TYROSINASE INHIBITION AND FREE RADICAL SCAVENGING ACTIVITIES OF BEE POLLEN FROM WESTERN HONEYBEE *Apis mellifera*

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ฤทธิ์ยับยั้งไทโรซิเนสและขจัดอนุมูลอิสระของเกสรผึ้งจากผึ้งพันธุ์ *Apis mellifera*

น.ส.พันทิวา คงคารัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	TYROSINASE INHIBITION AND FREE RADICAL SCAVENGING ACTIVITIES OF BEE POLLEN FRO M WESTERN HONEYBEE *Apis mellifera*
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พันทิวา คงคารัตน์ : ฤทธิ์ยับยั้งไทโรซิเนสและขจัดอนุมูลอิสระของเกสรผึ้งจากผึ้งพันธุ์ *Apis mellifera*. (TYROSINASE INHIBITION AND FREE RADICAL SCAVENGING ACTIVITIES OF BEE POLLEN FROM WESTERN H ONEYBEE *Apis mellifera*) อ.ที่ปรึกษาหลัก : ศ. ดร.จันทร์เพ็ญ จันทร์เจ้า, อ.ที่ปรึกษาร่วม : รศ. ดร.ปรีชา ภูวไพรศิริศาล

เกสรผึ้งเป็นผลิตภัณฑ์ชนิดหนึ่งของผึ้ง เป็นของผสมระหว่างเรณูของพืช น้ำหวาน และสารคัดหลั่งจากตัวผึ้ง สร้างโดยผึ้งงาน ในสกุลเอพิส เกสรผึ้งมีองค์ประกอบหลัก ได้แก่ โปรตีน กรดอะมิโนจำเป็น น้ำตาล ไขมัน กรดนิวคลีอิก และ ใยอาหาร ส่วน องค์ประกอบย่อย ได้แก่ แร่ธาตุและวิตามิน กรดไขมันอิ่มตัวและไม่อิ่มตัว พอลิฟีนอล ฟลาวานอยด์ โดยสารประกอบเหล่านี้ส่งผลให้เกสร ผึ้งมีฤทธิ์ทางชีวภาพที่หลากหลาย เช่น ฤทธิ์ต้านการอักเสบ ฤทธิขจัดอนุมูลอิสระ ฤทธิ์ต้านแบคทีเรียและเชื้อรา เป็นต้น งานวิจัยนี้ ศึกษา เกสรผึ้งของผึ้งพันธุ์ *A. mellifera* โดยเก็บตัวอย่างเกสรผึ้งจากดอกชา (*Camellia sinensis* (L.) Kuntze) และดอก ไมยราบยักษ์ (*Mimosa pigra* L.) จากจังหวัดเชียงใหม่ และ ดอกทานตะวัน (*Helianthus annuus* L.) จากจังหวัดลพบุรี ประเทศไทย ทำการสกัดเกสรผึ้งแต่ละชนิดด้วยเมทานอล จากนั้นทำการสกัดแยกส่วนโดยใช้ เฮกเซน ไดคลอโรมีเทนและเมทานอล นำแต่ ละส่วนที่แยกได้มาทำการทดสอบเพื่อตรวจวัดฤทธิ์ขจัดอนุมูลอิสระและฤทธิ์ยับยั้งการทำงานของเอนไซม์ไทโรซิเนส ซึ่งการทดสอบเพื่อ ตรวจวัดฤทธิ์ขจัดอนุมูลอิสระด้วยวิธีดีพีพีเอช พบว่าสารสกัดชั้นไดคลอโรมีเทนของเกสรผึ้งจากไมยราบมีฤทธิ์ในการกำจัดอนุมูลอิสระดี ที่สุด ซึ่งมีค่าอีซี 50 เท่ากับ 192.07 ไมโครกรัมต่อมิลลิลิตร จึงนำไปทำให้บริสุทธิ์ขึ้นโดยใช้ซิลิกาเจลคอลัมน์โครมาโทกราฟีและ ไซส์เอ็กซ์คลูชันโครมาโทกราฟี นำทุกแฟรกชันที่ได้มาทดสอบฤทธิ์ขจัดอนุมูลอิสระอีกครั้ง และวิเคราะห์โครงสร้างทางเคมีของสารใน ลำดับส่วนที่ออกฤทธิ์โดยใช้นิวเคลียร์แมกเนติกเรโซแนนซ์ (เอ็นเอ็มอาร์) ได้สารผสมซึ่งมีฤทธิ์ขจัดอนุมูลอิสระดีที่สุด ซึ่งมีค่าอีซี 50 เท่ากับ 121.29 ไมโครกรัมต่อไมโครลิตร และสารบริสุทธิ์ 1 ตัว คือ นารินเจนิน ซึ่งมีปริมาณน้อย เป็นสารในกลุ่มฟลาโวนอยด์ และมี ฤทธิ์ในการขจัดอนุมูลอิสระต่ำ ส่วนฤทธิ์ยับยั้งการทำงานของเอนไซม์ไทโรซิเนส ทดสอบโดยอาศัยปฏิกริยาทางชีวเคมีของสารสกัดที่มีผล ต่อเอนไซม์ไทโรซิเนส พบว่าสารสกัดชั้นไดคลอโรมีเทนของเกสรผึ้งจากทานตะวันมีฤทธิ์ยับยั้งการทำงานของเอนไซม์ไทโรซิเนสดีที่สุด ซึ่งมีค่าไอซี 50 เท่ากับ 159.39 ไมโครกรัมต่อมิลลิลิตร จึงนำไปทำให้บริสุทธิ์ขึ้นโดยใช้ซิลิกาเจลคอลัมน์โครมาโทกราฟีและเอชพี แอลซี นำทุกแฟรกชันที่ได้มาทดสอบฤทธิ์ยับยั้งการทำงานของเอนไซม์ไทโรซิเนสอีกครั้ง และวิเคราะห์โครงสร้างทางเคมีของสารในแฟรก ชันที่ออกฤทธิ์ โดยใช้เอ็นเอ็มอาร์ ได้สารประกอบในกลุ่มสเปอร์มิดีน ซึ่งมีฤทธิ์ยับยั้งการทำงานของเอนไซม์ไทโรซิเนสดีที่สุด ซึ่งมีค่าไอซี 50 เท่ากับ 6.65 ไมโครกรัมต่อมิลลิลิตร จึงสามารถสรุปได้ว่าเกสรผึ้งจากดอกไมยราบ มีฤทธิ์ในการขจัดอนุมูลอิสระได้ดี และเกสรผึ้ง จากดอกทานตะวันมีฤทธิ์ยับยั้งการทำงานของเอนไซม์ไทโรซิเนสได้ดี ตามลำดับ

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Bee pollen is one of bee products. It is the mixture of flower pollen, nectar, and bee secretion. It is produced by workers in the genus of *Apis*. The major chemical compositions of bee pollen are protein, essential amino acids, sugar, fat, nucleic acid, and fiber. Minor chemical compositions are minerals, vitamins, saturated/unsaturated fatty acids, polyphenol, and flavonoid. These compounds make bee pollen exhibit a wide range of bioactivities including anti-inflammatory, antioxidant, antibacterial, and antifungal activities. In this work, bee pollen of *A. mellifera* was studied. Tea flower (*Camellia sinensis* (L.) Kuntze) and mimosa flower (*Mimosa pigra* L.) were collected from Chiang Mai province and sunflower (*Helianthus annuus* L.) bee pollen was collected from Lopburi province, Thailand. All samples were extracted by methanol. Next, they were partitioned by hexane, dichloromethane, and methanol in order to isolate compounds depending on their polarities. The obtained partitioned extracts were tested for the free radical scavenging and antityrosinase activities. For the free radical scavenging activity by DPPH assay, dichloromethane partitioned extract of mimosa flower bee pollen (DCMMBP) provided the highest free radical scavenging activity at EC_{50} value of 192.07 μg/mL. Then, it was further purified by silica gel 60 column chromatography and size exclusion chromatography. All fractions were tested for the free radical scavenging activity and analysed for a chemical structure by nuclear magnetic resonance (NMR). The most active mixture had the EC_{50} value of 121.29 μg/mL. Additionally, a pure compound was found to be naringenin, belonging to flavonoid group. Naringenin was obtained in small amount and had low free radical scavenging activity. For the tyrosinase inhibitory activity, it was tested by a biochemical reaction of an extract on mushroom tyrosinase. The result showed that dichloromethane partitioned extract of sunflower bee pollen provided the highest mushroom tyrosinase inhibitory activity at IC_{50} value of 159.39 μg/mL. Thus, it was further purified by silica gel column chromatography and HPLC. All fractions were tested for antityrosinase activity and analysed a chemical structure of active fractions by NMR. The active mixtures were predicted to be spermidine derivatives which provided the highest tyrosinase inhibitory activity at IC_{50} value of 6.65 μg/mL. It could be concluded that the free radical scavenging and antityrosinase agents were rich in mimosa flower and sunflower bee pollen, respectively.

Field of Study:	Biotechnology	Student's Signature .....
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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
^{13}C NMR	carbon nuclear magnetic resonance
cm	centimeter
DCMMBP	dichloromethane partitioned extract of mimosa flower bee pollen
DCMMBP1-3	fraction number 1-3 originated from DCMMBP partitioned extract
DCMMBP3-1 to 3-2	fraction number 1-2 originated from fraction number DCMMBP3
DCMSBP	dichloromethane partitioned extract of sunflower bee pollen
DCMSBP1 – 5	fraction number 1-5 originated from DCMSBP partitioned extract
DCMTBP	dichloromethane partitioned extract of tea flower bee pollen
°C	degree Celsius
CD_3OD	deuterated methanol
CH_2Cl_2	dichloromethane or methylene chloride
d	doublet for NMR spectra
dd	doublet of doublet for NMR spectra
ddt	doublet of doublet of triplet for NMR spectra
DPPH	1,1-diphenyl-2-picrylhydrazyl or 2,2-diphenyl-1- picryl hydrazyl
dt	doublet of triplet for NMR spectra
EC_{50}	efficient concentration at 50%
EtOAc	ethyl acetate
γ	gamma
g	gram
H^+	proton
^1H NMR	proton nuclear magnetic resonance
HPLC	high performance liquid chromatography
HXMBP	hexane partitioned extract of mimosa flower bee pollen
HXSBP	hexane partitioned extract of sunflower bee pollen
HXTBP	hexane partitioned extract of tea flower bee pollen
Hz	hertz

h	hour
L-DOPA	L-3,4-dihydroxyphenylalanine
MeOH	methanol
µg	microgram
µL	microliter
µm	micrometer
m	multiplet for NMR spectra
m/z	m to z ratio of mass spectra
mg	milligram
mL	milliliter
mM	millimolar
min	minute
M.W.	molecular weight
MBP	mimosa flower bee pollen
MHz	megahertz
MTMBP	methanol partitioned extract of mimosa flower bee pollen
MTSBP	methanol partitioned extract of sunflower bee pollen
MTTBP	methanol partitioned extract of tea flower bee pollen
ND	no data
NMR	nuclear magnetic resonance
nm	nanometer
ppm	part per million
/	per
%	percentage
q	quartet
:	ratio
ROS	reactive oxygen species
RT	room temperature (25 °C)
rpm	revolution per minute
s	singlet for NMR spectra
sp.	species
SBP	sunflower bee pollen
TBP	tea flower bee pollen
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultra violet
v/v	volume by volume
w/w	weight by weight
w/v	weight by volume
IC_{50}	inhibitory concentration at 50%

CHAPTER I
INTRODUCTION

Bee pollen is one of bee products which include honey, propolis, bee pollen, royal jelly, bee venom, and wax, it is produced by honeybees. When workers collect nectar from flowers of plants 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 workers return to their hives, the collected bee pollen will be stored in a hive and used as food. Bee pollen contains various nutrients such as protein, lipid, carbohydrate, sugar, minerals, and vitamins which are useful for human health. However, the composition of bee pollen is variable depending on biogeographic (regional) origin, ecological habitat, or even the season.

Furthermore, bee pollen also contains secondary metabolites which are phenolic acid, flavonoids, catechins, kaempferol, quercetin, isorhamnetin, and carotenoid pigments such as lycopene and zeaxanthin. Because of a variable composition with various secondary metabolites, bee pollen can be consumed as supplementary food. A variety of primary and secondary metabolites contained in bee pollen exhibit a wide range of properties and bioactivities such as antioxidant, anti-inflammatory, anticarcinogenic, antibacterial, antifungicidal, hepatoprotective, and anti-atherosclerotic activities.

In this study, it is focused on the free radical scavenging activity of bee pollen. That is because, in everyday life, free radicals are derived either from metabolic processes in our body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals. These are involved in several diseases such as diabetes mellitus, Alzheimer’s disease, atherosclerosis, asthma, cataract cancers, and so on. A free radical can be defined as any molecular species contain an unpaired electron in an atomic orbital. The presence of an unpaired electron results free radicals to be unstable and highly reactive. These are highly reactive oxygen species including hydroxyl radical, superoxide anion radical, hydrogen peroxide, and oxygen singlet. In the nucleus and in the membrane of cells, highly reactive oxygen species are capable of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids. Free radicals attack important macromolecules leading to cell damage and homeostatic disruption.

In this work, it is additionally focused on the antityrosinase activity of bee pollen. As known, abnormal production or distribution of melanin is the cause of various dermatological disorders such as melasma, lentigines, age spots, and post-inflammatory hyperpigmentation. Due to the key role of tyrosinase in melanin pathway, searching for tyrosinase inhibitors are important for medicinal and cosmetic products that may be used as powerful skin whitening agents.

Interestingly, bioactivities of bee pollen depend on floral and honeybee species. Thus, in this work, it is focused on the free radical scavenging and antityrosinase activities of tea flower (*Camellia sinensis* (L.) Kuntze), mimosa flower (*Mimosa pigra* L.) bee pollen from Chaingmai province and sunflower (*Helianthus annuus* L.) bee pollen from Lopburi province. All samples are collected by *A. mellifera*. They are extracted by organic solvents and purified to get a pure active compound. At last, a chemical structure of the active compound is analysed by using spectroscopic techniques. Hopefully, a new free radical scavenging and antityrosinase compound will be obtained from the bee pollens and can be applied to be additive in cosmetic products.

CHAPTER II
LITERATURE REVIEWS

2.1 Biology of European honeybee (*Apis mellifera* Linnaeus)

Honeybees in the genus *Apis* are the most studied because of their fascinating and complex lifestyle with communication systems (Nieh, 1998). Four species are native to Thailand: *Apis andreniformis*, *A. cerana*, *A. dorsata*, and *A. florea* (Oldroyd & Wongsiri, 2009) while western honey bee, *A. mellifera*, was introduced for beekeeping. *A. mellifera* is native naturally to Europe, the Middle East, and Africa (Figure 2.1). However, *A. mellifera* has been spread extensively beyond its natural zone due to economic benefits. *A. mellifera* plays an important role as pollinators of native plants and agricultural crops. It produces the valuable hive products such as honey, royal jelly, bee wax, bee pollen, propolis, and even bee venom (Figure 2.2). As known, bee products are useful in food, cosmetics, and medical purposes. *A. mellifera* was brought to Thailand in the early 1940s for beekeeping. It is mainly cultured in bee farms because it can be managed in a hive box. Also, it has harmless behavior. Therefore, it is convenient to harvest bee products (Wongsiri, 1989). Beekeeping with *A. mellifera* in Thailand is quite successful. This species is used for honey production and is an integral part of Thai agriculture. It is used for pollination of longan, litchi, durian, rambutan, and other crops (Suwannapong et al., 2012).

Figure 2.1 European honey bees, *Apis mellifera* Linnaeus.

(A)

(B)

(C)

(D)

Figure 2.2 Bee products are economically important such as bee pollen (A), royal jelly (B), propolis (C), and beeswax (D). Figures are from https://cs.m.wikipedia .org/wiki/Soubor:Bienenwabe_mit_Eiern_und_Brut_5.jpg, https://www.indiamart.co m/proddetail/bee-propolis-19014952633.html, and http://www.huffshoneyfarm.com /product/pure-beeswax.

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 sections: head, thorax, and abdomen. (Figure 2.3, B). A head contains mouthparts, two compound eyes with three ocelli, and two antennae. A thorax contains appendages for locomotion, three pairs of legs and two pairs of wings. An abdomen contains organs for digestion, reproduction, and defense.

A head of honeybee is triangular when it is viewed from the front. Two antennae arise close together near the upper center of the head. There are two compound eyes and three simple eyes located on top of the head. The honeybee uses its proboscis, or long tongue, to feed liquids and its mandibles to manipulate pollen and work wax in comb building (Suwannapong et al., 2012).

A thorax is the middle part of the bee and contains thoracic glands, which are derived from a cocoon-spinning gland of the larva. Primarily, a thorax is the anchor point for a bee's locomotory appendages used for walking and flying. Three pairs of legs arise from the thorax: prothoracic (the closest to the head), mesothoracic (middle), and metathoracic (hind) legs. Honeybee metathoracic legs are modified to be pollen baskets. There are also two pairs of wings located on a thorax of adult bees (Suwannapong et al., 2012).

A honeybee abdomen is composed of nine segments and contains digestive organs, wax and some pheromone glands, reproductive organs, and a sting which is a modified ovipositor. As known, an ovipositor can be developed to sting and deposit eggs in adult females. The body color is a distinctive character among honeybee species and subspecies. The abdomen color of *A. mellifera*, which is similar in workers, queen, and drones, is yellow and black (Suwannapong et al., 2012).

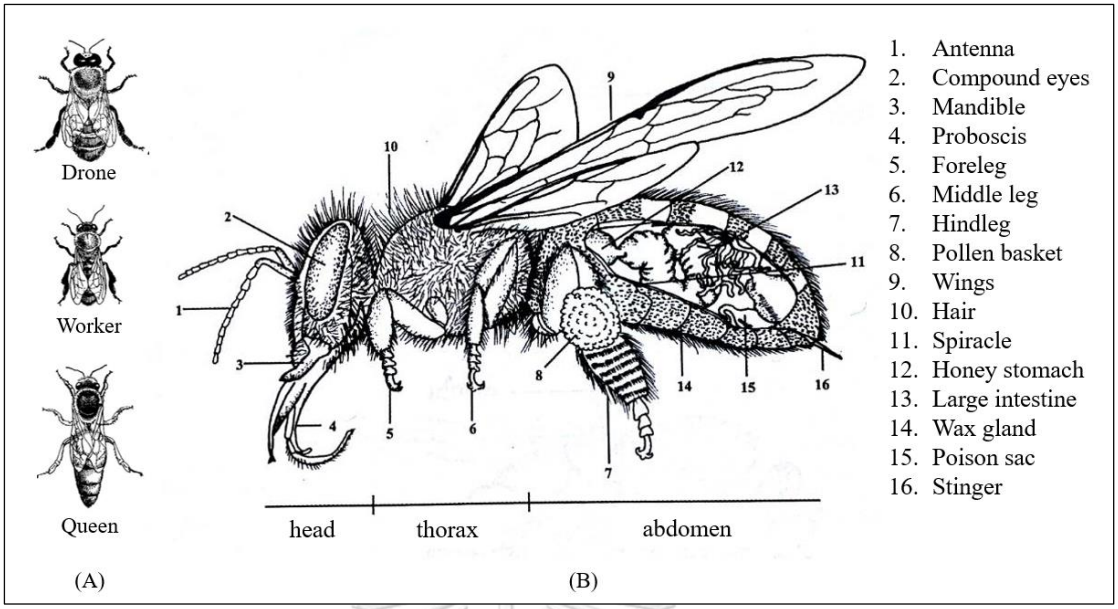


Figure 2.3 Social castes and characters of A. mellifera. Within a colony, there are 3 social castes which are drones, workers, and a queen (A). A bee body consists of 3 parts which are head, thorax, and abdomen (B) (Wongsiri & Deowanish, 2012).

2.1.2 Social caste and life cycle

There are three main castes of honeybees (a single queen, a few hundred drones, and several thousand workers) in a colony (Figure 2.3, A). A queen is a fertile and functional female that can produce males and females. A worker is an unfertilized female capable of only producing males (due to the haplodiploid sex determination system) and a drone is male (Tribe & Fletcher, 1977). Honeybees of all castes undergo complete metamorphosis (egg, larva, pupa, and adult). For all three castes, it spends three days for an egg stage (M. L. Winston, 1979). A larval stage lasts for different range of time, depending on caste, genetics, and the environment. The mean duration of the uncapped larval period is about 4.5 days for a queen, 5.5 days for workers, and 6.5 days for drones. Total developmental time takes 16, 21, and 24 days in average for a queen, workers, and drones, respectively (Tribe & Fletcher, 1977).

A queen is the only reproductive female in a colony in normal circumstances. There is generally one queen in one colony. Its main role is to lay eggs. Through the production of queen pheromone, it influences the physiology and behavior of workers. A queen lays an egg in a queen cup. Once it is hatched (3 -3½ days), a new larva is fed by a rich diet of royal jelly by nurse bees. A queen is diploid (2n = 32). Its

body is larger than a worker's body. That is due to the food it uptakes. 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 be fed by bee pollen and honey. A queen also has a sting but its barbs are reduced. Consequently, it does not die when it stings. An average life span of a queen is around 1-2 years.

A worker is a non-reproductive female and is diploid (2n = 32). Its body is specialized for pollen and nectar collection. It carries out almost duties in a colony like brood rearing, comb building, house cleaning, foraging, and defensing. A worker generally has a smaller body size than either a drone or a queen. There are about 40,000-50,000 workers in *A. mellifera* colony. A worker has a barbed sting, with a poison sac, at the end of its abdomen. Due to a barbed sting, a worker will die after stinging (Otis, 1990; M. Winston, 1992).

A drone is male and performs the mating behavior with a foreign queen only. That leads to the inheritance of its genes to honeybees in different colonies. The body size of a drone is larger than that of a worker, but still smaller than that of a queen. It arises from an unfertilized egg laid by a queen or a worker. Thus, a drone is haploid (n = 16) (Anderson, 1963; Mackensen, 1943; M. Winston, 1992).

2.2 Biological and chemical composition of bee pollen

Bee pollen is mixture of flower pollen from different species mixed with a small amount of the secretion from salivary glands or nectar. Then, it is stored in specific baskets (corbiculae) (Figure 2.4, A) which are situated on the tibia of honeybee's rear legs (Figure 2.4, B), and pollen-loads are formed (Figure 2.4, C). Later, a worker will transport the pollen-loads to its hive. In order to collect bee pollen, a pollen trap box will be put in front of an entrance of a hive. After foragers (workers) enter a hive, the pellets of bee pollen can be scraped from the legs and collected in a suitable tray (Jannesar et al., 2017).

The composition of bee pollen is variable depending on biogeographic (regional) origin, ecological habitat, or even the season. Although the chemical compounds of bee pollen are diverse, the major components generally include proteins (5–60%), essential amino acids, reducing sugars (13 – 55%), lipids (4 – 7%), nucleic acids (especially RNA), and crude fiber (0.3 – 20%). The minor components are minerals such as Ca, Mg, Fe, Zn, Cu, vitamins: provitamin A (β-carotene),

vitamin E (tocopherol), niacin, thiamine, biotin, folic acid, enzymes or co-enzymes, and bioactive substances (Denisow & Denisow-Pietrzyk, 2016).

(A) (B) (C)

Figure 2.4 Pollen basket and bee pollen of *A. mellifera*. Pollen basket on the tibia of honeybee's rear legs is shown in (A). With higher magnifocation, a lot of hair at a pollen basket is presented in order to provide the better attachment of bee pollen to a pollen basket (B). Pollen-loads are formed in the form of granules (C). Figures are from http://beekeeping.wikia.com/wiki/Apis_mellifera,https://www.gettyimages.ca/detail/photo/leg-of-honey-bee-high-res-stock-photography/523779790, and https://www.visionarycongress.org/shamanic-bee-keeping-shonagh-home/.

Bee pollen compositions have been reported widely for decades. It consisted of 10-40 g/ 100 g dry weight proteins, 1-13 g/100 g dry weight lipids, 13-55 g/100 g dry weight carbohydrates, 3-20 g/100 g dry weight fiber, and 2-6 g/100 g dry weight ash (Campos et al., 2008)

Considering minerals in bee pollen (100 g) from Brazil, it consisted of 400 – 2,000 mg potassium (K), 80 – 600 mg phosphorous (P), 20 – 300 mg calcium (Ca), 20 – 300 mg magnesium (Mg), 3 – 25 mg zink (Zn), 2 – 11 mg manganese (Mn), 1.1 – 17 mg iron (Fe), 0.2 – 1.6 mg copper (Cu), and 0.05-0.005 mg selenium (Se) (Morgano et al., 2012).

Bee pollen (100 g) also consisted of vitamins which were 7 – 56 mg ascorbic acid (C), 1 – 20 mg β-carotene (provitamin A), 4 – 32 mg tocopherol (vitamin E), 4 – 14.4 mg niacin (B3), 0.2 – 0.7 mg pyridoxine (B6), 0.6 – 1.3 mg thiamine (B1), 0.6 – 2.6 mg riboflavin (B2), 0.5 – 2 mg pantothenic acid, 0.3 – 1 mg folic acid, and 0.05 – 0.07 mg biotin (H) (de Arruda et al., 2013).

Furthermore, bee pollen also consisted of many bioactive substances. These included unsaturated/ saturated fatty acids (1–10%; linoleic, γ-linoleic, and archaic),

phospholipids (1.5%), phytosterols i.e. β-sitosterol, P-sitosterol (1.1%), and terpenes. Moreover, polyphenols, mainly flavonoids (3–8% dry weight) were relevant. In the group of flavonoids, catechins, kaempferol, quercetin, and isorhamnetin were the most frequently found. Bee pollen was also rich in organic carotenoid pigments (i.e. lycopene, zeaxanthin and β-carotene). Such a variable composition with various secondary metabolites made bee pollen a very valuable supplementation of the diet. A variety of primary and secondary metabolites in bee pollen exhibited a wide range of properties and bioactivities, i.e. antioxidant, anti-tyrosinase, anti-inflammatory, anticarcinogenic, antihypertensive, antibacterial, and antifungal activities. Given the nutritional properties of bee pollen compounds, it was recommended as a valuable dietary supplement (Denisow & Denisow-Pietrzyk, 2016).

2.3 Bioactivities of bee pollen

2.3.1 Anti-inflammatory activity

The anti-inflammatory activity of *Cistus* sp. bee pollen was studied by 95% ethanol extraction. After that, ethanol crude extract was tested for anti-inflammatory effect. The results showed ethanol crude extract inhibited the nitrogen oxide (NO) production and COX-2 activity. To identify an active compound, ethanol crude extract was further purified by LH-20 column chromatography and ODS or silica gel column chromatography, respectively. The active compounds were found to be flavonoids and their glycosides including isorhamnetin, kaempferol, quercetin, kaempferol-3-glucoside, and quercetin-7-rhamnoside (Maruyama et al., 2010).

Furthermore, there is the report of (7: 3) methanol: water extract of *Echium plantagineum* L. bee pollen on anti-inflammatory activity which was evaluated in lipopolysaccharide - stimulated macrophages. The levels of nitric oxide (NO) and L-citrulline was decreased for all extract concentrations tested, while the levels of prostaglandins, their metabolites, and isoprostanes evaluated by ultra performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS), was decreased by low extract concentrations of the mentioned compounds. Hence, *E. plantagineum* bee pollen extract could exert anti-inflammatory activity by reducing NO and prostaglandins (Moita et al., 2013).

2.3.2 Anticarcinogenic activity

The effect of a steroid fraction of *Brassica campestris* L. bee pollen on human cancer cells including PC-3, LNCaP, MCF-7, HeLa, BEL-7402, BCG-823, KB, A549, and HO8910 was studied. The result showed the steroid fraction provided strongest cytotoxicity on human prostate cancer PC-3 cells. The mode of cell death was appeared to be apoptosis in PC-3 cells. Caspase-3 activity was obviously enhanced after the cells were treated with the fraction. A time-dependent decrease in the expression of anti-apoptotic protein, Bcl-2, was also observed by western analysis. It was suggested that the steroid fraction could induce cytotoxicity in PC-3 cells by triggering apoptosis. The studies indicated that the steroid fraction of chloroform extract from bee pollen of *Brassica campestris* L. might be a promising candidate for the treatment of advanced prostate cancer (Wu & Lou, 2007).

2.3.3 Antihypertensive activity

The effect of six protease hydrolysates from bee pollen of *Cistus ladaniferus* on angiotensin I-converting enzyme (ACE) was studied. The large amounts of proteins were contained in these hydrolysates. The activities of these hydrolysates were extremely high, similar to various fermented foods such as fish sauce, mirin, sake, soy sauce, vinegar, cheese, miso, natto, and so on. These results suggested expected higher ACE inhibitory activities in hydrolysates from honeybee-collected pollen of *C. ladaniferus* in the body system since bee pollen would be digested to be useful components by gastrointestinal enzymes such as pepsin and trypsin and absorbed into a body easily. In this work, it indicated that honeybee-collected pollen derived peptides could be applied to various fields of functional foods and medicines (Nagai et al., 2007).

2.3.4 Antibacterial and antifungal activities

The antimicrobial activity of bee pollen was studied and found that the extracts of bee pollen was active against different Gram-positive and Gram negative pathogenic bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Escherichia coli*), microscopic fungi (*Aspergillus fumigatus, A. flavus,* and *A. niger),* and yeasts (*Candida krusei*, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Geotrichum candidum*, and *Rhodotorula mucilaginosa).* This activity was due to different phenolic compounds in bee pollen (Knazovicka, 2009).

Furthermore, the antibacterial activity of Turkish bee pollen against 13 different bacterial pathogens of plants (*Agrobacterium tumefaciens, A. vitis, Clavibacter michiganensis* subsp. *michiganensis, Erwinia amylovora, E. carotovora* pv. *carotovora, Pseudomonas corrugata, P. savastanoi* pv. *savastanoi, P. syringae* pv. *phaseolicola, P. syringae* pv. *syringae, P. syringae* pv. tomato, *Ralstonia solanacearum, Xanthomonas campestris* pv. *campestris*, and *X. axonopodis* pv. *vesicatoria*) was studied. The results showed that the Turkish bee pollen extract had the inhibitory effect against all pathogens (Basim et al., 2006).

2.4 Free radical, antioxidant, and antioxidative activity

2.4.1 Free radical

A free radical can be defined as any molecular species containing an unpaired electron in an atomic orbital. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecule, therefore, they behave as oxidants or reductants. Substances those have the ability to oxidize biological molecules in the body are called reactive species (RS) and most of them are in the form of reactive oxygen species (ROS) such as oxygen radical, singlet oxygen, superoxide radical, hydrogen peroxide, peroxyl radical, and hydroxyl radical. Free radicals and other ROS are derived either from normal essential metabolic processes in a human body or from external sources such as exposure to UV ray, X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals. If the accumulation of ROS is increased, it can cause oxidative stress

Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with a damage to a wide range of molecular species including lipids, proteins, and nucleic acids. Short-term oxidative stress does not affect health. On the other hand, if oxidative stress has persisted for a long time, there is a risk of damage in tissue, cell membrane, DNA, proteins, carbohydrates, and lipids. That can lead to many diseases such as cancer, atherosclerosis, respiratory diseases syndrome, heart diseases, Alzheimer's disease, Parkinson's disease, and muscular dystrophy (Lobo et al., 2010).

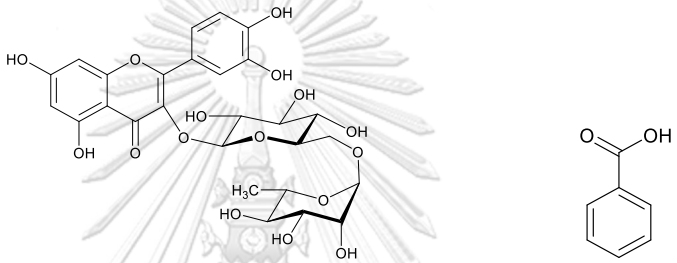
2.4.2 Antioxidant

An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it. An antioxidant delays or inhibits cellular

damage mainly through its free radical scavenging property. Antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Normally, antioxidants in a body are sufficient for the formation of free radicals within a body. In case, there are abnormal conditions in a body such as stress and sickness, they may cause an imbalance between free radical production and the amount of antioxidants, causing many diseases. Therefore, antioxidants in a body are important in preventing disease and deterioration of a body. The important characteristics of antioxidants are the conjugated double bond in the structure. When these substances donate an electron to or accept an electron from other molecules, free radicals are delocalized within the structure. The molecules are more stable than those without conjugated double bond. As a result, the intensity of free radicals is reduced. Some of such antioxidants, including glutathione, ubiquinol, and uric acid, are produced during normal metabolism in a body. Other lighter antioxidants are found in the diet. Beside several enzymes, principle micronutrients (vitamins) like vitamin E (α-tocopherol), vitamin C (ascorbic acid), and β-carotene are involved as antioxidants in scavenging free radicals within a body. The body cannot manufacture these micronutrients, so they must be supplied in the diet (Lobo et al., 2010). Furthermore, an antioxidant can be found in bee pollen which is one of supplementary food as follow.

The antioxidant effect of 14 types of monofloral bee pollen from Chaina was studied. They were extracted by using 75% ethanol. They were from flower pollen of ageratum, rape, camellia, hawthorn, chrysanthemum, buckwheat, sunflower, lotus, phellodendron, apricot, peach, rose, wuweizi, and dandelion. Those mentioned ethanol crude extracts were analysed for the antioxidant properties including trolox equivalent antioxidant capacity (TEAC), DPPH radical scavenging activity, and ferric reducing antioxidant power (FRAP). By TEAC, it showed ethanol crude extracts of wuweizi, phellodendron, dandelion, camellia, rape, and apricot flower pollen had the higher TEAC values. By DPPH radical scavenging activity, it showed ethanol crude extracts of rape, wuweizi, and phellodendron flower pollen had the stronger antioxidant activities at the IC_{50} values of 1.28, 1.66, and 2.50 mg/mL, respectively. By FRAP, it showed ethanol crude extracts of peach and wuweizi flower pollen significantly exhibited the highest reducing power, and, in order, it was followed by the ethanol crude extract of hawthorn and rape, camellia, phellodendron flower pollen. In this work, it presented that almost flower pollen extracts were powerful antioxidants (Zhang et al., 2015).

The antioxidative effect of rape bee pollen was also studied by using 70% methanol. The result showed methanol crude extract of rape bee pollen had high DPPH scavenging ability at IC_{50} value of 1.27 ± 0.00 µg/mL (lower than vitamin C) and ABTS radical scavenging activity. In addition, the extract had FRAP capacity at EC_{50} value of 3.19 ± 0.06µg/mL which was lower than that of trolox. It also had high ferric reducing antioxidant power (FRAP) at IC_{50} value of 0.12 ± 0.00 µg/mL which was lower than that of vitamin C. Considering phenolic composition in the extract, it contained high quantity of rutin (774.87 µg/g), quercetin (196.38 µg/g), benzoic acid (314.16 µg/g), kaempferol (9.26 µg/g), and resveratrol (242.88 µg/g) (Figure 2.5). (Sun et al., 2017).



(A) (B)

Figure 2.5 Chemical structures of rutin (A) and benzoic acid (B).

2.4.3 Antioxidative activity

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

An antioxidative activity can be detected by many methods, but a DPPH assay is the most common or popular. That is because this method is easy, inexpensive, fast, and accurate. This assay is based on a theory that a hydrogen donor is an antioxidant. Thus, it can be used to investigate whether an interesting compound is a radical scavenger or not. For a mechanism of this method, DPPH radical acts to accept hydrogen from an antioxidant (Figure 2.6). Thus, the antioxidative activity will be proportional to the disappearance of DPPH radical in test samples. DPPH radical shows a maximum absorption at 517 nm (purple). The color turns from purple to yellow by the formation of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidative activity can be easily evaluated by following the decrease of UV absorption at 517 nm.

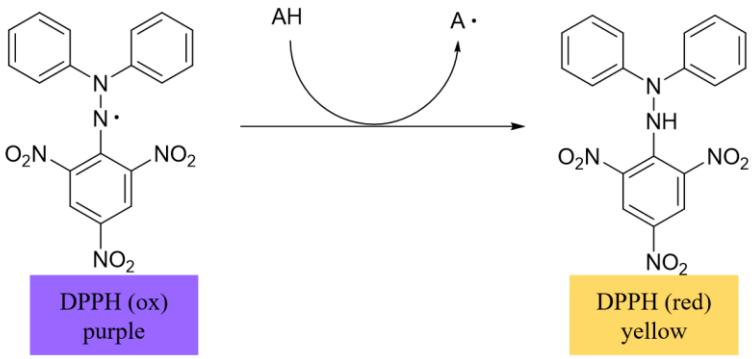


Figure 2.6 A reaction between DPPH radical and an antioxidant. DPPH free radical accepts hydrogen from an antioxidant and is conversed to DPPH.

2.5 Melanogenesis pathway, tyrosinase, tyrosinase inhibitor, and antityrosinase activity

2.5.1 Melanogenesis pathway

Melanogenesis is the production of the melanin pigments those are mostly produced by cells called melanocytes. Melanocytes are dendritic cells of the neuroectoderm. Melanoblasts, the precursor cells of melanocytes, are unpigmented cells originated from embryonic neural crest cells. After closure to the neural tube, melanoblasts migrate to various regions of a body and develop into melanocytes as well as cells of the peripheral nervous system, bone and cartilage of the head, and the choroid of the eye. Melanoblasts developed into melanocytes are predominantly found in the basal layer of skin epidermis and hair follicles. Melanocytes are also found in other tissues of the body such as the central nervous and cardiovascular systems, the uvea of eyes, cochlea, and even adipose tissue (D'Mello et al., 2016).

Melanin is primarily responsible for the pigmentation of human skin, eyes, and skin. It is produced by epidermis melanocytes. Melanocytes in the skin are surrounded by keratinocytes (one melanocyte is surrounded by approximately 36 keratinocytes). Melanocytes produce a specialized lysosomal related organelle termed the melanosome. Within the melanosome, biopolymers of the pigment melanin are synthesized to give hair and skin, as well as other tissue, its color. This melanin synthesis involves a bipartite process in which structural proteins are exported from the endoplasmic reticulum and fused with melanosome-specific regulatory glycoproteins released in coated vesicles from the Golgi apparatus. Melanin synthesis

ensues subsequent to the sorting and trafficking of these proteins to the melanosome. Each melanocyte resides in the basal epithelial layer and, by virtue of its dendrites, interacts with approximately keratinocytes to transfer melanosomes (Figure 2.7) and protect the skin from photo-induced carcinogenesis (Ebanks et al., 2009). A molecular structure of melanin is well suited to absorb ultraviolet (UV) and visible light. In response to ultraviolet B (UVB) irradiation, melanocyte synthesizes melanin through melanogenesis pathway. Under normally physiological conditions, pigmentation has a beneficial effect on the photo-protection of human skin against harmful UV injury and plays an importantly evolutionary role in camouflage and animal mimicry. Furthermore, the amount and type of melanin produced and transferred to the keratinocytes with subsequent incorporation, aggregation, and degradation influences skin complexion coloration (Pillaiyar et al., 2017).

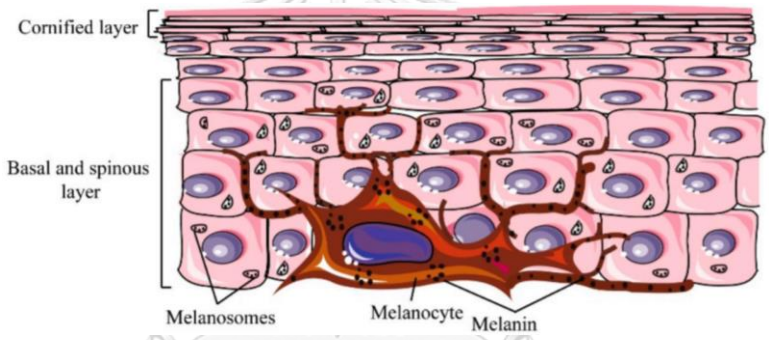


Figure 2.7 Association of keratinocytes and melanocytes. The dendritic melanocyte is located in the basal layer of skin and produces melanin. Melanin pigments in melanosomes are transferred (D'Mello et al., 2016)

Melanocytes produce melanosomes, which are intracellular organelles that produce melanin starting from the amino acid L-tyrosine. L-tyrosine oxidation to dopaquinone is catalyzed by the key enzyme, tyrosinase (TYR). The resulting quinone will serve as a substrate for the synthesis of eumelanin and pheomelanin. The formation of dopaquinone (DQ) is a rate-limiting step in the melanin synthesis because remainder of the reaction sequence can proceed spontaneously at a physiological pH value. After DQ formation, it undergoes intramolecular cyclization to produce indoline, leukodopachrome (cyclodopa). The redox exchange between leukodopachrome and DQ gives rise to dopachrome and L-3,4-dihydroxyphe nylalanine (L-DOPA), which is also a substrate for TYR and oxidized to DQ again by the enzyme. Dopachrome gradually decomposes to give dihydroxyindole (DHI) and dihydroxyindole-2-carboxylic acid (DHICA). The later process is catalyzed by TRP-

2, now known as dopachrome tautomerase (DCT). Ultimately, these dihydroxyindoles (DHI and DHICA) are oxidized to eumelanin. TRP-1 is believed to catalyze the oxidation of DHICA to eumelanin leading to brown-black skin. Alongside, DQ is converted to 5-S-cysteinyldopa or glutothionyldopa in the presence of cysteine or glutathione. Subsequent oxidation gives benzothiazine intermediates and finally produces pheomelanin leading to red-yellow pigmentation (Figure 2.8). Although three enzymes (TYR, TRP-1, and TRP-2) are involved in the melanogenesis pathway, tyrosinase is exclusively necessary for melanogenesis (Pillaiyar et al., 2017).

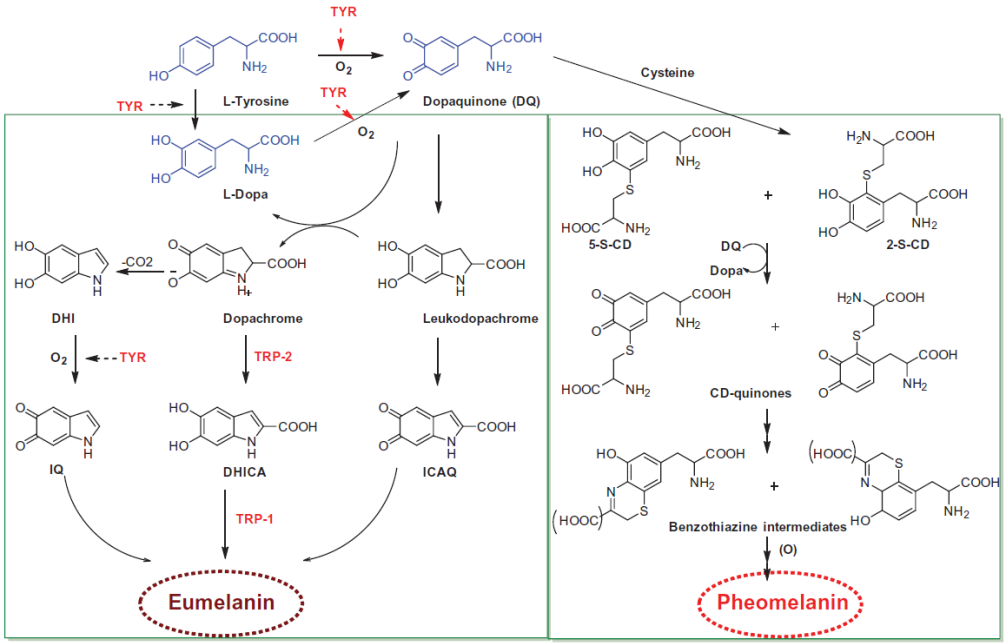


Figure 2.8 Melanogenesis pathway (production of eumelanin and pheomelanin). (Tyr: tyrosinase; DQ: dopaquinone; L-Dopa: L-3:4-dihydroxyphenylalanine; DHICA: 5,6- dihydroxyindole-2 carboxylic acid; DHI: 5,6-dihydroxyindole; ICAQ: indole-2-carboxylic acid-5,6-quinone; IQ: indole-5,6-quinone; HBTA: 5-hydroxy-1,4-benzothiazinylalanine) (Pillaiyar et al., 2017).

2.5.2 Tyrosinase

Tyrosinases (EC 1.14.18.1) catalyze the oxidations of both monophenols (cresolase or monophenolase activity) and o-diphenols (catecholase or diphenolase activity) into reactive o-quinones. Both tyrosinase activities appear to have broad substrate specificities, although the enzyme has a higher affinity for the L-isomers of the substrates than for the corresponding D-isomers. The best characterized tyrosinases are derived from *Streptomyces glausescens* (bacteria), *Neurospora crassa*

(fungi), and *Agaricus bisporus* (fungi). In fungi and vertebrates, tyrosinase catalyzes the initial step in the formation of the pigment melanin from tyrosine. The enzyme extracted from champignon mushroom *Agaricus bisporus* is highly homologous with the mammalian ones, and this renders it well suited as a model for studies on melanogenesis. In fact, mushroom tyrosinase has been used in almost studies on tyrosinase inhibition conducted so far because the enzyme is commercially available. The notable feature observed in tyrosinases from different sources is that the central copper-binding domain is conserved, which contains strictly conserved amino acid residues, including three histidines (Chang, 2009). For example, the structure and active site of a plant tyrosinase (leaves of walnut *Juglans regia*) contains two copper ions A and B which are coordinated by six histidine residues [His87, His108, and His117 for CuII (A) and His239, His243, and His273 for CuII (B)] (Figure 2.9) (Pillaiyar et al., 2017).

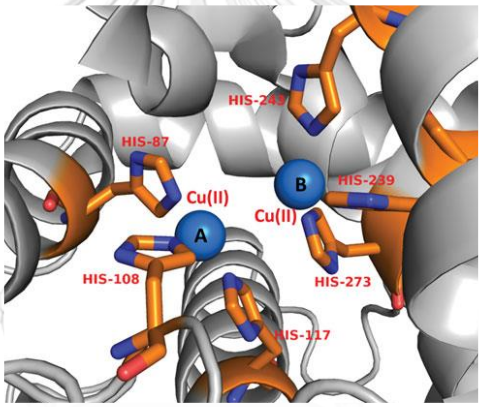


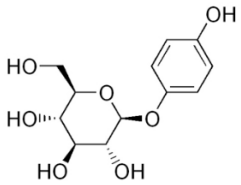
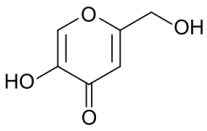
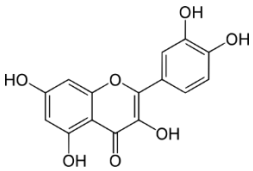
Figure 2.9 The structure and active site of plant tyrosinase (leaves of walnut, Juglans regia). It contains two copper ions A and B which are coordinated by six histidine residues [His87, His108, and His117 for CuII (A) and His239, His243, and His273 for CuII (B)] (Pillaiyar et al., 2017).

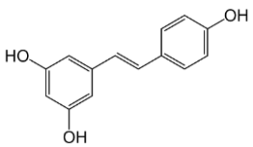
2.5.3 Tyrosinase inhibitors

Since tyrosinase is a rate limiting enzyme of melanogenesis, it becomes the most prominent and successful target for melanogenesis inhibitors that directly inhibit the tyrosinase catalytic activity. Most of cosmetics or skin lightening agents commercially available are tyrosinase inhibitors. Inhibitors are classified into four types, including competitive inhibitors, uncompetitive inhibitors, mixed (competitive and uncompetitive) inhibitors, and non-competitive inhibitors. A competitive inhibitor is a substance that combines with a free enzyme in a manner that prevents substrate

binding. An uncompetitive inhibitor can bind only to the enzyme-substrate complex. A mixed (competitive and uncompetitive) inhibitor can bind not only to a free enzyme but also to the enzyme-substrate complex. A noncompetitive inhibitor binds reversibly to the enzyme at a site away from the active site, thus, this allows the substrate to bind normally. However, the enzyme is completely inactivated when the inhibitor is bound. Many tyrosinase inhibitors such as hydroquinone (HQ), arbutin, kojic acid, azelaic acid, L-ascorbic acid, ellagic acid, tranexamic acid, quercetin, and resveratrol (Table 1) have been used as skin-whitening agents (Chang, 2009).

Table 2.1 Tyrosinase inhibitors.

Compound	**Source**	**Type of inhibition**	**Side effects**	**Reference**
Hydroquinone 	wheat, tea, berries, beer, and coffee	competitive inhibition	exogenous ochronosis and permanent depigmentation following long term use	(Ebanks et al., 2009)
Arbutin 	cranberries, blueberries, wheat, and pears	competitive inhibition	paradoxical hyperpigmentation	(Ebanks et al., 2009)
Kojic acid 	Acetobacter, Aspergillus, and Penicillium	competitive inhibition	carcinogenicity and instability during storage	(Ebanks et al., 2009)
Quercetin 	tea, onions, black tea, and apples	competitive inhibition	damage of normal cells and nutritional problems	(Fan et al., 2017)

Compound	Source	Type of inhibition	Side effects	Reference
Resveratrol 	mulberries, peanuts, and grapes	competitive inhibition	-	(Bernard & Berthon, 2000)

Furthermore, tyrosinase inhibitor can be found in bee pollen as below.

The antityrosinase activity of 75% ethanol crude extracts from 14 types of bee pollens from rape, wuweizi, phellodendron, apricot, dandelion, hawthorn, peach, buckwheat, chrysanthemum, sunflower, ageratum, camellia, rose, and lotus flower pollen was studied. All of the crude extracts showed the tyrosinase inhibitory activity, especially the ethanol crude extracts of apricot and sunflower bee pollen provided the strongest tyrosinase inhibitory activity against monophenolase at IC_{50} value of 0.12 ± 0.02 mg/mL and diphenolase at IC_{50} value of 0.30 ± 0.00 mg/mL, respectively. It was also reported that the active compounds were polyphenol (Zhang et al., 2015).

The anti-tyrosinase activity of camellia bee pollen was studied. It was extracted by using five solvents including ethanol, petroleum ether, ethyl acetate, n-butanol, and water. Then, those crude extracts were purified by using high speed counter current chromatography (HSCCC), macroporous adsorptive resin chromatography. The result showed ethyl acetate was the most appropriate solvent for the extraction because ethyl acetate crude extract provide the highest tyrosinase inhibition of 65.61% at 10 mg ethyl acetate crude extract. After purification, it was found that the active compound was kaempferol (Figure 2.10) that had the IC_{50} value at 0.05 mg/mL (Yang et al., 2016).

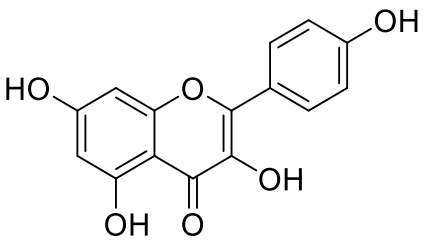
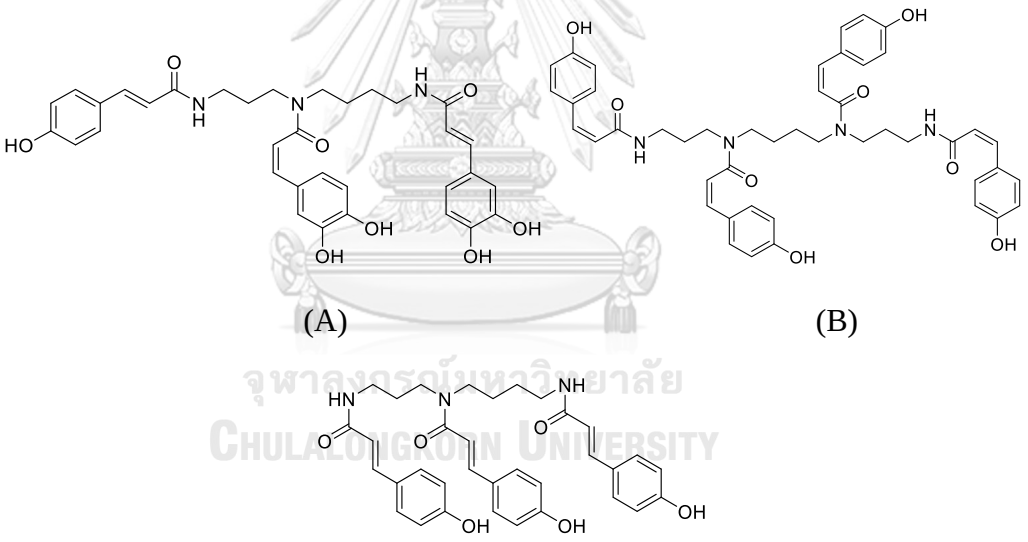


Figure 2.10 A chemical structure of kaempferol.

The effect of *Quercus mongolica* bee pollen on tyrosinase inhibitory activity was studied. Bee pollen was extracted by 80% MeOH and partitioned successively by n-hexane, CH_2Cl_2, EtOAc, and n-BuOH. After that, all crude extracts were purified by silica gel column chromatography and high-performance liquid column chromatography (HPLC). Eighteen constituents isolated from the bee pollen were identified to be polyamide derivatives. Evaluation of the biological activity of isolated compounds was revealed that N_1-(E)-p-coumaroyl-N_5-(Z)-N_{10}-(E)-di-caffeoylspermi dine (Figure 2.11, A) showed the highest tyrosinase inhibitory activity at IC_{50} value of 18.9 μM followed by N_1,N_5,N_{10},N_{14}-(Z)-tetra-p-coumaroylspermine (mongoline A) (Figure 2.11, B) at IC_{50} value of 19.5 μM, and N_1,N_5,N_{10}-(E)-tri-p-coumaroyl spermidine (Figure 2.11, C) at IC_{50} value of 20.3 μM. All of three compounds provided the stronger tyrosinase inhibitory activity than kojic acid. Therefore, polyamines, including the compounds reported in this study, could be used as functional whitening cosmetic materials (Kim et al., 2018).



(A) (B)

(C)

Figure 2.11 Chemical structures of polyamide derivatives isolated from Quercus mongolica bee pollen. N_1-(E)-p-coumaroyl-N_5-(Z)-N_{10}-(E)-di-caffeoylspermidine (A), N_1,N_5,N_{10},N_{14}-(Z)-tetra-p-coumaroylspermine (mongoline A) (B), and N_1,N_5,N_{10}-(E)-tri-p-coumaroylspermidine (C) (Kim et al., 2018).

2.5.4 Antityrosinase activity

The tyrosinase inhibitory activity is examined by the DOPAchrome enzymetic method using tyrosine or dihydroxyphenylalamine (L-DOPA) as a substrate of tyrosinase. Dopaquinone which can chemically change to DOPAchrome, one of the intermediate substances in melanin synthesis pathway, can be detected in visible light with red in color at 475 nm using UV-vis spectrophotometry (Figure 2.12). The potential tyrosinase inhibitor results in a decrease in DOPAchrome absorption (Micillo et al., 2017).

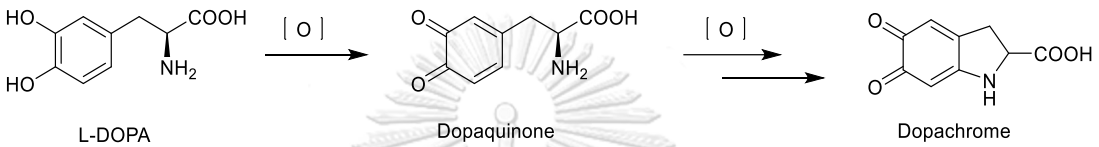


Figure 2.12 Dopachrome formation by tyrosinase catalyzed oxidation of 3,4-dihydroxy-l-phenylalanine (L-DOPA). λmax: Wavelength of maximum absorbance

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 and antityrosinase activities. This inspiration led to the interest in studying chemical constituents and their active free radical scavenging and antityrosinase activities from bee pollen of *A. mellifera.* The benefit of this work may be that new active free radical scavenging and antityrosinase compounds could be obtained and may be developed to be free radical scavenging agent and tyrosinase inhibitor useful in pharmaceutical and cosmetic industry. Finally, this may be benefit to the promotion of bee industry in Thailand which brings the increased income to bee keepers.

CHAPTER III
MATERIALS AND METHODS

3.1 Sample collection

Bee pollen of *Apis mellifera* was collected from camellia (*Camellia sinensis* L.), mimosa (*Mimosa pigra* L.) flowers in Chiangmai province and from sunflower (*Helianthus annuus* L.) in Lopburi province, Thailand in 2017. Bee pollen was stored at room temperature (25 ℃) until used.

3.2 Morphology study

Morphology of bee pollens were observed under a light microscope. Briefly, bee pollens were dissolved in water and dropped on a glass slide. After that, pictures and characteristics of bee pollens were recorded at 400X magnification.

3.3 Extraction

Bee pollen (140 g) was mixed with 800 ml of methanol. After it was shaken at 100 rpm, 15 ℃ for 18 h, it was centrifuged at 5500 × g, 4 ℃ for 15 min. The supernatant was collected. The solid residue was re-extracted 3 times by 800 mL of methanol. All supernatants were combined together and evaporated under reduced pressure and a maximum temperature of 40–45 °C. The sample from this step was named to be methanol crude extract of sunflower bee pollen (MTSBP), methanol crude extract of mimosa flower bee pollen (MTMBP) and methanol crude extract of tea flower bee pollen (MTTBP). It was kept at -20 ℃ in the dark until used.

3.4 Partition

Each methanol crude extract was separately partitioned by three organic solvents with different polarities (methanol, high polar; dichloromethane, medium polar; and hexane, low polar). At the beginning, crude extract was dissolved in methanol until it was not sticky. The volume of methanol was recorded. The mixture was poured into a separating funnel. Later, it was partitioned with the equal volume of hexane. After two phases were clearly separated, the upper phase (hexane part) was removed from the funnel and collected. After that methanol part 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 under reduced pressure and a maximum temperature of 40–45 °C. The sample from this step was named to be hexane partitioned extract of tea flower, sunflower and mimosa flower bee pollen or HXTBP, HXSBP and HXMBP, respectively. After that, methanol part was added with the

equal volume of dichloromethane to re-partition. After two phases were clearly separated, the lower phase (dichloromethane part) was removed from the funnel and collected. The left-over upper phase (methanol part) was re-extracted by dichloromethane at least 2 times. The dichloromethane parts were pooled together and evaporated under reduced pressure and a maximum temperature of 40–45 °C. The sample from this step was named to be dichloromethane partitioned extract of tea flower, sunflower and mimosa flower bee pollen or DCMTBP, DCMSBP and DCMMBP, respectively. At last, the methanol part was evaporated under reduced pressure and a maximum temperature of 40–45 °C. The obtained sample was named to be methanol partitioned extract of tea flower, sunflower and mimosa flower bee pollen or MTTBP, MTSBP and MTMBP, respectively. Then, all 9 partitioned extracts were tested for the free radical scavenging activity by 1,1-Diphyenyl-2-picrylhydrazyl (DPPH) assay (as mentioned in 3.5) and tyrosinase inhibitory activity assay (as mentioned in 3.6)

3.5 Free radical scavenging activity

3.5.1 The 1,1-Diphyenyl-2-picrylhydrazyl (DPPH) assay

The method was modified from Chantarudee et al. (2012). Five different concentrations of sample were prepared in dimethyl sulfoxide (DMSO). For each concentration, 20 µL of sample was mixed with 80 µL of 0.15 mM DPPH in methanol (as mentioned in Appendix A) and incubated at room temperature for 30 min. The absorbance of each well was measured at 517 nm by using microplate reader. Each sample was performed and measured in triplication. Ascorbic acid (vitamin C) was used as the standard reference. Triplication of experiments was performed. By using the Microsoft Excel 2016, the data was recorded as mean ± standard deviation (mean ± SD). The free radical scavenging activity was calculated by the formular below:

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

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.

3.5.2 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. Linear, nonlinear 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 2016.

3.6 Tyrosinase inhibitory activity

3.6.1 Tyrosinase inhibitory activity

The tyrosinase inhibitory activity was determined according to Zhang et al. (2015) with minor modifications. Five different concentrations of sample in DMSO were prepared. A reaction mixture contained 120 µL of 2.5 mM L-DOPA (as mentioned in Appendix A) in phosphate buffer, 30 µL of 80 mM phosphate buffer (pH 6.8) (as mentioned in Appendix A) and 10 µL of the sample solution in DMSO. The mixtures were mixed in 96-well plate and pre-incubated at 25 ℃ for 10 min. After that, 40 µL of 165 units/mL of mushroom tyrosinase in phosphate buffer (as mentioned in Appendix A) was added and incubated at 25 ℃ for 5 min. The absorbance of sample in each well was measured at 475 nm by using microplate reader. Kojic acid was used as the positive standard of a diphenolase inhibitor. Each sample was performed and measured in triplication. By using the Microsoft Excel 2016, the data was recorded as mean ± standard deviation (mean ± SD). The inhibitory percentage of tyrosinase was calculated as followed:

$$\% \text{ Tyrosinase inhibition} = \frac{(A–B) – (C–D)}{(A–B)} \times 100$$

Where: A is the absorbance at 475 nm after incubation without the test substance.

B is the absorbance at 475 nm after incubation without the test substance and tyrosinase.

C is the absorbance at 475 nm after incubation with the test substance and tyrosinase.

D is the absorbance at 475 nm after incubation with the test substance, but without tyrosinase.

3.6.2 The inhibitory concentration at 50% (IC_{50})

The inhibitory concentration at 50% (IC_{50}) was obtained by plotting a graph. The percentage of tyrosinase inhibition was on a Y-axis while the concentration of each sample was on an X-axis. Linear, nonlinear 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 2016.

3.7 Purification

The partitioned extracts providing the highest free radical scavenging activity and/or the highest tyrosinase inhibitory activities were further purified.

3.7.1 Silica gel 60 column chromatography

3.7.1.1 DCMSBP partitioned extract

A silica gel 60 column chromatography at the volume of 500 mL was packed with fine silica gel 60 (Merck's silica gel 60 for column chromatography). The targeted partitioned extract (4.93 g) was dissolved in 40 mL of methanol (MeOH) and combined with 10 g of rough silica gel (Merck's silica gel 60 for column chromatography). After drying, it was gradually poured over the surface of the packed silica gel 60 column chromatography. The packed chromatography was firstly eluted by 500 mL of dichloromethane (CH_2Cl_2). After that, the column was eluted by 2,000 mL of 10: 1 (v/v) CH_2Cl_2: MeOH and 1,000 mL of MeOH, respectively. Eluted fractions (10 mL each) were collected. Later, all fractions were evaporated under reduced pressure and a maximum temperature of 40–45 °C. The profile of chemical each fraction was tested by thin layer chromatography or TLC (as mentioned in 3.7.3). Fractions providing the same pattern of chemical compounds were pooled and tested for the free radical scavenging activity by DPPH assay (as mentioned in 3.5) and tyrosinase inhibitory activity assay (as mentioned in 3.6).

3.7.1.2 DCMMBP partitioned extract

A silica gel 60 column chromatography at the volume of 500 mL was packed with fine silica gel 60 (Merck's silica gel 60 for column chromatography). The targeted partitioned extract (7.24 g) was dissolved in 40 mL of methanol (MeOH) and combined with 20 g of rough silica gel (Merck's silica gel 60 for column chromatography). After drying, it was gradually poured over the surface of the packed

silica gel 60 column chromatography. The packed chromatography was firstly eluted by 800 mL of dichloromethane (CH_2Cl_2). After that, the column was eluted by 1,000 mL of 10: 1 (v/v) CH_2Cl_2: MeOH and 1,000 mL of MeOH, respectively. Eluted fractions (10 mL each) were collected. Later, all fractions were evaporated under reduced pressure and a maximum temperature of 40–45 °C. The profile of chemical each fraction was tested by TLC (as mentioned in 3.7.3). Fractions providing the same pattern of chemical compounds were pooled and tested for the free radical scavenging activity by DPPH assay (as mentioned in 3.5) and tyrosinase inhibitory activity assay (as mentioned in 3.6).

3.7.2 Size exclusion chromatography (Sephadex LH-20 chromatography)

Before packing the column, Sephadex LH-20 gel was immersed in MeOH overnight to make the beads swollen. Cotton was placed at the bottom of the column (250 mL in size). Prepared sephadex LH-20 gel was packed into the column at 4/5 of the column height. After that, the active fraction was be dissolved in 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 partitioned extract be absorbed into the texture of a gel better. After that, 500 mL of absolute MeOH was added to elute. Fractions (5 mL each) were collected. The chemical profile in each fractions was tested by TLC (as mentioned in 3.7.3). Fractions providing the same pattern of chemical compounds were pooled together and tested for the free radical scavenging activity by DPPH assay (as mentioned in 3.5) and tyrosinase inhibitory activity assay (as mentioned in 3.6).

3.7.3 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. While the TLC plate containing samples was dried at room temperature, the appropriate solvent such as 10: 1 (v/v) CH_2Cl_2: MeOH and 10: 0.5: 1 (v/v) CH_2Cl_2: ethyl acetate or EtOAc: MeOH was used as a mobile phase and 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 of TLC plate front line. After the TLC plate was dried, the result of migrating chemical compounds on the plate could be

visualized under ultraviolet light at the wavelength of 2 5 4 nm or under dipping anisaldehyde and heat over the hot plate.

3.8 High performance liquid chromatography (HPLC) analysis

To determine a target compound, an HPLC method was developed and modified from the method reported by Lv et al. (2015). At last, the operating condition could be achieved as below.

Column:	C18 column (5 µm, 10 x 250 mm)
Mobile phase:	(A) = Milli Q water and (B) = Acetonitrile
	(Isocratic; 60% A: 40% B)
Detection:	UV-visible spectroscopy at 254 nm
Injection volume:	20 µL
Column temperature:	25°C
Flow rate:	1 mL/minute

A target compound in an enriched fraction was analyzed by using the developed HPLC conditions. The enriched fraction was dissolved in absolute methanol at concentration of 50 mg/mL and filtered by using 0.2 µm pore size syringe filter. The sample solutions were analyzed, and the retention time of the extract was determined.

3.9 Chemical structure analysis

Pure compounds or partially pure fractions were 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) at the ratio of 5-20 mg of compound to 600 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 plus 400 or a Bruker AVANCE 400 spectrometer. It was operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei using tetramethylsilane (TMS) as an internal standard. The value of chemical shift in δ (ppm) was assigned with the reference to the signal or the residual protons in deuterated solvents. Chemical shifts and J coupling values were reported in ppm and Hz, respectively. The molecular weight of active fractions was analyzed by mass spectrometer. Briefly, enriched fractions were mixed with α-cyano-4-hydroxycinnamic acid (CCA) solution. After that, the mixed solution was spotted

on target. Then, the mass spectra were recorded by Bruker microflex mass spectrometer.

3.10 Statistical Analysis

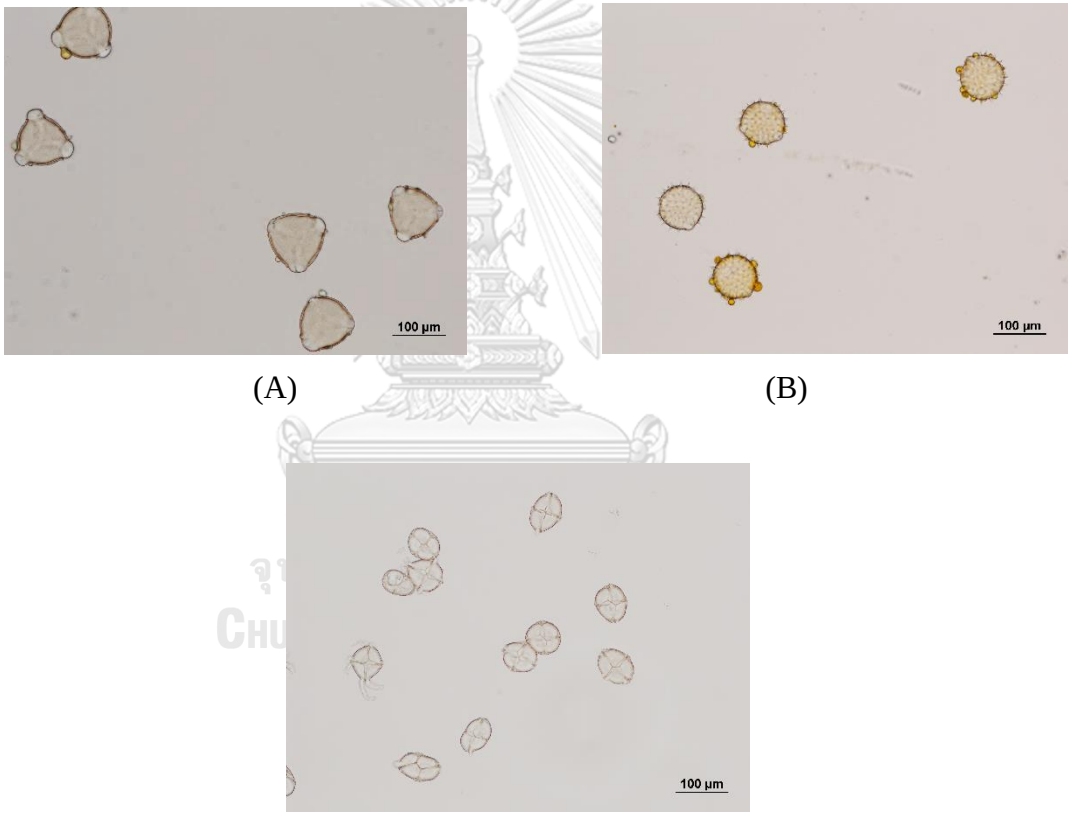
The results are presented as mean±standard deviation (SD) of three replicates. One-way ANOVA and T-test were used to test for significant differences in percentages of inhibition, inhibitory concentration at 50% (IC_{50}) and efficient concentration at 50% (EC_{50}). Tukey's test ($p<0.05$) were applied for the pairwise multiple comparisons. The statistical analyses were performed using IBM SPSS statistics version 22 for windows.

CHAPTER IV

RESULTS

4.1 Bee pollen morphology

By using light microscope, the morphology of tea flower (*Camellia sinensis* (L.) Kuntze), sunflower (*Helianthus annuus* L.) and Mimosa flower (*Mimosa pigra* L.) bee pollen were different. The morphology of tea flower bee pollen showed oblate to spherical shape with three farrows (tricolporate pollen). Sunflower bee pollen showed spherical shape with three farrows (tricolporate pollen) and spine and mimosa flower bee pollen showed oblate to spherical shape with four pollen subunits (tetrad).



(A) (B)

(C)

Figure 4.1 The morphology of tea flower (*Camellia sinensis* (L.) Kuntze) (A), sunflower (*Helianthus annuus* L.) (B) and mimosa flower (*Mimosa pigra* L.) (C) bee pollen under light microscope at 400X magnification

4.2 Bee pollen crude extracts

After bee pollen (sunflower bee pollen, mimosa bee pollen, and tea bee pollen) was extracted and evaporated (as mentioned in 3.3), the MTSBP of 113.85 g (yield, 81.32%) was obtained. It was turbid brown oil in color. The MTTBP of 93.48 g (yield, 66.77%) was obtained. It was turbid brown oil in color. The MTMBP of 85.76 g (yield, 61.26%) was obtained. It was turbid dark brown oil in color.

4.3 The partitioned extracts of MTTBP, MTSBP, and MTMBP

For MTTBP, MTSBP and MTMBP, they were separately partitioned by three organic solvents with different polarity. Those solvents were methanol, dichloromethane, and hexane. In total, nine partitioned extracts were evaporated and weighed. The data were recorded in Table 4.1. After that, all of nine partitioned extracts (MTTBP, DCMTBP, HXTBP, MTSBP, DCMSBP, HXSBP, MTMBP, DCMMBP, and HXMBP) were tested for the free radical scavenging activity and tyrosinase inhibitory activity.

Table 4.1 The weight, yield and the character of the partitioned extracts.

Sample	Weight (g)	Yield (%)	Character
MTTBP	64.79	69.31	Pale brown oil
DCMTBP	3.12	3.34	Sticky dark brown solid
HXTBP	7.97	8.53	Dark brown oil
MTSBP	83.91	73.70	Dark brown oil
DCMSBP	4.93	4.33	Sticky dark brown solid
HXSBP	9.66	8.48	Dark brown oil
MTMBP	51.87	60.48	Dark brown oil
DCMMBP	7.24	8.44	Sticky dark brown solid
HXMBP	8.14	9.49	Dark brown oil

4.4 Free radical scavenging activity of the partitioned extracts

All of nine partitioned extracts (MTTBP, DCMTBP, HXTBP, MTSBP, DCMSBP, HXSBP, MTMBP, DCMMBP, and HXMBP) were tested for the free radical scavenging activity by DPPH assay. The obtained absorbance was calculated for the percentage of free radical scavenging activity (as mentioned in 3.5.1). The results were presented as mean ± SD. (Table 4.2). The free radical scavenging activity

of DCMMBP (1,000 µg/mL) was the highest at 87.17 ± 2.49%. In contrast, the lowest free radical scavenging activity or no free radical scavenging activity were from MTTBP (62.5 µg/mL), HXTBP (15.625, 62.5, and 250 µg/mL), DCMSBP (15.625 and 62.5 µg/mL), HXSBP (15.625 and 62.5 µg/mL), MTMBP (62.5 and 250 µg/mL), and HXMBP (15.625 µg/mL), respectively. As a reference, the highest free radical scavenging activity of ascorbic acid (1,000 µg/mL) was 95.93 ± 1.67%.

Table 4.2 The percentage of free radical scavenging activity of nine partitioned extracts.

Sample	The percentage of free radical scavenging activity			
	15.625 µg/mL	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
MTTBP	0.24 ± 0.55	0.00	2.61 ± 2.31	19.17 ± 0.86
DCMTBP	2.79 ± 2.84	3.49 ± 1.21	10.95 ± 0.95	33.92 ± 0.24
HXTBP	0.00	0.00	0.00	7.70 ± 1.36
MTSBP	2.89 ± 1.22	1.80 ± 1.12	3.59 ± 1.94	16.06 ± 2.14
DCMSBP	0.00	0.00	4.01 ± 2.21	23.06 ± 3.11
HXSBP	0.00	0.00	2.69 ± 7.26	10.19 ± 3.97
MTMBP	0.96 ± 0.53	0.00	0.00	6.51 ± 1.23
DCMMBP	5.44 ± 2.69	18.82 ± 1.33	53.83 ± 2.07	87.17 ± 2.49
HXMBP	0.00	0.24 ± 0.42	1.21 ± 0.22	9.56 ± 0.52
Ascorbic acid*	3.06 ± 1.57	36.22 ± 4.14	97.18 ± 1.17	95.93 ± 1.67

Remark: * referred to a standard reference.

According to Table 4.2, the data were plotted (Figure 4.1). The EC_{50} values were estimated and recorded in Table 4.3. In addition, ascorbic acid was used as the standard reference. It was found that DCMMBP provided the EC_{50} value of 192.07 µg/mL, which was still high, comparing to the EC_{50} value of ascorbic acid (89.82 µg/mL). However, its value was the best among the rest which the EC_{50} values were about > 1,000 µg/mL.

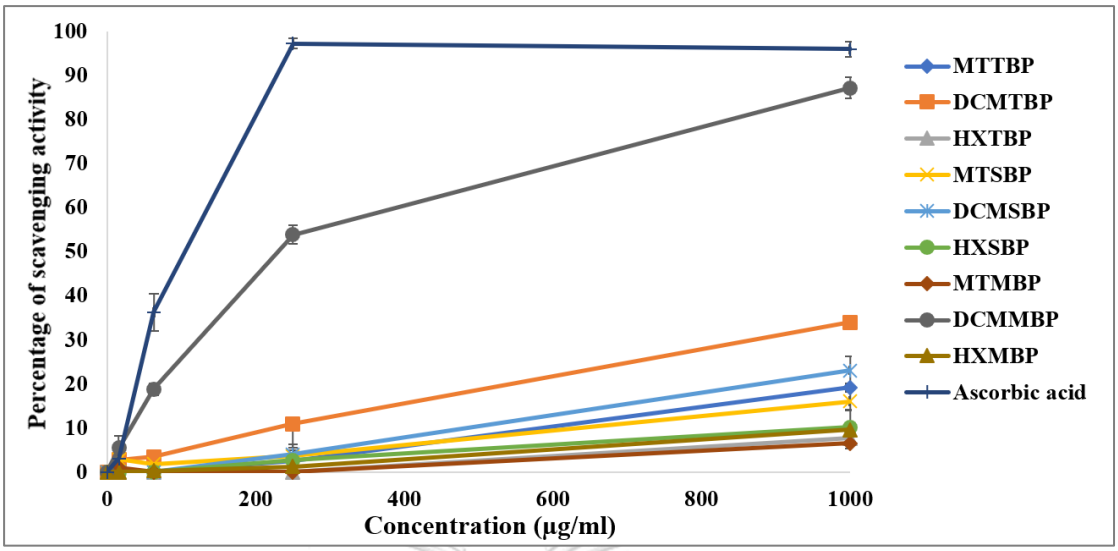


Figure 4.2 The free radical scavenging activity of MTTBP, DCMTBP, HXTBP, MTSBP, DCMSBP, HXSBP, MTMBP, DCMMBP, and HXMBP. Ascorbic acid was used as a positive control

Table 4.3 The EC_{50} values of nine partitioned extracts.

Sample	EC_{50} (µg/mL)*	r^2
MTTBP	> 1,000[c]	ND
DCMTBP	> 1,000[c]	ND
HXTBP	> 1,000[c]	ND
MTSBP	> 1,000[c]	ND
DCMSBP	> 1,000[c]	ND
HXSBP	> 1,000[c]	ND
MTMBP	> 1,000[c]	ND
DCMMBP	192.07[b]	0.9687
HXMBP	> 1,000[c]	ND
Ascorbic acid	89.82[a]	0.8916

Remark: * indicated the concentration sufficient to obtain 50% of a maximum free radical scavenging capacity as described in materials and methods. The EC_{50} values were calculated from a nonlinear regression and the r^2 represented the correlation coefficient. In addition, ND represented no available data. A different superscript letter is significantly different ($p \leq 0.05$; One-way ANOVA)

4.5 Tyrosinase inhibitory activity of nine partitioned extracts

After tested for the free radical scavenging activity, all of nine partitioned extracts (MTTBP, DCMTBP, HXTBP, MTSBP, DCMSBP, HXSBP, MTMBP, DCMMBP, and HXMBP) were also tested for the tyrosinase inhibitory activity by *in vitro* mushroom tyrosinase inhibitory assay. The obtained absorbance was calculated for the percentage of tyrosinase inhibition (as mentioned in 3.6.1). The results were presented as mean ± SD. as in Table 4.4. The percentage of tyrosinase inhibition of DCMSBP (500 µg/mL) was the highest at 66.96 ± 4.61. In contrast, the lowest tyrosinase inhibitory activity or no tyrosinase inhibitory activity were from HXTBP (62.5, 125, 250, and 500 µg/mL), HXSBP (62.5, 250, and 500 µg/mL), MTMBP (62.5, 250, and 500 µg/mL), and HXMBP (62.5, 125, 250, and 500 µg/mL). As a reference, the highest tyrosinase inhibitory activity of kojic acid (500 µg/mL) was 98.63 ± 0.53% (Table 4.4).

Table 4.4 The percentage of tyrosinase inhibition of nine partitioned extracts.

Sample	The percentage of tyrosinase inhibition			
	62.5 µg/mL	**125 µg/mL**	**250 µg/mL**	**500 µg/mL**
MTTBP	5.14 ± 1.03	9.58 ± 3.29	21.86 ± 3.44	38.75 ± 0.64
DCMTBP	15.41 ± 7.74	33.85 ± 5.96	49.06 ± 3.42	56.26 ± 2.47
HXTBP	0.00	0.00	0.00	0.00
MTSBP	3.95 ± 0.22	14.36 ± 5.66	15.47 ± 2.76	26.84 ± 2.35
DCMSBP	45.17 ± 1.19	49.07 ± 0.81	53.46 ± 0.97	66.96 ± 4.61
HXSBP	0.00	2.58 ± 1.62	0.00	0.00
MTMBP	0.00	2.96 ± 3.49	0.00	0.00
DCMMBP	8.01 ± 2.96	15.56 ± 3.97	9.80 ± 11.26	12.22 ± 4.91
HXMBP	0.00	0.00	0.00	0.00
Kojic acid*	91.15 ± 0.10	95.69 ± 0.49	96.78 ± 1.81	98.63 ± 0.53

Table 4.5 The percentage of tyrosinase inhibition of kojic acid.

Sample	The percentage of tyrosinase inhibition				
	0.32 µg/mL	**1.60 µg/mL**	**8 µg/mL**	**40 µg/mL**	**200 µg/mL**
Kojic acid*	8.56± 0.43	11.11±1.58	43.93±1.69	84.60±0.52	96.34±0.72

Remark: * referred to a standard reference.

According to Tables 4.4 and 4.5, the data were plotted (Figure 4.3). The IC_{50} values were estimated and recorded in Table 4.6. In addition, kojic acid was used as the standard reference. It was found that DCMSBP provided the IC_{50} value (159.39 µg/mL) which was high, comparing to kojic acid (8.58 µg/mL). Nonetheless, its value was still much better than DCMTBP (IC_{50}, 312.98 µg/mL) and another partitioned extracts (IC_{50}, > 500 µg/mL).

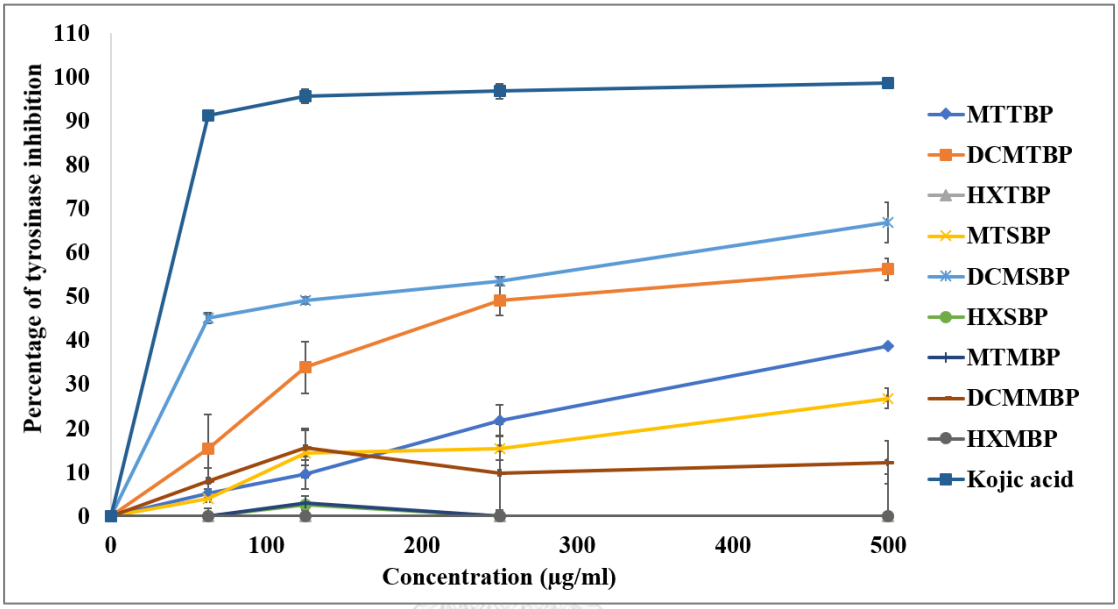


Figure 4.3 The tyrosinase inhibitory activity of MTTBP, DCMTBP, HXTBP, MTSBP, DCMSBP, HXSBP, MTMBP, DCMMBP, and HXMBP. Kojic acid was used as a positive control

Table 4. 6 The IC_{50} values of nine partitioned extracts.

Sample	IC_{50} (µg/mL) *	r^2
MTTBP	$> 500^{d}$	ND
DCMTBP	312.98^{c}	0.9666
HXTBP	$> 500^{d}$	ND
MTSBP	$> 500^{d}$	ND
DCMSBP	159.39^{b}	0.9941
HXSBP	$> 500^{d}$	ND
MTMBP	$> 500^{d}$	ND
DCMMBP	$> 500^{d}$	ND
HXMBP	$> 500^{d}$	ND
Kojic acid	8.58^{a}	0.9391

Remark: * indicated the concentration sufficient to obtain 50% maximal inhibitory concentration as described in materials and methods. The IC_{50} values of kojic acid and DCMTBP were calculated from a nonlinear regression and the r^2 represented the correlation coefficient. The IC_{50} value of DCMSBP was calculated from a linear regression and the r^2 represented the correlation coefficient. In addition, ND represented no available data. A different superscript letter is significantly different ($p \leq 0.05$; One-way ANOVA)

Since the DCMMBP provided the best free radical scavenging activity (192.07 µg/mL, the lowest value of EC_{50}) and DCMSBP provided the best tyrosinase inhibitory activity (159.39 µg/mL, the lowest value of IC_{50}). Therefore, both DCMMBP and DCMSBP were further isolated or purified by silica gel 60 column chromatography.

4.6 Purification of DCMMBP

4.6.1 Silica gel 60 column chromatography

From partition, DCMMBP at 7.24 g was obtained. After further purified by using silica gel 60 column chromatography, total of 96 fractions were collected. Considering the compound profile on TLC plate, fractions performing a similar pattern of compounds were pooled together. Finally, 3 different fractions (number

DCMMBP1 – DCMMBP3) were obtained. Their weight and characters were recorded in Table 4.7.

Table 4.7 The weight, yield and character of pooled fractions after silica gel 60 column chromatography.

Fraction	Weight (g)	Yield (%)	Fraction character
DCMMBP1	0.42	5.80	Sticky dark brown solid
DCMMBP2	0.69	9.53	Sticky dark brown solid
DCMMBP3	1.92	26.52	Pale brown solid

4.6.2 Free radical scavenging activity of pooled fractions after silica gel 60 column chromatography

Three pooled fractions from 4.6.1 were separately tested for the percentage of free radical scavenging activity. The result was shown in Table 4.8.

Table 4.8 The percentage of free radical scavenging activity of fraction number DCMMBP1 – DCMMBP3.

Sample	The percentage of free radical scavenging activity			
	15.625 µg/mL	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
DCMMBP1	1.37 ± 2.99	8.74 ± 1.95	26.36 ± 0.18	59.66 ± 1.07
DCMMBP2	0.00	9.40 ± 1.36	38.08 ± 0.65	85.36 ± 2.28
DCMMBP3	9.03 ± 1.18	35.32 ± 1.19	79.79 ± 1.60	83.37 ± 2.11
Ascorbic acid*	7.22 ± 0.52	41.87 ± 0.51	95.11 ± 0.18	95.54 ± 0.33

Remark: * referred to standard reference.

Due to Table 4.8, the percentage of free radical scavenging activity was plotted. The EC_{50} value was determined from the graph. The data were reported in Figure 4.4 and Table 4.9. Considering the EC_{50} value, fraction number DCMMBP3 gave the lowest EC_{50} value (or the highest free radical scavenging activity) at 112.19 µg/mL. It was followed by fraction number DCMMBP2 and DCMMBP1 which provided the EC_{50} values at 284.06 and 819.46 µg/mL, respectively. The ascorbic acid used as a reference presented the EC_{50} value at 81.07 µg/mL.

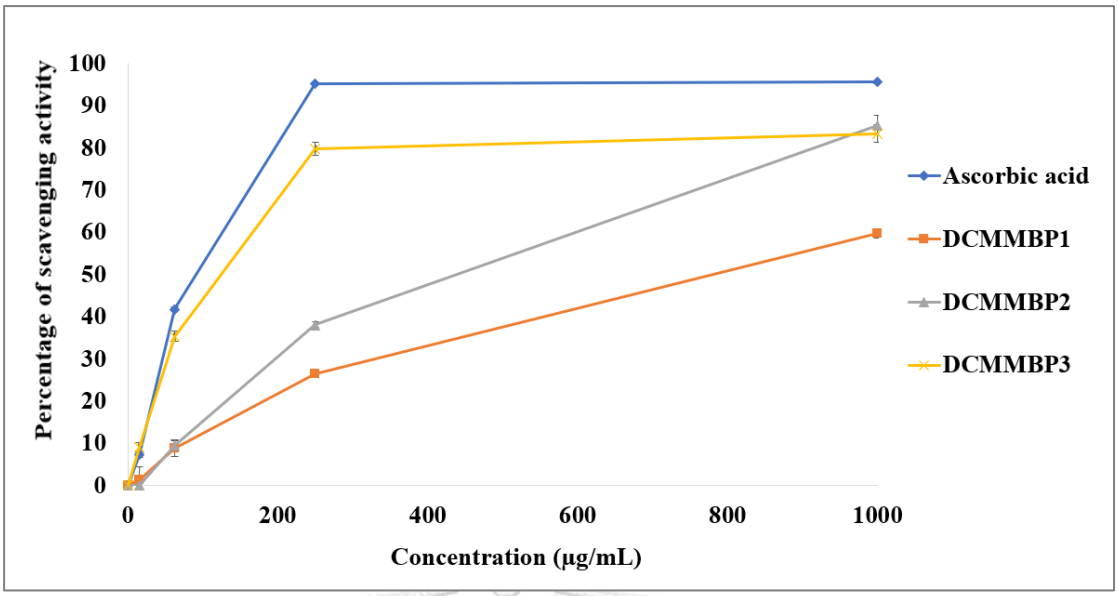


Figure 4.4 Free radical scavenging activity of fraction number DCMMBP1-DCMMBP3

Table 4.9 The EC_{50} values of pooled fractions after silica gel 60 column chromatography.

Sample	EC_{50} (µg/mL) *	r^2
DCMMBP1	819.46^{c}	0.9126
DCMMBP2	284.06^{b}	0.9225
DCMMBP3	112.19^{a}	0.9229
Ascorbic acid*	81.07^{a}	0.9024

Remark: * indicated the concentration sufficient to obtain 50% of a maximum free radical scavenging capacity as described in materials and methods. The EC_{50} values were calculated from a nonlinear regression and the r^2 represented the correlation coefficient. A different superscript letter is significantly different ($p \leq 0.05$; One-way ANOVA)

Since fraction number DCMMBP3 provided the best free radical scavenging activity (112.19 µg/mL, the lowest value of EC_{50}), therefore, fraction number DCMMBP3 was further purified by size exclusion chromatography.

4.6.3 Tyrosinase inhibitory activity of fraction number DCMMBP1-DCMMBP3

After tested for free radical scavenging activity, fraction number DCMMBP1-DCMMBP3 at 50 µg/mL were also tested for tyrosinase inhibitory activity. The result was shown in Figure 4.5. All three fractions provided very low tyrosinase inhibitory activity. This showed that a tyrosinase inhibitor was not available in all three fractions.

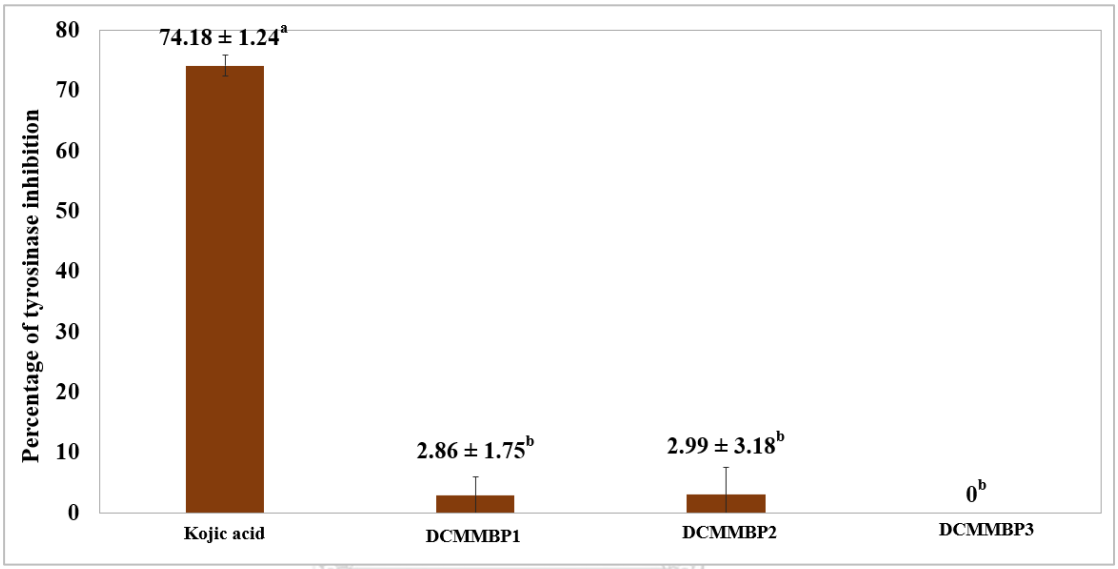


Figure 4.5 Tyrosinase inhibitory activity of fraction number DCMMBP1-DCMMBP3 at concentration of 50 µg/mL. A different superscript letter is significantly different ($p \leq 0.05$; One-way ANOVA)

4.6.4 Size exclusion chromatography (sephadex LH-20 chromatography) of DCMMBP3

The fraction number DCMMBP3 was further purified by sephadex LH-20 chromatography. After the column, total 66 fractions were obtained. By TLC, the fractions presenting the similar compound profile were pooled together. Finally, 2 different fractions of DCMMBP3-1 and DCMMBP3-2 were obtained and weighed. The data together with the character of both mentioned fractions were recorded in Table 4.10. After that, both fraction number DCMMBP3-1 and DCMMBP3-2 were tested for the free radical scavenging activity.

Table 4.10 The weight, yield and character of pooled fractions after size exclusion chromatography (sephadex LH-20 chromatography).

Fraction	Weight (g)	Yield (%)	Character
DCMMBP3-1	1.6599	86.45	Pale brown solid
DCMMBP3-2	0.0176	0.92	Yellow solid

4.6.5 Free radical scavenging activity of pooled fractions after size exclusion chromatography (sephadex LH-20 chromatography)

Two pooled fractions from 4.6.4 were separately tested for the percentage of free radical scavenging activity. The result was shown in Table 4.11.

Table 4.11 The percentage of free radical scavenging activity of fraction number DCMMBP3-1 and DCMMBP3-2

Sample	The percentage of free radical scavenging activity			
	15.625 µg/mL	**62.5 µg/mL**	**250 µg/mL**	**1000 µg/mL**
DCMMBP3-1	9.68 ± 1.23	35.67 ± 1.58	75.12 ± 2.11	81.75 ± 1.82
DCMMBP3-2	0.00	0.93 ± 1.55	5.48 ± 1.16	22.75 ± 1.18
Ascorbic acid*	7.22 ± 0.52	41.87 ± 0.51	95.11 ± 0.18	95.54 ± 0.33

Remark: * referred to standard reference.

Due to Table 4.11, the percentage of free radical scavenging activity was plotted. The EC_{50} value was determined from the graph. The data were reported in Figure 4.6 and Table 4.12. Considering the EC_{50} value, fraction number DCMMBP3-1 gave the lowest EC_{50} value (or the highest free radical scavenging activity) at 121.29 µg/mL which was high, comparing to ascorbic acid which was used as a reference (81.07 µg/mL).

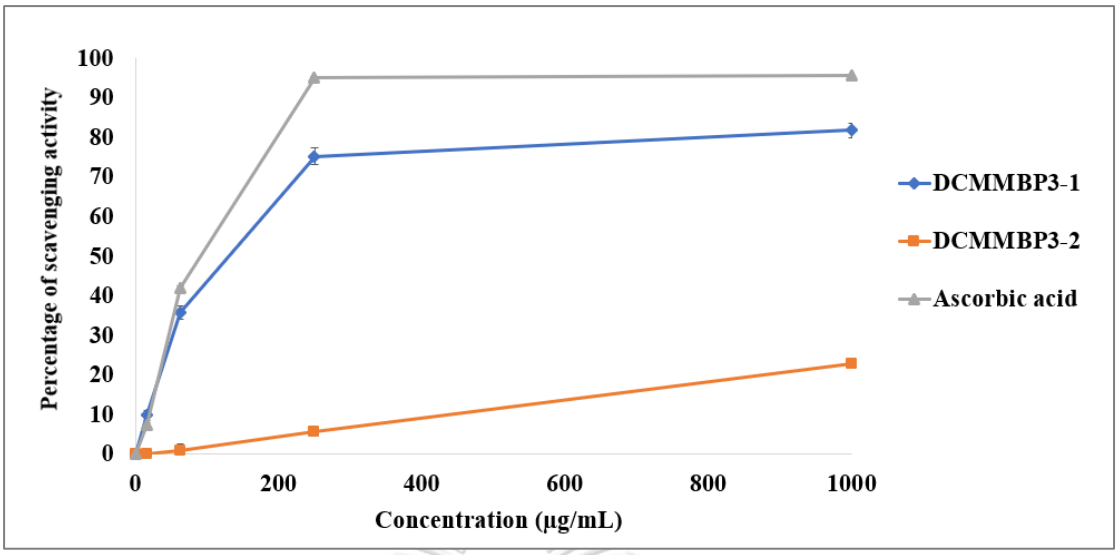


Figure 4.6 Free radical scavenging activity of fraction number DCMMBP3-1 and DCMMBP3-2**.**

Table 4.12 The EC_{50} values of pooled fractions after size exclusion chromatography (sephadex LH-20 chromatography).

Sample	EC_{50} (µg/mL)*	r^2
DCMMBP3-1	121.29^{b}	0.9421
DCMMBP3-2	$> 1000^{c}$	ND
Ascorbic acid*	81.07^{a}	0.9024

Remark: * indicated the concentration sufficient to obtain 50% of a maximum free radical scavenging capacity as described in materials and methods. The EC_{50} values were calculated from a nonlinear regression and the r^2 represented the correlation coefficient. In addition, ND represented no available data. A different superscript letter is significantly different ($p \leq 0.05$; One-way ANOVA)

Since fraction number DCMMBP3-1 provided the best free radical scavenging activity (EC_{50} value, 121.29 µg/mL), its EC_{50} value was higher than that of fraction number DCMMBP3, the starting sample (EC_{50} value, 112.19 µg/mL). It could be explained that some active compound was separated to fraction number DCMMBP3-2. This behavior was called synergistic effect of active compounds.

4.6.6 Thin layer chromatography (TLC) of fraction number DCMMBP3-1 and DCMMBP3-2

After purified by size exclusion chromatography, chemical compositions of two obtained fractions were tested by TLC. The result was shown in Figure 4.7. Fraction number DCMMBP3-1 seemed to contain more than one chemical composition because a smear band on a TLC plate was observed. It indicated that the polarity and molecular weight of chemical compositions in fraction number DCMMBP3-1 were very similar. Therefore, fraction number DCMMBP3-1 was not further purified and reported as mixture. In contrast, only a sharp band on a TLC plate was observed from fraction number DCMMBP3-2 which indicated pure compound. Therefore, the chemical structure of pure compound in fraction number DCMMBP3-2 was analyzed by using nuclear magnetic resonance spectroscopy (NMR) and it was named compound I. After that, the chemical structure of compound I was elucidated from NMR spectrums.

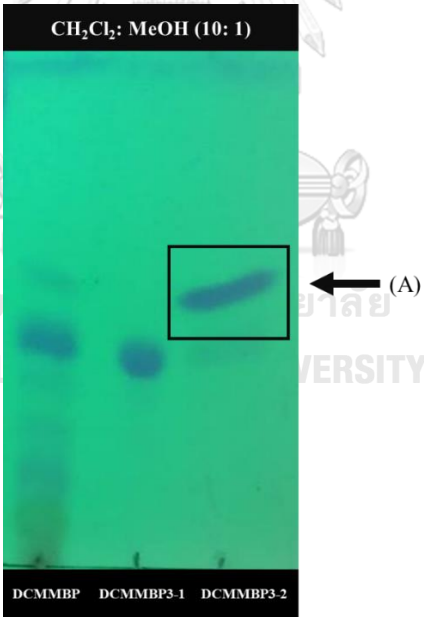


Figure 4.7 A TLC plate showed the compound profile of DCMMBP, fraction number DCMMBP3-1, and fraction number DCMMBP3-2. An arrow of (A) indicated compound I

4.6.7 Chemical structure analysis

4.6.7.1 Compound I

Compound I which was yellow solid was Naringenin (Figure 4.8). After it was analysed for a chemical structure by NMR, the obtained NMR peaks of chemical shift pattern (appendix B) were ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.30 (d, J = 8.5 Hz, 2H), 6.81 (d, 8.5 Hz, 2H), 5.88 (q, J = 2.2 Hz, 2H), 5.31 (dd, J = 13.0, 3.0 Hz, 1H), 3.09 (dd, J = 17.1, 13.0 Hz, 1H), 2.67 (dd, J = 17.1, 3.0 Hz, 1H). ^{13}C NMR (100 MHz, MeOD) δ 197.8, 168.4, 164.9, 159.0, 131.1, 129.0, 116.4, 103.4, 97.1, 96.3, 80.5, 44.0.

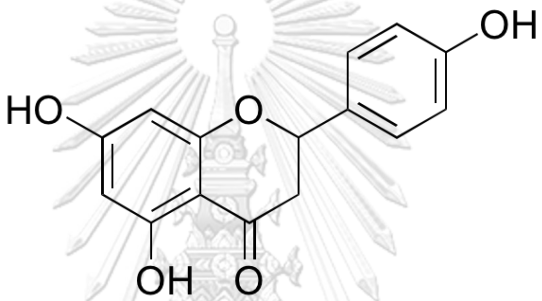


Figure 4.8 The formula structure of naringenin found in fraction number DCMMBP3-2 from DCMMBP3

4.6.7.2 Fraction number DCMMBP3-1

Fraction number DCMMBP3-1 was pale brown solid. After it was analysed for a chemical structure by NMR, the obtained NMR peaks of chemical shift pattern (appendix C) were ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.56 – 7.41 (m, 3H), 7.39 (dd, J = 9.2, 5.5 Hz, 1H), 7.13 – 7.05 (m, 2H), 7.05 – 7.02 (m, 1H), 7.02 – 6.91 (m, 4H), 6.91 – 6.65 (m, 7H), 6.40 (ddt, J = 20.6, 15.7, 5.5 Hz, 3H), 4.64 (s, 1H), 3.86 – 3.71 (m, 14H), 3.52 (s, 2H), 3.46 (dd, J = 18.5, 7.7 Hz, 3H), 3.34 (s, 13H), 1.87 (dt, J = 26.9, 7.1 Hz, 3H), 1.67 (s, 1H), 1.66 – 1.51 (m, 4H). ^{13}C NMR (100 MHz, Methanol-d_4) δ 169.2, 149.8, 149.5, 149.3, 144.7, 142.4, 142.1, 128.6, 128.3, 123.5, 123.3, 121.3, 116.6, 116.3, 115.4, 112.3, 111.7, 106.9, 106.5, 56.9, 56.6, 56.5, 49.9, 49.7, 49.5, 49.3, 49.1, 48.9, 48.6, 48.4, 47.6, 47.1, 45.7, 40.2, 39.9, 38.3, 38.1, 28.9, 27.9, 27.7.

4.6.7.3. Mass spectroscopy of fraction number DCMMBP3-1

Molecular weight of compounds in fraction number DCMMBP3-1 was analyzed by mass spectrometry. The result showed that the molecular weight of major compound in fraction number DCMMBP3-1 was 226.829 (appendix D).

4.7 Purification of DCMSBP

4.7.1 Silica gel 60 column chromatography

Since the DCMSBP provided the best tyrosinase inhibitory activity (IC_{50} value, 159.39 µg/mL, the lowest value of IC_{50}), the sample (4.93 g) was further purified by using silica gel 60 column chromatography. Total 155 fractions were collected. Considering the compound profile on a TLC plate, fractions performing a similar pattern of compounds were pooled together. Finally, 5 different fractions of DCMSBP1 – DCMSBP5 were obtained. Their weight and characters were recorded in Table 4.13.

Table 4.13 The weight, yield and character of pooled fractions after silica gel 60 column chromatography.

Fraction	Weight (g)	Yield (%)	Character
DCMSBP1	0.51	10.34	Sticky dark brown solid
DCMSBP2	0.76	15.42	Sticky dark brown solid
DCMSBP3	0.25	5.07	Sticky dark brown solid
DCMSBP4	0.67	13.59	Sticky dark brown solid
DCMSBP5	1.04	21.10	Pale yellow solid

4.7.2 Tyrosinase inhibitory activity of pooled fractions after silica gel 60 column chromatography

Five pooled fractions from 4.7.1 were separately tested for the percentage of tyrosinase inhibition at concentration of 50 µg/mL. The result was shown in Figure 4.9. Fraction number DCMSBP5 provided the highest tyrosinase inhibitory activity at 52.42 ± 4.59%. It was followed by fraction number DCMSBP4 at 34.59 ± 5.41%. Next, the percentage of tyrosinase inhibitory activity of fraction number DCMSBP5 with various concentrations was plotted in order that the IC_{50} value could be determined from the graph. The data was reported in Figure 4.10, Table 4.14 and 4.15.

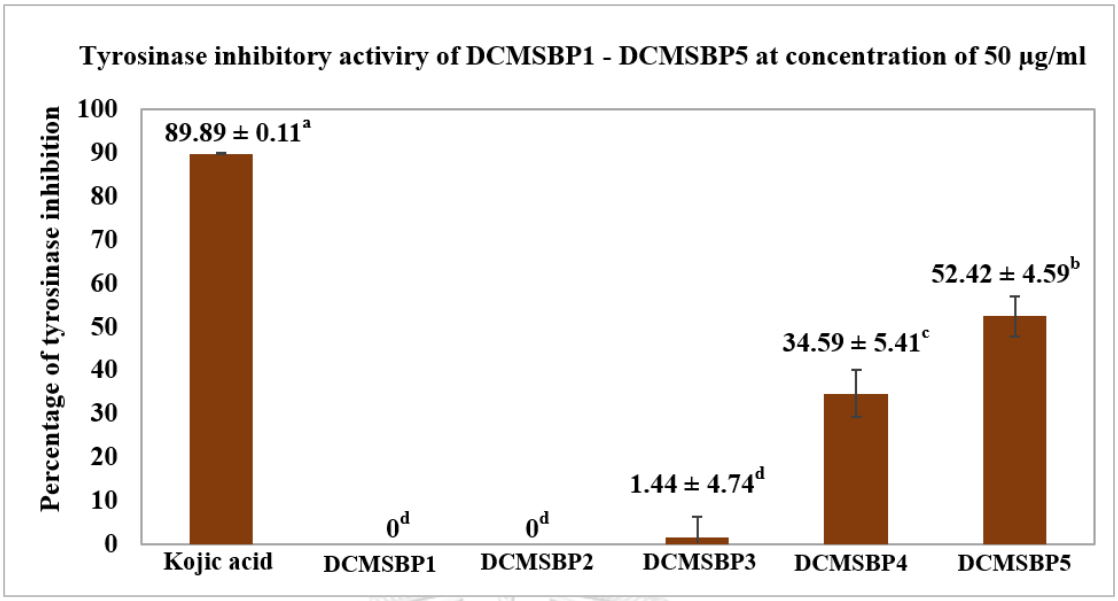


Figure 4.9 Tyrosinase inhibitory activity of fraction number DCMSBP1 – DCMSBP5 at concentration of 50 µg/mL. A different superscript letter is significantly different ($p \leq 0.05$; One-way ANOVA)

Table 4.14 The percentage of tyrosinase inhibition of fraction number DCMSBP5

Sample	The percentage of tyrosinase inhibition			
	3.13 µg/mL	**6.25 µg/mL**	**12.5 µg/mL**	**25 µg/mL**
DCMSBP5	25.78 ± 1.54	39.07 ± 0.59	45.48 ± 0.61	52.29 ± 0.29
Kojic acid*	14.04 ± 2.11	32.39 ± 1.51	56.79 ± 3.93	76.99 ± 1.06

Remark: * referred to a standard reference.

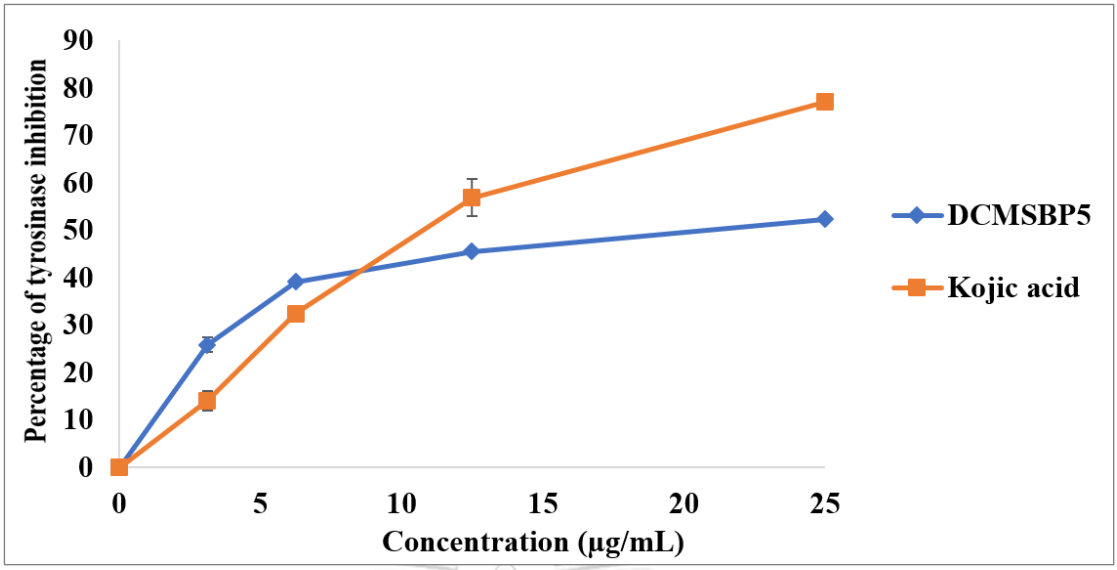


Figure 4.10 Tyrosinase inhibitory activity of fraction number DCMSBP5

Table 4.15 The IC_{50} values of fraction number DCMSBP5 after silica gel 60 column chromatography.

Sample	IC_{50} (µg/mL) *	r^2
DCMSBP5	18.78^{b}	0.9656
Kojic acid*	10.38^{a}	0.9973

Remark: * indicated the concentration sufficient to obtain 50% maximal inhibitory concentration as described in materials and methods. The IC_{50} values were calculated from a nonlinear regression and the r^2 represented the correlation coefficient. A different superscript letter is significantly different ($p \leq 0.05$; T-Test)

Due to Table 4.15, the result showed fraction number DCMSBP5 provided tyrosinase inhibitory activity at IC_{50} value of 18.78 µg/mL. It was higher than that of kojic acid (IC_{50} value, 10.38 µg/mL) but it was lower than that of the starting DCMSBP (IC_{50} value, 159.39 µg/mL). It presented that an active compound was enriched after purified by silica gel 60 column chromatography. The pattern of fraction number DCMSBP5 on TLC plate (Figure 4.11) showed that almost chemical compositions of starting DCMSBP were found in fraction number DCMSBP5. Thus, they could not be separated by silica gel 60 column chromatography because of the similarity in polarity. Therefore, fraction number DCMSBP5 was further purified by HPLC.

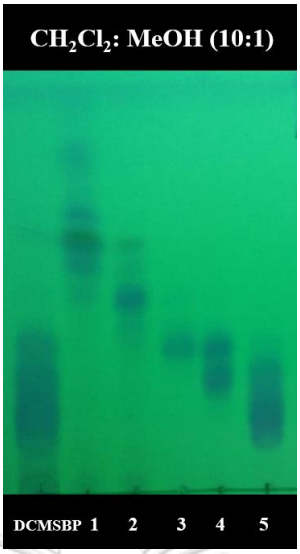


Figure 4.11 A TLC plate showed the chemical profile of DCMSBP, fraction number DCMSBP1- DCMSBP5

4.7.3 High performance liquid chromatography (HPLC) of fraction number DCMSBP5

Since the fraction number DCMSBP5 provided the best tyrosinase inhibitory activity (IC_{50} 18.78 µg/mL, the lowest value of IC_{50}), it was further purified by HPLC. The conditions used for the mobile phase were water and acetonitrile, with isocratic elution ranging from 0 to 70% water. However, the condition used to separate the sample (fraction number DCMSBP5) was 60% water: 40% acetonitrile. The HPLC chromatogram of fraction number DCMSBP5 was shown in Figure 4.12. Two major peaks were eluted at retention time of 24.354 and 27.496 minutes with peak area of 57.12 and 42.88%, respectively, and were detected at 254 nm. Finally, 2 fractions of DCMSBP5-1 and DCMSBP5-2 were obtained. Their weight and characters were recorded in Table 4.16.

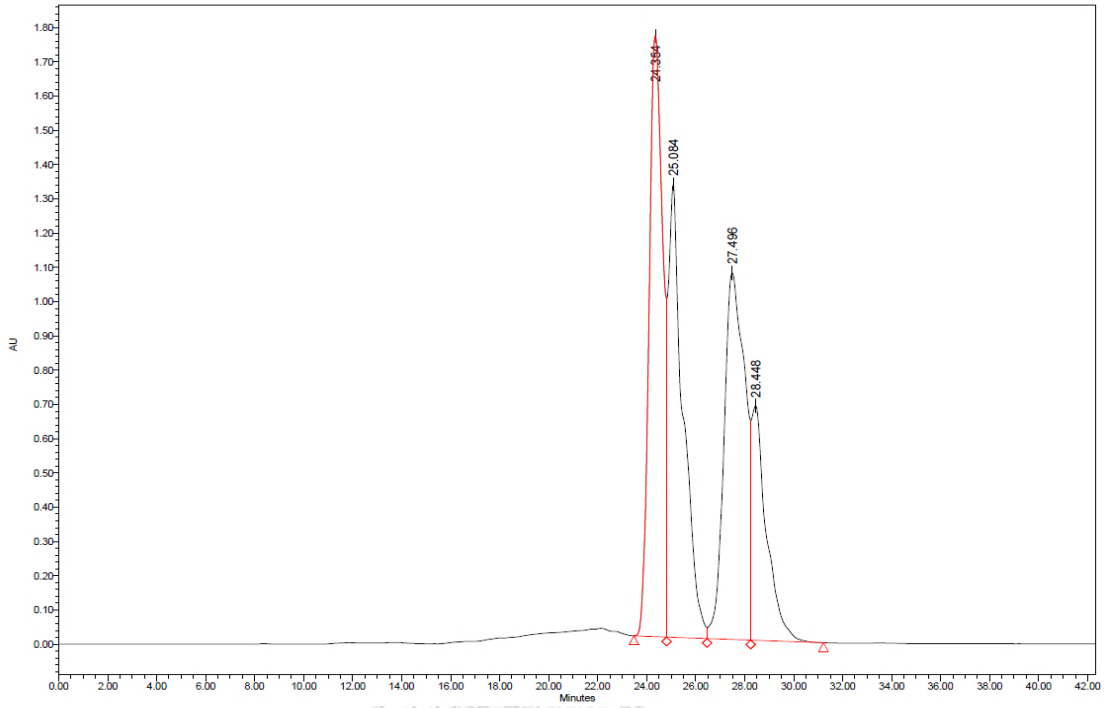


Figure 4.12 The HPLC chromatogram of fraction number DCMSBP5-1 and DCMSBP5-2 by HPLC isocratic condition detected at 254 nm

Table 4.16 The weight, yield, and character of fraction number DCMSBP5-1 and DCMSBP5-2 after HPLC.

Fraction	Weight (mg)	Yield (%) *	Character
DCMSBP5-1	6.8	48.6	Pale yellow solid
DCMSBP5-2	6.0	42.9	Pale yellow solid

*20 µL of 50 mg/mL of DCMSBP5 was injected for 14 times.

Then chemical compositions of fraction number DCMSBP5, DCMSBP5-1, and DCMSBP5-2 were analyzed by TLC using 10: 1 methanol: dichloromethane as mobile phase. The result showed two spots in fraction number DCMSBP5-1 lane and smear band was found in fraction number DCMSBP5-2 lane (Figure 4.13). It implied that the main compounds were separated to fraction number DCMSBP5-1. Also, HPLC could not be used to separate the mixture to pure compound because the HPLC condition was not appropriate. After that, fraction number DCMSBP5-1 and DCMSBP5-2 were tested for tyrosinase inhibitory activity.

Figure 4.13 A TLC plate showed the compound profile of fraction number DCMSBP5, DCMSBP5-1, and DCMSBP5-2

4.7.4 Tyrosinase inhibitory activity of fraction number DCMSBP5-1 and DCMSBP5-2 after HPLC

Fraction number DCMSBP5-1 and DCMSBP5-2 were separately tested for the percentage of tyrosinase inhibition. The percentage of tyrosinase inhibition of fraction number DCMSBP5-1 and DCMSBP5-2 were plotted and the IC_{50} value was determined from the graph. The data were reported in Figure 4.14, Table 4.17 and 4.18.

Table 4.17 The percentage of tyrosinase inhibition of fraction number DCMSBP5-1 and DCMSBP5-2 after HPLC.

Sample	The percentage of tyrosinase inhibition			
	3.13 µg/mL	**6.25 µg/mL**	**12.5 µg/mL**	**25 µg/mL**
DCMSBP5-1	39.50 ± 0.34	51.85 ± 0.89	58.85 ± 2.79	62.08 ± 2.07
DCMSBP5-2	37.38 ± 4.82	42.89 ± 5.27	44.81 ± 4.23	48.20 ± 1.10
Kojic acid*	28.02 ± 1.79	44.20 ± 1.01	63.29 ± 1.96	81.64 ± 1.35

Remark: * referred to a standard reference.

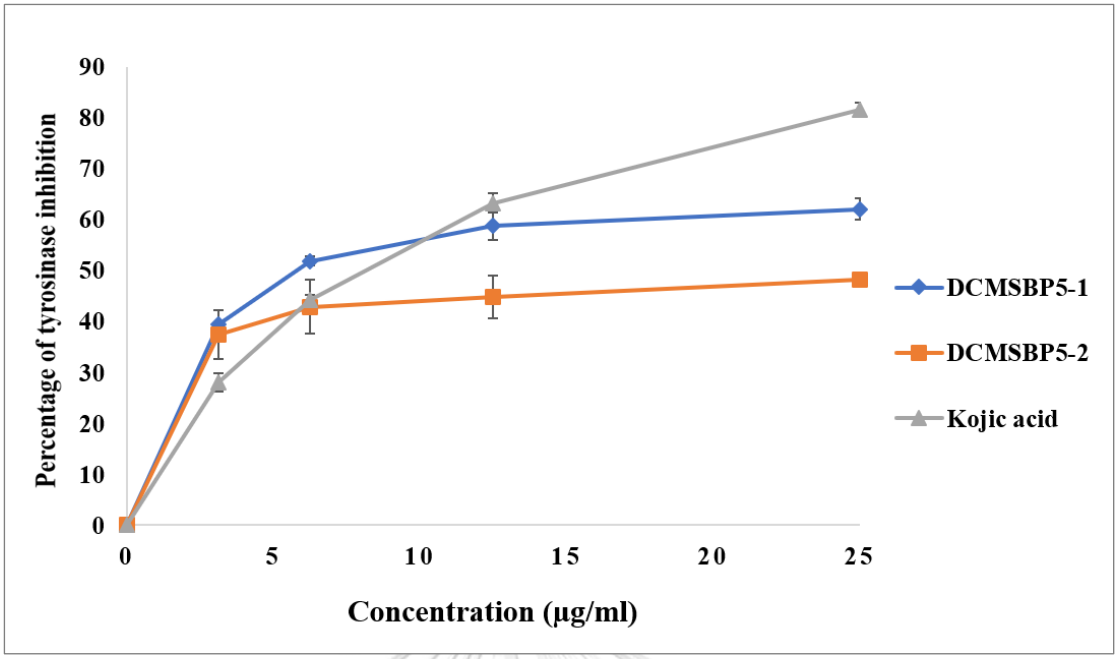


Figure 4.14 Tyrosinase inhibitory activity of fraction number DCMSBP5-1 and DCMSBP5-2

Table 4.18 The IC50 values of fraction number DCMSBP5-1 and DCMSBP5-2 after HPLC.

Fraction	IC_{50} (µg/mL) *	r^2
DCMSBP5-1	6.65^{a}	0.9302
DCMSBP5-2	34.02^{b}	0.9610
Kojic acid*	7.49^{a}	0.9989

Remark: * indicated the concentration sufficient to obtain 50% maximal inhibitory concentration as described in materials and methods. The IC_{50} values were calculated from a nonlinear regression and the r^2 represented the correlation coefficient. A different superscript letter is significantly different ($p \leq 0.05$; One-way ANOVA)

Due to Table 4.18, the result showed fraction number DCMSBP5-1 provided tyrosinase inhibitory activity at IC_{50} value of 6.65 µg/mL which was low, comparing to kojic acid (IC_{50} value, 7.49 µg/mL). Furthermore, it was much lower than that of the starting DCMSBP (IC_{50} value, 159.39 µg/mL) and that of fraction number DCMSBP5 (IC_{50} value, 18.78 µg/mL). It meant that an active compound was enriched after purification by HPLC. After that, the structure of chemical composition

in fraction number DCMSBP5-1 and DCMSBP5-2 were analyzed by using nuclear magnetic resonance spectroscopy (NMR). Then, the structure of chemical compositions was elucidated from NMR spectrums and it was found that the compounds in fraction number DCMSBP5-1 and DCMSBP5-2 were spermidine derivatives.

4.7.5 Chemical structure analysis

4.7.5.1 Fraction number DCMSBP5

Fraction number DCMSBP5 was pale yellow solid. After it was analysed for a chemical structure by NMR, the obtained NMR peaks of chemical shift pattern (appendix E) were ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.79 (s, 4H), 7.60 – 7.44 (m, 2H), 7.47 – 7.36 (m, 3H), 7.23 (dd, *J* = 15.0, 6.2 Hz, 1H), 6.87 (s, 2H), 6.91 – 6.71 (m, 6H), 6.49 – 6.35 (m, 1H), 6.03 – 5.79 (m, 1H), 4.65 – 4.60 (m, 1H), 3.50 (ddt, *J* = 20.9, 14.3, 7.7 Hz, 2H), 3.37 (s, 3H), 3.35 (s, 2H), 3.30 (s, 8H), 1.90 – 1.82 (m, 1H), 1.67 (s, 2H), 1.62 (s, 1H), 1.42 (s, 4H), 1.29 (s, 1H), 0.89 (s, 1H). ^{13}C NMR (100 MHz, MeOD) δ 172.0, 169.3, 160.6, 159.3, 144.5, 141.9, 141.9, 134.9, 132.2, 131.3, 130.9, 130.7, 128.2, 127.9, 127.7, 121.0, 118.5, 116.9, 116.8, 116.5, 116.5, 116.1, 114.9, 49.9, 49.7, 49.5, 49.3, 49.1, 48.9, 48.6, 48.45, 47.8, 45.8, 44.1, 39.9, 38.3, 37.9, 30.7, 29.8, 28.9, 28.4, 27.9, 27.7, 26.9, 26.4.

4.7.5.2 Fraction number DCMSBP5-1

Fraction number DCMSBP5-1 was pale yellow solid. After it was analysed for a chemical structure by NMR, the obtained NMR peaks of chemical shift pattern (appendix F) were ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.58 – 7.43 (m, 1H), 7.47 – 7.32 (m, 3H), 7.26 – 7.15 (m, 1H), 6.90 – 6.67 (m, 4H), 6.67 – 6.48 (m, 0H), 6.46 – 6.31 (m, 1H), 4.84 (s, 10H), 4.63 (s, 6H), 3.51 (ddt, *J* = 29.5, 14.5, 7.9 Hz, 1H), 3.35 (q, *J* = 4.0 Hz, 2H), 3.24 (s, 1H), 1.93-1.58 (m, 2H).

4.7.5.3 Fraction number DCMSBP5-2

Fraction number DCMSBP5-2 was pale yellow solid. After it was analysed for a chemical structure by NMR, the obtained NMR peaks of chemical shift pattern (appendix F) were ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.54 – 7.41 (m, 1H), 7.39-7.20 (m, 6H), 6.81 – 6.76 (m, 6H), 6.75-6.68 (m, 1H), 6.58 – 6.47 (m, 1H), 6.46 – 6.38 (m,

1H), 5.88 – 5.78 (m, 1H), 3.50-3.33 (m, 1H), 1.84 (s, 2H), 1.61 (d, *J* = 15.4 Hz, 3H), 1.29 (d, *J* = 7.4 Hz, 4H), 1.19 (s, 5H).

4.7.6 Mass spectroscopy of fraction number DCMSBP5-1 and DCMSBP5-2

To confirm that the chemical structures found in fraction number DCMSBP5-1 and DCMSBP5-2 were spermidine derivatives, molecular weight of compounds were analyzed by mass spectrometry (appendix G). Next, the chemical structures were predicted. The result was shown in Table 4.19 and 4.20, respectively.

Table 4.19 Molecular mass and a possible structure of chemical composition in fraction number DCMSBP5-1.

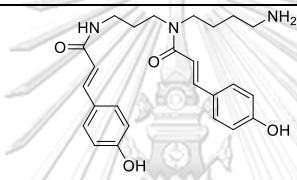
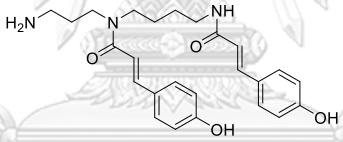
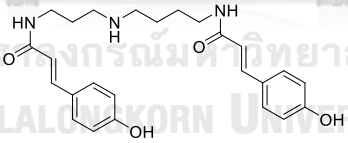
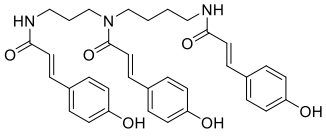
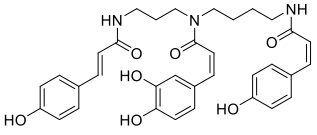
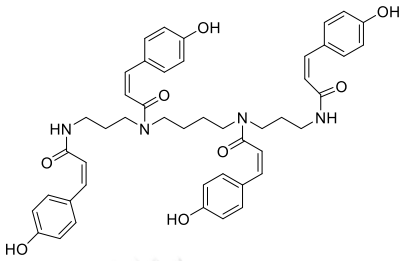
Molecular mass	Possible structure	Reference
438.354 $[M+1]^+$	 N_1,N_5-Di-coumaroylspermidine  N_5,N_{10}-Di-coumaroylspermidine  N_1,N_{10}-Di-coumaroylspermidine	(Werner et al., 1995)
584.431 $[M+1]^+$ 606.459 $[M+Na]^+$	 N_1,N_5,N_{10}-Tri[(E)-p-coumaroyl] spermidine	
622.439 $[M+1]^+$	 N_1-(E)-N_{10}-(Z)-di-p-coumaroyl-N_5-(Z)-caffeoyl spermidine	(Kim et al., 2018)

Table 4.20 Molecular mass and a possible structure of chemical composition in fraction number DCMSBP5-2.

Molecular mass	Possible structure	Reference
787.611 $[M+1]^+$ 809.652 $[M+Na]^+$	 N_1, N_5,N_{10},N_{14}-(Z)-tetra-p-coumaroyl spermine	(Kim et al., 2018)

4.7.7 Free radical scavenging activity of fraction number DCMSBP1 - DCMSBP5

After tested for tyrosinase inhibitory activity, fraction number DCMSBP1 - DCMSBP5 were also tested for free radical scavenging activity at 1,000 µg/mL. The result was shown in Figure 4.15 which fraction number DCMSBP2 provided the highest free radical scavenging activity at 87.84 ± 1.19%. It was followed by fraction number DCMSBP3, DCMSBP4, DCMSBP1, and DCMSBP5 at 73.48 ± 1.86, 57.24 ± 3.95, 50.16 ± 2.93, and 15.54 ± 3.81%, respectively. Then, various concentrations of these four fractions were further tested for the percentage of free radical scavenging activity. The result was shown in Table 4.21.

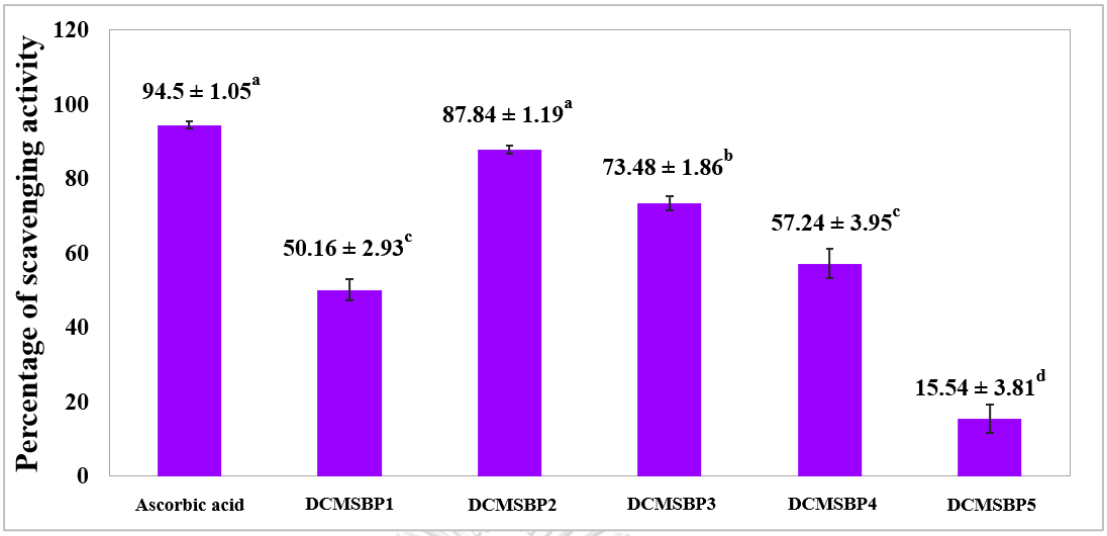


Figure 4.15 Free radical scavenging activity of fraction number DCMSBP1-DCMSBP5 at concentration of 1,000 µg/mL. A different superscript letter is significantly different ($p \leq 0.05$; One-way ANOVA)

Table 4.21 The percentage of free radical scavenging activity of fraction number DCMSBP1 - DCMSBP4.

Sample	The percentage of free radical scavenging activity			
	15.625 µg/mL	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
DCMSBP1	0.85±1.29	2.13±0.66	14.89±0.38	50.32±0.48
DCMSBP2	2.45±1.91	7.98±0.78	30.70±2.33	88.23±0.20
DCMSBP3	1.77±1.91	8.08±0.34	30.15±1.07	74.37±2.26
DCMSBP4	1.94±0.77	5.41±0.77	20.12±0.46	59.55±0.99
Ascorbic acid*	10.81±1.82	46.22±1.20	95.84±1.10	96.63±1.36

Remark: * referred to a standard reference.

According to Table 4.21, the data were plotted (Figure 4.16). The EC_{50} values were estimated and recorded in Table 4.22. In addition, ascorbic acid was used as the standard reference. It was found that fraction number DCMMBP2 provided the EC_{50} value (538.53 µg/mL) which was high, comparing to ascorbic acid. (EC_{50} value, 71.49 µg/mL). However, its value was still much better than the EC_{50} values of fraction number DCMSBP3 (633.00 µg/mL), fraction number DCMSBP4 (822.51 µg/mL), and fraction number DCMSBP1 (986.00 µg/mL), respectively.

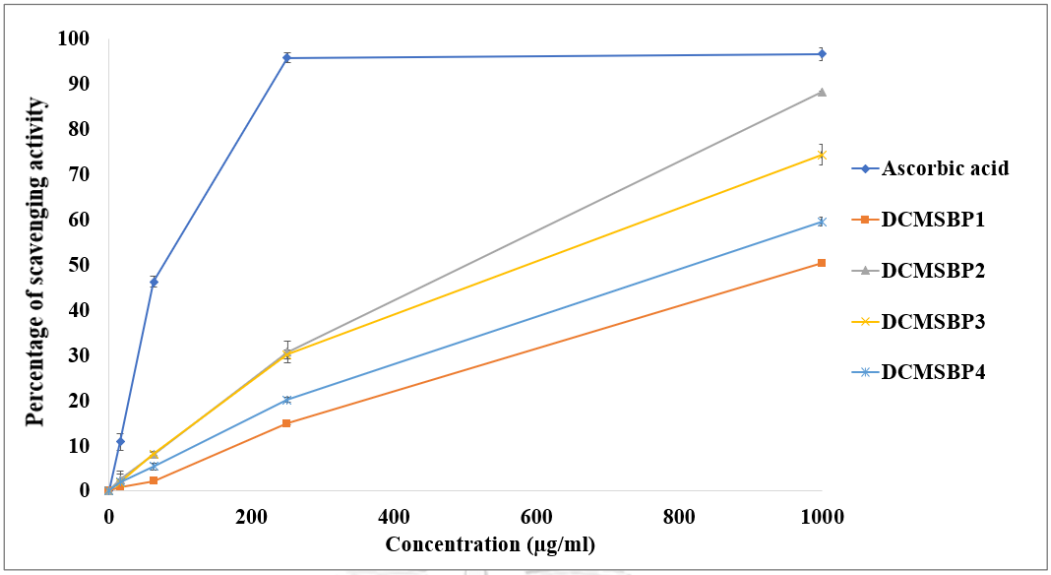


Figure 4. 16 Free radical scavenging activity of fraction number DCMSBP1–DCMMBP4

Table 4.22 The EC_{50} values of pooled fractions after silica gel 60 column chromatography**.**

Sample	EC_{50} value (µg/mL)*	r^2
DCMSBP1	986.00^{e}	0.9963
DCMSBP2	538.53^{b}	0.9913
DCMSBP3	633.00^{c}	0.9759
DCMSBP4	822.51^{d}	0.9937
Ascorbic acid	71.49^{a}	0.9044

Remark: * indicated the concentration sufficient to obtain 50% of a maximum free radical scavenging capacity as described in materials and methods. The EC_{50} values of ascorbic acid was calculated from a nonlinear regression and the r^2 represented the correlation coefficient. The EC_{50} values of fraction number DCMSBP1 – DCMSBP4 were calculated from a linear regression and the r^2 represented the correlation coefficient. A different superscript letter is significantly different ($p \leq 0.05$; One-way ANOVA)

Due to Table 4.22, it showed that, after purified by silica gel 60 column chromatography, fraction number DCMSBP1-DCMSBP4 had higher free radical scavenging activity than starting DCMSBP. It indicated that antioxidants were available in these four fractions. However, free radical scavenging activity of these was still lower than the activity of DCMMBP (EC_{50} value, 192.07 µg/mL, Table 4.3).

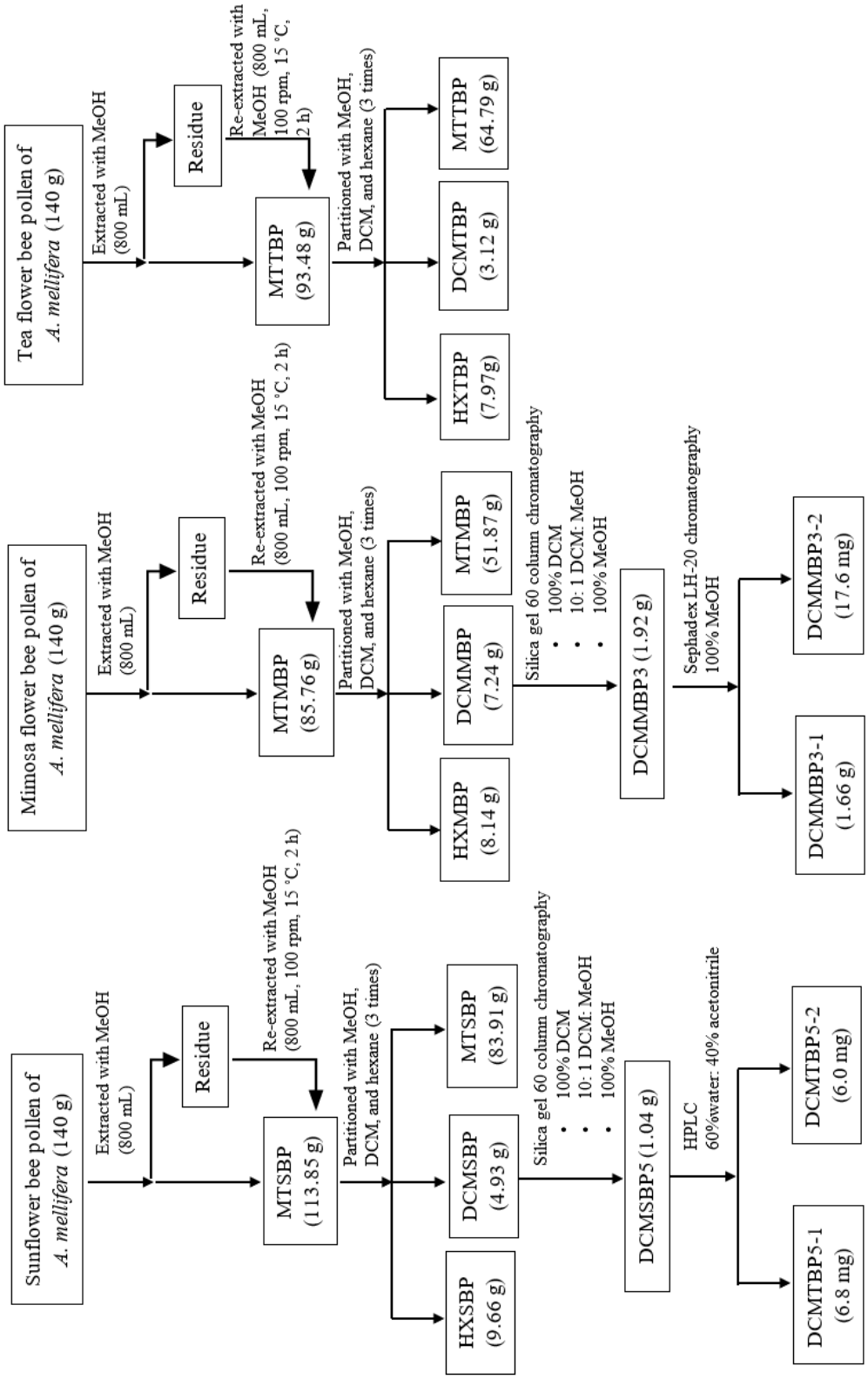


Figure 4.17 The extraction and purification procedure of *A. mellifera* bee pollens.

CHAPTER V
DISCUSSION

5.1 Bee pollen morphology

In this research, bee pollen from tea flower, sunflower, and mimosa flower were studied under a light microscope since, considering by naked eyes at the beginning, the shape of bee pollen looks similar to each other. Only a little bit difference in color could be noticed. According to a figure from a light microscope, the morphology of bee pollens was compared (Figure 4.1). The bee pollen from each particular plant was different to the others but it was related to floral pollen of plant sources. Morphology of tea flower bee pollen is similar to that of tea floral pollen (Zavada & Wei, 1993). Morphology of sunflower bee pollen was consistent with that of sunflower pollen (Klimko et al., 2000). And morphology of mimosa bee pollen was consistent with that of mimosa floral pollen (Medina-Acosta et al., 2018). Considering morphology of bee pollen, it could be confirmed that bee pollen in this work were originated from tea flower, sunflower, and mimosa flower bee pollen.

5.2 Extraction and partition

Three types of bee pollen were separately extracted by 100% methanol and partitioned by methanol, dichloromethane, and hexane. After that, all nine partitioned extracts were tested for the free radical scavenging and tyrosinase inhibitory activities. The EC_{50} value against free radical scavenging activity obtained from the DCMMBP partitioned extract (originated from MTMBP crude extract) was 192.07 µg/mL (Table 4.3) which was higher than the EC_{50} value of ascorbic acid (89.82 µg/mL). The obtained data was coincided to previous which reported corn bee pollen was similarly extracted by 3 types of solvents (methanol, dichloromethane, and hexane). The result showed dichloromethane crude extract of corn bee pollen provided the highest free radical scavenging activity at the EC_{50} value of 7.47 ± 0.12 µg/mL which was lower than the value in this study (Chantarudee et al., 2012). It could be because of the difference in types of bee pollen. It was reported that the polyphenol and the active antioxidants in bee pollen were very significantly varied according to the floral species (Zhang et al., 2015). The IC_{50} value against the tyrosinase inhibitory activity obtained from the DCMSBP partitioned extract (originated from MTSBP crude extract) was 159.39 µg/mL which was higher than the IC_{50} value of kojic acid (8.58 µg/mL). The obtained data was coincided to the previous. However, the solvents used

in the extraction were different to those used in this study. It was reported that camellia bee pollen was extracted by water, ethanol, petroleum ether, ethyl acetate, and n-butanol. It showed the ethyl acetate crude extract of camellia bee pollen had the highest tyrosinase inhibitory activity (65.61%) at concentration of 10 mg/mL (Yang et al., 2016). Considering dichloromethane and ethyl acetate, both are organic solvents with medium polarity. This could be indicated that a free radical scavenging agent and a tyrosinase inhibitor should contain medium polarity. Since the DCMMBP (originated from MTMBP) and the DCMSBP (originated from MTSBP) were active, both were chosen for further purification.

5.3 Compounds purified by chromatography

5.3.1 DCMMBP partitioned extract

After purified by silica gel 60 column chromatography, the active fractions were eluted by dichloromethane (CH_2Cl_2), 10: 1 (v/v) CH_2Cl_2: MeOH, and MeOH solvent system. Fraction number DCMMBP2 and DCMMBP3 were derived at 0.69 and 1.92 g, respectively. Both fractions showed the good free radical scavenging activity due to the EC_{50} values (Figure 4.4 and Table 4.9). The activity from fraction number DCMMBP3 was higher than that from the starting material, DCMMBP. Although fraction number DCMMBP3 provided the best free radical scavenging activity, its tyrosinase inhibitory activity was not improved after purification by silica gel 60 chromatography. In order to select which column should be used to isolate an interesting compound, the separating pattern of compounds as spots on a TLC plate was considered. The separating pattern of compounds in fraction number DCMMBP3 were visible as smear, it might indicate that the polarity of those compounds was close. Therefore, fraction number DCMMBP3 was selected for further purification by size exclusion chromatography (sephadex LH-20). After size exclusion chromatography, two fractions (DCMMBP3-1 and DCMMBP3-2) were obtained and tested for the free radical scavenging activity. The result showed fraction number DCMMBP3-1 provided the better free radical scavenging activity, but its potential was lower than that of the starting fraction number DCMMBP3. It might be the active compounds were separated already into fraction number DCMMBP3-2. Therefore, the free radical scavenging activity of fraction number DCMMBP3-1 was lower than that of the starting fraction number DCMMBP3. This obtained data could be explained by synergism between components that were partially/totally lost upon the separation (Chantarudee et al. (2012). Nonetheless, fraction number DCMMBP3-1

and DCMMBP3-2 originated from fraction number DCMMBP3 were collected and used for the chemical compound analysis. At the end, the mixture was obtained from fraction number DCMMBP3-1 while compound I was obtained from fraction number DCMMBP3-2. Considering the separating pattern of compounds in fraction number DCMMBP3-1, it still appeared as smear. It indicated size exclusion chromatography could not separate the mixture in fraction number DCMMBP3-1 to pure compounds. Therefore, fraction number DCMBP3-1 was reported as the mixture.

5.3.2 DCMSBP partitioned extract

After purified by silica gel 60 column chromatography, the active fractions were eluted by dichloromethane (CH_2Cl_2), CH_2Cl_2: MeOH, and MeOH solvent system. Fraction number DCMSBP1, DCMSBP2, DCMSBP3, DCMSBP4, and DCMSBP5 were derived. Fraction number DCMSBP4 and DCMSBP5 showed the good tyrosinase inhibitory activity due to the percentage in inhibition and IC_{50} values (Figure 4.9 and Table 4.14). Fraction number DCMSBP5 provided the best tyrosinase inhibitory activity which was higher than that of the starting DCMSBP. Therefore, active compounds were concentrated by silica gel 60 column chromatography. In contrast, the free radical scavenging activity of fraction number DCMSBP5 was still worse than that of the starting DCMSBP. The free radical scavenging activity of fraction number DCMSBP1, DCMSBP2, DCMSBP3, and DCMSBP4 was improved after purified by silica gel 60 chromatography. Since the activity of these four fractions was still lower than that of DCMMBP, these four fractions were not further purified in order to search for a free radical scavenging agent. Meantime, fraction number DCMSBP5 was selected to further purification by HPLC using 40% (v/v) acetonitrile: water as eluent. Considering the HPLC chromatogram, it was obvious that two main peaks were completely separated eventhough each main peak was still composed of two sub peaks. In overall, two fractions were obtained from HPLC including fraction number DCMSBP5-1 and DCMSBP5-2. After tested for the tyrosinase inhibitory activity, fraction number DCMSBP5-1 provided the activity better than fraction number DCMSBP5-2, the starting fraction number DCMSBP5, and kojic acid. It presented that active compounds were enriched into fraction number DCMSBP5-1. However, the HPLC with 40% (v/v) acetonitrile: water as eluent could not provide pure active compounds. Thus, both fractions were report as the mixture.

In the future, in order to purify a pure compound out of the mixture, more and new types of chromatography should be tried. Recently, there are many purification technics used to purify an active compound from bee pollen. For example, Kaempferol could be purified from camellia bee pollen by using high speed counter current chromatography (HSCCC) (Yang et al., 2016). Furthermore, The eighteen polyamine derivatives were successfully isolated form *Quercus mongolica* bee pollen by using HPLC with gradient elution of methanol: water (Kim et al., 2018). Both mentioned technics could be used to purify an active compound from bee pollen in this study.

5.4 Chemical structure analysis of Naringenin and active mixture (fraction number DCMMBP3-1)

After bee pollen was extracted and purified as mentioned earlier, compound I and the active mixture were obtained. Compound I was found in small amount and provided low free radical scavenging activity. However, the chemical structure of compound I was analyzed. It was analyzed to be naringenin which was flavonoid belonging to the flavanone group. Briefly about the analysis, the obtained chemical shifts (δ) of ^{1}H NMR and ^{13}C NMR were compared to the previous study. The flavonoid was isolated from aerial parts of *Cyclotrichium origanifolium* (Guzel et al., 2017). Between naringenin in bee pollen and that in aerial parts of *C. origanifolium*, the chemical shift value of naringenin in bee pollen was a little bit shifted from the other in ppm unit. The slightly difference in chemical shifts might be derived from a different deuterated solvents used for running ^{1}H NMR and ^{13}C NMR. Due to Guzel et al. (2017), DMSO-d_6 was used as a deuterated solvent for running ^{1}H NMR and ^{13}C NMR while in this research, methanol-d4 (CD_3OD) was used as a deuterated solvent for running ^{1}H NMR and ^{13}C NMR (Table 5.1). Then, the active mixture (fraction number DCMMBP3-1) was also analyzed by ^{1}H NMR, ^{13}C NMR, and mass spectrometry. The result of ^{1}H and ^{13}C NMR could be concluded that the active mixture was composed of aromatic ring because the chemical shift in the range of 7.56 to 6.40 ppm and of 149.83 to 128.29 ppm, respectively, was revealed. The result from mass spectroscopy showed a major molecular peak at m/z of 226.811. Although the structure of the active mixture could not be elucidated from ^{1}H NMR, ^{13}C NMR, and mass spectrometry data, it could be concluded that the active mixture was composed of a phenol derivative with molecular weight around 226.811.

Table 5.1 The chemical shifts (δ_H and δ_C) of naringenin (compound I) and the referenced naringenin (Guzel et al., 2017).

Naringenin (CD_3OD)			Reference naringenin (DMSO-d6)		
Position	**δ_H (ppm)**	**δ_C (ppm)**	**Position**	**δ_H (ppm)**	**δ_C (ppm)**
2	5.31	80.5	**2**	5.30	79.0
3	2.67/3.09	44.0	**3**	2.67/3.08	42.5
4	-	197.8	**4**	-	196.2
5	-	165.4	**5**	-	164.1
6	5.88	96.3	**6**	5.90	95.8
7	-	164.9	**7**	-	163.4
8	5.88	97.1	**8**	5.89	94.9
9	-	168.5	**9**	-	167.3
10	-	103.4	**10**	-	101.8
1'	-	131.1	**1'**	-	129.7
2'	7.30	129.0	**2'**	7.30	127.6
3'	6.81	116.4	**3'**	6.83	114.9
4'	-	159.0	**4'**	-	157.5
5'	6.81	116.4	**5'**	6.83	114.9
6'	7.30	129.0	**6'**	7.30	127.6

5.5 Chemical structure analysis of active compounds in fraction number DCMSBP5-1 and DCMSBP5-2

In this study, fraction number DCMSBP5-1 provided the highest tyrosinase inhibition. It was followed by fraction number DCMSBP5-2, therefore, both fractions were analyzed for a chemical structure by using three spectroscopic analyses including ^{1}H NMR, ^{13}C NMR, and mass spectrometry. The data from ^{1}H NMR showed a chemical shift was similar to spermidine derivatives reported by previous study (Kim et al., 2018). The data from ^{13}C NMR could be used to confirm the active compounds in fraction number DCMSBP5-1 and DCMSBP5-2 which were spermidine derivatives. The ^{13}C NMR data showed several chemical shifts between 190 to 165 ppm referred to carbonyl groups of spermidine derivatives, between 170 to 125 ppm referred to an aromatic ring of spermidine derivatives, between 160 to 120 ppm referred to alkene of spermidine derivatives, and between 50 to 10 ppm referred to aliphatic backbone of spermidine derivatives. After that, mass spectroscopic data

were used to determine the molecular weight of spermidine and identify the structure of spermidine derivatives. The result showed a molecular peak at m/z of 438.354, which was consistent to N_1,N_5-di-coumaroylspermidine or N_5,N_{10}-di-coumaroyl spermidine or N_1,N_{10}-di-coumaroyl spermidine, at m/z of 584.431 and 606.459, which were consistent to N_1,N_5,N_{10}-tri[(E)-p-coumaroyl]spermidine, at m/z of 622.439, which was consistent to N_1-(E)-N_{10}-(Z)-di-p-coumaroyl-N_5-(Z)-caffeoylspermidine, and at m/z of 787.611 and 809.652, which were consistent to N_1,N_5,N_{10},N_{14}-(Z)-tetra-p-coumaroylspermine. Furthermore, N_1,N_5,N_{10}-tri[(E)-p-coumaroyl]spermidine could be isolated from sunflower pollen by using silica gel 60 chromatography and C-18 column chromatography (Lin & Mullin, 1999). Thus, active compounds isolated from bee pollen were probably related to compounds isolated from floral pollen.

5.6 Relationship between a chemical structure and the activity

5.6.1 Free radical scavenging activity

In this research, DPPH assay was chosen for the detection of the free radical scavenging capacity in ROS groups because it was reported to be successful to detect the activity of phenolic compounds like flavonoids (Silva et al., 2009). The hydroxyl group of phenolic compounds or an electron donor would be able to react with DPPH. The color will be changed after a DPPH radical receives an electron.

Naringenin could be separated from mimosa bee pollen (Figure 5.1). After tested for the free radical scavenging activity by DPPH assay, Naringenin showed the weak free radical scavenging activity. Naringenin also showed the free radical scavenging activity sixty-time lower than quercetin (Choi et al., 2002). The relationship between chemical structure of naringenin and its potency was explained by the previous reports. Naringenin lacks the C3 hydroxyl group from the flavanol basic moiety but contains a single 4’ hydroxyl group and the saturated heterocyclic C ring, leading to the consequent lack of conjugation between the A and B rings. Additionally, it is found that the metal chelation can enhance the antioxidant activity of naringenin and the solvent can play an important role in this enhancement (Uivarosi et al., 2016). Although naringenin provided the low free radical scavenging in this study, it was presented to have another bioactivities including hepatoprotective, anti-inflammatory, anticarcinogenic effect, and so on (Rao et al., 2017).

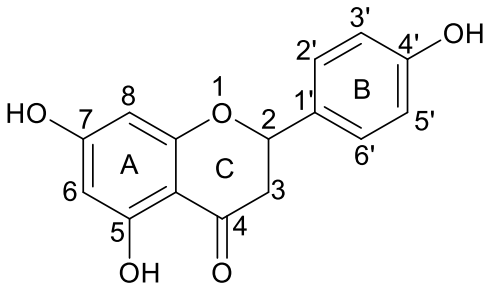


Figure 5.1 A chemical structure of naringenin.

The active mixture in fraction number DCMMBP3-1 of mimosa flower bee pollen provided the highest free radical scavenging activity. It might be a phenol derivative according to ^{1}H, ^{13}C NMR, and mass spectrometry data. These data were consistent with the study of antioxidant activity of six bee pollens which were mesquite, yucca, palm, turpentine, bush, mimosa, and chenopod. The result showed that the mimosa bee pollen provided the highest antioxidant activity among the tested bee pollens by either the DPPH or FRAP assays. Also, it had the highest amount of polyphenols (LeBlanc et al., 2009). Several types of phenolic compounds have a different substitution group including a position of a hydroxyl group and double bond. Accordingly, the antioxidant and free radical scavenging capacities have an unequal activity. When a phenolic compound donates a hydrogen atom to a free radical or a DPPH molecule, the internal structure of a phenolic compound will be delocalized an electron and make a molecule stable (Heim et al., 2002).

5.6.2 Tyrosinase inhibitory activity

In this research, dopachrome assay was chosen for the detection of the tyrosinase inhibitory activity. The commercial tyrosinase in this method was extracted from mushroom which was highly homologous with the mammalian tyrosinase (Chang, 2009). Therefore, this method can be used as a screening method for searching a tyrosinase inhibitor. The active compounds obtained in this study were polyamine derivatives which were spermidine and spermine conjugated with coumaroyl or caffeoyl moieties (Figure 5.2). Considering the coumaroyl and caffeoyl moieties of polyamine derivatives, both coumaroyl and caffeic structures and orientations were similar to tyrosine (Figure 5.3, A) and L-dopa (Figure 5.3, B), respectively. Therefore, polyamine derivatives extracted from sunflower bee pollen could bind to an active site of tyrosinase like lock and key. Since they block tyrosine and L-dopa well, tyrosine and L-dopa would not be converted to dopachrome and

melanin. It could be concluded that coumaroyl and caffeoyl moieties of polyamine derivatives played the key role in this inhibition. These data were consistent with the study of tyrosinase inhibitory activity of polyamine derivatives from the bee pollen of *Quercus mongolica*. It was revealed that the polyamine derivatives with coumaroyl and caffeoyl moieties showed the tyrosinase inhibition and also concluded that polyamines with phenolic groups were good candidates for tyrosinase inhibition. Their inhibitory activity might be different depending on the numbers and types of phenolic moieties (Kim et al., 2018).

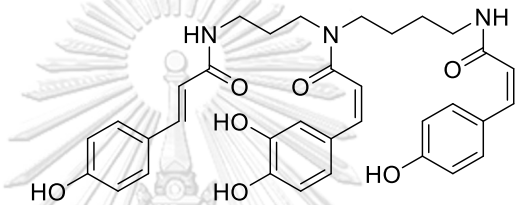


Figure 5.2 Spermidine conjugated with coumaroyl or caffeoyl moieties.

(A) (B)

Figure 5.3 Chemical structures of tyrosine (A) and L-dopa (B)

Moreover, the tyrosinase inhibitory activity of *p*-coumaric acid (Figure 5.4) which was similarly presented in a molecule of spermidine in this work was reported. It was presented that *p*-coumaric acid acted as a high inhibitor to human or murine tyrosinase in comparison with kojic acid and arbutin. In addition, *p*-coumaric acid inhibited human tyrosinase at much lower concentrations than those required for the inhibition of murine tyrosinase. Enzyme kinetics was also further analysed. The result indicated that *p*-coumaric acid was a mixed type (for tyrosine) or a competitive inhibitor (for L-DOPA) of human tyrosinase. The antimelanogenic effect of *p*-coumaric acid was observed in human epidermal melanocytes exposed to UVB. It was reported that *p*-coumaric acid was a potent and selective inhibitor of human tyrosinase and was potentially useful as a hypopigmenting agent (An et al., 2010).

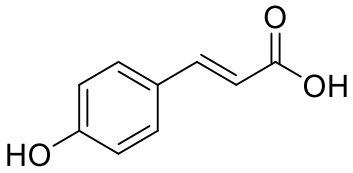


Figure 5.4 A chemical structure of *p*-coumaric acid

CHAPTER VI
CONCLUSION

In overall, the obtained data showed that bee pollens provided the *in vitro* free radical scavenging and tyrosinase inhibitory activities as follow.

1) Considering pollen morphology under a light microscope, tea flower, sunflower, and mimosa flower bee pollen collected by *Apis mellifera* were similar to flower pollen.

2) For free radical scavenging activity, DCMMBP partitioned extract provided the best free radical scavenging activity at EC_{50} value of 192.07 μg/mL.

3) For tyrosinase inhibitory activity, DCMSBP partitioned extract provided the best tyrosinase inhibitory activity at IC_{50} value of 159.39 μg/mL. Then, it was followed by DCMTBP partitioned extract.

4) The mixture of fraction number DCMMBP3-1 provided the best free radical scavenging activity at EC_{50} value of 121.29 μg/mL. Furthermore, the active mixture of fraction number DCMSBP5-1 provided the best tyrosinase inhibitory activity at IC_{50} value of 6.65 μg/mL which was higher than that of kojic acid. It was followed by the mixtures of fraction number DCMSBP5-2 at IC_{50} value of 34.02 μg/mL.

5) The active mixture of fraction number DCMMBP3-1 was expected to be a phenolic compound according to ^{1}H, ^{13}C NMR, and mass spectroscopy. Also, the active mixture of fraction number DCMSBP5-1 and DCMSBP5-2 were expected to be polyamine conjugated with coumaric acid and caffeic acid according to ^{1}H, ^{13}C NMR and mass spectroscopy.

6) A phenolic compound could inhibit the chain reaction of free radical by donating the hydrogen atom to a free radical or DPPH molecule. The internal structure of phenolic compound would be delocalized by an electron, resulting in a stable molecule.

7) Coumaroyl and caffeoyl moieties were the key part for inhibiting tyrosinase because their structures were similar to tyrosine and L-dopa, respectively. Therefore, they could bind to an active site of tyrosinase and inhibit the melanin synthesis.

8) Naringenin could be purified from mimosa flower bee pollen. It showed low free radical scavenging activity, comparing to ascorbic acid. However, it was found to have another activities like hepatoprotective, anti-inflammatory, and anticarcinogenic activities

These were suggested that consuming bee pollens as supplementary food could help to repair the damage and prevent a disease caused by a free radical in our bodies. Besides the free radical scavenging activity, bee pollen also provided the antityrosinase activity. Therefore, bee pollen could be used to reduce the excess production of melanin or used as a skin whitening agent. However, an *in vivo* experiment is needed to confirm the activity in a biological system. At least, it is important to present that an active compound in bee pollens is not toxic to and still active in our cells. Since bee pollen extracts can be developed to be an additive in cosmetic products, bee management and industry can be promoted at the same time. They support each other that will lead to the increase of income for bee farmers eventually.

APPENDICES

Appendix A: **Preparation of solutions**

1. Preparation of 0.15 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 20 mL MeOH

1) Stock solution of 15 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH, $C_{18}H_{12}N_5O_6$, MW 394.32) is prepared by dissolving 11.83 mg DPPH in 2 mL MeOH.

2) Keep in the dark at 4 ℃ for 1 month.

3) For working solution, dilute stock solution to 0.15 mM of DPPH.

$$C_1V_1 = C_2V_2$$

$$15\ \text{mM} \times V_1 = 0.15\ \text{mM} \times 20\ \text{mL}$$

$$V_1 = 0.2\ \text{mL}$$

Hence, 0.2 mL of 15 mM DPPH stock solution is mixed with 19.8 mL MeOH to provide 0.15 mM DPPH working solution.

2. Preparation of potassium phosphate buffer (pH 6.8)

1) Stock solution of 200 mM potassium phosphate buffer (pH 6.8)

Solution A: 200 mM K_2HPO_4 (MW: 174.18) is prepared by dissolving K_2HPO_4 (17.418 g) in 500 mL of d-H_2O.

Solution B: 200 mM KH_2PO_4 (MW: 136.08) is prepared by dissolving KH_2PO_4 (13.608 g) in 500 mL of d-H_2O.

Then, solution A and solution B are mixed together at equal volume to provide 200 mM potassium phosphate buffer (pH 6.8).

2) Dilute to 80 mM potassium phosphate buffer (pH 6.8) from stock solution.

$$C_1V_1 = C_2V_2$$

$$200\ \text{mM} \times V_1 = 80\ \text{mM} \times 45\ \text{mL}$$

$$V_1 = 18\ \text{mL}$$

Hence, 18 mL of 200 mM potassium phosphate buffer (pH 6.8) is mixed with 27 mL sterile d-H_2O to provide 80 mM potassium phosphate buffer (pH 6.8).

3. Preparation of 165 units (U)/mL of mushroom tyrosinase

Mushroom tyrosinase (labeled activity as 2,687 U/mg)

1) Stock of 5,000 U/mL of mushroom tyrosinase is prepared by dissolving 9.31 mg mushroom tyrosinase in 5 mL of 80 mM potassium phosphate buffer (pH 6.8).

2) Keep in the dark at -20 ℃ until use.

3) Dilute to 165 U/mL of mushroom tyrosinase from stock.

$$C_1V_1 = C_2V_2$$
$$5000\ \text{U/mL} \times V_1 = 165\ \text{U/mL} \times 4\ \text{mL}$$
$$V_1 = 0.132\ \text{mL}$$

Hence, 0.132 mL of stock is mixed with 3.868 mL of 80 mM potassium phosphate buffer (pH 6.8) to provide 165 U/mL of mushroom tyrosinase

4. Preparation of 2.5 mM L-DOPA ($C_9H_{11}NO_4$, MW: 197.19)

L-DOPA (14.79 mg) is dissolved in 30 mL of 80 mM phosphate buffer (pH 6.8) to provide 2.5 mM L-DOPA.

Appendix B: Chemicals and Equipments

1. Chemicals

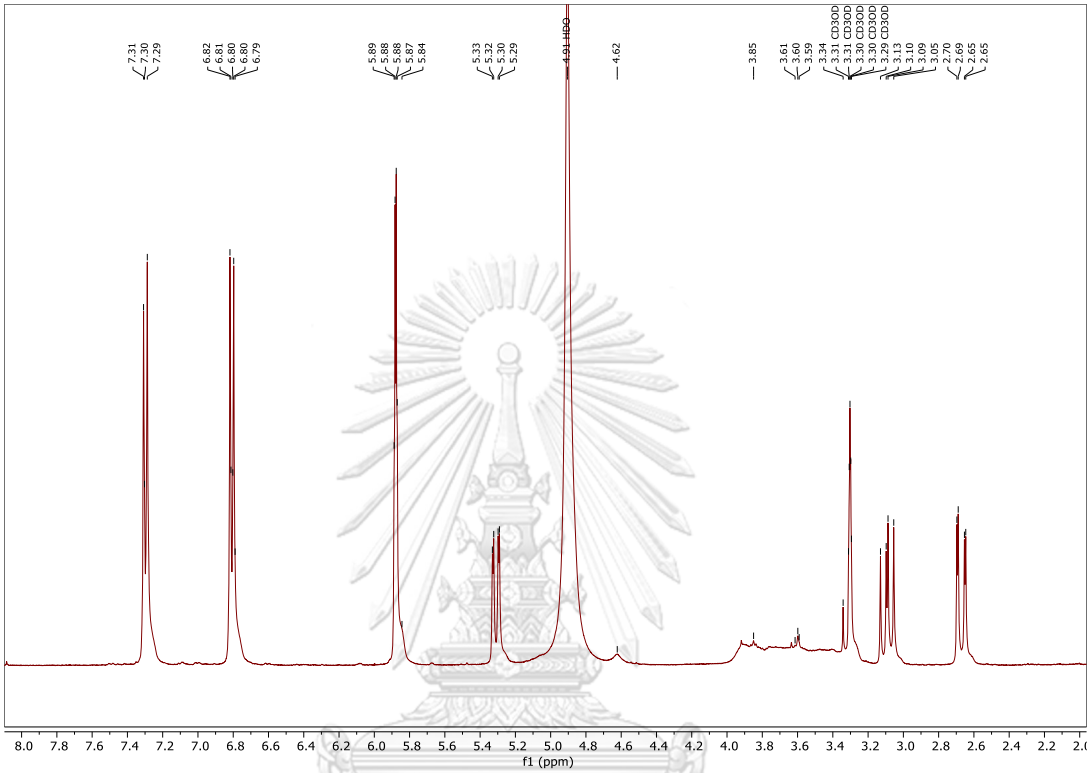
- 2,2-Diphenyl-1-picrylhydrazyl, $C_{18}H_{12}N_5O_6$, M.W. = 394.32, Sigma-Aldrich, USA
- Acetonitrile for analysis, CH_3CN, M.W. = 41.05, Merck KGaA Darmstadt, Germany
- Apollo C18 5u column (250 mm length, 10 mm i.d.), AllTech Associates, New Zealand
- Di potassium hydrogen phosphate, K_2HPO_4, M.W. = 174.18, Ajax Finechem Pty Ltd., Australia
- Dimethyl sulfoxide, $(CH_3)_2SO$, M.W. = 78.13, Sigma-Aldrich, USA
- Hexane, C_6H_{14}, M.W. = 86.18, TSL chemical, Thailand
- Kojic acid, $C_6H_6O_4$, M.W. = 142.11, Sigma-Aldrich, USA
- L-3,4-Dihydroxyphenylalanine (L-DOPA), $C_9H_{11}NO_4$, M.W. = 197.19, Abcam Plc., UK
- L-Ascorbic acid, $C_6H_8O_6$, M.W.= 176.12, Sigma-Aldrich, USA
- Methanol, CH_3OH, M.W. = 32.04, TSL chemical, Thailand
- Methanol-D4, CD_3OD, M.W. = 36.07, Merck KGaA Darmstadt, Germany
- Methanol for analysis, CH_3OH, M.W. = 32.04, Merck KGaA Darmstadt, Germany
- Methylene chloride, CH_2Cl_2, M.W. = 84.93, TSL chemical, Thailand
- Potassium dihydrogen phosphate, KH_2PO_4, M.W. = 136.08, Merck KGaA Darmstadt, Germany
- Sephadex LH-20, GE Healthcare Bio-sciences AB, Sweden
- SGE Analytical Science Syringe, Trajan Scientific Pty Ltd., Australia
- Silica gel 60 for column chromatography (0.063-0.200 mm), SiO_2, M.W. = 60.08, Merck KGaA Darmstadt, Germany
- TLC silica gel 60 F_{254}, Merck KGaA Darmstadt, Germany
- Tyrosinase from mushroom, Sigma-Aldrich, USA

2. Equipments

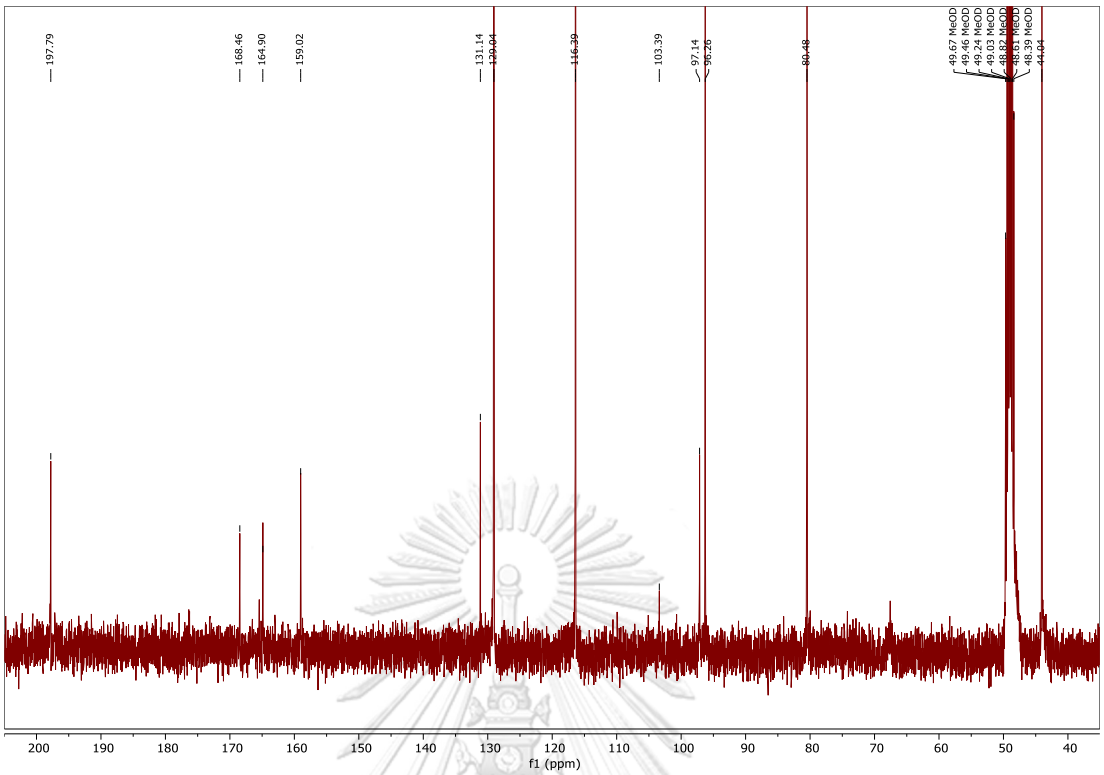
- Freezer (-20 ℃), model: MDF-U332, Sanyo, Japan
- Refrigerator (4 ℃), model: NR-BT262, Panasonic, Thailand
- 96-well microplate, Thermo scientific, Germany
- Automatic micropipette, model: P10, P20, P100, P200 and P1,000 µL, Gilson, France
- Beaker, model: 50, 250, 600 and 1000 mL, Pyrex®, Germany
- Superspeed centrifuge, model: Sorvall™ RC 6 Plus Thermo scientific, Germany
- Centrifuge tube, model: 15 and 50 mL, Sarstedt, Germany
- Circulating aspirator, model: WJ-20, Sibata, Japan
- Column chromatography, model: 250 mL, Schott Duran, Germany
- Flask, model: 50, 250, 500 and 1,000 mL, Schott Duran, Germany
- High performance liquid chromatography (HPLC), Waters Corporation, Anaheim, California, USA
- Measuring cylinder, model: 10, 100, 500 and 1,000 mL, Witex, Germany
- Microplate reader, model: Sunrise, Tecan, Austria
- Microtube, model: 1.5 mL, Sarstedt, Germany
- Rotary evaporator, model: Basis Hei-VAP Value, Heidolph, Germany
- 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
- Ultraviolet light, model: AB-409U electronic money detector, China
- Vortex mixer, model: KMC-1300V, Vision Scientific Co., Ltd., South Korea
- Weighing machine, model: 240A , Precisa instrument Ltd., Switzerland

Appendix C: ^{1}H and ^{13}C NMR of naringenin in CD_3OD

C1: ^{1}H NMR peak data at chemical shift (δ), 2.0-8.0 ppm in length
C2: ^{13}C NMR peak data at chemical shift (δ), 40-200 ppm in length



(C1)

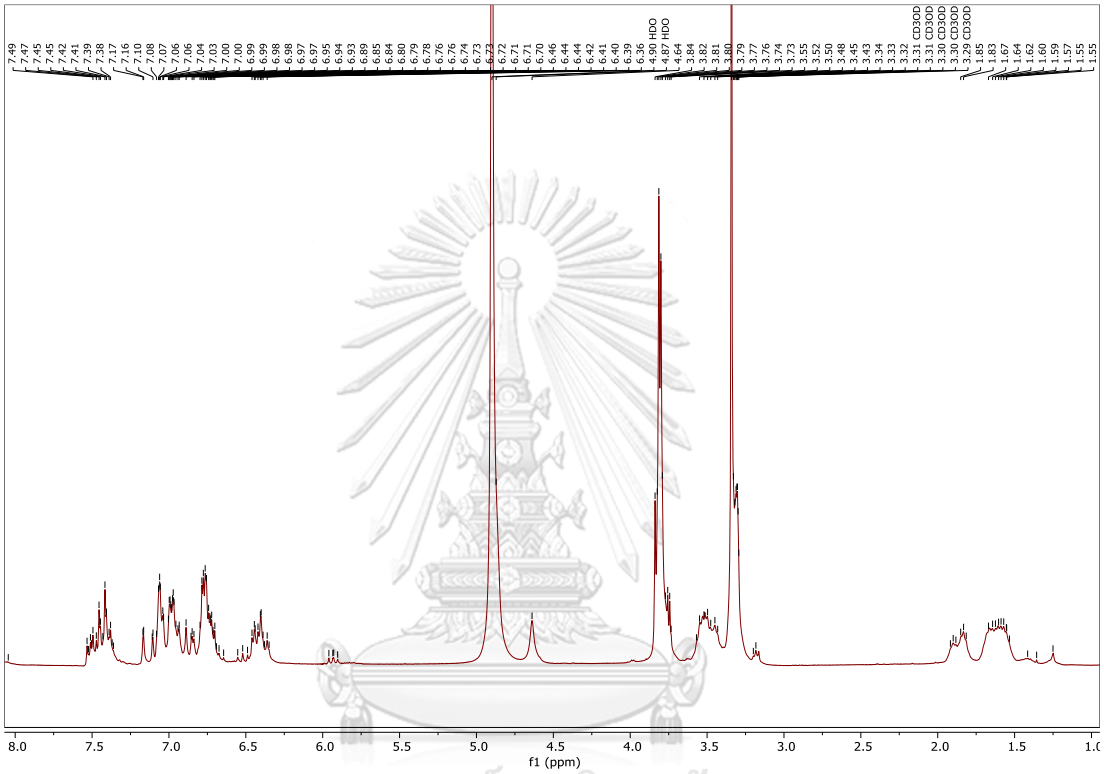


(C2)

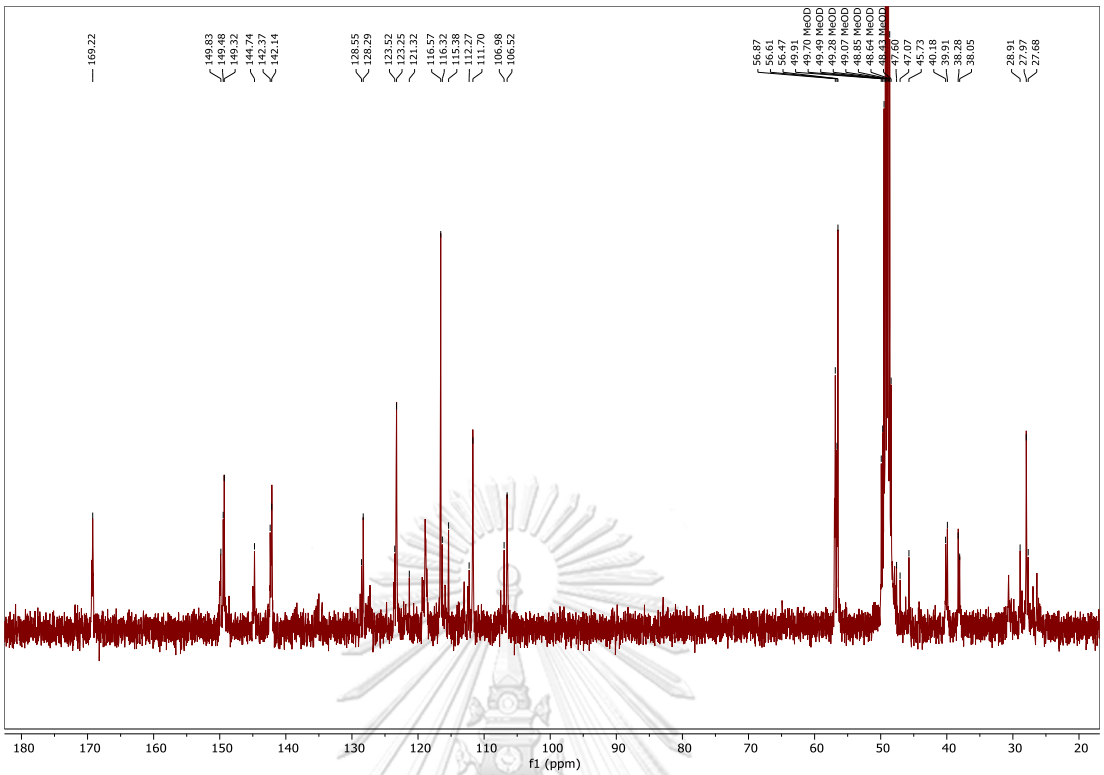
Appendix D: ^{1}H and ^{13}C NMR of fraction number DCMMBP3-1 in CD_3OD

D1: ^{1}H NMR peak data at chemical shift (δ), 1.0-8.0 ppm in length

D2: ^{13}C NMR peak data at chemical shift (δ), 20-180 ppm in length



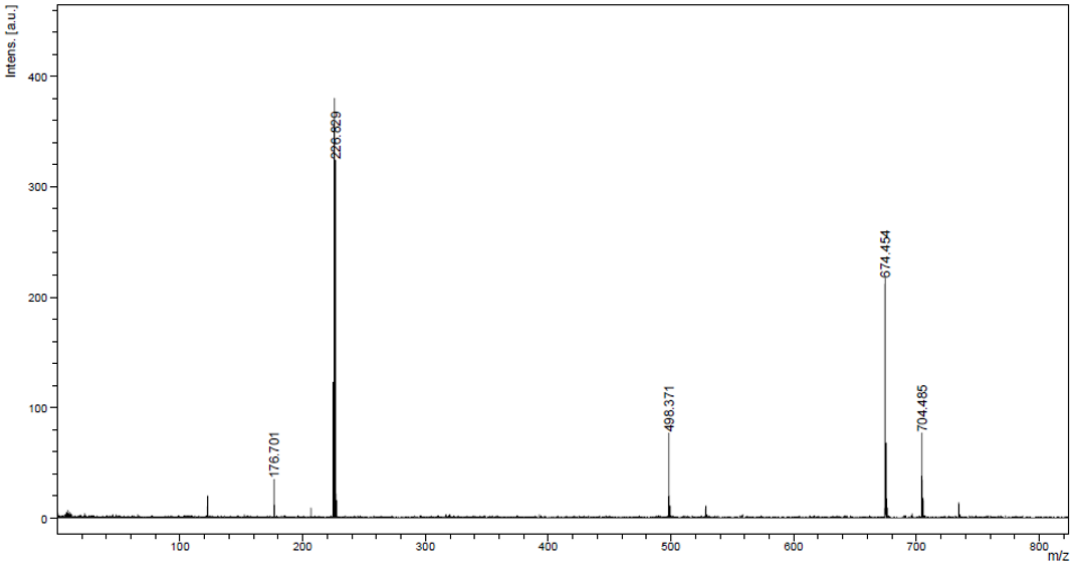
(D1)



(D2)

Appendix E: Mass spectrum of fraction number DCMMBP3-1

E1: Mass spectrum of fraction number DCMMBP3-1 at m/z 100-800

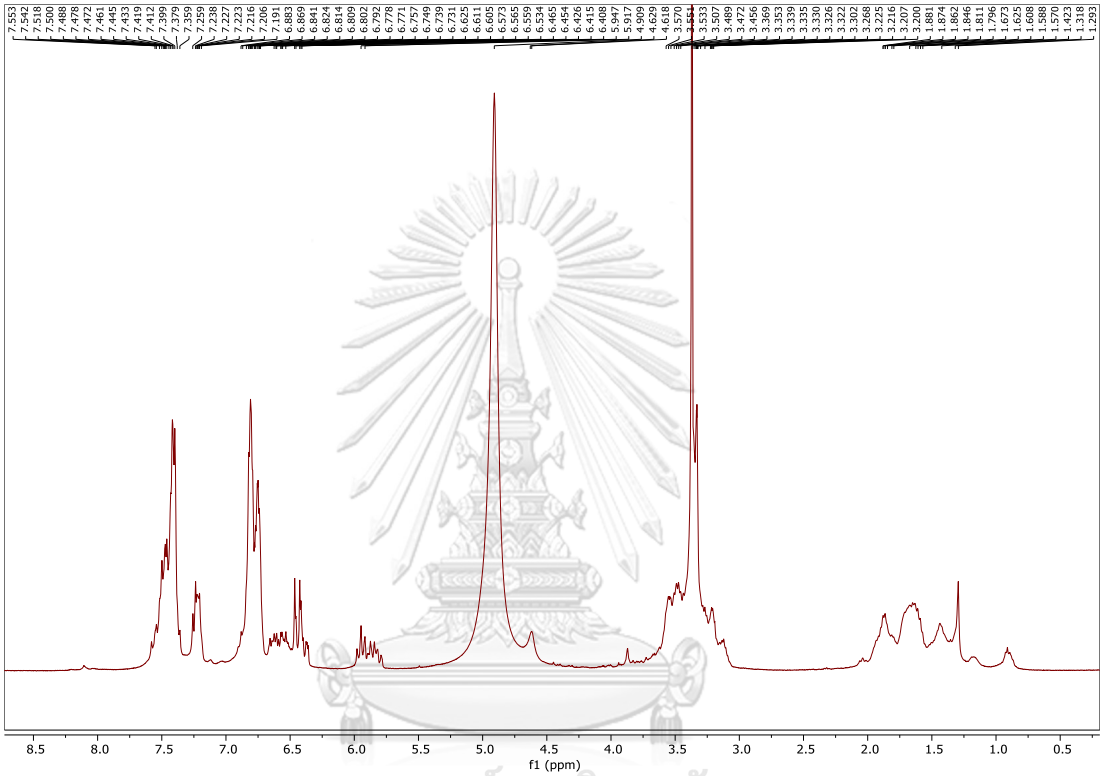


(E1)

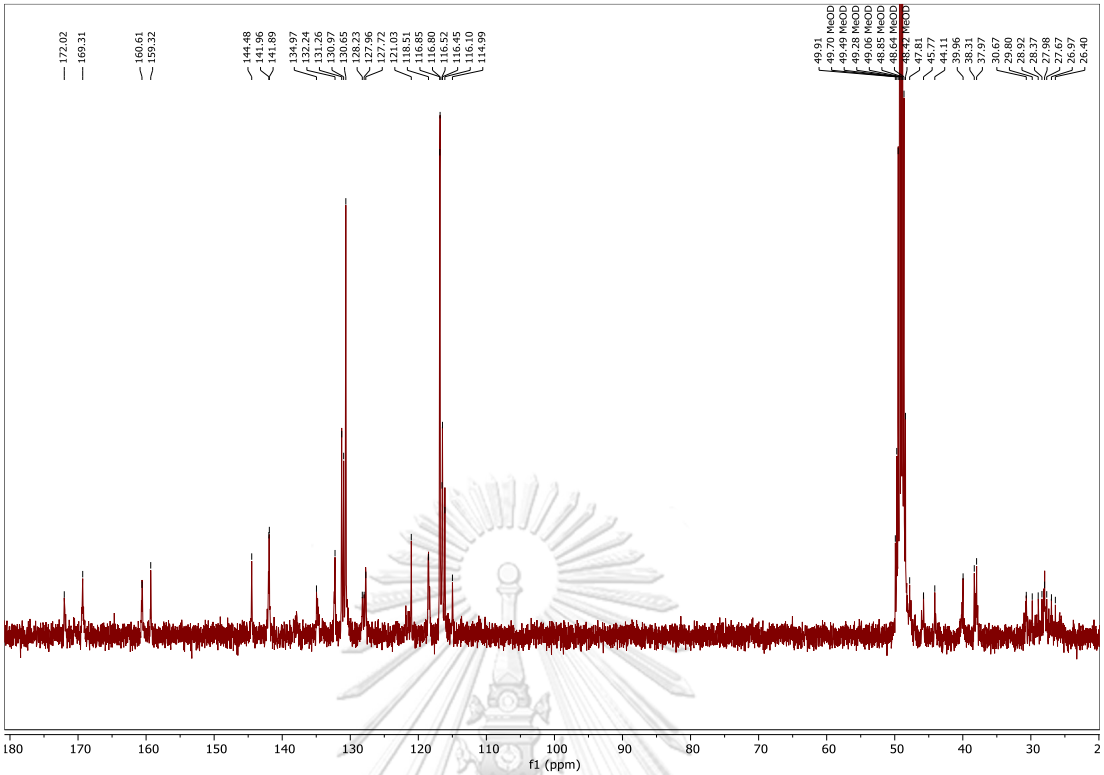
Appendix F: ^{1}H and ^{13}C NMR of fraction number DCMSBP5 in CD_3OD

F1: ^{1}H NMR peak data at chemical shift (δ), 0.0-8.5 ppm in length

F2: ^{13}C NMR peak data at chemical shift (δ), 2-180 ppm in length



(F1)

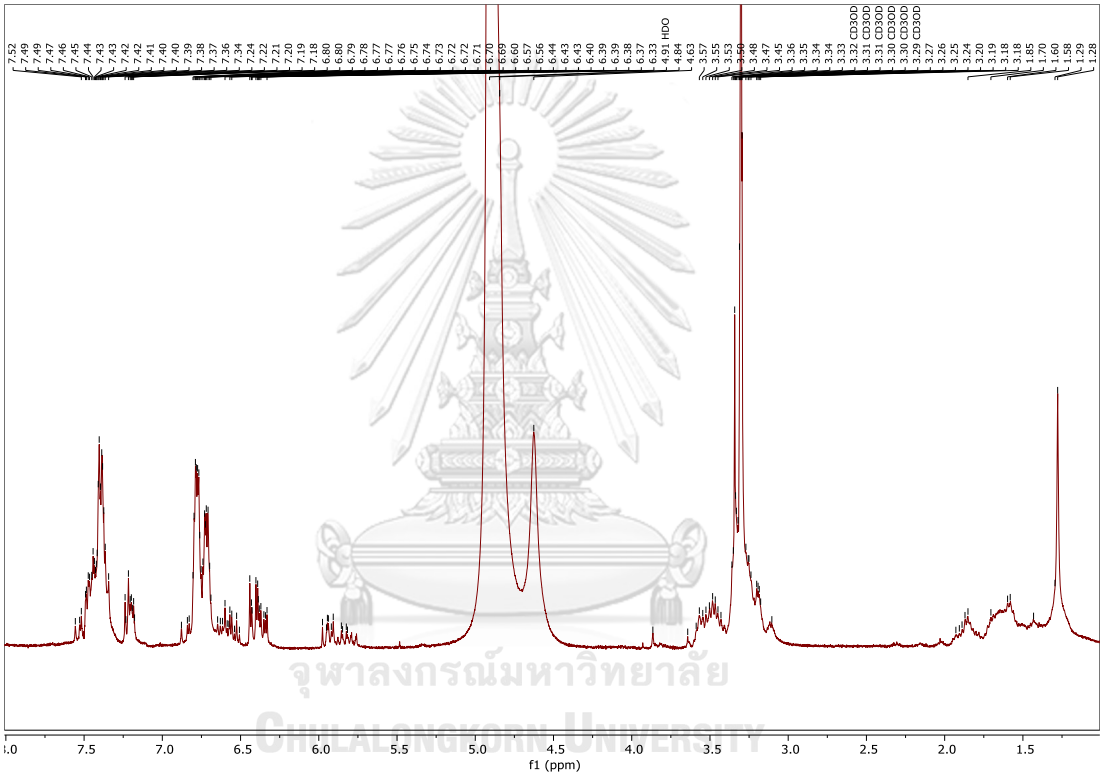


(F2)

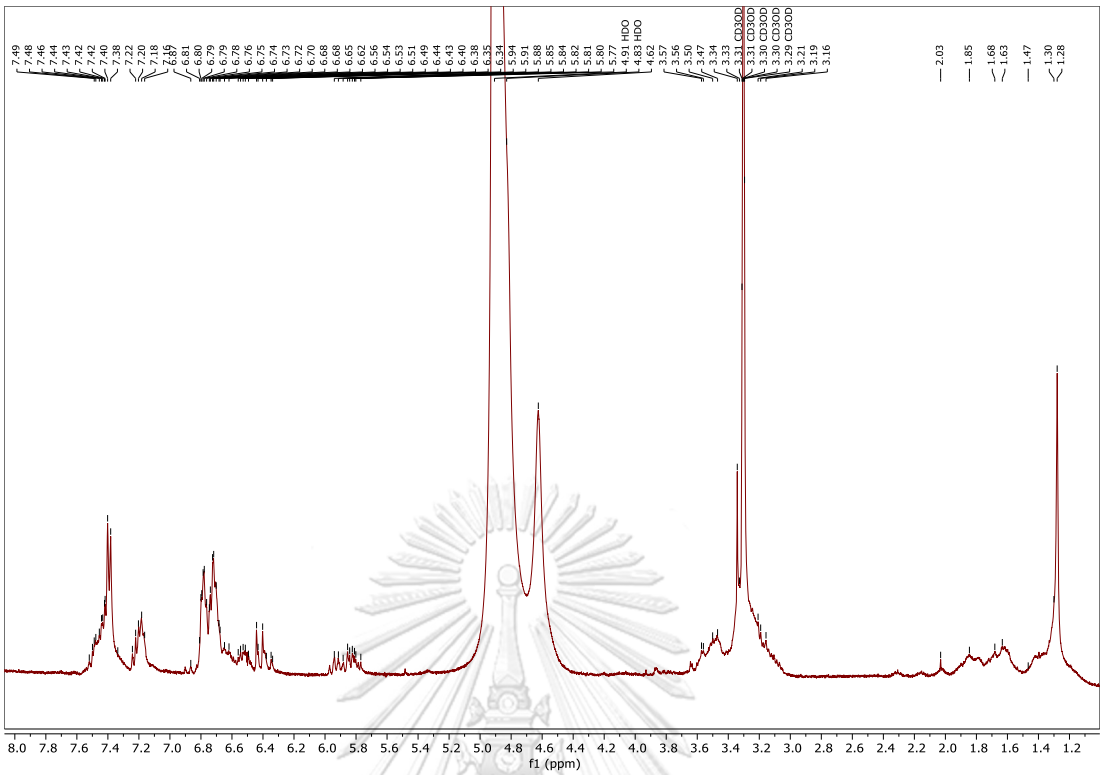
Appendix G: ^{1}H NMR of fraction number DCMSBP5-1 and DCMSBP5-2 in CD_3OD

G1: ^{1}H NMR peak data at chemical shift (δ), 1.0-8.0 ppm in length of fraction number DCMSBP5-1

G2: ^{1}H NMR peak data at chemical shift (δ), 1.0-8.0 ppm in length of fraction number DCMSBP5-2



(G1)



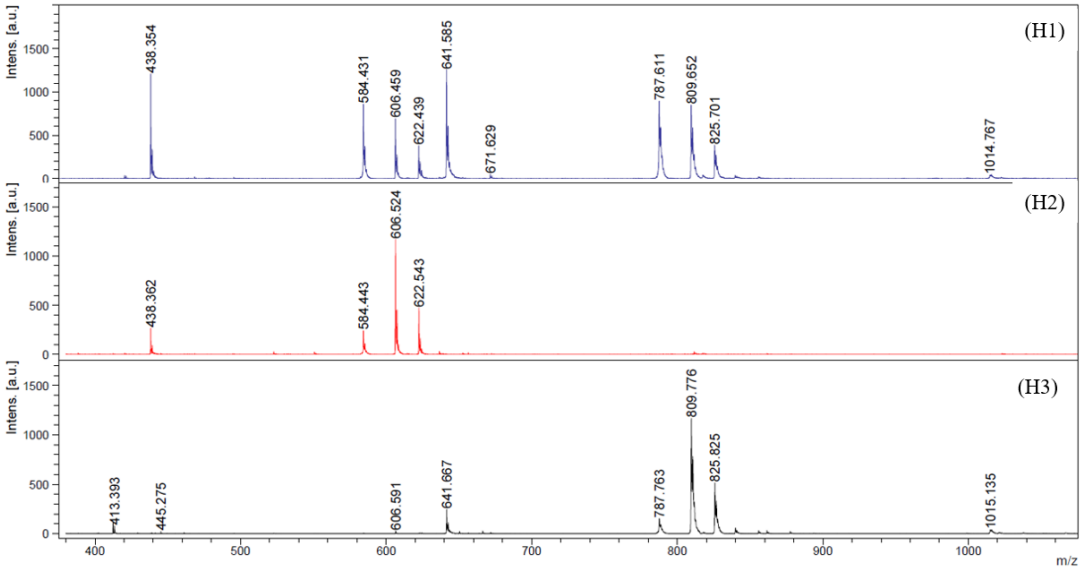
(G2)

Appendix H: Mass spectrum of fraction number DCMSBP5, DCMSBP5-1, and DCMSBP5-2

H1: Mass spectrum of fraction number DCMSBP5 at m/z 400-1060

H2: Mass spectrum of fraction number DCMSBP5-1 at m/z 400-1060

H3: Mass spectrum of fraction number DCMSBP5-2 at m/z 400-1060



Appendix I: Raw data of the percentage of free radical scavenging activity of MTTBP, DCMTBP, and HXTBP (Remark: TBP as tea flower bee pollen)

Table I1: The percentage of free radical scavenging activity of MTTBP

MTTBP	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	0.71	-0.83	0.24	18.19
Rep. 2	0	-0.36	-1.56	2.76	19.83
Rep. 3	0	0.35	-0.47	4.84	19.48
Mean	0	0.24	-0.96	2.61	19.17
SD.	0	0.55	0.56	2.31	0.86

Table I2: The percentage of free radical scavenging activity of DCMTBP

DCMTBP	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	-0.47	2.34	9.85	33.99
Rep. 2	0	4.77	3.37	11.52	34.11
Rep. 3	0	4.06	4.76	11.48	33.64
Mean	0	2.79	3.49	10.95	33.92
SD.	0	2.84	1.21	0.95	0.24

Table I3: The percentage of free radical scavenging activity of HXTBP

HXTBP	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	-1.78	0.89	-4.01	8.03
Rep. 2	0	-1.84	-1.49	0.34	6.21
Rep. 3	0	-0.11	-0.46	0.34	8.87
Mean	0	-1.25	-0.35	-1.11	7.70
SD.	0	0.98	1.19	2.52	1.36

Appendix J: Raw data of the percentage of free radical scavenging activity of MTSBP, DCMSBP, and HXSBP (Remark: SBP as sunflower bee pollen)

Table J1: The percentage of free radical scavenging activity of MTSBP

MTSBP	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	1.99	1.33	3.21	16.06
Rep. 2	0	4.28	0.99	1.86	13.93
Rep. 3	0	2.39	3.07	5.69	18.20
Mean	0	2.89	1.80	3.59	16.06
SD.	0	1.22	1.12	1.94	2.14

Table J2: The percentage of free radical scavenging activity of DCMSBP

DCMSBP	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	2.01	1.79	3.24	23.13
Rep. 2	0	-4.46	-4.69	2.29	19.91
Rep. 3	0	-1.32	0.44	6.50	26.13
Mean	0	-1.26	-0.82	4.01	23.06
SD.	0	3.24	3.42	2.21	3.11

Table J3: The percentage of free radical scavenging activity of HXSBP

HXSBP	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	-1.27	-1.90	2.53	10.01
Rep. 2	0	-1.57	0.72	10.02	14.25
Rep. 3	0	-3.41	-4.74	-4.50	6.33
Mean	0	-2.08	-1.97	2.69	10.19
SD.	0	1.16	2.74	7.26	3.97

Appendix K: Raw data of the percentage of free radical scavenging activity of MTMBP, DCMMBP, and HXMBP (Remark: MBP as mimosa flower bee pollen)

Table K1: The percentage of free radical scavenging activity of MTMBP

MTMBP	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	0.5	-0.5	-0.38	5.75
Rep. 2	0	0.84	0.24	-1.08	5.86
Rep. 3	0	1.54	-0.83	-1.06	7.03
Mean	0	0.96	-0.36	-0.84	6.51
SD.	0	0.53	0.55	0.40	1.23

Table K2: The percentage of free radical scavenging activity of DCMMBP

DCMMBP	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	3.32	17.29	51.66	84.59
Rep. 2	0	8.47	19.37	54.06	87.36
Rep. 3	0	4.55	19.78	55.77	89.56
Mean	0	5.44	18.82	53.83	87.17
SD.	0	2.69	1.33	2.07	2.49

Table K3: The percentage of free radical scavenging activity of HXMBP

HXMBP	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	-0.24	0.49	1.47	9.16
Rep. 2	0	-0.60	0.48	1.08	9.39
Rep. 3	0	-1.33	-0.24	1.09	10.14
Mean	0	-0.72	0.24	1.21	9.56
SD.	0	0.55	0.42	0.22	0.52

Table K4: The percentage of free radical scavenging activity of ascorbic acid

Ascorbic acid	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	1.32	32.93	97.25	95.57
Rep. 2	0	4.36	40.87	95.98	94.47
Rep. 3	0	3.49	34.86	98.32	97.75
Mean	0	3.06	36.22	97.18	95.93
SD.	0	1.57	4.14	1.17	1.67

Appendix L: Raw data of the percentage of tyrosinase inhibition of MTTBP, DCMTBP, and HXTBP (Remark: TBP as tea flower bee pollen)

Table L1: The percentage of tyrosinase inhibition of MTTBP

MTTBP	The percentage of tyrosinase inhibition				
	0 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
Rep. 1	0	5.86	5.99	25.72	38.31
Rep. 2	0	5.59	10.27	19.09	38.45
Rep. 3	0	3.96	12.47	20.78	39.48
Mean	0	5.14	9.58	21.86	38.75
SD.	0	1.03	3.29	3.44	0.64

Table L2: The percentage of tyrosinase inhibition of DCMTBP

DCMTBP	The percentage of tyrosinase inhibition				
	0 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
Rep. 1	0	6.47	26.99	45.28	55.25
Rep. 2	0	19.86	37.67	49.97	54.45
Rep. 3	0	19.89	36.89	51.93	59.07
Mean	0	15.41	33.85	49.06	56.26
SD.	0	7.74	5.96	3.42	2.47

Table L3: The percentage of tyrosinase inhibition of HXTBP

HXTBP	The percentage of tyrosinase inhibition				
	0 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
Rep. 1	0	-3.32	-1.53	2.21	-3.47
Rep. 2	0	-4.93	0.37	-1.11	-3.34
Rep. 3	0	-9.01	-5.74	-1.61	-12.23
Mean	0	-5.75	-2.29	-0.17	-6.34
SD.	0	2.94	3.13	2.08	5.09

Appendix M: Raw data of the percentage of tyrosinase inhibition of MTSBP, DCMSBP, and HXSBP (Remark: SBP as sunflower bee pollen)

Table M1: The percentage of tyrosinase inhibition of MTSBP

MTSBP	The percentage of tyrosinase inhibition				
	0 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
Rep. 1	0	3.74	8.59	16.92	24.41
Rep. 2	0	4.18	19.92	17.20	29.11
Rep. 3	0	3.94	14.57	12.29	27.00
Mean	0	3.95	14.36	15.47	26.84
SD.	0	0.22	5.66	2.76	2.35

Table M2: The percentage of tyrosinase inhibition of DCMSBP

DCMSBP	The percentage of tyrosinase inhibition				
	0 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
Rep. 1	0	43.83	48.71	52.58	72.09
Rep. 2	0	46.09	50.00	53.31	65.61
Rep. 3	0	45.58	48.50	54.49	63.18
Mean	0	45.17	49.07	53.46	66.96
SD.	0	1.19	0.81	0.97	4.61

Table M3: The percentage of tyrosinase inhibition of HXSBP

HXSBP	The percentage of tyrosinase inhibition				
	0 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
Rep. 1	0	-1.17	3.13	-2.74	-11.61
Rep. 2	0	-1.15	3.85	-0.38	2.82
Rep. 3	0	1.88	0.75	-2.25	-22.58
Mean	0	-0.15	2.58	-1.79	-10.46
SD.	0	1.76	1.62	1.24	12.74

Appendix N: Raw data of the percentage of tyrosinase inhibition of MTMBP, DCMMBP, and HXMBP (Remark: MBP as mimosa flower bee pollen)

Table N1: The percentage of tyrosinase inhibition of MTMBP

MTMBP	The percentage of tyrosinase inhibition				
	0 µg/mL	**62.5 µg/mL**	**125 µg/mL**	**250 µg/mL**	**500 µg/mL**
Rep. 1	0	2.42	3.30	1.98	-2.93
Rep. 2	0	-3.84	-0.68	-1.58	-1.88
Rep. 3	0	-0.22	6.27	-0.43	-0.29
Mean	0	-0.55	2.96	-0.01	-1.70
SD.	0	3.14	3.49	1.82	1.33

Table N2: The percentage of tyrosinase inhibition of DCMMBP

DCMMBP	The percentage of tyrosinase inhibition				
	0 µg/mL	**62.5 µg/mL**	**125 µg/mL**	**250 µg/mL**	**500 µg/mL**
Rep. 1	0	6.94	13.53	-2.10	13.32
Rep. 2	0	5.73	13.01	11.24	6.86
Rep. 3	0	11.35	20.14	20.27	16.49
Mean	0	8.01	15.56	9.80	12.22
SD.	0	2.96	3.97	11.26	4.91

Table N3: The percentage of tyrosinase inhibition of HXMBP

HXMBP	The percentage of tyrosinase inhibition				
	0 µg/mL	**62.5 µg/mL**	**125 µg/mL**	**250 µg/mL**	**500 µg/mL**
Rep. 1	0	-5.25	-5.32	-6.46	-16.47
Rep. 2	0	-5.17	-7.97	-9.09	-0.91
Rep. 3	0	-6.69	-6.97	-8.03	-18.65
Mean	0	-5.70	-6.75	-7.95	-12.01
SD.	0	0.86	1.34	1.35	9.67

Table N4: The percentage of tyrosinase inhibition of kojic acid

Kojic acid	The percentage of tyrosinase inhibition						
	0 µg/mL	**0.32 µg/mL**	**1.60 µg/mL**	**8 µg/mL**	**40 µg/mL**	**200 µg/mL**	**1,000 µg/mL**
Rep. 1	0	9.03	12.93	43.09	84.95	96.17	98.97
Rep. 2	0	8.48	10.34	42.83	84	95.72	98.76
Rep. 3	0	8.17	10.06	45.88	84.85	97.14	97.78
Mean	0	8.56	11.11	43.93	84.60	96.34	98.50
SD.	0	0.43	1.58	1.69	0.52	0.72	0.64

Appendix O: Raw data of the percentage of free radical scavenging activity of fraction number DCMMBP1-DCMMBP3 after silica column chromatography

Table O1: The percentage of free radical scavenging activity of fraction number DCMMBP1

DCMMBP1	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	2.19	9.69	26.56	59.38
Rep. 2	0	3.88	10.03	26.21	60.84
Rep. 3	0	-1.95	6.49	26.30	58.77
Mean	0	1.37	8.74	26.36	59.66
SD.	0	2.99	1.95	0.18	1.07

Table O2: The percentage of free radical scavenging activity of fraction number DCMMBP2

DCMMBP2	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	-0.66	10.93	38.41	83.77
Rep. 2	0	-2.06	8.93	38.49	87.97
Rep. 3	0	-1.33	8.33	37.33	84.33
Mean	0	-1.35	9.40	38.08	85.36
SD.	0	0.7	1.36	0.65	2.28

Table O3: The percentage of free radical scavenging activity of fraction number DCMMBP3

DCMMBP3	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	8.02	33.95	79.94	85.80
Rep. 2	0	10.33	35.87	78.12	82.07
Rep. 3	0	8.72	36.14	81.31	82.24
Mean	0	9.03	35.32	79.79	83.37
SD.	0	1.18	1.19	1.60	2.11

Table O4: The percentage of free radical scavenging activity of ascorbic acid

Ascorbic acid	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	7.28	42.41	95.25	95.89
Rep. 2	0	7.72	41.80	95.18	95.50
Rep. 3	0	6.69	41.40	94.90	95.22
Mean	0	7.22	41.87	95.11	95.54
SD.	0	0.52	0.51	0.18	0.33

Appendix P: Raw data of the percentage of free radical scavenging activity of fraction number DCMMBP3-1 and DCMMBP3-2 after size exclusion column chromatography

Table P1: The percentage of free radical scavenging activity of fraction number DCMMBP3-1

DCMMBP 3-1	The percentage of free radical scavenging activity				
	0 µg/mL	15.625 µg/mL	62.5 µg/mL	250 µg/mL	1,000 µg/mL
Rep. 1	0	8.31	33.85	72.92	80.92
Rep. 2	0	10.67	36.59	77.13	80.49
Rep. 3	0	10.06	36.59	75.30	83.84
Mean	0	9.68	35.67	75.12	81.75
SD.	0	1.23	1.58	2.11	1.82

Table P2: The percentage of free radical scavenging activity of fraction number DCMMBP3-2

DCMMBP 3-2	The percentage of free radical scavenging activity				
	0 µg/mL	15.625 µg/mL	62.5 µg/mL	250 µg/mL	1,000 µg/mL
Rep. 1	0	-3.11	-0.62	4.67	21.50
Rep. 2	0	-1.24	2.48	6.81	23.84
Rep. 3	0	-0.62	0.93	4.95	22.91
Mean	0	-1.66	0.93	5.48	22.75
SD.	0	1.30	1.55	1.16	1.18

Appendix Q: Raw data of the percentage of tyrosinase inhibition of fraction number DCMMBP1-DCMMBP3 at concentration of 50 µg/mL after silica column chromatography

Table Q1: The percentage of tyrosinase inhibition of fraction number DCMMBP1-DDCMMBP3 and kojic acid at concentration of 50 µg/mL

	The percentage of tyrosinase inhibition at 50 µg/mL			
	Kojic acid	DCMMBP1	DCMMBP2	DCMMBP3
Rep. 1	72.79	1.16	0.93	-3.95
Rep. 2	74.60	2.77	1.39	-3.93
Rep. 3	75.17	4.66	6.65	3.99
Mean	74.18	2.86	2.99	-1.30
SD.	1.24	1.75	3.18	4.58

Appendix R: Raw data of the percentage of tyrosinase inhibition of fraction number DCMSBP1-DCMMBP5 at concentration of 500 µg/mL after silica column chromatography

Table R1: The percentage of tyrosinase inhibition of fraction number DCMSBP1-DCMSBP5 and kojic acid at concentration of 50 µg/mL

	The percentage of tyrosinase inhibition at 50 µg/mL					
	Kojic acid	**DCM SBP1**	**DCM SBP2**	**DCM SBP3**	**DCM SBP4**	**DCM SBP5**
Rep. 1	89.95	-5.50	-3.11	-3.83	28.95	47.61
Rep. 2	89.77	-5.81	-8.60	2.79	35.12	56.74
Rep. 3	89.96	6.70	-1.34	5.36	39.73	52.90
Mean	89.89	-1.54	-4.35	1.44	34.60	52.42
SD.	0.11	7.13	3.79	4.74	5.41	4.59

Appendix S: Raw data of the percentage of tyrosinase inhibition of fraction number DCMSBP5 after silica column chromatography

Table S1: The percentage of tyrosinase inhibition of fraction number DCMSBP5

DCMSBP5	**The percentage of tyrosinase inhibition**				
	0 µg/mL	**3.13 µg/mL**	**6.25 µg/mL**	**12.5 µg/mL**	**25 µg/mL**
Rep. 1	0	24.65	38.46	45.96	52.47
Rep. 2	0	25.15	39.10	44.79	52.46
Rep. 3	0	27.54	39.65	45.70	51.95
Mean	0	25.78	39.07	45.48	52.29
SD.	0	1.54	0.59	0.61	0.29

Table S2: The percentage of tyrosinase inhibition of kojic acid

Kojic acid	**The percentage of tyrosinase inhibition**				
	0 µg/mL	**3.13 µg/mL**	**6.25 µg/mL**	**12.5 µg/mL**	**25 µg/mL**
Rep. 1	0	11.92	30.77	52.31	75.77
Rep. 2	0	16.15	33.76	59.63	77.61
Rep. 3	0	14.04	32.64	58.44	77.61
Mean	0	14.04	32.39	56.79	76.99
SD.	0	2.11	1.51	3.93	1.06

Appendix T: Raw data of the percentage of tyrosinase inhibition of fraction number DCMMBP5-1 and DCMSBP5-2 after high pressure liquid chromatography (HPLC)

Table T1: The percentage of tyrosinase inhibition of fraction number DCMSBP5-1

DCMSBP 5-1	The percentage of tyrosinase inhibition				
	0 µg/mL	3.13 µg/mL	6.25 µg/mL	12.5 µg/mL	25 µg/mL
Rep. 1	0	39.25	52.66	55.82	60.55
Rep. 2	0	39.36	52.01	59.44	61.24
Rep. 3	0	39.88	50.88	61.29	64.44
Mean	0	39.50	51.85	58.85	62.08
SD.	0	0.34	0.89	2.79	2.07

Table T2: The percentage of tyrosinase inhibition of fraction number DCMSBP5-2

DCMSBP 5-2	The percentage of tyrosinase inhibition				
	0 µg/mL	3.13 µg/mL	6.25 µg/mL	12.5 µg/mL	25 µg/mL
Rep. 1	0	38.48	47.53	45.27	47.94
Rep. 2	0	41.57	43.98	48.80	49.40
Rep. 3	0	32.11	37.16	40.37	47.25
Mean	0	37.38	42.89	44.81	48.20
SD.	0	4.82	5.27	4.23	1.10

Table T3: The percentage of tyrosinase inhibition of kojic acid

Kojic acid	The percentage of tyrosinase inhibition				
	0 µg/mL	3.13 µg/mL	6.25 µg/mL	12.5 µg/mL	25 µg/mL
Rep. 1	0	28.23	43.14	61.03	83.10
Rep. 2	0	26.15	44.31	64.27	80.44
Rep. 3	0	29.70	45.15	64.55	81.39
Mean	0	28.02	44.20	63.29	81.64
SD.	0	1.79	1.01	1.96	1.35

Appendix U: Raw data of the percentage of free scavenging activity of fraction number DCMSBP1-DCMMBP5 at concentration of 1,000 µg/mL after silica column chromatography

Table U1: The percentage of scavenging activity of fraction number DCMSBP1-DCMSBP5 and ascorbic acid at concentration of 1000 µg/mL

	The percentage of free radical scavenging activity at 1,000 µg/mL					
	Ascorbic acid	**DCM SBP1**	**DCM SBP2**	**DCM SBP3**	**DCM SBP4**	**DCM SBP5**
Rep. 1	93.31	53.53	88.85	72.12	52.79	11.15
Rep. 2	95.29	48.15	86.53	72.72	58.59	17.51
Rep. 3	94.92	48.81	88.14	75.59	60.34	17.97
Mean	94.50	50.16	87.84	73.48	57.24	15.54
SD.	1.05	2.93	1.19	1.86	3.95	3.81

Appendix V: Raw data of the percentage of free radical scavenging activity of fraction number DCMSBP1-DCMMBP4 after silica column chromatography

Table V1: The percentage of free radical scavenging activity of fraction number DCMSBP1

DCMSBP1	**The percentage of free radical scavenging activity**				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1000 µg/mL**
Rep. 1	0	2.23	2.87	14.65	50.32
Rep. 2	0	0.64	1.60	14.70	50.80
Rep. 3	0	-0.32	1.92	15.34	49.84
Mean	0	0.85	2.13	14.89	50.32
SD.	0	1.29	0.66	0.38	0.48

Table V2: The percentage of free radical scavenging activity of fraction number DCMSBP2

DCMSBP2	**The percentage of free radical scavenging activity**				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1000 µg/mL**
Rep. 1	0	1.85	7.69	28.92	88
Rep. 2	0	0.92	7.38	29.85	88.31
Rep. 3	0	4.59	8.87	33.33	88.38
Mean	0	2.45	7.98	30.70	88.23
SD.	0	1.91	0.78	2.33	0.20

Table V3: The percentage of free radical scavenging activity of fraction number DCMSBP3

DCMSBP3	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1000 µg/mL**
Rep. 1	0	-0.29	8.48	30.12	73.10
Rep. 2	0	2.12	7.88	29.10	73.03
Rep. 3	0	3.47	7.89	31.23	76.97
Mean	0	1.77	8.08	30.15	74.37
SD.	0	1.91	0.34	1.07	2.26

Table V4: The percentage of free radical scavenging activity of fraction number DCMSBP4

DCMSBP4	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1000 µg/mL**
Rep. 1	0	2.75	5.50	20.18	58.41
Rep. 2	0	1.23	4.60	19.63	60.12
Rep. 3	0	1.84	6.13	20.55	60.12
Mean	0	1.94	5.41	20.12	59.55
SD.	0	0.77	0.77	0.46	0.99

Table V5: The percentage of free radical scavenging activity of ascorbic acid

Ascorbic acid	The percentage of free radical scavenging activity				
	0 µg/mL	**15.625 µg/mL**	**62.5 µg/mL**	**250 µg/mL**	**1,000 µg/mL**
Rep. 1	0	9.93	46.81	95.39	96.45
Rep. 2	0	9.60	47.02	95.03	95.36
Rep. 3	0	12.90	44.84	95.84	96.63
Mean	0	10.81	46.22	95.84	96.63
SD.	0	1.82	1.20	1.10	1.36

Appendix W: Regression analysis of free radical scavenging and tyrosinase inhibitory activities with correlation coefficient (r^2)

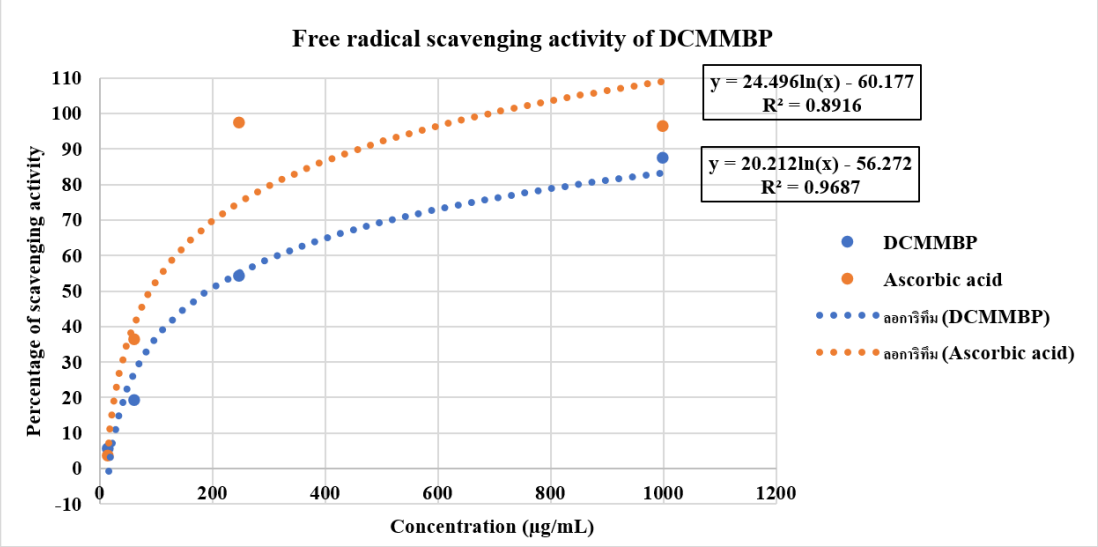


Figure 1 Free radical scavenging activity of DCMMBP

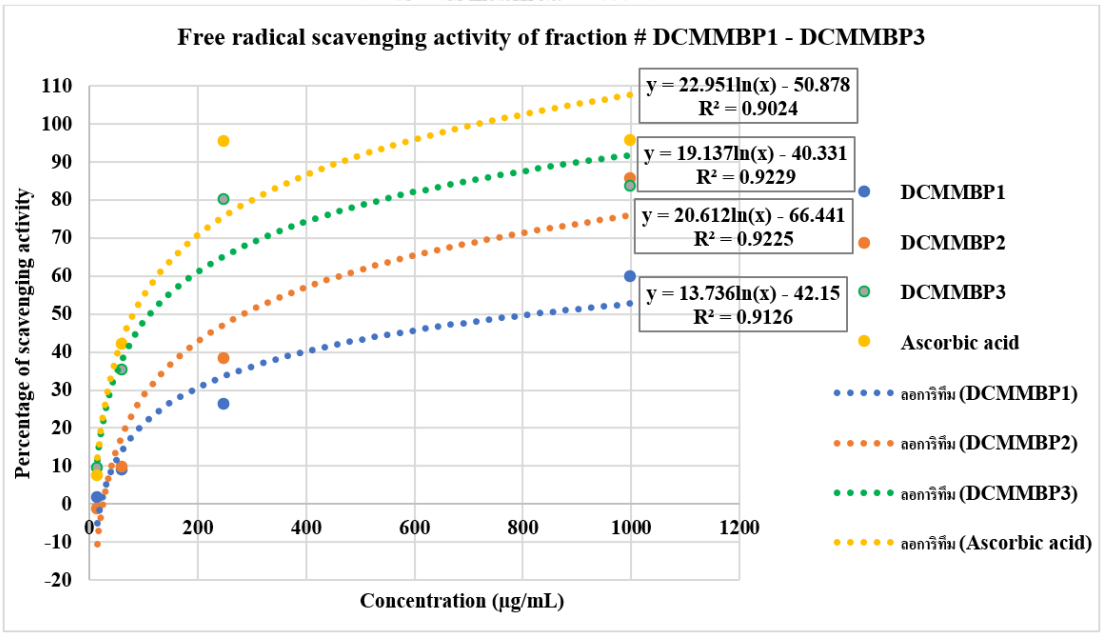


Figure 2 Free radical scavenging activity of fraction number DCMMBP1 – DCMMBP3

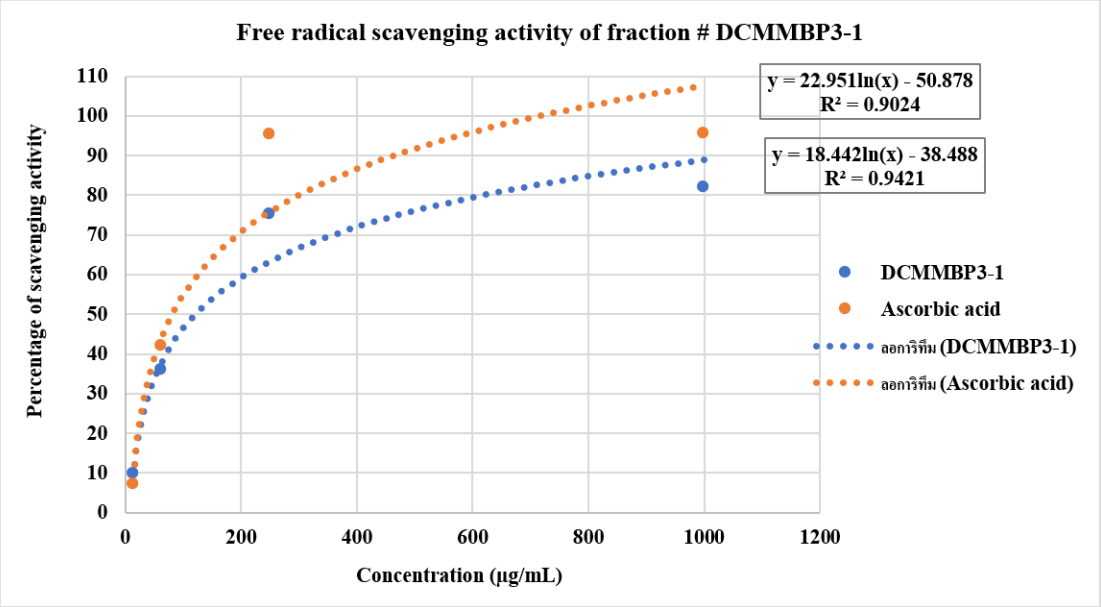


Figure 3 Free radical scavenging activity of fraction number DCMMBP3-1

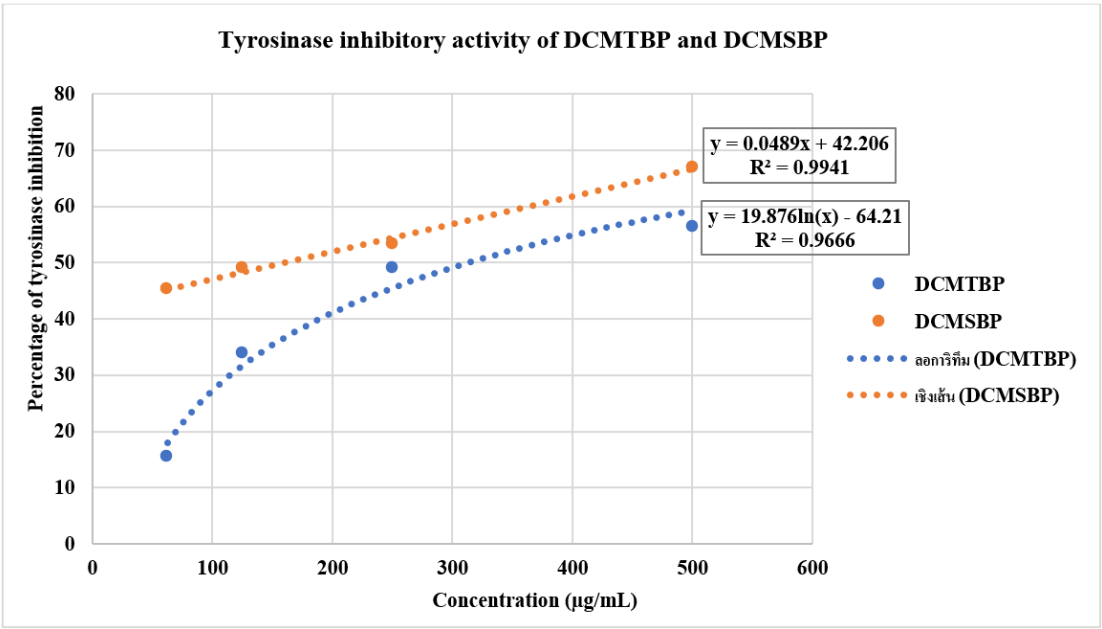


Figure 4 Tyrosinase inhibitory activity of DCMTBP and DCMSBP

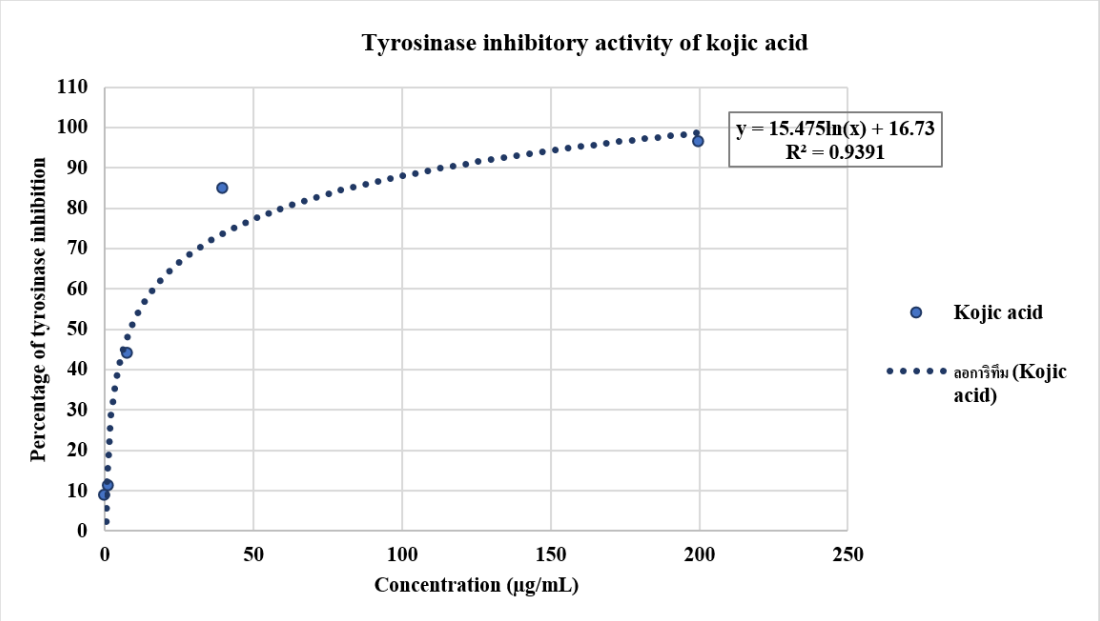


Figure 5 Tyrosinase inhibitory activity of kojic acid

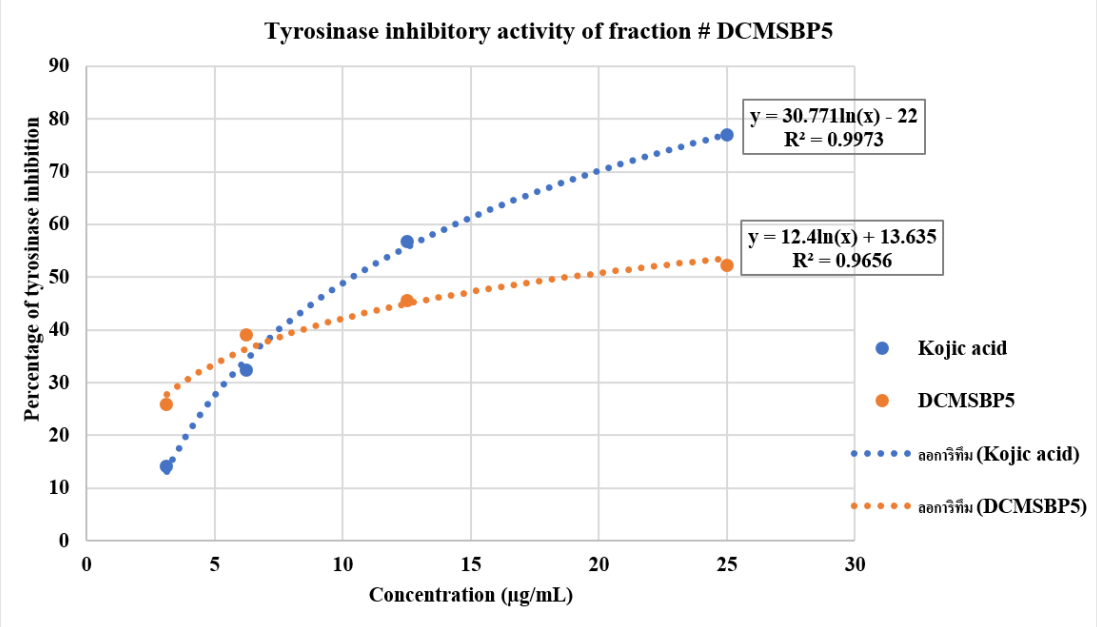


Figure 6 Tyrosinase inhibitory activity of fraction number DCMSBP5

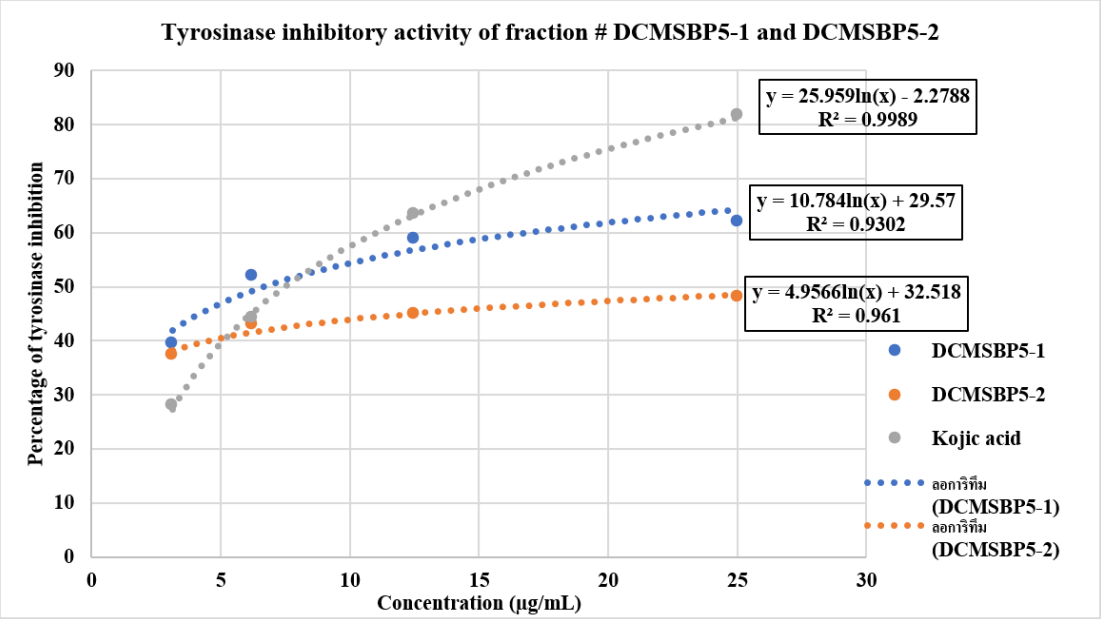


Figure 7 Tyrosinase inhibitory activity of fraction number DCMSBP5-1 and DCMSBP5-2

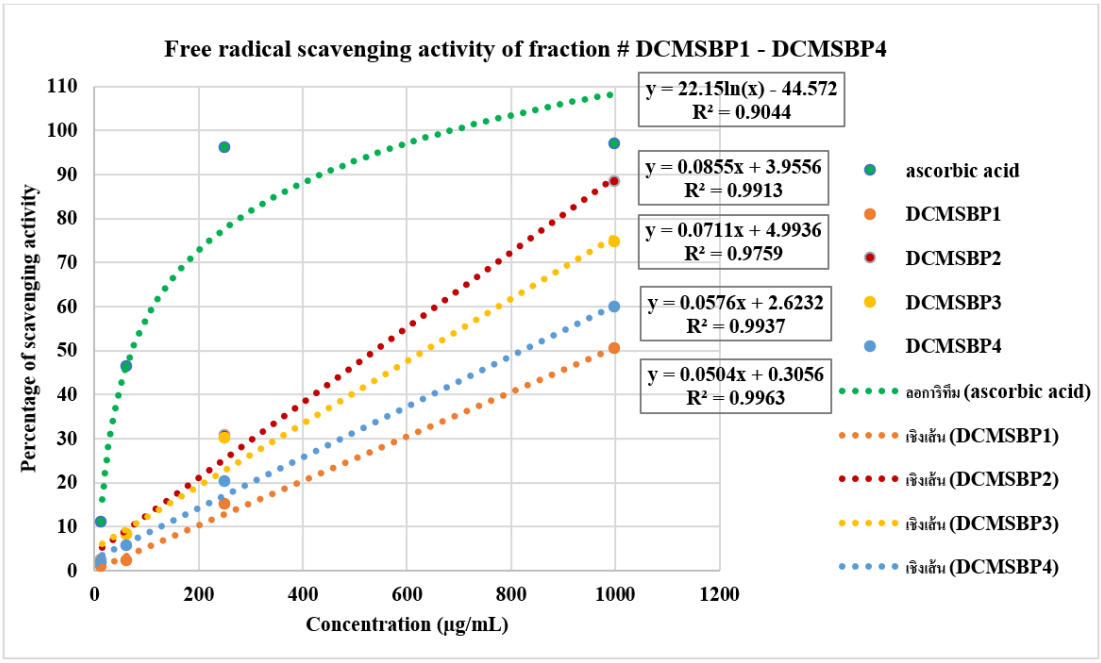


Figure 8 Free radical scavenging activity of fraction number DCMSBP1 – DCMSBP4

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