH in MeOH (as mentioned in Appendix B) in a 96 well-plate. It was incubated at RT for 1 h in the dark. After that, the absorbance was measured at 517 nm by a microplate reader. Ascorbic acid (vitamin C) was used as the standard reference. Triplication of experiments was performed. By using the Microsoft Excel 2007, the data was recorded as mean ± standard error (S.E.). The free radical scavenging activity was calculated by the formular below:

Percentage of free radical scavenging activity = $\frac{\text{(ABS control – ABS sample)}}{\text{ABS control}} \times 100$

where: ABS control is defined as the absorbance at 517 nm of control.

ABS sample is defined as the absorbance at 517 nm of sample.

3.8.2 The efficient concentration at 50% (EC_{50})

The efficient concentration at 50% (EC_{50}) was obtained by plotting a graph. The percentage of free radical scavenging activity was on a Y-axis while the concentration of each sample was on an X-axis. A linear regression formular and a correlation of both parameters (which r^2 was in the range of 0.8 and 1) were calculated by Microsoft Excel 2007.

3.8.3 DPPH spray

The method was followed by Braca *et al*. (2002). The 0.2% (w/v) DPPH in MeOH was prepared and poured into a spray bottle. After being sprayed onto the developed TLC plate, an active spot would turn from violet to be yellow.

3.9 Chemical structure analysis

A pure fraction was evaporated and sent for the analysis service at Department of Chemistry, Faculty of Science, Chulalongkorn University. Briefly, the evaporated sample was dissolved in an appropriate deuterated solvent (methanol-D4, Merck, catalog# 106028) at the ratio of 2-5 mg of compound to 500 µl of the deuterated solvent. Then, it was transferred to an NMR tube. It was shaken until they were well combined. The NMR spectra were recorded by a Varian Mercury$^+$ 400. It was operated at 400 MHz for ^{1}H and 2D NMR (COSY, HSQC, and HMBC) and 100 MHz for ^{13}C nuclei in order to detect a functional group by using Tetramethylsilane (TMS) as an internal standard. The value of chemical shift in δ (ppm) was assigned with the reference to the signal from the residual protons in deuterated solvents.

3.10 Contents of chemical compounds

3.10.1 Total disaccharide sugar

The method was followed by Dubois *et al*. (1956). Total disaccharide sugar content was measured by using phenol sulphuric acid assay. For drawing a standard curve, glucose was diluted to be 20, 40, 60, and 80 µg/ml by using deionized water as a solvent, respectively. Also, 2.5 mg/ml of CWE was prepared. The prepared CWE (100 µl), blank (100 µl), and glucose at various dilutions (100 µl) was separately mixed with 50 µl of 80% phenol and 2 ml of H_2SO_4. The mixture was incubated at RT for 10 min. Then, 150 µl of the mixture was transferred into a well of a 96-well plate. The absorbance was measured at 490 nm by a microplate reader. An experiment was repeated three times. Total disaccharide sugar content was calculated by a linear regression from Microsoft Excel 2007 which r^2 was in the range of 0.8 and 1.

3.10.2 Reducing sugar

Total reducing sugar content was measured by using dinitrosalicylic acid assay (DNSA) (Miller, 1959). For drawing a standard curve, glucose was diluted to be 200, 400, 600, 800, and 1,000 µg/ml, respectively. The CWE was prepared to be 2.5 mg/ml in water. The prepared extract (500 µl), blank (500 µl) and glucose at various dilutions (500 µl) was separately mixed with 500 µl of DNSA reagent (Appendix B). The mixture was boiled at 100°C for 10 min, rapidly chilled on ice for 2 min, and quick spun. The mixture (100 µl) was transferred into a well of a 96-well plate. The absorbance was measured at 540 nm by a microplate reader. Each experiment was repeated three times. Total reducing sugar content was calculated by a linear regression from Microsoft Excel 2007 which r^2 was in the range of 0.8 and 1.

3.10.3 Total flavonoid

Total flavonoid content was measured by using the aluminum chloride photometry assay (Woisky *et al*., 1998). For drawing a standard curve, quercetin was diluted to be 10, 20, 30, 40, and 50 µg/ml in methanol as a solvent, respectively. The CWE was prepared to be 20 µg/ml in MeOH. The prepared extract (1.5 ml), blank (1.5 ml), and quercetin at various dilutions (1.5 ml) were separately mixed with 1.5 ml of 20 mg/ml of $AlCl_3$. The mixture was incubated at RT for 1 h in the dark. The absorbance was measured at 420 nm by spectrophotometer. In addition, the solution

of 1.5 ml of MeOH and 1.5 ml of $AlCl_3$ was used as blank. Triplication of an experiment was done. Total flavonoid content was calculated by a linear regression from Microsoft Excel 2007 which r^2 was in the range of 0.8 and 1.

3.10.4 Total phenolic compound

Total phenolic compound was measured by using Follin-ciocalteu reagent (Eraslan *et al*., 2009). For drawing a standard curve, gallic acid was diluted to be 10, 20, 30, 40, and 50 µg/ml in MeOH as a solvent, respectively. The CWE was prepared to be 500 µg/ml in MeOH. The prepared extract (1 ml), blank (1 ml), and gallic acid at various dilutions (1 ml) were separately mixed with 1 ml of Follin-ciocalteu reagent. Then, it was mixed with 1 ml of 100 mg/ml Na_2CO_3. The mixture was incubated at RT for 1 h in the dark. The absorbance was measured at 760 nm by spectrophotometer. Also, the solution of 1 ml MeOH, 1 ml Follin-ciocalteu reagent, and 1 ml of Na_2CO_3 was used as blank. The experiment was repeated three times. The content of total phenolic compound was calculated by a linear regression from Microsoft Excel 2007 which r^2 was in the range of 0.8 and 1.

3.11 Free radical scavenging of *Zea mays* (corn) pollen

3.11.1 Sample collection

Corn flowers (1.5 kg) were collected directly from a corn field surrounding an apiary in Chedichai sub-district, Pua district, Nan province in June, 2010. The freshly harvested corn flowers were dried in an oven at 40°C for 2 days. Only corn pollen was kept at RT until used.

3.11.2 Morphology analysis

The preparation of corn pollen was done at the Scientific and Technological Research Equipment Centre (STREC) of Chulalongkorn University. The morphology of corn pollen was analysed by SEM. The comparison in morphology between bee pollen and corn pollen was performed.

3.11.3 Extraction and isolation

For extraction, corn pollen (350 g) was stirred with 2,000 ml of 96% (v/v) EtOH at 100 rpm, 15°C for 18 h. Then, it was centrifuged at 7,000 rpm, 20°C for 15 min. The supernatant was collected. The pellet was re-extracted by 500 ml of 96% (v/v) EtOH. Both supernatants were pooled together and that brought to the final

volume of 2,500 ml. Then, it was evaporated. The obtained sample from this step was named to be "crude ethanol extract of corn pollen" or "CEC". It was stored at –20°C in the dark until used. Later, it was tested for the free radical scavenging activity by DPPH assay (as mentioned in 3.8.1).

The CEC was next partitioned by three organic solvents with different polarities (MeOH, CH_2Cl_2, and hexane) as mentioned previously in 3.6. All obtained extracts were tested for the free radical scavenging activity by DPPH assay (as mentioned in 3.8.1). The result was compared to that of bee pollen.

3.12 Nutritional composition analysis

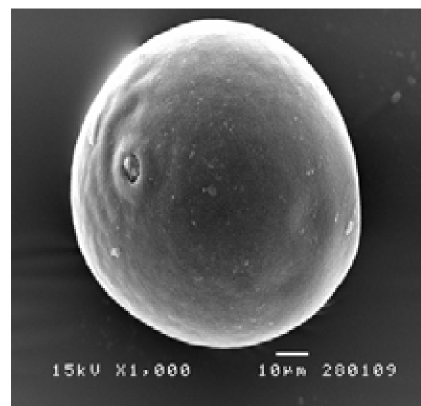
Bee pollen was analysed for nutritional compositions. The sample was sent to the Central Laboratory (Thailand) Co., Ltd, Kasetsart University, Thailand for ash, biotin, calories, calories from fat, carbohydrate, crude fiber, fat, folic acid, invert sugar, moisture, vitamin complex, fructose, glucose, sucrose, maltose, and lactose analyses. In addition, the sample was sent to the Institute of Food Research and Product Development, Kasetsart University, Thailand for co-enzyme Q10 analysis.

CHAPTER IV
RESULTS

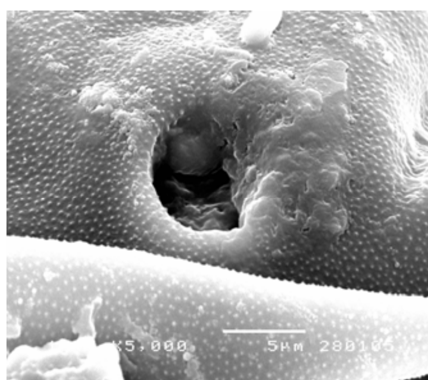
4.1 Pollen morphology

By using scanning electron microscope (SEM), the morphology of bee pollen and the morphology of corn pollen were much similar (Figures 4.1A and 4.1B). Pollen from both sources was in a sphere shape and the surface of outer wall (exine) looked rough. Considering germination pore and ornamentation of exine, they also looked alike (Figures 4.1C and 4.1D). In this research, the change of germination pore and exine were chosen due to the easiness in observation. As known, a pollen grain is a male gametophyte. When it is ready to fertilize an egg (a female gamete), the germination pore and exine will be dramatically changed and can be easily observed, especially in the growing pollen tube. Due to Figure 4.1, it could be implied that the botanical origin of bee pollen of *A. mellifera* was from corn pollen.

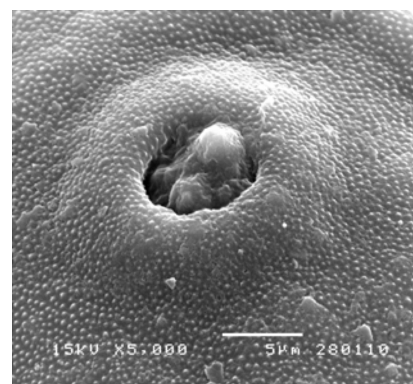
(A)



(B)



(C)



(D)

Figure 4.1 Comparison of bee pollen of *A. mellifera* and corn pollen by SEM. The external morphology of bee pollen was presented in (A) while the external morphology of corn pollen was presented in (B). Furthermore, germination pore and ornamentation of exine of bee pollen (C) and those of corn pollen (D) were shown.

4.2 *A. mellifera*'s bee pollen extraction

4.2.1 EtOH extraction

After bee pollen was extracted and evaporated (as mentioned in 3.5.1), the CEE of 3.45 g was obtained. It was a sticky solid and dark brown in color.

4.2.2 Water extraction

After bee pollen was extracted and evaporated (as mentioned in 3.5.2), the CWE of 4.14 g was obtained. It was a sticky solid and dark brown in color.

4.3 Free radical scavenging activity of CEE and CWE

The CEE was tested for the free radical scavenging activity by DPPH assay. Later, the obtained absorbance would be calculated and interpreted to be the percentage of scavenging activity (as mentioned in 3.8.1). The result indicated that 25 µg/ml of CEE had the percentage of scavenging activity at 57.93 ± 1.29%. The CEE (50 µg/ml) had the activity at 58.81 ± 0.80%. The CEE (75 µg/ml) had the activity at 60.84 ± 0.76%. The CEE (100 µg/ml) had the activity at 60.05 ± 1.51%. The CEE (200 µg/ml) had the activity at 62.37 ± 1.3%. The CEE (300 µg/ml) had the activity at 63.02 ± 0.86% and the CEE (400 µg/ml) had the percentage of scavenging activity at 64.79 ± 2.17%. All values were plotted in order to calculate the EC_{50} value (Figure 4.1). The data were shown in Table 4.1. The EC_{50} of CEE was 21.58 ± 0.48 µg/ml.

The CWE was also tested for the free radical scavenging activity by DPPH assay. Later, the percentage of scavenging activity was calculated (as mentioned in 3.8.1). The result revealed that 25 µg/ml of CWE had the percentage of scavenging activity at 51.84 ± 2.54%. The CWE (50 µg/ml) had the activity at 49.88 ± 1.74%. The CWE (75 µg/ml) had the activity at 46.72 ± 3.72%. The CWE (100 µg/ml) had the activity at 50.02 ± 1.26%. The CWE (200 µg/ml) had the activity at 51.84 ± 0.52%. The CWE (300 µg/ml) had the activity at 46.28 ± 0.72% and the CWE (400 µg/ml) had the percentage of scavenging activity at 50.98 ± 1.71%. All values were plotted in order to calculate the EC_{50} value (Figure 4.2). The obtained data were shown in Table 4.1. The EC_{50} of CWE was 24.12 ± 6.12 µg/ml.

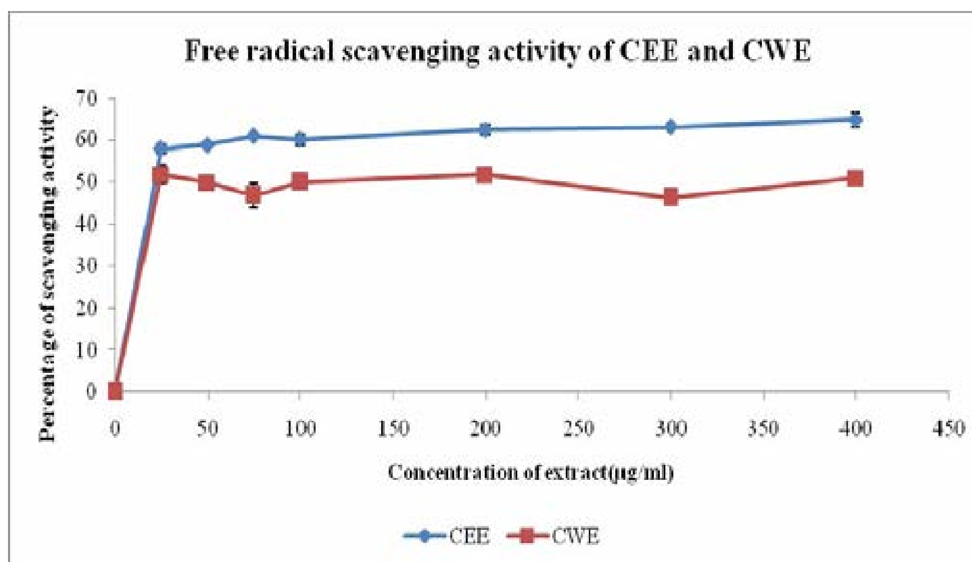


Figure 4.2 The free radical scavenging activity of CEE and CWE.

By DPPH assay, the trend lines of the free radical scavenging activity looked similar in both CEE and CWE. They provided the good percentage of scavenging activity (about 50% and 60%, respectively) with the close EC_{50} values about 21-25 µg/ml (Figure 4.2).

Table 4.1 The EC_{50} values of crude extracts. Ascorbic acid was used as a positive control.

Sample	EC_{50} (µg/ml)*	r^2
Ascorbic acid	8.57 ± 0.24	1
CEE	21.58 ± 0.48	1
CWE	24.12 ± 6.12	1

Remark: * indicated the concentration sufficient to obtain 50% of a maximum scavenging capacity as described in Materials and Methods. The EC_{50} values were calculated from a linear regression and the r^2 represents the correlation coefficient.

4.4 The partitioned extracts from CEE and CWE

Since both extracts (CEE and CWE) presented the good free radical scavenging activity, they were separately partitioned by three organic solvents with different polarity. Those solvents were MeOH, CH_2Cl_2, and hexane. In total, six crude extracts were evaporated and weighed. The data were recorded in Table 4.2.

Table 4.2 The weight and the character of the partitioned extracts.

Sample	**Weight (g)**	**Crude character**
CME (originated from CEE)	3.087	Dark brown sticky solid
CDE (originated from CEE)	0.332	Dark brown solid
CHE (originated from CEE)	0.229	Yellow solid
CME (originated from CWE)	2.410	Dark brown sticky solid
CDE (originated from CWE)	0.035	Dark brown solid
CHE (originated from CWE)	0.342	Yellow solid

4.5 Free radical scavenging activity of the partitioned crudes

All six partitioned crudes (CME, CDE, and CHE originated from CEE and those originated from CWE) were tested for the free radical scavenging activity by DPPH assay. The obtained absorbance was calculated for the percentage of scavenging activity (as mentioned in 3.8.1). The results were presented as mean ± S.E. (Table 4.3). The free radical scavenging activity of CDE (originated from CEE, 400 μg/ml) was the highest at 72.86 ± 1.12%. In contrast, the lowest free radical scavenging activity or no free radical scavenging activity were from CME (originated from CEE: 50 μg/ml), CHE (originated from CEE: 10 and 50 μg/ml) and CHE (originated from CWE: 10, 50, 100, and 400 μg/ml), respectively. As a reference, the highest free radical scavenging activity of ascorbic acid (50 μg/ml) was 76.9 ± 1.27%.

Table 4.3 The percentage of scavenging activity of six partitioned extracts.

Sample	The percentage of scavenging activity			
	10 μg/ml	50 μg/ml	100 μg/ml	400 μg/ml
CME (from CEE)	3.98 ± 0.81	0.00	17.58 ± 1.89	47.19 ± 1.96
CDE (from CEE)	66.9 ± 1.08	63.84 ± 2.85	69.22 ± 0.19	72.86 ± 1.12
CHE (from CEE)	0.00	0.00	4.61 ± 2.86	6.56 ± 2.10
CME (from CWE)	1.99 ± 3.12	0.08 ± 3.73	0.66 ± 3.50	4.74 ± 1.76
CDE (from CWE)	15.53 ± 2.81	24.41 ± 2.35	49.17 ± 5.89	72.72 ± 1.08
CHE (from CWE)	0.00	0.00	0.00	0.00
Ascorbic acid*	58.36 ± 1.66	76.90 ± 1.27	76.79 ± 1.05	76.12 ± 0.72

Remark: * refers to a standard reference.

According to Table 4.3, the data were plotted (Figure 4.3). The EC_{50} values were estimated and recorded in Table 4.4. In addition, ascorbic acid was used as the standard reference. It was found that CDE (originated from CEE) provided the best EC_{50} value (7.47 ± 0.12 μg/ml) which was also better than the EC_{50} value of ascorbic acid (8.57 ± 0.24 μg/ml). Considering the CDE originated from CWE, although its EC_{50} value (101.83 ± 38.94 μg/ml) was high, comparing to ascorbic acid, its value was still much better than the rest which the EC_{50} values were about > 400 μg/ml.

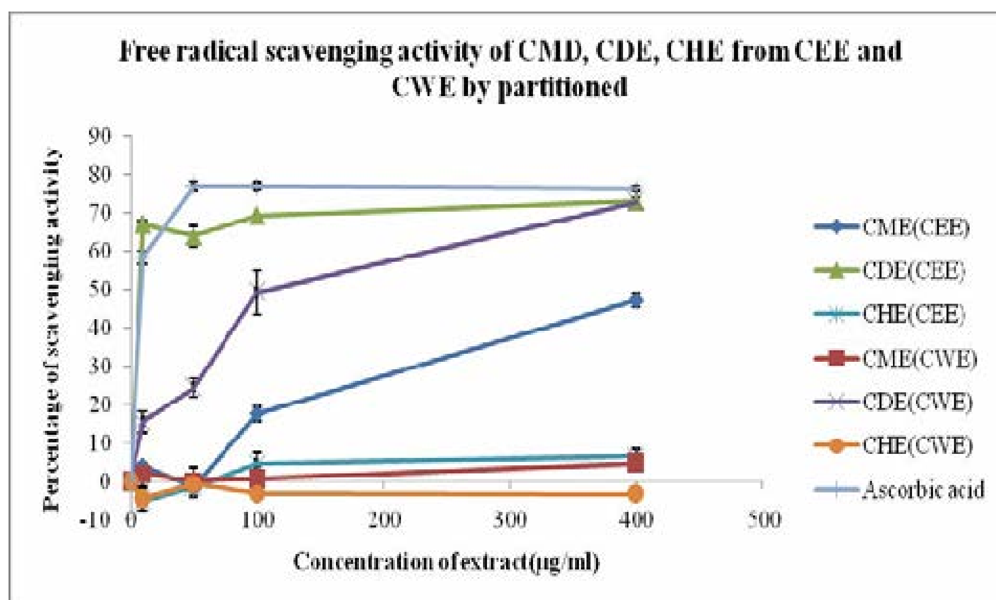


Figure 4.3 The free radical scavenging activity of CMD, CDE and CHE originated from CEE and those originated from CWE. Ascorbic acid was used as a positive control.

Table 4.4 The EC_{50} values of six partitioned crudes.

Sample	EC_{50} (µg/ml)*	r^2
Ascorbic acid	8.57 ± 0.24	1
CME (originated from CEE)	> 400	ND
CDE (originated from CEE)	7.47 ± 0.12	1
CHE (originated from CEE)	> 400	ND
CME (originated from CWE)	> 400	ND
CDE (originated from CWE)	101.83 ± 38.94	1
CHE (originated from CWE)	> 400	ND

Remark: * indicated the concentration sufficient to obtain 50% of a maximum scavenging capacity as described in Materials and Methods. The EC_{50} values were calculated from a linear regression and the r^2 represents the correlation coefficient. In addition, ND represented no available data.

4.6 Compound purification

4.6.1 Quick column chromatography

Since the CDE (originated from CEE) provided the best free radical scavenging activity (21.58 ± 0.48 µg/ml), the lowest value of EC_{50}), more bee pollen (350 g) was extracted for the second time by EtOH in order to gain more sample. It yielded more CEE (89.09 g). Then, CEE was partitioned to provide CDE originated from CEE due to the highest free radical scavenging activity (7.47 ± 0.12 µg/ml), the lowest value of EC_{50}). The sample was obtained at 2.06 g (Scheme 4.1). Then, it was further purified by using quick column chromatography. Total 39 fractions were collected. Considering the compound profile on TLC plate, fractions performing similar compounds were pooled together. Finally, 7 different fractions of CDE1–CDE7 were obtained. Their weight and characters were recorded in Table 4.5.

Table 4.5 The weight and character of pooled active fractions after quick column chromatography.

Fraction	Weight (g)	Crude character
CDE1	0.062	Clear white solid
CDE2	0.042	Pale yellow solid
CDE3	0.006	Pale brown solid
CDE4	0.005	Pale brown solid
CDE5	0.776	Dark brown solid
CDE6	0.430	Sticky and dark brown solid
CDE7	0.760	Dark brown solid

4.6.2 Free radical scavenging activity of pooled active fractions after quick column chromatography

Seven pulled active fractions from 4.6.1 were separately tested for the percentage of scavenging activity. The result was shown in Table 4.6. Among the fractions, the best free radical scavenging activity was from CDE6 (78.39 ± 1.44% at 1,000 µg/ml and 76.66 ± 1.2% at 400 µg/ml). The worst free radical scavenging activity were from CDE1 (400 µg/ml), CDE2 (50, 100, and 400 µg/ml), CDE3 (100 and 400 µg/ml) and CDE4 (10, 50, and 100 µg/ml), respectively. As the references,

the ascorbic acid and CDE (originated from CEE) were used. The free radical scavenging activity of ascorbic acid was 76.9 ± 1.3% at 50 µg/ml while the activity of CDE (originated from CEE) was 70.79 ± 0.73% at 100 µg/ml.

Table 4.6 The percentage of scavenging activity of fraction CDE1 - CDE7.

Fraction	The percentage of scavenging activity				
	10 µg/ml	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1,000 µg/ml**
CDE1	3.76 ± 4.27	0.04 ± 2.05	0.31 ± 2.54	0.00	8.29 ± 2.41
CDE2	3.16 ± 1.47	0.00	0.00	0.00	5.92 ± 1.85
CDE3	1.43 ± 2.07	0.1 ± 1.58	0.00	0.00	9.05 ± 0.54
CDE4	0.00	0.00	0.00	14.35 ± 5.91	12.04 ±0.54
CDE5	5.15 ± 0.87	14.03 ± 3.04	22.52 ± 1.86	59.78 ± 3.98	65.27 ± 0.8
CDE6	3.69 ± 3.7	19.43 ± 3.8	37.6 ± 1.33	76.66 ± 1.2	78.39 ± 1.44
CDE7	3.34 ± 1.33	6.19 ± 2.13	9.69 ± 0.8	38.31 ± 4.8	58.51 ± 1.71
CDE (CEE)*	66.9 ± 0.88	68.8 ± 0.47	70.79 ± 0.73	70.05 ± 0.47	61.82 ± 0.7
Ascorbic acid*	58.36 ± 1.66	76.9 ± 1.3	76.79 ± 1.05	76.12 ± 0.72	65.19 ± 6.5

Remark:* referred to standard reference.

Due to Table 4.6, the percentage of scavenging activity was plotted, the EC_{50} was determined from the graph. The data was reported in Figure 4.4 and Table 4.7. Considering the EC_{50}, CDE6 gave the lowest EC_{50} value (or the highest free radical scavenging activity) at 195.54 ± 9.86 µg/ml. It was followed by CDE5 which provided the EC_{50} at 321.77 ± 27.35 µg/ml. In addition, CDE7 presented the EC_{50} at 762.12 ± 67.37 µg/ml. The rest of CDE1 to CDE4 presented the high value of EC_{50} at > 1,000 µg/ml. These indicated the very low free radical scavenging activity. The ascorbic acid and CDE (originated from CEE) which were used as references presented the EC_{50} at 8.57 ± 0.24 and 7.47 ± 0.12 µg/ml, respectively.

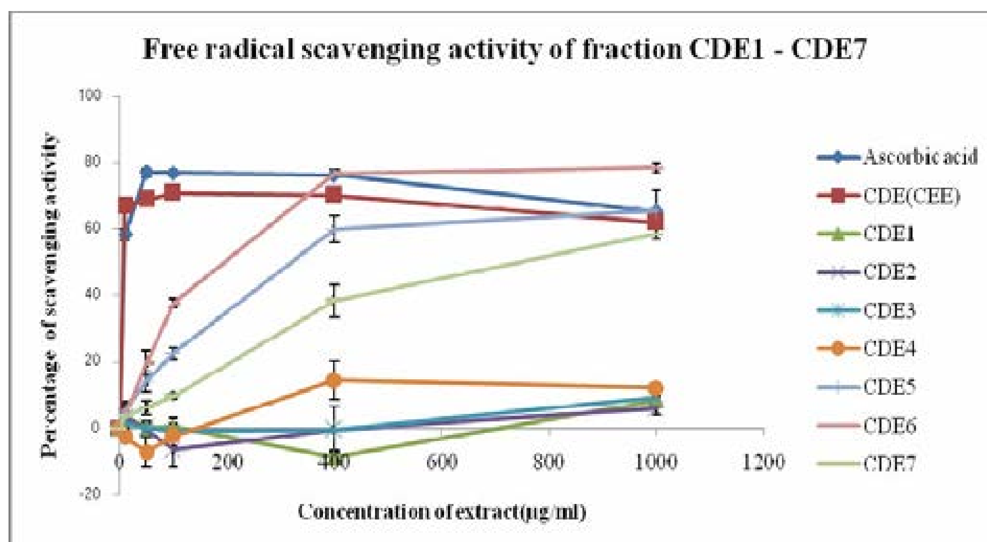


Figure 4.4 Free radical scavenging activity of fraction CDE1 - CDE7.

Table 4.7 The EC_{50} values of pooled active fractions after quick column chromatography**.**

Fraction	EC_{50} (µg/ml)*	r^2
Ascorbic acid	8.57 ± 0.24	1
CDE (originated from CEE)	7.47 ± 0.12	1
CDE1	>1000	ND
CDE2	>1000	ND
CDE3	>1000	ND
CDE4	>1000	ND
CDE5	321.77 ± 27.35	1
CDE6	195.54 ± 9.86	1
CDE7	762.12 ± 67.37	1

Remark: * indicated the concentration sufficient to obtain 50% of a maximum scavenging capacity as described in Materials and Methods. The EC_{50} values were calculated from a linear regression and the r^2 represented the correlation coefficient. In addition, ND represented no available data.

4.6.3 Size exclusion chromatography (sephadex LH-20 chromatography)

The CDE5 fraction was further purified by sephadex LH-20 chromatography. After the column, total 58 fractions were obtained. By TLC, the fractions presenting the similar compound profile were pooled together. Finally, 6 different fractions of CDE5-1 to CDE5-6 were obtained and weighed. The data together with the character of those mentioned fractions were recorded Table 4.8.

Table 4.8 The weight and character of pooled active fractions after size exclusion chromatography (sephadex LH-20 chromatography).

Fraction	Weight (g)	Crude character
CDE5-1	0.0281	Brown solid
CDE5-2	0.1884	Dark brown solid
CDE5-3	0.0078	Dark brown solid
CDE5-4	0.0039	Brown solid
CDE5-5	0.0028	Brown solid
CDE5-6	0.0099	Yellow-green solid

The fractions (CDE5-1 to CDE5-6) were re-confirmed for the active activity by DPPH spray. The result was shown that fractions CDE5-5 and CDE5-6 gave the best activity so both were selected for further purification but separately.

4.6.4 Re-purification of CDE5-5 by sephadex LH-20 chromatography

The fraction CDE5-5 was loaded onto sephadex LH-20 column again. The procedure of purification was the same as mentioned in scheme 4.1. After DPPH assay and TLC analysis, it revealed that fractions# 33-45 (Figure 4.5) were pure and presented the same pattern of chemical compounds so they were pooled together. The pooled compound could be obtained with the weight of 3.2 mg and was named as compound I. Compound I was a clear yellow solid.

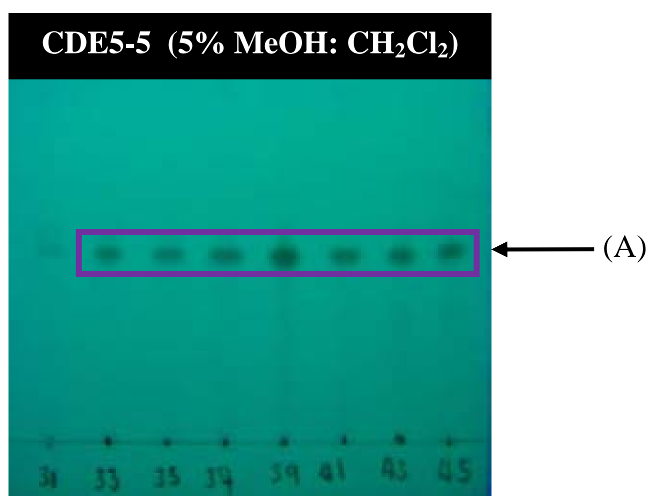


Figure 4.5 The TLC plate showed the compound profile of fractions# 33-45. The arrow of (A) indicated compound I.

4.6.5 Purification of CDE5-6 by adsorption chromatography (silica gel chromatography)

The fraction CDE5-6 was loaded onto a silica gel chromatography. The procedure of purification was the same as mentioned in scheme 4.1. After DPPH assay and TLC analysis, it revealed that fractions# 1-69 had many compounds and the chemical pattern was not clear. In contrast, in fractions# 70-109, the same pattern of chemical compounds was presented and clearly visible (Figure 4.6). The compounds in fractions# 70-90 could be obtained with the weight of 6 mg and was named as compound II. It was green-yellow solid.

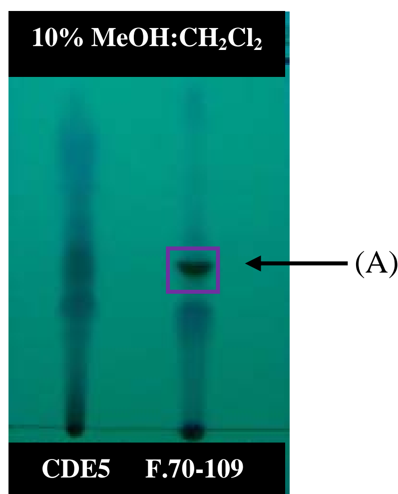


Figure 4.6 The TLC plate showed the chemical compound profile of fractions# 70-109. The arrow of (A) presented compound II.

4.7 Chemical structure analysis

4.7.1 Compound I

Compound I which was clear yellow solid was hydroquinone (Figure 4.7). After it was analysed for a chemical structure by NMR, the obtained NMR peaks of chemical shift pattern (appendix C) were ^{1}H NMR (CD_3OD, 400 MHz) δ7.22 (2H, d, J = 8.8 Hz, H-2 and H-6) and 6.72 (2H, d, J =8.4 Hz, H-3 and H-5).

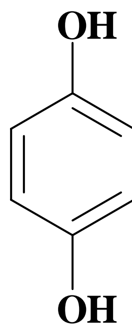


Figure 4.7 The formular structure of hydroquinone found in fractions# 33-45 from CDE5-5.

4.7.2 Compound II

Compound II which was green-yellow solid was apigenin (Figure 4.8). When it was analysed for a chemical structure by NMR, the obtained NMR peak of chemical shift pattern (appendix D) were ^{1}H NMR (CD_3OD, 400 MHz) δ_H 7.77 (1H, s, H-3), 7.63 (2H, d, *J*= 8.8 Hz, H-2´, and H-6´), 6.83 (2H, d, *J* = 8.4 Hz, H-3´, and H-5´), 6.32 (1H, s, H-8), and 6.09 (1H, s, H-6).

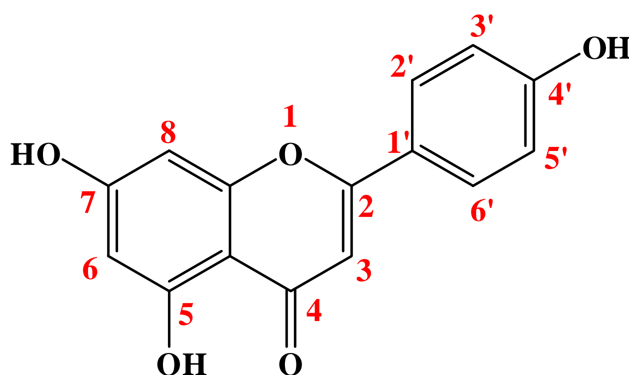


Figure 4.8 The formular structure of apigenin found in fraction# 70-109 from CDE5-6.

4.8 The content of chemical compounds

4.8.1 Total disaccharide sugar

The standard curve for neutral sugar was drawn by the known concentrations of glucose [Appendix E(A)]. A linear regression was estimated by Microsoft excel 2007. Then, the equation of Y = 0.001X and correlation coefficient value (r^2 = 0.99) were obtained. Since the absorbance value at 490 nm of CWE was 0.553), total disaccharide sugar content was 22.12% per 1 g of CWE (Table 4.9, Appendix F).

4.8.2 Total reducing sugar

The standard curve for reducing sugar was drawn by the known concentrations of glucose [Appendix E(B)]. A linear regression was estimated by Microsoft excel 2007. Then, the equation of Y = 0.0000695X and correlation coefficient value (r^2 = 0.98) were obtained. Since the absorbance value at 540 nm of

CWE was 0.659), total reducing sugar content was 37.9% per 1 g of CWE (Table 4.9, Appendix F).

4.8.3 Total flavonoid content

The standard curve for total flavonoid was drawn by the known concentrations of quercetin [Appendix E(C)]. A linear regression was estimated by Microsoft excel 2007. Then, the equation of Y = 0.012X and correlation coefficient value (r^2 = 0.99) were obtained. Since the absorbance value at 420 nm of CWE was 0.0093), total flavonoid content was 3.875% per 1 g of CWE (Table 4.9, Appendix F).

4.8.4 Total phenolic compound

The standard curve for total phenolic compound was drawn by the known concentrations of gallic acid [Appendix E (D)]. A linear regression was estimated by Microsoft excel 2007. Then, the equation of Y = 0.010X and correlation coefficient value (r^2 = 0.99) were obtained. Since the absorbance value at 760 nm of CWE was 0.223), total phenolic content was 4.46% per 1 g of CWE (Table 4.9, Appendix F).

Table 4.9 The content of chemical compounds in bee pollen of *A. mellifera* from Nan province, Thailand.

Compound	% in 1 g of CWE	r^2*
Disaccharide sugar	22.12	0.99
Reducing sugar	37.9	0.98
Flavonoid	3.875	0.99
Phenolic compound	4.46	0.99

Remark: * Total disaccharide sugar, reducing sugar, phenolic compound, and flavanoid contents were calculated by comparing to the standard curve of references as mentioned in Materials and Methods together with a linear regression. The r^2 represented the correlation coefficient of the linear regression.

4.9 Free radical scavenging of *Zea mays* (corn) pollen

4.9.1 Extraction of *Zea mays* (corn)'s pollen

After corn's pollen was extracted and evaporated (as mentioned in 3.5.1), the weight of crude EtOH extract of corn pollen (CEC) at 16.8 g was obtained. The crude was a sticky solid and dark green in color.

4.9.2 Free radical scavenging activity of CEC

It was assayed by DPPH method. Also, the percentage of scavenging activity was determined as mentioned previously in 3.8.1. The result was shown that 10 µg/ml of CEC performed the highest percentage of scavenging activity at 0.86 ± 2.28%. The CEC at 50, 100, 400, and 1,000 µg/ml provided the activity at 14.60 ± 1.91%, 28.35 ± 1.55%, 65.48 ± 0.95%, and 73.15 ± 1.20%, respectively. All values were plotted in order to determine the EC_{50}. Due to Figure 4.9, the EC_{50} of CEC was 276.67 ± 10.03 µg/ml while the EC_{50} of ascorbic acid as a reference was 8.57 ± 0.24 µg/ml.

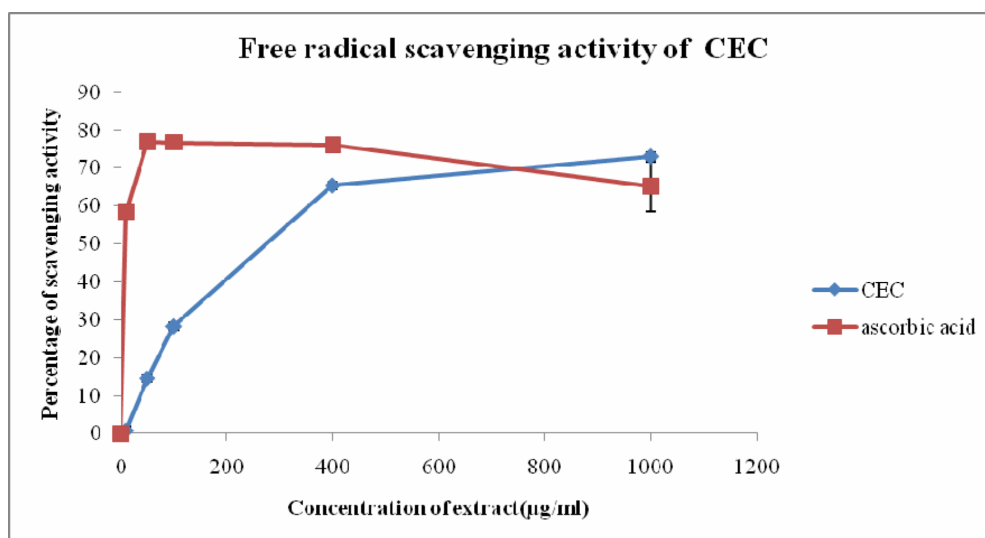


Figure 4.9 The percentage of free radical scavenging activity of CEC, comparing to the activity of ascorbic acid.

4.9.3 Partition of CEC

The CEC was partitioned by using the protocol described in section 3.6 of Materials and Methods. After being partitioned and evaporated, crude methanol extract of CEC (CMC), crude dichloromethane extract of CEC (CDC), and crude hexane extract of CEC (CHC) were obtained. The weight and character were recorded in Table 4.10.

Table 4.10 The weight and character of three crude extracts of CEC.

Crude extract	Weight (g)	Crude character
CMC	7.52	Dark brown solid
CDC	4.02	Brown solid
CHC	6.56	Dark green oil or wax

4.9.4 Free radical scavenging activity of CMC, CDC, and CHC

The CMC, CDC, and CHC originated from CEC were tested for the free radical scavenging activity by DPPH assay (as mentioned in 3.8.1). Later, the percentage of scavenging activity could be obtained. The data was reported as mean ± S.E. (Table 4.11).

Table 4.11 The percentage of scavenging activity of CMC, CDC, and CHC originated from CEC.

Crude	The percentage of scavenging activity				
	10 µg/ml	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1,000 µg/ml**
CMC	0.00	0.00	12.94 ± 5.96	55.17 ± 4.01	72.38 ± 3.02
CDC	0.00	15.82 ± 3.40	37.31 ± 1.94	71.19 ± 1.16	69.59 ± 2.38
CHC	0.00	0.00	0.00	0.00	3.51 ± 2.41
Ascorbic acid*	58.36 ± 1.66	76.9 ± 1.3	76.79 ± 1.05	76.12 ± 0.72	65.19 ± 6.5

Remark: * Standard reference by ascorbic acid

Due to Table 4.11, the percentage of scavenging activity was plotted. Then, the EC_{50} was estimated from the graph. The data was recorded in Figure 4.10 and Table 4.12. It was presented that CDC had the lowest EC_{50} (212.30 ± 13.58 µg/ml) which meant the highest free radical scavenging activity. In addition, CMC and CHC had the EC_{50} of 365.24 ± 38.81 and >1000 µg/ml, respectively. The EC_{50} of ascorbic acid as a standard was 8.57 ± 0.24 µg/ml.

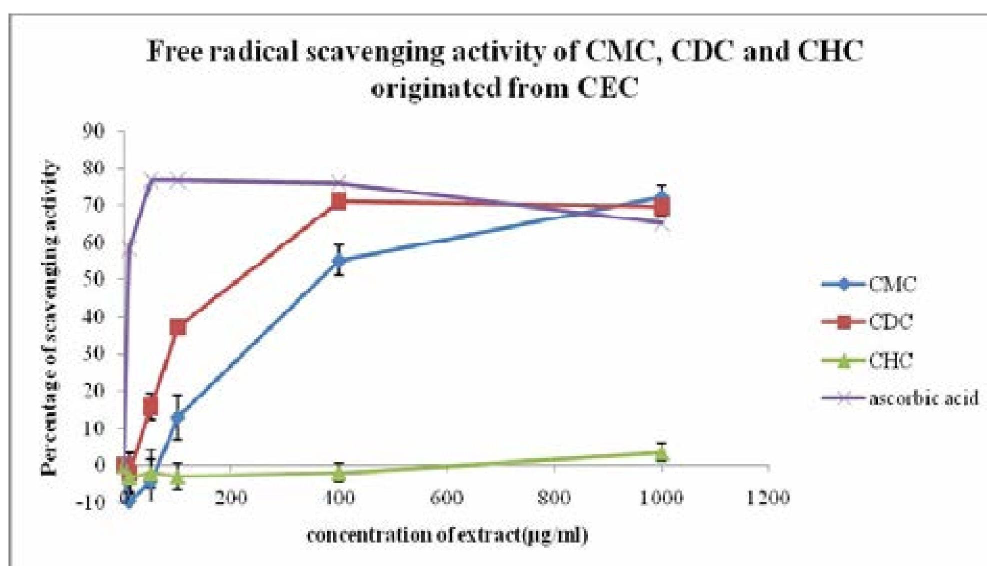


Figure 4.10 The percentage of free radical scavenging activity of CMC, CDC, and CHC originated from CEC.

Table 4.12 The EC_{50} of CMC, CDC, and CHC originated from CEC.

Crude extract	EC_{50} (µg/ml)*	r^2
CMC	365.24 ± 38.81	1
CDC	212.30 ± 13.58	1
CHC	> 1,000	ND
Ascorbic acid	8.57 ± 0.24	1

Remark: * indicated the concentration sufficient to obtain 50% of a maximum scavenging capacity as described in Materials and Methods. The EC_{50} were calculated from a linear regression and the r^2 represented the correlation coefficient. In addition, ND represented no available data.

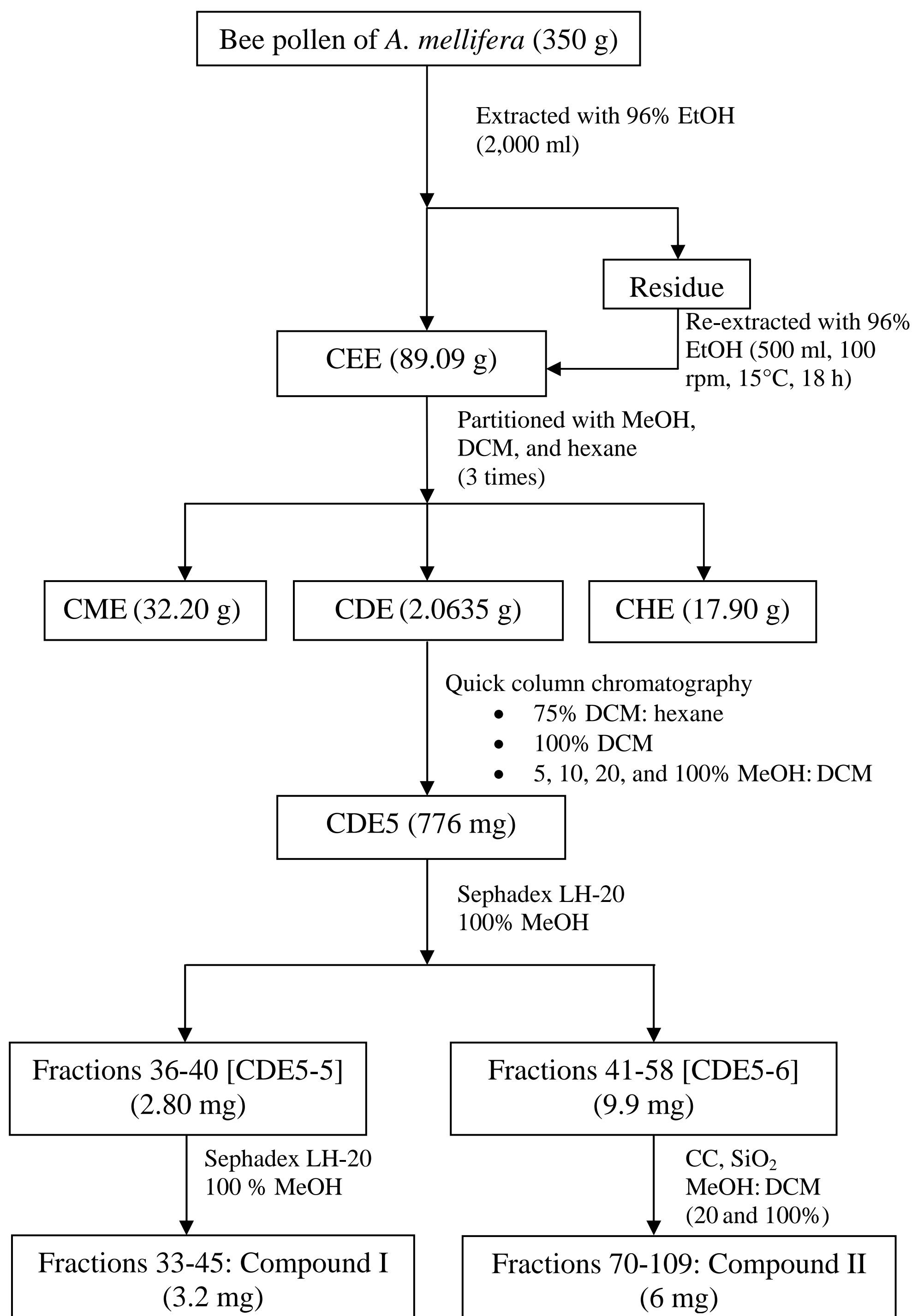
4.10 Nutritional composition analysis

Bee pollen was analysed for nutritional compositions. The sample was sent to the Central Laboratory (Thailand) Co., Ltd, Kasetsart University, Thailand. The results of analysis were reported in Table 4.13.

Table 4.13 Nutritional composition analysis of bee pollen of *A. mellifera* from Nan province, Thailand.

Analyzed item	Result	Unit
Ash	2.43	g/100g
Biotin	56.69	µg/100g
Calories	397.16	Kcal/100g
Calories from fat	63.00	Kcal/100g
Carbohydrate	64.42	g/100g
Crude fiber	0.86	g/100g
Fat	7.00	g/100g
Folic acid	54.70	µg/100g
Invert sugar	19.85	g/100g
Moisture	7.03	g/100g
Protein (%N x 6.25)	19.12	g/100g
Reducing sugar	14.11	g/100g
Total sugar (HPLC)		
Total sugar	14.71	g/100g
Fructose	7.16	g/100g
Glucose	6.42	g/100g
Sucrose	0.60	g/100g
Maltose	0.53	g/100g
Lactose	<0.1	g/100g
VitaminA- β carotene	1,530.4	µg/100g
Vitamin B_1	0.20	mg/100g
Vitamin B_2	0.50	mg/100g
Vitamin B_3 (Niacin)	7.03	mg/100g
Vitamin B_5	0.39	mg/100g
Vitamin B_6	Not detected	mg/100g
Vitamin B_{12}	1.87	µg/100g
Vitamin E (α-Tocopherol)	6.21	mg/100g

In addition, the sample was sent to the Institute of Food Research and Product Development, Kasetsart University, Thailand for co-enzyme Q10 analysis. Bee pollen extract of *A. mellifera* from Nan province, Thailand had co-enzyme Q10 at the concentration of 0.12 mg/100ml.



Scheme 4.1 The extraction and purification procedure of bee pollen of *A. mellifera*.

CHAPTER V
DISCUSSIONS

5.1 Pollen morphology

In this research, bee pollen and pollen from corn (*Zea mays* L.) were chosen for SEM analysis since, considering by eyes at the beginning, the shape and color of bee pollen looks similar to corn pollen. Also, considering the agricultural area, it was surrounding by corn fields. In addition, during the bee pollen collecting period, it was the season that farmers in Nan province grew corn. Thus, it was highly possible that *Apis mellifera* collected pollen from corn. Pollen counting for isolated types of pollen grain was not performed because it was obvious that the composition of corn pollen was higher than 80 %. Thus, it was possible that corn was dominant.

According to SEM analysis, the morphology of bee pollen and corn pollen, in term of ornamentation of exine and germination pore, were compared (Figure 4.1 as mentioned in 4.1). Since the pollen of each plant was very unique and typical, its morphology was always analysed to classify the type of plants. There were many methods used to analyse the morphology of pollens. For example, Silva *et al.* (2009) was successfully catagolized bee pollen of *Melipona rufiventris* by using a glycerin jelly method which was explained previously in Maurizio and Louveaux (1965). After that, the morphology was analysed by a light microscope. The sample morphology was compared to the morphology of plant pollen from a slide standard and the recorded figures in the Palynotheca book.

5.2 Extraction and partition

When bee pollen was extracted by EtOH and water extraction, they were tested for the free radical scavenging activity by DPPH assay. The EC_{50} values were shown that the activity from both EtOH and water extracts were not different (The EC_{50} value of CEE was 21.58 ± 0.48 μg/ml and the EC_{50} value of CWE was 24.12 ± 6.12 μg/ml). Considering the graph mentioned in Figure 4.2 of 4.3, that led to the decision to partition both samples.

After being partitioned, the CDE (originated from CEE) turned to be the best source for the free radical scavenging activity. The EC_{50} value obtained from the CDE (originated from CEE) was 7.47±0.12 μg/ml (Table 4.4 as mentioned in 4.5) which was lower than the EC_{50} value of ascorbic acid (8.57±0.24 μg/ml). The obtained data was coincided to Silva *et al*. (2009). Their pollen was similarly extracted by 3 types of solvents (EtOH, hexane, and EtOAc). The result revealed that crude ethyl acetate extract of bee pollen (CAP) had the best value of EC_{50} (15.3±0.4 μg/ml). Considering CH_2Cl_2 and EtOAc, both are organic solvents with medium polarity. This could be indicated that the free radical scavenging agent should contain medium polarity. Between the work of Silva *et al*. (2009) and our work, the similar results were also obtained in CME and CHE. In overall, since the free radical scavenging activity was found in CDE (originated from CEE) and CME (originated from CEE), this indicated that active compounds in bee pollen were polar. From the above, among the three crude extracts, since the CDE (originatedly from CEE) provided the best activity extract, it was chosen for further purification.

5.3 Compounds purified by chromatographies

After purified by quick column chromatography, the active fractions were eluted by 5-20% (v/v) of MeOH: CH_2Cl_2 solvent system. Fraction CDE5, CDE6, and CDE7 showed the good activity due to the EC_{50} values (Figure 4.4 and Table 4.7 as mentioned in 4.6.2). Although fraction CDE6 provided the best activity, it was followed by fraction CDE5 and CDE7. Among those fractions, fraction CDE5 was the most suitable for continuing an isolation because it had enough quantity. Considering the chemical compound profile on the TLC plate, compounds in fraction CDE5 was also the easiest separated. After fraction CDE5 was purified by size exclusion chromatography, all of fractions could not be tested for the free radical scavenging activity by DPPH assay because of low obtained quantity. Thus, the assay technique was changed to use the DPPH spraying directly onto the TLC plate. A fraction which had an activity would turn the background color of TLC plate from violet to be pale yellow. According to the mentioned technique, fraction CDE5-5 and CDE5-6 originated from CDE5 were collected and used for the chemical compound analysis. At the end, compound I and compound II were obtained from both fractions.

In order to decide which column should be used to isolate an interesting compound, the separating pattern of compounds as spots on a TLC plate was considered. If spots were visible as smear or looked connected to each other, it might indicate that the molecular size of those compounds was close. They should be further isolated by size exclusion chromatography (sephadex LH-20). In case, each spot had a wide distance, the adsorbtion chromatography should be chosen for polarity isolation.

Moreover, there still had another method to isolate a chemical constituent of bee pollen (Leblance *et al*., 2009). By using Gas Chromatography-Mass Spectrometry (GC-MS), they successfully isolated chemical constituents belonging to phenolic compound group in bee pollen from Sonoran desert, USA. Those phenolic compounds were reported in Table 2.1 as mentioned in 2.2. In addition, they found that dominant plant pollen in this bee pollen were from two plant species.

In addition, the High Performance Liquid Chromatography/Diodade Array Detector (HPLC/DAD) was successfully used to categorize types of phenolic compounds and flavanoids in bee pollen (Abarca *et al*., 2007). The phenolic compound types were reported in Table 2.1 as mentioned in 2.2. It was also presented that main plant pollen in this bee pollen was from *Prosopis juliflora*.

Both techniques could be analyzed within a short period when compared to a column chromatography which took a longer time. However, their cost was much higher and many standard compounds had to be used. Thus, column chromatography was still the most suitable technique in this research.

5.4 The relationship of active compounds of bee pollen of *A. mellifera* from Nan province, Thailand

When CDE (originated from CEE) was isolated by polarity isolation from quick column chromatography, it could be noticed that fraction CDE1-CDE7 provided the decreased activity. While the EC_{50} value of CDE (originated from CEE) was 7.47 ± 0.12 μg/ml, the EC_{50} values of CDE1-CDE7 were much higher (>100 μg/ml, Table 4.7 and Figure 4.4 as mentioned in 4.6.2). It could be summarized that many active compounds were needed to work in group. That was why crude extract could perform the better activity than the more purified compound.

5.5 Free radical scavenging activity by DPPH assay

In this research, DPPH assay was chosen for the detection of the free radicals scavenging capacity in ROS group because it was reported to be successful to detect the activity of phenolics compounds like flavonoids (Silva *et al*., 2006; 2009). Several types of phenolic compounds would have a different substitution group including a position of hydroxyl group and double bond. Accordingly, the antioxidant and free radical scavenger capacities had an unequal activity. In the B-ring of flavonoid, the hydroxyl group of this ring was the most significant determinant of scavenging of ROS and Reactive Nitrogen Species (RNS) (Heim *et al*., 2002). When the flavonoid gave a donation to a hydrogen atom to a free radical or DPPH molecule, the internal structure of flavonoid would be delocalized of electron and made a molecule stable. The hydroxyl group of flavonoid or electron donor would be able to react with DPPH and change the color when DPPH radicals received an electron. Within our body, the flavonoid and radical molecule were stable, so they were eliminated by mechanisms within the body.

Moreover, DPPH assay was used to determine the free radical scavenging activity of bee pollen extract before (Silva *et al*., 2006; 2009; Marghitas *et al*., 2009; Leblanc *et al*., 2009).

5.6 Chemical structural analysis

After bee pollen was extracted and purified as mentioned earlier, two active compounds were obtained. Compound II was the main active compound in the bee pollen of *A. mellifera*. It was analysed to be apigenin which was flavanoid belonging to the flavone group. Briefly about the analysis, the obtained chemical shift (δ) of ^{1}H NMR was compared to apigenin isolated by Fathiazad *et al*. (2006). They isolated flavanoid from tobacco leave waste and found the combination of major compounds of apigenin, quercetin, and rutin. Between apigenin in bee pollen and that in tobacco leave waste, the chemical shift value of apigenin of bee pollen was slightly different from the other in ppm unit. That might be from a different deuterated solvent used for running ^{1}H NMR. Due to Fathiazad *et al*. (2006), DMSO-d_6 was used as a deuterated solvent for running ^{1}H NMR while in our research, methanol-d4 (CD_3OD) was used as a deuterated solvent for running ^{1}H NMR (Table5.1).

Table 5.1 The chemical shifts (δ_H) of apigenin (compound II) and the referenced apigenin (Fathiazad *et al*., 2006).

Apigenin (CD_3OD)		**Referenced apigenin**	
position	**δ_H (ppm)**	**position**	**δ_H (ppm)**
3	7.77	**3**	6.81
6	6.09	**6**	6.21
8	6.32	**8**	6.50
2´, 6´	7.63	**2´, 6´**	7.95
3´, 5´	6.83	**3´, 5´**	6.94

For ^1H NMR analysis, it was suggested that methanol-d4 (CD_3OD) was suitably used for high polar target sample which was in small amount since the sample could be re-evaporated and re-used. In contrast, DMSO-d_6 was suitably used for very high polar target sample which was in large amount since the dissolved compound could not be re-used (Gottlieb *et al*., 1997; Beyer *et al*., 2010). In this research, small amount of compound (6 mg) was isolated from bee pollen so CD_3OD was used.

Previously, apigenin in the glycoside form had been reported from bee pollen collected by *A. mellifera* in Mexico (Abarca *et al*., 2007). The dominant plant pollen in the mentioned bee pollen was from *Prosopis juliflora* plants. In this research, it could be firstly reported that there was 7-*O*-R derivatived apigenin in bee pollen collected by *A. mellifera*. The dominant plant pollen was from corn pollen from Nan province, Thailand.

In our research, we found apigenin from corn pollen. It was flavonoid in a flavone group. The obtained data was supported by Ceska and Styles (1984). They also reported the chemical constituents of corn pollen were varieties of flavanoids such as quercetin, isorhamnetin, kaemferol, and flavanol glycoside.

The minor compound (compound I) in our research was analysed to be hydroquinone which was phenolic compound. It was previously reported that hydroquinone had an antioxidant activity but only for medical purpose. The use of hydroquinone needed to be controlled by a specialist only. Moreover, hydroquinone could be supplied in cosmetics like skin care products (Hu *et al*., 2009).

5.7 The content of chemical compounds

It was reported that bee pollen was full of nutrients which were polysaccharide (50%), simple sugar (4-10%), fats (1-20%), protein (6-28%), and amino acid (6%) (Rice- Evans *et al*., 1997), together with moisture (7.4%) and ash (2.2%) (Almeida-Muradian *et al*., 2005). In our work, we focused on other nutritionally chemical compounds. Due to Table 4.9 as mentioned in 4.8, disaccharide sugar, reducing sugar, phenolic compound, and flavanoid contents could be reported. For each gram of CWE, it consisted of 22.12% of disaccharide sugar, 37.9% of reducing sugar, 4.46% of total phenolic content, and 3.88% of total flavanoid. It could be said that nutrient supplements of bee pollen were firstly reported in our work. Furthermore, searching for phenolic compounds and their derivatives in bee pollen was very supportive for bioactivities since Morais *et al.* (2011) reported antioxidant and antimicrobial activities from bee pollen in Portugal. Main chemical compounds providing such bioactivities were phenolic compounds.

In this research, bee pollen was sent to the Institute of Food Research and Product Development, Kasetsart University, Thailand and the Central Laboratory (Thailand) Co., Ltd, Kasetsart University, Thailand in order to find the quantity of nutritionally chemical compounds in term of biotin, crude fiber, folic acid, vitamin B_3, vitamin B_5, vitamin B_{12}, and co-enzyme Q10. Our work could be said to be firstly reported in Thailand again as mentioned in 2.2.

5.8 Comparison of free radical scavenging activity between bee pollen and corn pollen

Bee pollen and corn pollen were similarly extracted as mentioned in Materials and Methods. Interestingly, it was found that, in both samples, CDE had the best free radical scavenging activity. Then, it was followed by CME and CHE, respectively. However, if the EC_{50} value was considered, it could be easily observed that bee pollen extract performed the better free radical scavenging activity than corn pollen extract (as mentioned in Table 5.2).

Table 5.2 Comparison of the EC_{50} value between bee pollen and corn pollen.

EC_{50} of bee pollen (µg/ml)		**EC_{50} of corn pollen (µg/ml)**	
CEE	21.58 ± 0.48	**CEC**	276.67 ± 10.03
CME (CEE)	>400	**CMC (CEC)**	365.24 ± 38.81
CDE (CEE)	7.47 ± 0.12	**CDC (CEC)**	212.30 ± 13.58
CHE (CEE)	> 400	**CHC (CEC)**	> 1000

Due to Table 5.2, it could be summarized that bee pollen which consisted of packed or modified corn pollen had the better free radical scavenging activity than natural form of corn pollen. It could be possible that honeybees could modify the chemical structure of active compounds by the functional combination of nectar, enzymes, and bee secretion when they packed the corn pollen. In addition, it could be summarized that bee pollen collected by *A. mellifera* from Nan province in Thailand could be used as an alternative source for the free radical scavenging activity. In the future, it is possible to develop bioactive compounds in bee pollen to be an antioxidant agent.

5.9 Research benefit

In overall, the obtained data showed that bee pollen from Nan province, Thailand during corn season not only gave a good free radical scavenging activity but it also contained varieties of nutritional supplements. The research data brought to the promotion of bee apiaries in Nan province and also to all apiaries in other provinces. It could be said that corn cultivation together with bee industry can be managed within the same area. They can support each other. Both can help in the increase of income for farmers. Furthermore, it should be possible to apply bee pollen and its extract to food and cosmetic products with the free radical scavenging activity.

CHAPTER VI
CONCLUSIONS

1) By considering pollen morphology from Scanning Electron Microscope, bee pollen of *Apis mellifera* from Nan province, Thailand was mainly from corn pollen. External morphology which is ornamentation of exine (outer membrane) and germination pore could be obviously noticed.

2) Considering the free radical scavenging activity (EC_{50} value), the best crude extract for bee pollen is crude methylene chloride or dichloromethane extract (7.47 ± 0.12 µg/ml). Then, it was followed by crude ethanol extract (21.58 ± 0.48 µg/ml), crude water extract (24.12 ± 6.12 µg/ml), crude methanol extract (420.17 ± 25.88 µg/ml), and crude hexane extract (> 450 µg/ml), respectively.

3) The major active compound from bee pollen of *A. mellifera* from Nan province, Thailand was apigenin which belonged to flavone group. The other active flavanoid compound, hydroquinone, was also found although it was the minor one.

4) Chemical compositions of *A. mellifera* bee pollen from Nan province, Thailand were 22.12% (w/w) of total disaccharide sugar content, 37.9% (w/w) of total reducing sugar content, 3.87% (w/w) of total flavonoid content, and 4.46% (w/w) of total phenolic compound contents.

5) Bee pollen of *A. mellifera* from Pua district, Nan province, Thailand performed the better free radical scavenging activity than natural corn pollen due to the EC_{50} value.

6) The less free radical scavenging activity was observed when the sample was more purified. This indicated that active compounds in *A. mellifera* bee pollen from Nan Province, Thailand needed to work all together.

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APPENDICES

Appendix A: Preparation of 200 ml of 20 mM phosphate buffer (pH 7.0)

1. Preparation of 20 mM phosphate buffer

1) Monobasic ($NaH_2PO_4.2H_2O$) MW = 156.01
2) Diabasic (Na_2HPO_4) MW = 141.96

2. Monobasic calculation (100 mM)

1,000 mM: weigh 156.01 g of monobasic and dissolve in d-H_2O with the final volume of 1,000 ml

∴ 100 mM: weigh 1.56 g of monobasic and dissolve in d-H_2O with the final volume of 100 ml

3. Diabasic calculation (100 mM)

1,000 mM: weigh 141.96 g of diabasic and dissolve in d-H_2O with the final volume of 1,000 ml

∴ 100 mM: weigh 1.42 g of diabasic and dissolve in d-H_2O with the final volume of 100 ml

4. Mix of monobasic and diabasic solutions

100 mM monobasic (A) + 100 mM diabasic (B), adjust pH to be ≈ 7

∴ 39 ml of A + 61 ml of B to make pH 7 of the buffer

200 ml of 20 mM phosphate buffer (pH 7) can be prepared as the formula below:

$$C_1V_1 = C_2V_2$$

$$100 \text{ mM} \times V_1 = 20 \text{ mM} \times 200 \text{ ml}$$

$$V_1 = 40 \text{ ml}$$

∴ 40 ml of 100 mM phosphate buffer (pH 7) is mixed with 160 ml of d-H_2O to provide 20 mM phosphate buffer (pH 7).

Appendix B: Preparation of solutions

1. Preparation of 0.15 mM 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in 10 ml MeOH

1) the formular of DPPH = $C_{18}H_{12}N_5O_6$

MW = 394.32 g

2) Weigh 1 mg DPPH and dissolve in 20 ml MeOH.

3) Keep in the dark at 4°C for 1 month.

2. Preparation of dinitrosalicylic acid (DNSA) reagent

1) Weigh 1 g DNSA, 30 g of Na-K tartrate, and 1.6 g NaOH.

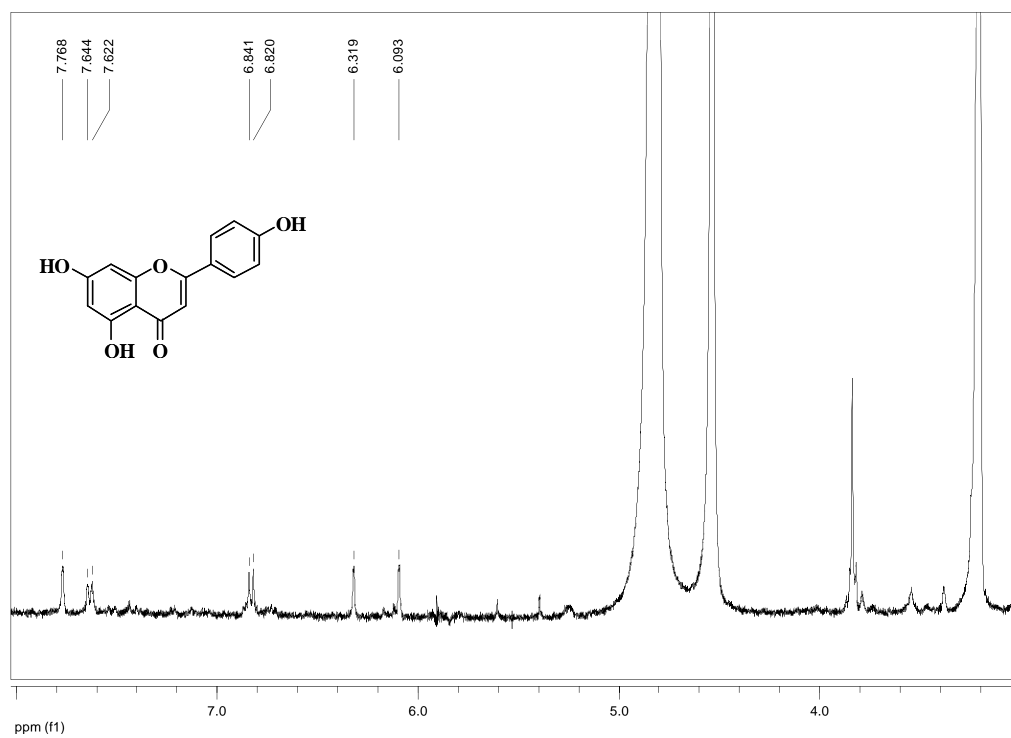
2) Combine 3 compounds together and adjust the final volume to be 100 ml. Melt the compounds by stirring on a hot plate.

3) Keep in the dark at RT for 1 month.

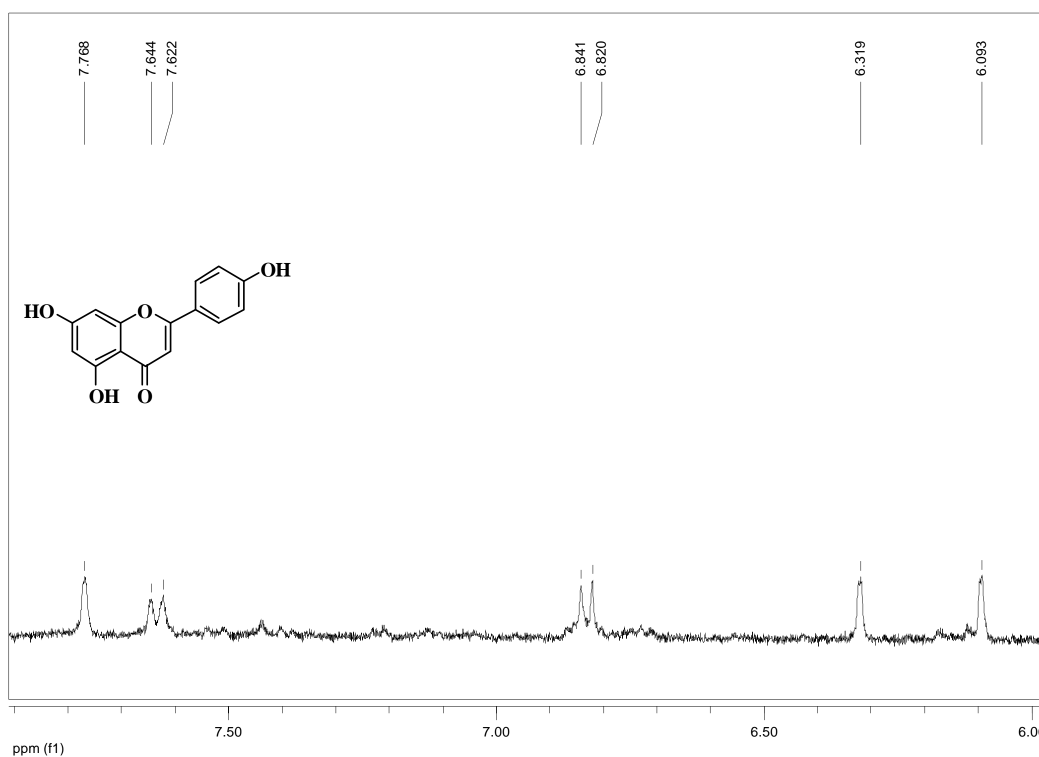
Appendix C: ^{1}H NMR of apigenin in CD_3OD

A: ^{1}H NMR peak data at chemical shift (δ), 3.0-8.0 ppm in length

B: ^{1}H NMR peak data at chemical shift (δ), 6.0-8.0 ppm in length



(A)



(B)

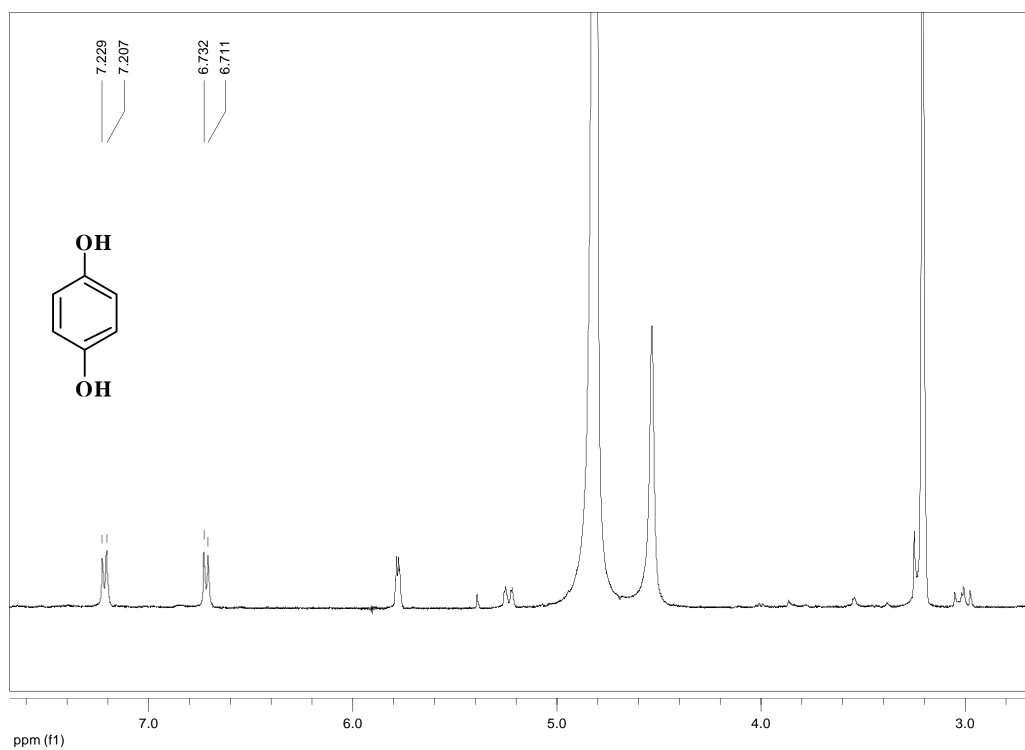
Appendix D: ^{1}H NMR and COSY of hydroquinone in CD_3OD

A: ^{1}H NMR peak data at chemical shift (δ), 2.6-7.6 ppm in length

B: ^{1}H NMR peak data at chemical shift (δ), 6.56-7.3 ppm in length

D: COSY peak data at chemical shift (δ), 2.6-8.0 ppm in length

E: COSY peak data at chemical shift (δ), 6.52–7.36 ppm in length



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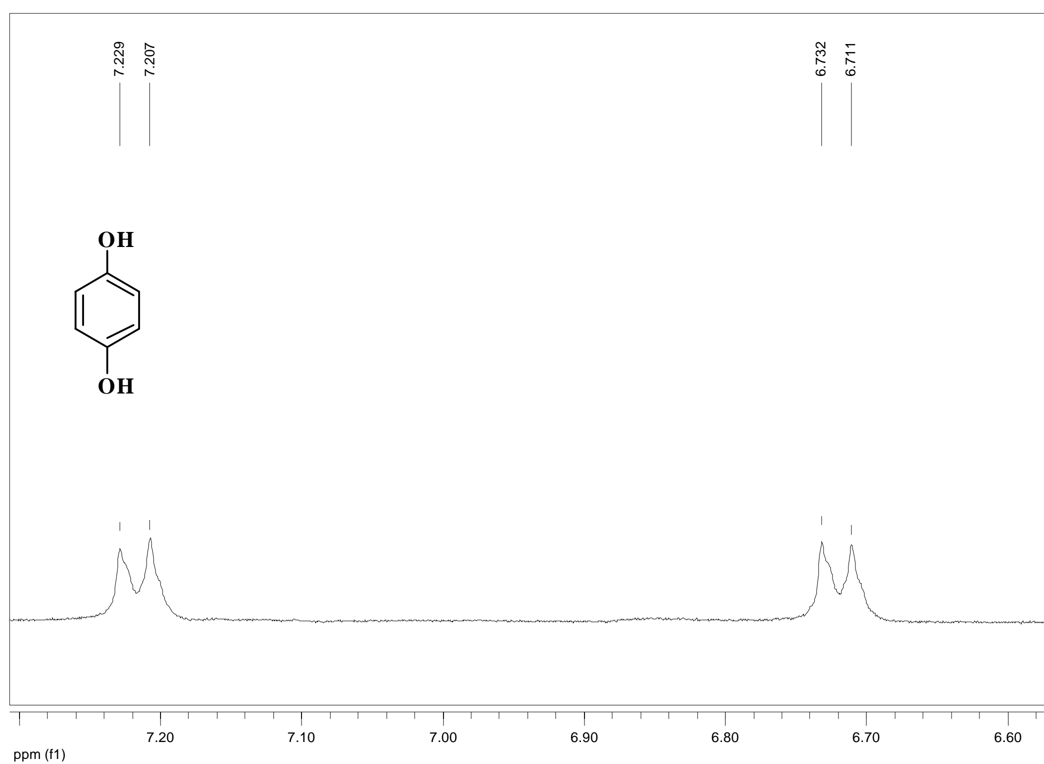

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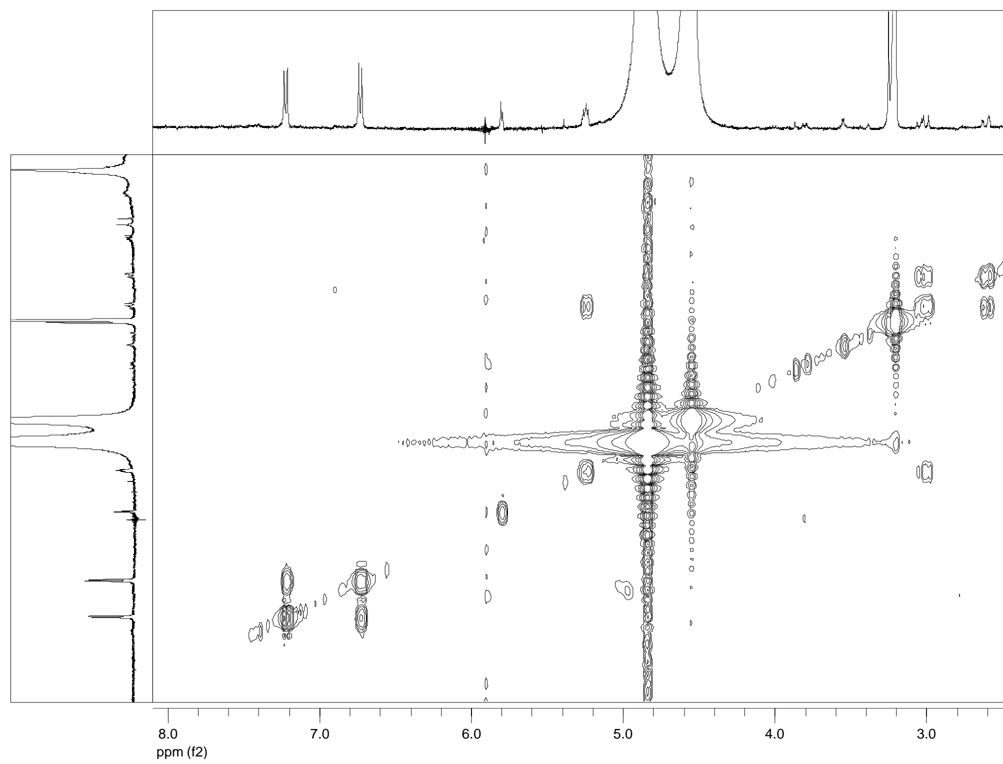
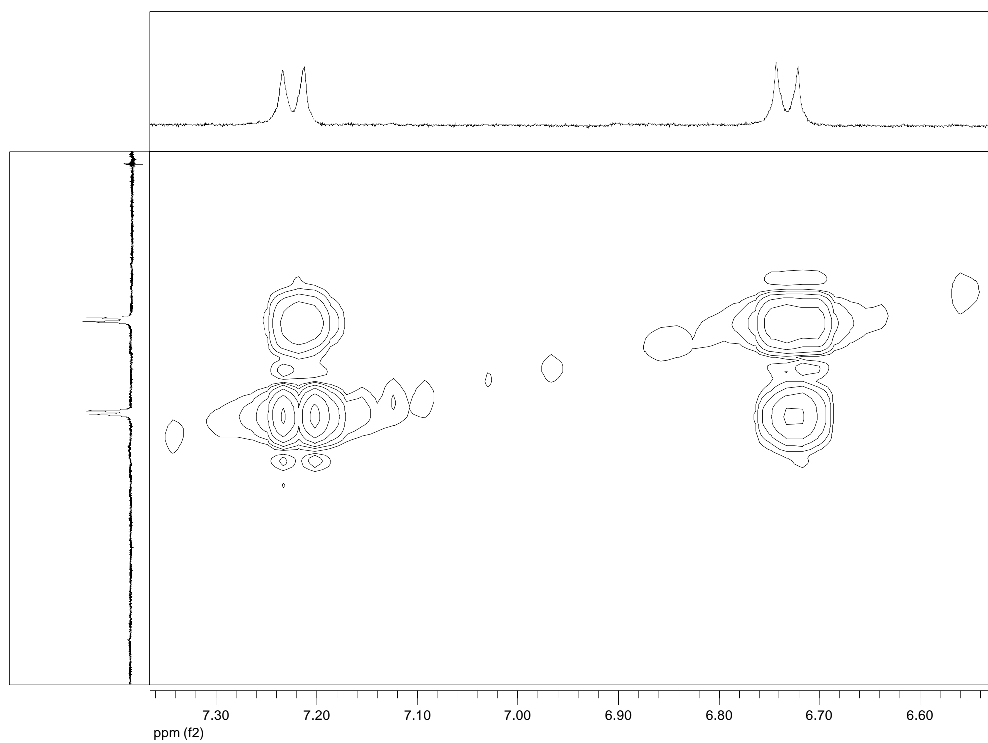


(C)



(D)

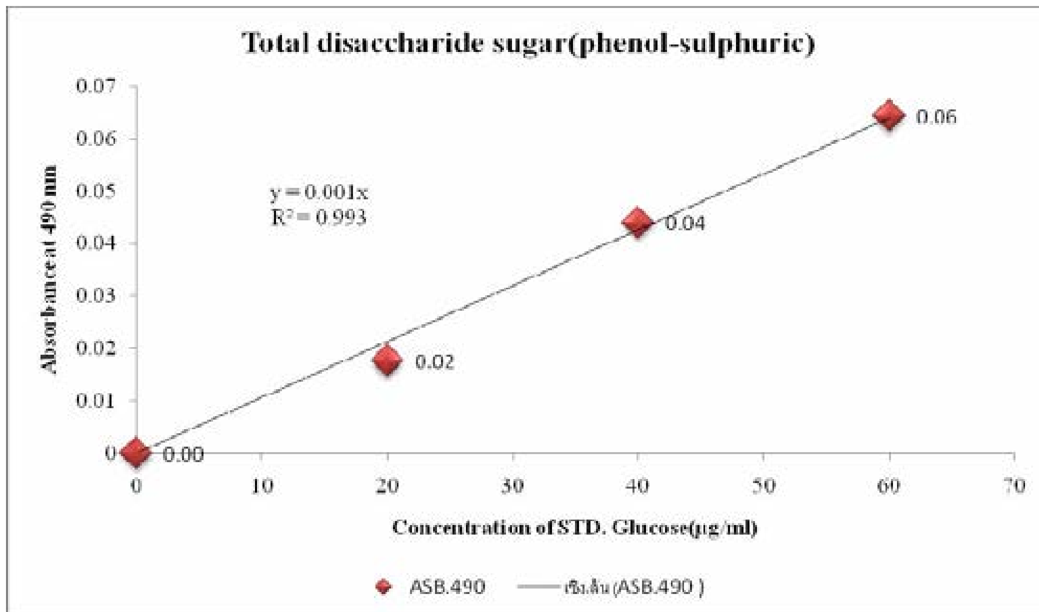
Appendix E: Standard curve for the content of chemical compounds

A: Standard curve for total disaccharide sugar content (phenol-sulphuric assay)

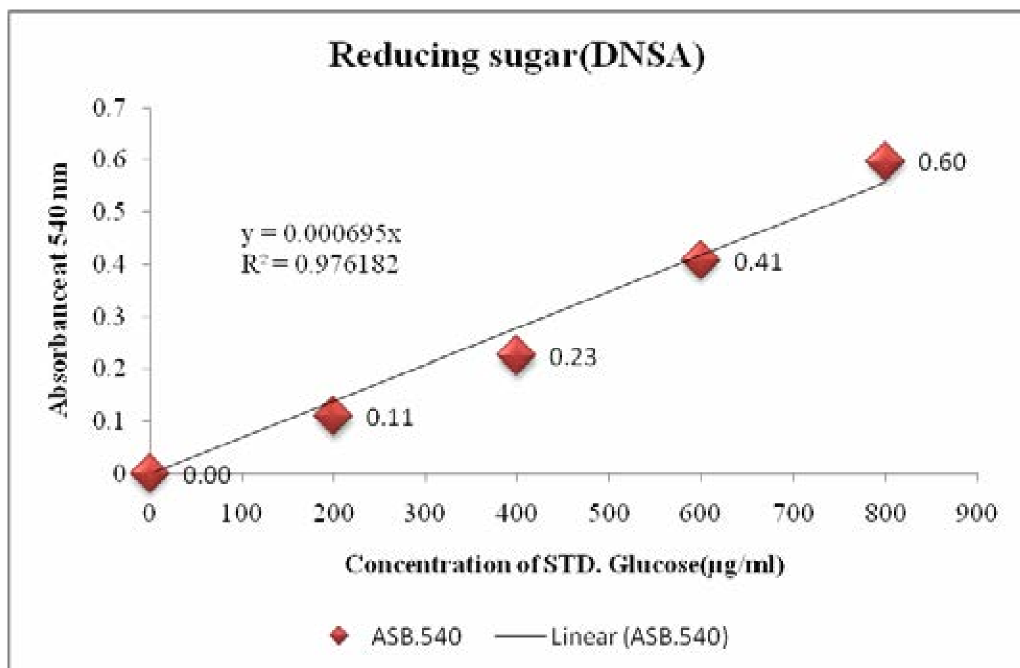
B: Standard curve for total reducing sugar content (DNSA assay)

C: Standard curve for total flavonoid content (aluminum chloride photometry assay)

D: Standard curve for total phenolic compound content (folin-ciocalteu assay)



(A)



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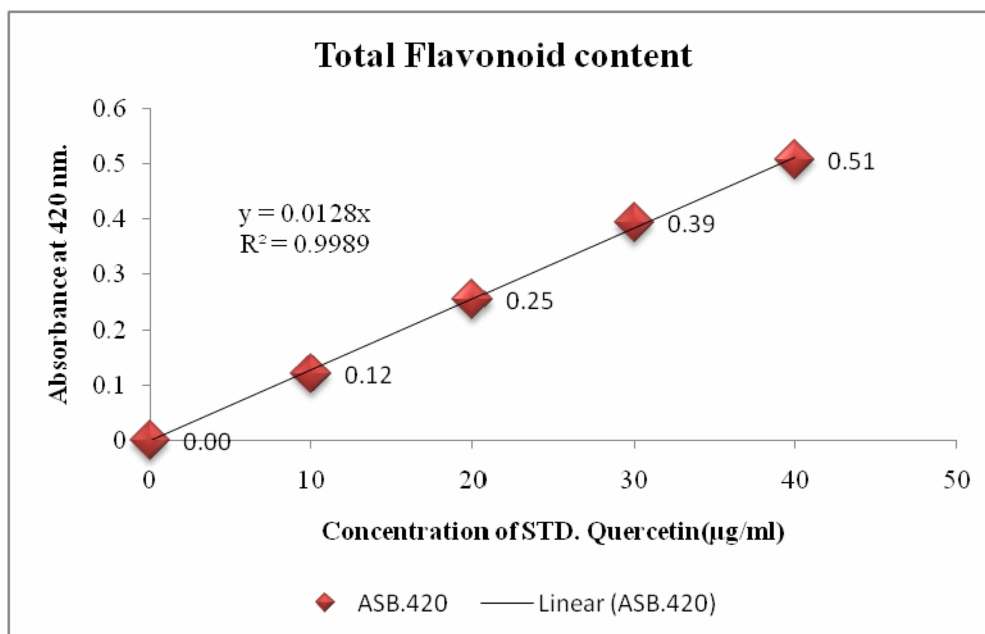

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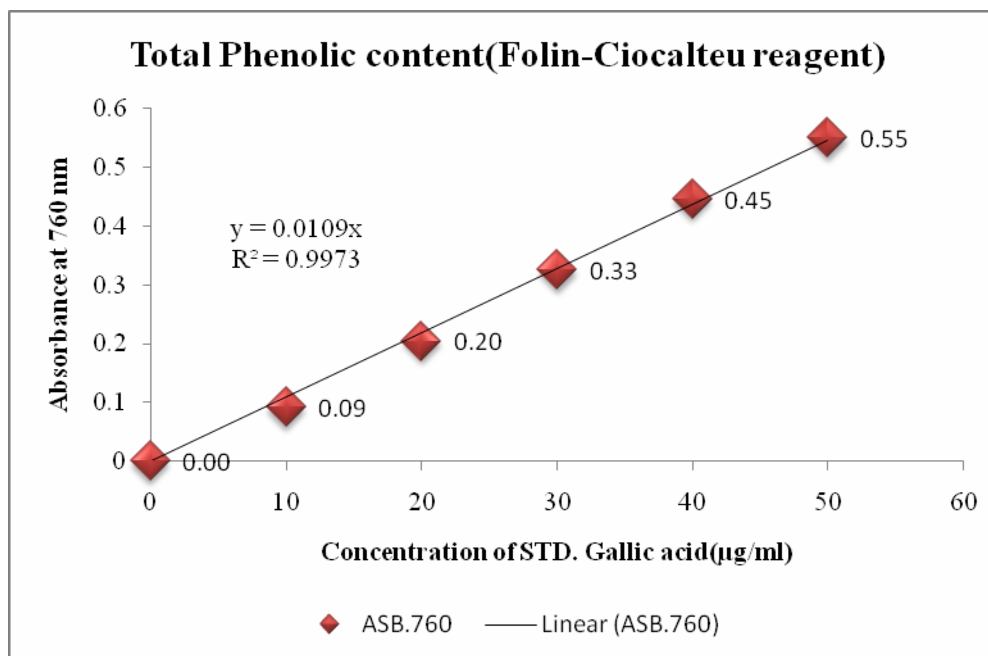

(D)

Appendix F: Calculation of content of chemical compounds

1. Total disaccharide sugar content (phenol-sulphuric assay)

From the equation, y = 0.001x ABS of 2.5 mg/ml of CWE = 0.553 (y)

∴ x = 553 µg/ml

∴ CWE of 2.5 mg/ml, total disaccharide sugar content = 0.553 mg/ml

CWE of 1 mg/ml, total disaccharide sugar content = 0.2212 mg/ml

∴ Calculate to be % of CWE in g = 22.12%.

2. Total reducing sugar content (DNSA assay)

From the equation, y = 0.0000695x ABS of 2.5 mg/ml of CWE = 0.659 (y)

∴ x = 948.20 µg/ml

∴ CWE of 2.5 mg/ml, total reducing sugar content = 0.9482 mg/ml

CWE of 1 mg/ml, total reducing sugar content = 0.379 mg/ml

∴ Calculate to be % of CWE in g = 37.9%.

3. Total flavonoid content (aluminum chloride photometry assay)

From the equation, y = 0.012x ABS of 20 µg/ml of CWE = 0.0093 (y)

∴ x = 0.775 µg/ml

∴ CWE of 20 µg/ml, total flavonoid content = 0.775 µg/ml

CWE of 1 µg/ml, total flavonoid content = 0.03875 µg/ml

∴ Calculate to be % of CWE in g = 3.875%.

4. Total phenolic compound content (Folin-ciocalteu assay)

From the equation, y = 0.010x ABS of 500 µg/ml of CWE = 0.223 (y)

∴ x = 22.3 µg/ml

∴ CWE of 500 µg/ml, total phenolic compound content = 0.553 µg/ml

CWE of 1 µg/ml, total phenolic compound content = 0.0446 µg/ml

∴ Calculate to be % of CWE in g = 4.46%.

Appendix G: Raw data of the percentage of scavenging activity of CEE and CWE

Table A: The percentage of scavenging activity of CEE

Table B: The percentage of scavenging activity of CWE

Table A

CEE	The percentage of scavenging activity							
	0 µg/ml	**25 µg/ml**	**50 µg/ml**	**75 µg/ml**	**100 µg/ml**	**200 µg/ml**	**300 µg/ml**	**400 µg/ml**
Rep. 1	0	60.28	60	62.36	62.64	64.17	63.9	69.03
Rep. 2	0	57.7	57.3	60.19	57.12	59.22	61.31	61.9
Rep. 3	0	55.82	59.14	59.97	60.4	63.17	63.85	63.43
Mean	0	57.93	58.81	60.84	60.05	62.19	63.02	64.79
S.E.	0	1.29	0.80	0.76	1.60	1.51	0.86	2.17

Table B

CWE	The percentage of scavenging activity							
	0 µg/ml	**25 µg/ml**	**50 µg/ml**	**75 µg/ml**	**100 µg/ml**	**200 µg/ml**	**300 µg/ml**	**400 µg/ml**
Rep. 1	0	47.57	51.28	42.86	49.43	52.86	47.71	52.86
Rep. 2	0	51.57	46.43	43.14	52.43	51.14	45.43	47.57
Rep. 3	0	56.37	51.94	54.15	48.2	51.52	45.7	52.5
Mean	0	51.84	49.88	46.72	50.02	51.84	46.28	50.98
S.E.	0	2.54	1.74	3.72	1.26	0.52	0.72	1.71

Appendix H: Raw data of the percentage of scavenging activity of CME, CDE, and CHE originated from CEE and CWE

Table A: The percentage of scavenging activity of CME (CEE)

Table B: The percentage of scavenging activity of CME (CWE)

Table C: The percentage of scavenging activity of CDE (CEE)

Table D: The percentage of scavenging activity of CDE (CWE)

Table E: The percentage of scavenging activity of CHE (CEE)

Table F: The percentage of scavenging activity of CHE (CWE)

Table G: The percentage of scavenging activity of ascorbic acid (10, 50, 100, and 400 μg/ml)

Table A

CME (CEE)	The percentage of scavenging activity				
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**
Rep. 1	0	5.26	-3.16	13.83	49.65
Rep. 2	0	2.48	-2.48	19.13	43.32
Rep. 3	0	4.21	0.93	19.79	48.61
Mean	0	3.98	-1.57	17.59	47.19
S.E.	0	0.81	1.27	1.89	1.96

Table B

CME (CWE)	The percentage of scavenging activity				
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**
Rep. 1	0	-3.16	-7.37	7.66	7.66
Rep. 2	0	1.52	3.54	-2.90	4.98
Rep. 3	0	7.61	4.06	-2.77	1.58
Mean	0	1.99	0.08	0.66	4.74
S.E.	0	3.12	3.73	3.50	1.76

Table C

CDE (CEE)	The percentage of scavenging activity				
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**
Rep. 1	0	64.88	69.23	69.23	70.90
Rep. 2	0	68.56	59.53	68.90	72.91
Rep. 3	0	67.24	62.76	69.54	74.77
Mean	0	66.90	63.84	69.22	72.86
S.E.	0	1.08	2.85	0.19	1.12

Table D

CDE (CWE)	The percentage of scavenging activity				
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**
Rep. 1	0	18.99	19.83	58.12	72.56
Rep. 2	0	17.62	25.82	51.33	74.67
Rep. 3	0	9.96	27.59	38.06	70.93
Mean	0	15.53	24.41	49.17	72.72
S.E.	0	2.81	2.35	5.89	1.08

Table E

CHE (CEE)	The percentage of scavenging activity				
	0 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	400 µg/ml
Rep. 1	0	-10.92	-6.90	7.30	10.48
Rep. 2	0	-3.14	1.57	-1.10	3.30
Rep. 3	0	-0.53	1.06	7.64	5.90
Mean	0	-4.86	-1.42	4.61	6.56
S.E.	0	3.12	2.74	2.86	2.10

Table F

CHE (CWE)	The percentage of scavenging activity				
	0 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	400 µg/ml
Rep. 1	0	-0.53	1.06	-3.60	-1.60
Rep. 2	0	-10.80	-2.27	-2.38	-4.37
Rep. 3	0	-2.13	-0.53	-3.59	-3.98
Mean	0	-4.48	-0.58	-3.19	-3.32
S.E.	0	3.19	0.96	0.40	0.87

Table G

Ascorbic acid	The percentage of scavenging activity				
	0 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	400 µg/ml
Rep. 1	0	55.56	74.40	74.88	74.89
Rep. 2	0	58.23	78.48	78.48	76.09
Rep. 3	0	61.29	77.82	77.02	77.39
Mean	0	58.36	76.90	76.79	76.12
S.E.	0	1.66	1.27	1.05	0.72

Appendix I: Raw data of the percentage of scavenging activity of CDE1-CDE7 after quick column chromatography

Table A: The percentage of scavenging activity of CDE1

Table B: The percentage of scavenging activity of CDE2

Table C: The percentage of scavenging activity of CDE3

Table D: The percentage of scavenging activity of CDE4

Table E: The percentage of scavenging activity of CDE5

Table F: The percentage of scavenging activity of CDE6

Table G: The percentage of scavenging activity of CDE7

Table H: The percentage of scavenging activity of ascorbic acid(10, 50, 100, 400, and 1,000 μg/ml)

Table I: The percentage of scavenging activity of CDE (CEE)

Table A

CDE1	The percentage of scavenging activity					
	0 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	400 µg/ml	1000 µg/ml
Rep. 1	0	12.24	3.80	4.64	-11.81	5.65
Rep. 2	0	0.45	-0.45	0.45	-5.33	13.11
Rep. 3	0	-1.39	-3.24	-4.17	-9.44	6.10
Mean	0	3.76	0.04	0.31	-8.86	8.29
S.E.	0	4.27	2.05	2.54	1.89	2.42

Table B

CDE2	The percentage of scavenging activity					
	0 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	400 µg/ml	1000 µg/ml
Rep. 1	0	2.64	0.00	-11.89	0.00	2.49
Rep. 2	0	5.93	2.54	3.81	-0.43	6.45
Rep. 3	0	0.91	-4.55	-11.82	-0.89	8.82
Mean	0	3.16	-0.67	-6.63	-0.44	5.92
S.E.	0	1.47	2.07	5.22	0.26	1.85

Table C

CDE3	The percentage of scavenging activity					
	0 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	400 µg/ml	1000 µg/ml
Rep. 1	0	5.24	3.06	0.00	-14.22	8.71
Rep. 2	0	-1.86	-2.33	-5.12	8.68	8.33
Rep. 3	0	0.90	-0.45	2.26	3.95	10.13
Mean	0	1.43	0.09	-0.95	-0.53	9.06
S.E.	0	2.07	1.58	2.18	6.98	0.55

Table D

CDE4	The percentage of scavenging activity					
	0 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	400 µg/ml	1000 µg/ml
Rep. 1	0	-2.38	-16.19	-8.57	14.23	12.45
Rep. 2	0	-2.29	-3.67	0.46	4.17	10.96
Rep. 3	0	-3.51	-1.75	0.88	24.66	12.71
Mean	0	-2.73	-7.20	-2.41	14.35	12.04
S.E.	0	0.39	4.53	3.08	5.92	0.54

Table E

CDE5	The percentage of scavenging activity					
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1000 µg/ml**
Rep. 1	0	3.96	8.81	20.26	52.32	63.79
Rep. 2	0	6.85	19.35	26.21	61.13	66.38
Rep. 3	0	4.64	13.92	21.10	65.90	65.67
Mean	0	5.15	14.03	22.52	59.78	65.28
S.E.	0	0.87	3.04	1.86	3.98	0.77

Table F

CDE6	The percentage of scavenging activity					
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1000 µg/ml**
Rep. 1	0	11.07	26.94	39.48	77.86	80.07
Rep. 2	0	0.00	14.77	35.02	74.26	75.53
Rep. 3	0	0.00	16.60	38.30	77.87	79.57
Mean	0	3.69	19.43	37.60	76.66	78.39
S.E.	0	3.69	3.79	1.33	1.20	1.44

Table G

CDE7	The percentage of scavenging activity					
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1000 µg/ml**
Rep. 1	0	1.78	2.22	8.44	33.19	60.45
Rep. 2	0	5.98	6.84	11.11	33.88	60.00
Rep. 3	0	2.26	9.50	9.50	47.89	55.09
Mean	0	3.34	6.19	9.69	38.32	58.51
S.E.	0	1.33	2.13	0.78	4.79	1.71

Table H

Ascorbic acid	The percentage of scavenging activity					
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1000 µg/ml**
Rep. 1	0	55.56	74.40	74.88	74.89	61.30
Rep. 2	0	58.23	78.48	78.48	76.09	56.37
Rep. 3	0	61.29	77.82	77.02	77.39	77.90
Mean	0	58.36	76.90	76.79	76.12	65.19
S.E.	0	1.66	1.27	1.05	0.72	6.51

Table I

CDE (CEE)	The percentage of scavenging activity					
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1000 µg/ml**
Rep. 1	0	64.88	67.86	69.44	69.44	60.71
Rep. 2	0	68.56	69.37	71.96	69.74	63.10
Rep. 3	0	67.24	69.18	70.97	70.97	61.65
Mean	0	66.90	68.80	70.79	70.05	61.82
S.E.	0	1.08	0.48	0.73	0.47	0.69

Appendix J: Raw data of the percentage of scavenging activity of corn's pollen extract

Table A: The percentage of scavenging activity of CEC

Table B: The percentage of scavenging activity of CMC

Table C: The percentage of scavenging activity of CDC

Table D: The percentage of scavenging activity of CHC

Table A

CEC	The percentage of scavenging activity					
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1000 µg/ml**
Rep. 1	0	-3.63	10.88	25.39	63.73	70.98
Rep. 2	0	2.38	15.71	29.05	65.71	73.33
Rep. 3	0	3.83	17.22	30.62	66.99	75.12
Mean	0	0.86	14.61	28.35	65.48	73.15
SE	0	2.28	1.91	1.55	0.95	1.20

Table B

CMC	The percentage of scavenging activity					
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1000 µg/ml**
Rep. 1	0	-27.95	-21.74	1.24	47.20	66.46
Rep. 2	0	-2.97	3.47	16.83	58.42	74.26
Rep. 3	0	1.89	5.19	20.75	59.91	76.42
Mean	0	-9.68	-4.36	12.94	55.18	72.38
S.E.	0	9.24	8.70	5.96	4.01	3.02

Table C

CDC	The percentage of scavenging activity					
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1000 µg/ml**
Rep. 1	0	-7.80	13.66	34.15	70.73	64.88
Rep. 2	0	8.72	22.48	40.83	73.39	72.48
Rep. 3	0	-7.39	11.33	36.95	69.46	71.43
Mean	0	-2.16	15.82	37.31	71.19	69.59
S.E.	0	5.44	3.39	1.94	1.16	2.38

Table D

CHC	The percentage of scavenging activity					
	0 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**400 µg/ml**	**1000 µg/ml**
Rep. 1	0	-10.49	-9.09	-9.32	-6.76	-0.70
Rep. 2	0	-1.11	-2.23	-2.67	-0.89	3.56
Rep. 3	0	1.53	4.60	2.84	1.53	7.66
Mean	0	-3.36	-2.24	-3.05	-2.04	3.51
S.E.	0	3.65	3.95	3.52	2.46	2.41

Appendix K: Raw data of absorbance values of standard and CWE for the content of chemical compounds

Table A: ABS of standard glucose at 490 nm for total disaccharide sugar content(phenol- sulphuric assay)

Table B: ABS of standard glucose at 540 nm for total reducing sugar content (DNSA assay)

Table C: ABS of standard quercetin 420 nm for total flavonoid content (aluminum chloride photometry assay)

Table D: ABS of standard gallic acid 760 nm for total phenolic compound content (Folin-ciocalteu assay)

Table A

STD. Glucose	Absorbance at 490 nm				
	Blank	20 µg/ml	40 µg/ml	60 µg/ml	CWE (2.5mg/ml)
Rep. 1	0	0.018	0.046	0.062	0.541
Rep. 2	0	0.018	0.041	0.064	0.59
Rep. 3	0	0.017	0.045	0.067	0.527
Mean	0	0.018	0.044	0.064	0.553

Table B

STD. Glucose	Absorbance at 540 nm					
	Blank	200 µg/ml	400 µg/ml	600 µg/ml	800 µg/ml	CWE (2.5mg/ml)
Rep. 1	0	0.11	0.221	0.408	0.598	0.653
Rep. 2	0	0.11	0.225	0.411	0.597	0.658
Rep. 3	0	0.11	0.23	0.403	0.596	0.666
Mean	0	0.11	0.225	0.407	0.597	0.659

Table C

STD. Quercetin	Absorbance at 420 nm					
	Blank	10 µg/ml	20 µg/ml	30 µg/ml	40 µg/ml	CWE (20 µg/ml)
Rep. 1	0	0.129	0.264	0.405	0.536	0.013
Rep. 2	0	0.117	0.253	0.394	0.501	0.007
Rep. 3	0	0.114	0.244	0.38	0.484	0.008
Mean	0	0.12	0.254	0.393	0.507	0.009

Table D

STD. Gallic acid	Absorbance at 760 nm						
	Blank	10 µg/ml	20 µg/ml	30 µg/ml	40 µg/ml	50 µg/ml	CWE (500µg/ml)
Rep. 1	0	0.095	0.208	0.344	0.45	0.556	0.23
Rep. 2	0	0.092	0.21	0.323	0.451	0.555	0.217
Rep. 3	0	0.087	0.195	0.308	0.435	0.535	0.223
Mean	0	0.091	0.204	0.325	0.445	0.549	0.223

BIOGRAPHY

Mr. Atip Chantarudee was born on October 24, 1984 in Bangkok, Thailand. He graduated with Bachelor degree of Science from Department of Marine Science, Faculty of Science, Chulalongkorn University in 2008. Then, he continued his graduate study for the Master degree of Science in Biotechnology program, Faculty of Science, Chulalongkorn University in 2009.

Academic presentations:

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