สารต้านออกซิเดชันจากรากต้นพีพวนน้อย *Uvaria rufa* Blume

นางสาวกนกภรณ์ พยาฆรินทรังกูร

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ANTIOXIDANTS FROM *Uvaria rufa* Blume ROOTS

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กนกภรณ์ พยาฆรินทรังกูร: สารต้านออกซิเดชันจากรากต้นพีพวนน้อย *Uvaria rufa* Blume (ANTIOXIDANTS FROM *Uvaria rufa* Blume ROOTS) อ. ที่ปรึกษา: รศ.ดร.สันติ ทิพยางค์ อ. ที่ปรึกษาร่วม: ผศ.ดร. ปรีชา ภูวไพรศิริศาล, 67 หน้า. ISBN 974-53-2637-2

ในการค้นหาสารที่มีฤทธิ์ต้านออกซิเดชั่นจากพืชสมุนไพรไทยจำนวน 40 ชนิด โดยทดสอบฤทธิ์เบื้องต้นในการต้านอนุมูลอิสระ DPPH(2,2-Diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl) ซึ่งเป็นอนุมูลอิสระที่เสถียร พบว่าสิ่งสกัดไดคลอโรมีเทนและสิ่งสกัดบิวทานอลของรากต้นพีพวนน้อย (*Uvaria rufa*) แสดงฤทธิ์ที่ดี ดังนั้นจึงเลือกนำมาศึกษาต่อไป โดยการแยกองค์ประกอบทางเคมีด้วยวิธีทางโครมาโทกราฟิก แยกสารได้ทั้งหมด 15 ชนิด ในส่วนของสิ่งสกัดไดคลอโรมีเทนพบสารในกลุ่มฟลาโวนอยด์ 6 ชนิด ได้แก่ 2,5-dihydroxy-7-methoxy flavanone (1), tectochrysin (2), 5-hydroxy-7-methoxy flavanone (3), 6,7-*O*,*O*-dimethylbaicalein (6), 7-*O*-methylwogonine (7) และ 2,5-dihydroxy-6,7-dimethoxy flavanone (8) และสารในกลุ่มอะโรแมติก 2 ชนิด ได้แก่ benzyl benzoate (4) และ 2-methoxybenzyl benzoate (5) ในส่วนของสิ่งสกัดบิวทานอลพบสารในกลุ่มแอลคาลอยด์ 7 ชนิด ได้แก่ liriodenine (9), lanuginosine (10), oxoanolobine (11), roemerine (12), anonaine (13), xylopine (14) และ roemeroline (15) โดยสาร 1, 2, 6 และ 7 เคยแยกได้จากรากต้นพีพวนน้อย นอกนั้นรายงานเป็นครั้งแรก การหาสูตรโครงสร้างของสารใช้วิธีทางสเปกโทรสโคปีและเปรียบเทียบกับข้อมูลที่มีรายงานไว้แล้ว การศึกษาฤทธิ์ต้านออกซิเดชั่นของสารที่แยกได้ใช้ 3 วิธี คือ การทดสอบฤทธิ์ต้านอนุมูลอิสระ DPPH การทดสอบฤทธิ์ต้านอนุมูลอิสระ superoxide และการทดสอบฤทธิ์ยับยั้งการทำงานของเอนไซม์ xanthine oxidase จากผลการศึกษาพบว่า สาร 8 แสดงฤทธิ์ต้านอนุมูลอิสระ DPPH และ superoxide ดีที่สุด โดยมีค่า IC_{50} = 0.16 และ 1.03 mg/mL ตามลำดับ ในส่วนของฤทธิ์ยับยั้งการทำงานของเอนไซม์ xanthine oxidase ไม่มีสารใดแสดงฤทธิ์ที่ดี

สาขาวิชา.....เทคโนโลยีชีวภาพ.... ลายมือชื่อนิสิต.....กนกภรณ์ พยาฆรินทรังกูร.....

ปีการศึกษา.....2548..... ลายมือชื่ออาจารย์ที่ปรึกษา.....สันติ ทิพยางค์.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....ปรีชา ภูวไพรศิริศาล.....

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KEY WORD: ANTIOXIDANT ACTIVITY /*Uvaria rufa*

KANOGPORN PAYAKARINTARUNGKUL: ANTIOXIDANTS FROM *Uvaria rufa* Blume ROOTS THESIS ADVISOR: ASSOC.PROF. SANTI TIP-PYANG, Ph.D. THESIS CO-ADVISOR: ASSIST.PROF. PREECHA PHUWAPRAISIRISAN, Ph.D., 67 pp. ISBN 974-53-2637-2

In the research for antioxidant from medicinal herbs, the 40 Thai medicinal plants were preliminary evaluated using TLC autographic assay with 2,2-Diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl (DPPH) as a stable radical. The CH_2Cl_2 and butanolic extracts of *Uvaria rufa* roots (Pee-Puan-Noi) were found to have a promising activity. Therefore, this plant was selected for futher investigation. Chromatographic separation of these two extracts led to the isolation of fifteen known compounds. Six flavonoids, 2,5-dihydroxy-7-methoxy flavanone (**1**), tectochrysin (**2**), 5-hydroxy-7-methoxy flavanone (**3**), 6,7-*O*,*O*-dimethylbaicalein (**6**), 7-*O*-methylwogonine (**7**) and 2,5-dihydroxy-6,7-dimethoxy flavanone (**8**), together with two aromatic compounds, benzyl benzoate (**4**) and 2-methoxybenzyl benzoate (**5**), were isolated from the CH_2Cl_2 crude extract. While seven alkaloids, liriodenine (**9**), lanuginosine (**10**), oxoanolobine (**11**), roemerine (**12**), anonaine (**13**), xylopine (**14**) and roemeroline (**15**) were isolated from butanolic crude extract. All the compounds, except for **1**, **2**, **6** and **7**, were isolated from this plant for the first time. The structures of all isolated compounds were elucidated on the basis of spectroscopic methods, as well as comparison with previously reported. The antioxidant capability was determined using three complementary in vitro assays, the DPPH radicals scavenging activity, the superoxide radicals scavenging activity and the inhibition of xanthine oxidase activity. Compound **8** showed the highest scavenging activity on DPPH and superoxide radicals with IC_{50} value 0.16 and 1.03 mg/mL, respectively. In addition, the inhibitory activity against xanthine oxidase, no compounds displayed good activity as a xanthine oxidase inhibitor.

Field of study..... Biotechnology..... Student's signature.....Kanogporn Payakarintarungkul

Academic year.....2005..... Advisor's signature.....Santi Tip-pyang.....

Co-advisor's signature.....Precha Phuwapraisirisan

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List of Abbreviations

$[M+H]^+$	molecular ion plus hydrogen
°C	degree of Celsius
^{13}C NMR	carbon 13 nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
2D NMR	two dimensional nuclear magnetic resonance
A	absorbance
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
brs	broad singlet (NMR)
CD_3OD	deuterated methanol
$CDCl_3$	deuterated chloroform
CH_2Cl_2	dichloromethane, methylene chloride
cm	centimeter
COSY	homonuclear correlated spectroscopy
d	doublet (NMR)
δ	chemical shift
dd	doublet of doublet (NMR)
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl
dt	double triplet (NMR)
EC.1.1.3.22	
	EC enzyme code
	1 oxidoreductases (enzyme class), oxidation/reduction reaction
	1.1 acting on the CH-OH group of donors
	1.1.3 with oxygen as acceptor
	1.1.3.22 xanthine oxidase
EIMS	electron impact mass spectroscopy
ESIMS	electrospray ionization mass spectrometry
EtOAc	ethyl acetate
g	gram
H_2SO_4	sulfuric acid

HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum correlation
Hz	hertz
IC_{50}	inhibition concentration at 50 %
J	coupling constant
kg	kilogram
L	liter
λ_m	maximum wavelength
m	meter
m	multiplet (NMR)
M	molar
m/z	mass to charge ratio
M^+	molecular ion
MeOH	methanol
mg	milligram
μg	microgram
MHz	megahertz
min	minute chromatography
mL	milliliter
μL	microliter
mm	millimeter
mM	millimolar
μM	micromolar
mp	melting point
MS	mass spectrometry
NBT	nitroblue tetrazolium
nm	nanometer
NMR	nuclear magnetic resonance
no.	number
ppm	part per million
R_f	retardation factor
ROS	reactive oxygen species

RPHPLC	reversed phase high performance liquid chromatography
s	singlet (NMR)
SDS	sodium dodecyl sulfate
SiO_2	silica gel
TBHQ	tertiary butylhydroquinone
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultraviolet
w/w	weight by weight
XOD	xanthine oxidase

CHAPTER I

INTRODUCTION

There is now increasing evidence to suggest that free radicals, chemical species which have one or more unpaired electrons in the outer orbit, can cause oxidative damage to several cell components and may thus play a causative role in a variety of diseases such as aging, cancer, cardiovascular disease, brain dysfunction, cataract, inflammation, arthritis and immune system impairment (Papas, 1999; Franke *et al.*, 2004; Zou *et al.*, 2004). Biomolecules that can be attacked by free radicals are carbohydrates, proteins in tissues or enzymes, lipids in cell membrane, DNA and small cellular molecules (Pietta, 2000).

Most free radical reactions involve the reduction of molecular oxygen, leading to the formation of oxygen-centered free radicals, known as reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and nitric oxide (Franke *et al.*, 2004; Zou *et al.*, 2004). ROS are formed by various endogenous and exogenous sources. They generated by cells come from the following four sources: 1) normal aerobic respiration in mitochondria, which produces superoxide radicals ($O_2^{\bullet -}$) and the ensuring toxic products, hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($HO^{\bullet}$); 2) stimulated macrophages and polymorphonuclear leukocytes, which release superoxide and the nitric oxide radical ($NO^{\bullet}$), which in turn can react to form the nonradical destructive peroxynitrite; 3) degradation of fatty acid and other molecules in peroxisomes, which produce H_2O_2 as a by-product; 4) induction of cytochrome P_{450} enzymes, which cause oxidant as by-products (Borek, 2001). However, in aerobic organisms, ROS are constantly generated in small amounts during normal aerobic metabolism and can be regulated by antioxidant systems in the body (endogenous antioxidants), including enzymatic defenses such as superoxide dismutase, catalase and glutathione peroxidase, and nonenzymatic defenses such as the iron-binding proteins transferrin and ferritin, melatonin and urate (Pietta, 2000; Lee *et al.*, 2002). Even so, humans do

not have a large excess of antioxidant defenses, oxidative damage is not completely prevented, and there are the existence of some physiopathological situations that include the following: tobacco smoke; UV light; ozone (O_3) and oxides of nitrogen in polluted air; industrial toxins such as carbon-tetrachloride; drugs such as phenobarbital, which is a known tumor promoter in liver; and charcoal-broiled foods, which form a variety of carcinogens, notably benzo(a)pyrene (Borek, 2001; Pietta, 2000). Hence, ROS are produced in excess at the wrong time and place. Therefore, exogenous antioxidants are essential for diminishing the cumulative effects of oxidative damage over the life span (Pietta, 2000). They include natural **(Figure 1.1)** and phytochemicals from plants with antioxidant activity, and synthetic (Papas, 1999) **(Figure 1.2)**.

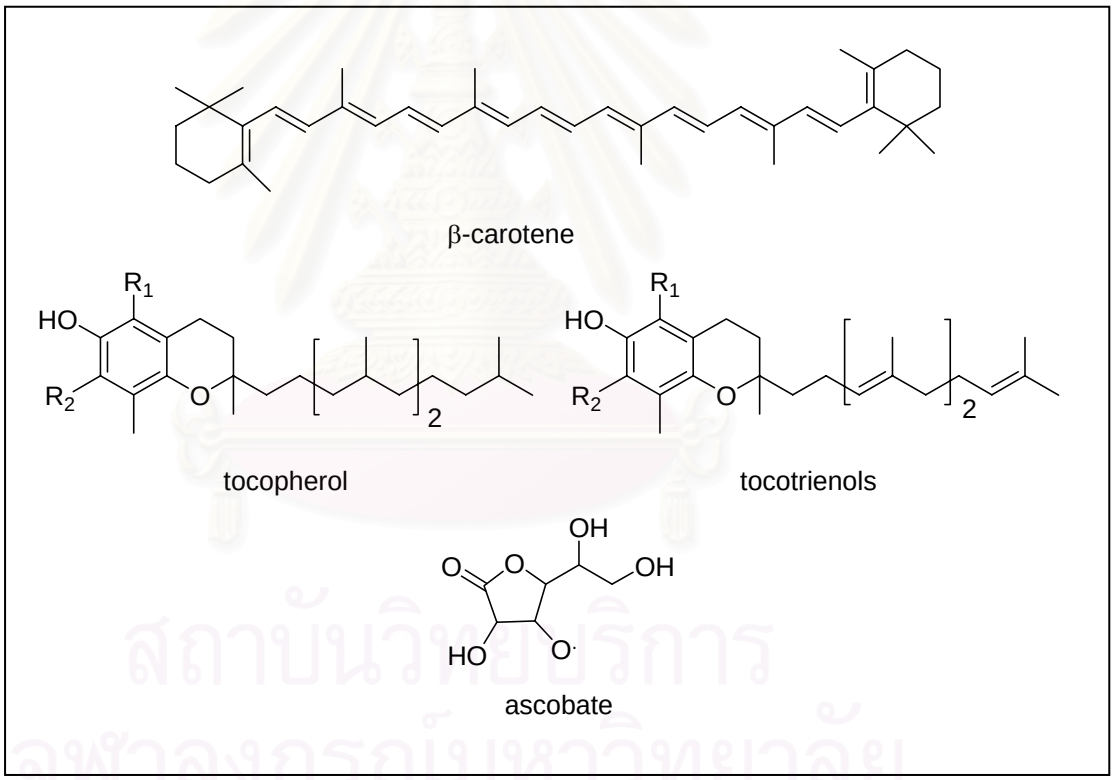


Figure 1.1 Natural antioxidants

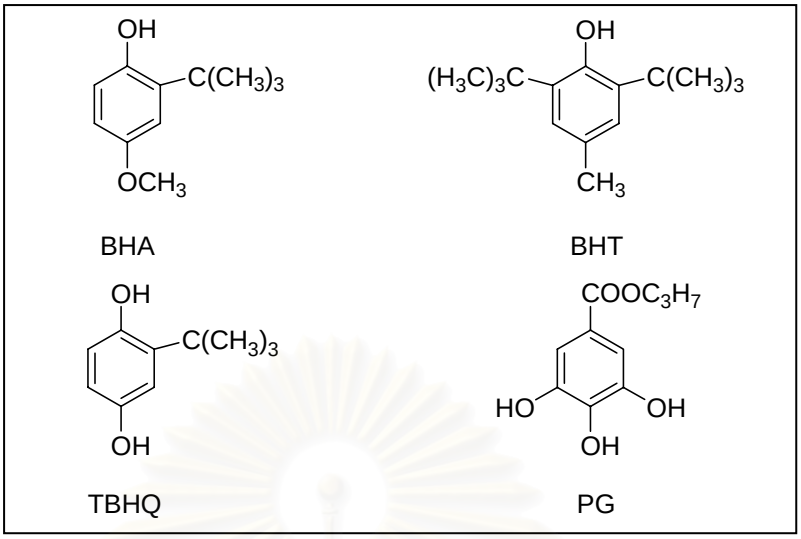


Figure 1.2 Synthetic antioxidants

The synthetic antioxidants are suspected of being carcinogenic and causing liver damage (Zou *et al.*, 2004). Therefore, botanical sources are of considerable interest for potential antioxidant principles. In addition, some of natural antioxidant compounds such as flavonoids, tannins, coumarins, curcuminoids, xanthones, phenolics, and terpenoids, all of which are found in the fruits, leaves, seeds, and oils of various plant products are as much effective as synthetic antioxidants in model systems (Lee *et al.*, 2006).

Medicinal herbs have been used in primary health care over many centuries before the advent of modern medicine (Rahman, 2003), and half of the top 50 drugs sold in Europe and many herbal/food supplements in present are natural products (Zhu *et al.*, 2004). Current studies have shown that a number of plant constituents and plant extracts exert an antioxidant action. For example, *Phyllanthus niruri*, a perennial herb in India, has several bioactive molecules such as lignans, phyllanthin, hypophyllanthin, flavonoids, glycosides and tannins in the extracts (Rajeshkumar *et al.*, 2002). *P. niruri* is used as one of the components of a multiherbal preparation for treating liver ailments (Kapur *et al.*, 1994), and the hepatoprotective effect is associated with antioxidant rich plant extracts. The alcoholic extract of medicinal herb feverfew (*Tanacetum parthenium*) possessed a strong free radical scavenging activity in a DPPH (2,2-Diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl) test, and this capacity might result from luteolin and other components (Wu *et al.*, 2006). Flavonoids,

dihydroquercetin and ethyl protocatechuate were identified as antioxidant components in peanut seed (Pratt & Miller, 1984; Huang *et al.*, 2003). Pomegranates (*Punica granatum*), the folk medicine, has been demonstrated to be high in antioxidant activity and is effective in the prevention of atherosclerosis. These activities may be related to diverse phenolic compounds present in pomegranate juice; punicalagins, ellagic acid derivatives and anthocyanins, which are known for their properties in scavenging free radicals and inhibiting lipid oxidation in vitro (Gil *et al.*, 2000; Schubert *et al.*, 1999; Aviram *et al.*, 2000; Kaplan *et al.*, 2001; Noda *et al.*, 2002). Moreover, many plants have long been recognized to contain potent antioxidants for long time such as catechin from tea as well as rosmanol, isoromanol and rosmariquinone from rosemary.

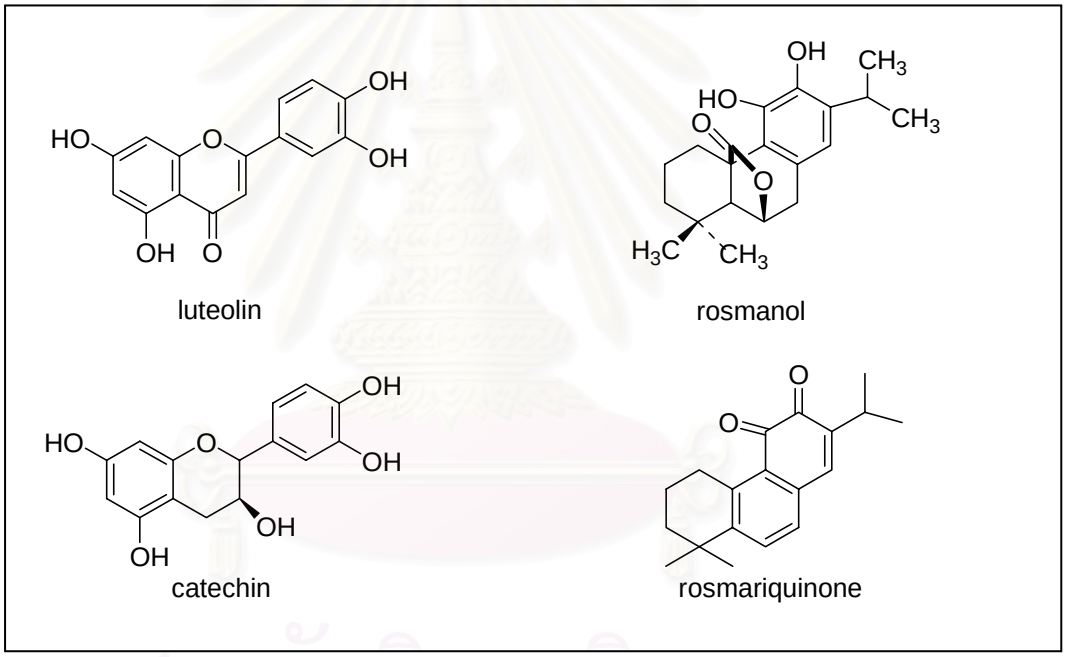


Figure 1.3 Plant antioxidants

In Thailand, many herbs have long been used to prevent and cure certain diseases. In this research, the screening was focused on Thai medicinal plants, of which the phytochemical information have not been widely investigated. The chemical compositions of plants were investigated by chromatographic and spectroscopic techniques. The antioxidant activities were determined by using three complementary in vitro assays, DPPH radicals scavenging activity, superoxide radicals scavenging activity and inhibition of xanthine oxidase activity.

The antioxidant activity were determined against DPPH (2,2-Diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl) radicals. It is a rapid and low cost assay, so it has been widely used to test the free radical scavenging ability of various natural products (Fazilatun *et.al*, 2004; Aligiannis *et.al*, 2003). DPPH is classified as a nitrogen-centered radical. It is stable at room temperature because of virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise (Molyneux, 2004). The delocalization also gives rise to the deep violet, characterized by an absorption band at 517 nm. The reduction capability of DPPH radicals is determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. Antioxidants scavenge DPPH radical by converting DPPH to α,α-diphenyl-β-picryl hydrazine, the pale yellow nonradical form, due to their H-donation ability **(Figure 1.4)**. The degree of discoloration indicates the scavenging potential of the antioxidant compounds. Radical scavenging activity was expressed in term of IC_{50} (concentration in mM/L required for a 50% decrease in absorbance of DPPH radical). In this method, a compound with high antioxidant potential effectively traps the radical, thereby preventing its propagation and the resultant chain reaction (Fazilatun *et.al*, 2004).

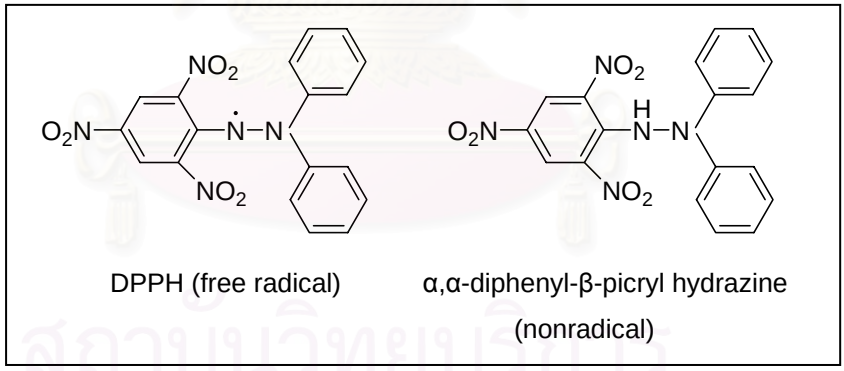
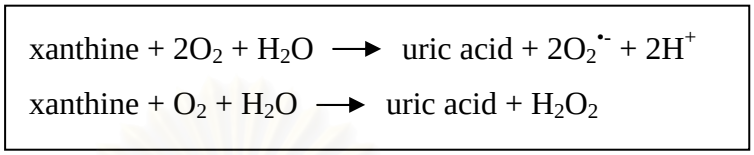


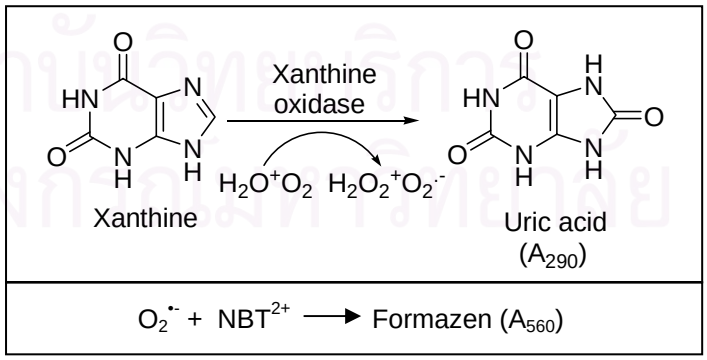
Figure 1.4 Structures of DPPH and DPPHn

The enzyme xanthine oxidase (EC 1.1.3.22) catalyses the oxidation of hypoxathine and xanthine to uric acid in the catabolic pathways for purine nucleotide degradation in humans **(Scheme 1.2)**. Excess uric acid production leads to a disease called gout. In the blood, uric acid is present as a relatively insoluble monosodium salt, sodium urate (Tropp, 1997). The urate crystals may be deposited in a joint, leading to an acute inflammatory response, or in soft tissues, such as cartilage,

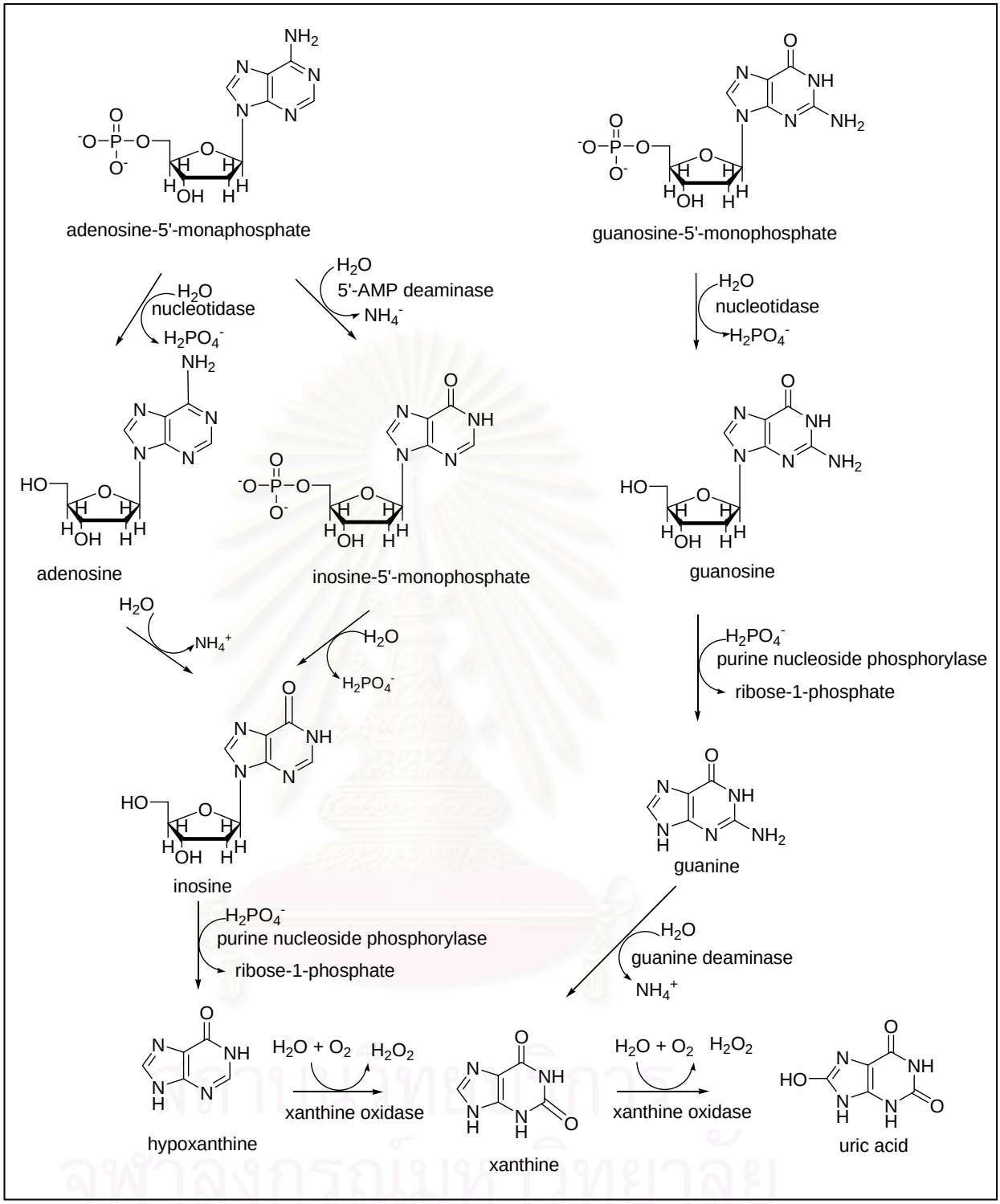
causing no inflammation. Most cases of gout are characterized by the sudden onset of severe acute monarticular arthritis in a peripheral joint in the leg (Emmerson, 1996). Moreover, during the reoxidation of xanthine oxidase, molecular oxygen acts as electron accepter, producing superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) according to the following equations:



Consequently, this enzyme is considered to be an important biological source of superoxide radicals that contribute to the oxidative stress on the organism and are involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging (Cos *et al.*, 1998). The antioxidant potential is evaluated using the xanthine-xanthine oxidase system in vitro by spectrophotometric measurement **(Scheme 1.1)**. For estimation of the scavenging effect on the superoxide anion, nitroblue tetrazolium (NBT) reduction method is used. In this method, $O_2^{\bullet-}$ reduces the yellow dye (NBT^{2+}) to produce the blue formazen, which is measured spectrophotometrically at 560 nm (Parejo *et al.*, 2004). Antioxidants that are able to scavenge the superoxide anions can reduce the blue formazen in reaction, so absorbance at 560 nm is decrease. For evaluation of the inhibitory effects on xanthine oxidase, a decreased production of uric acid is measured by UV absorption method at absorbance 290 nm.



Scheme 1.1 The xanthine-xanthine oxidase system



Scheme 1.2 Major pathways for purine nucleotide degradation in humans and other primate (Tropp, 1997).

As a part of our ongoing investigations of antioxidant agents from Thai medicinal plants, the CH_2Cl_2 extracts from 40 Thai medicinal plants were screened using DPPH TLC autography. Among plants screened, *Uvaria rufa* root extracts were found to have a promising activity; however, biological activity has not been reported. Therefore, this plant was selected for further investigation of the chemical constituents and their antioxidant activity.

Botanical aspects and distribution of *Uvaria rufa*

Uvaria rufa Blume or *Uvaria ridleyi* King **(Figure 1.5)** is one of the the larger palaeotropical genera in the Annonaceae. The genus *Uvaria* comprises approximately 150 species. It is most diverse in continental Southeast Asia and Malesia but it also occurs in the wet tropical regions of Africa, Madagascar, Indomalaya and Northern Australia (Willis, 1973; Meade, 2005). Fourteen species have been found in Thailand. *Uvaria rufa*, locally known as Pe-Puan-Noi "พีพวนน้อย", Nom-Kwai "นมควาย" (south), Bu-Nga-Yai "บุหงาใหญ่" (north), Nom-Maew-Pa "นมแมวป่า" (Chiangmai) and Ting-Tung "ติงตัง" (Nakhonrachsima), is a climber shrub that can climb to farness of 5-9 m (เต็ม สมิตินันท์, 2523).

Its branch and plumule have dense stellate hairs. It leaves have an elliptical shape with 3-6 cm width and 7-15 cm long, acute to acuminate apex, hairs on upper blade (scabrid group) on mid rib and secondary veins, 12-15 pairs of secondary veins. Its petiole is 0.5-1 cm long. Its flower is short inflorescences opposite leaves with magenta petals. Its petals have an elliptical shape with 6 mm width and 1-1.2 cm long. It has 6 petals, obtuse apex, imbricate perianth. Its sepals have an oval shape with 5 mm width and long. Its fruit is aggregate fruit, elliptical to cylinder shape with 1.5-2 cm width and 3-4 cm long. It has 6-9 fruits. Its immature fruit is green and turn to red when mature. It is many-seeded apocarpous fruits. (ปิยะ เฉลิมกลิ่น, 2544; Meade, 2003, 2005).

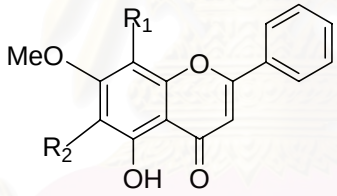
Uvaria rufa widely distributes in the tropical rainforest and tropical evergreen forest areas of Thailand (around 50-600 m above the sea level). It starts blooming in April to June (ปิยะ เฉลิมกลิ่น, 2544).

Uvaria rufa is a traditional Thai herb. Its roots and heartwood have been used as a folk medicine to treat fever from eating poisoning and nourish milk in woman. Its fruits have been used to relieve irritant (คณะเภสัชศาสตร์ ม.มหิดล, 2538).

Figure 1.5 *Uvaria rufa*

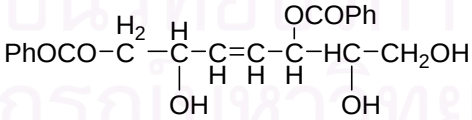
Phytochemical investigation of *Uvaria rufa*

According to report of Jack R. Cannon *et al.* (1980), *Uvaria rufa* gave positive tests for alkaloid. Subsequently, an attempt to isolate the alkaloids using the acid extraction by Lojanapiwatna and coworkers (1989) was failed; however three flavonoids named tectochrysin **(1)** 7-*O*-methylwogonine **(2)** and 6,7-*O*,*O*-dimethylbaicalein **(3)** were identified in non-basic portion.



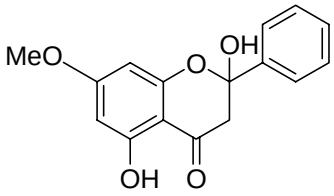
	R_1	R_2
(1)	H	H
(2)	OMe	H
(3)	H	OMe

In 1984, Chayunkiat *et al.* found a new compound, (*E*)-3,7-bisbenzoyloxyhept-4-en-1,2,6-triol, from the CH_2Cl_2-MeOH extract of leaves of *Uvaria rufa*.



(E)-3,7-bisbenzoyloxyhept-4-en-1,2,6-triol

In 1989, Chantrapromma *et al.* could isolate 2,5-dihydroxy-7-methoxy flavanone from *Uvaria rufa* roots, in addition to three known flavonoids previously reported by Lojanapiwatna.



2,5-dihydroxy-7-methoxy flavanone

To date few literature has been reported regarding phytochemical investigation and antioxidant activity of isolated compounds from this plant. Thus, the present study is undertaken to further investigate chemical constituents of *U. rufa* roots and determine their antioxidant activity.

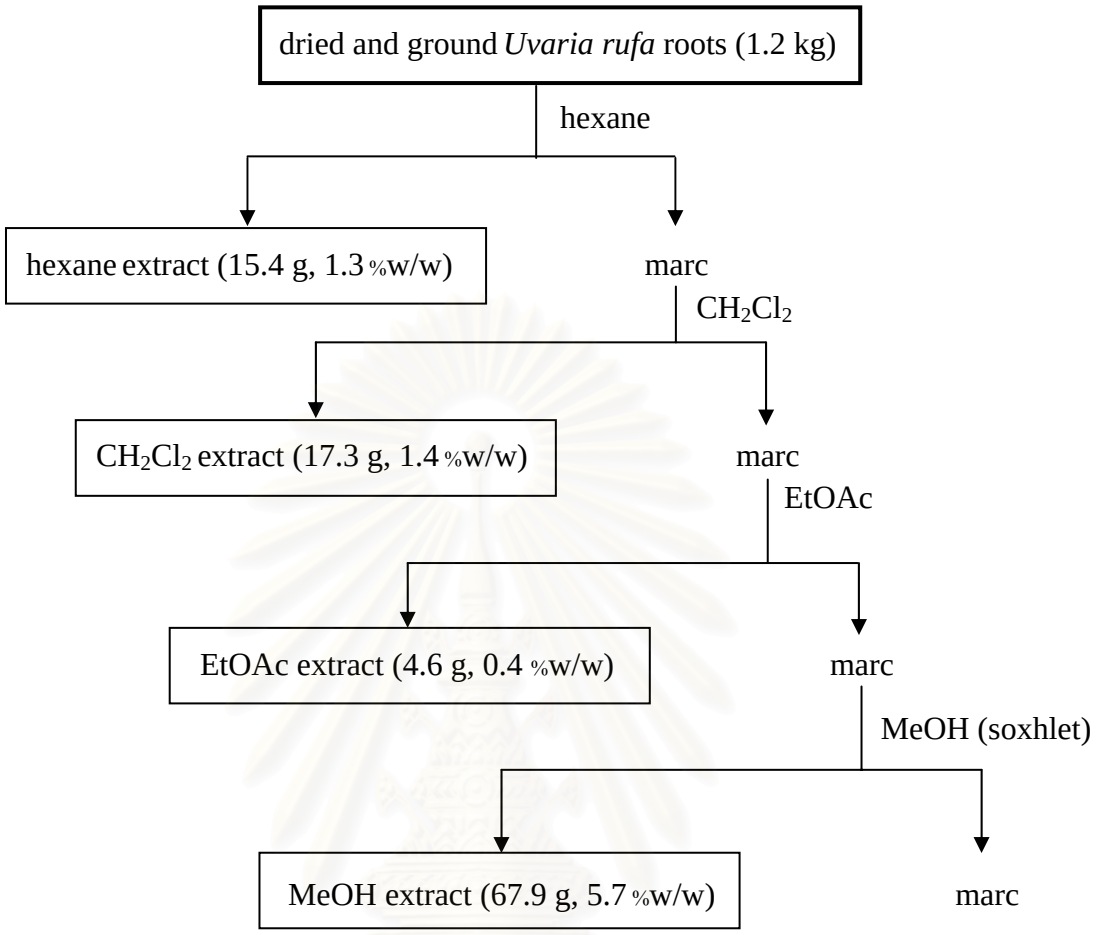
CHAPTER II

ISOLATION AND CHARACTERIZATION OF ANTIOXIDANT COMPOUNDS FROM *Uvaria rufa*

In the course of searching for antioxidant compounds from plant sources, the CH_2Cl_2 extracts from approximately 40 Thai medical plants were screened using TLC autographic assay (Maver *et.al*, 2005). The extract of *Uvaria rufa* roots (Pee-Puan-Noi) exhibited promising activity and showed several spots of antioxidant compounds as indicated by white spots on purple DPPH background. Therefore, an attempt to identify antioxidant compounds presented in this plant was carried out.

2.1 Extraction and Isolation

The sun-dried and ground roots (1.2 kg) of *Uvaria rufa* were twice extracted with hexane, CH_2Cl_2 and EtOAc at room temperature. The extract was filtered though a Buchner funnel, and the filtrate was concentrated to dryness under reduced pressure, yielding hexane (15.4 g), CH_2Cl_2 (17.3 g) and EtOAc (4.6 g) crude extracts. The extraction procedure was summarised in Scheme 2.1



Scheme 2.1 Extraction procedure of *Uvaria rufa* roots

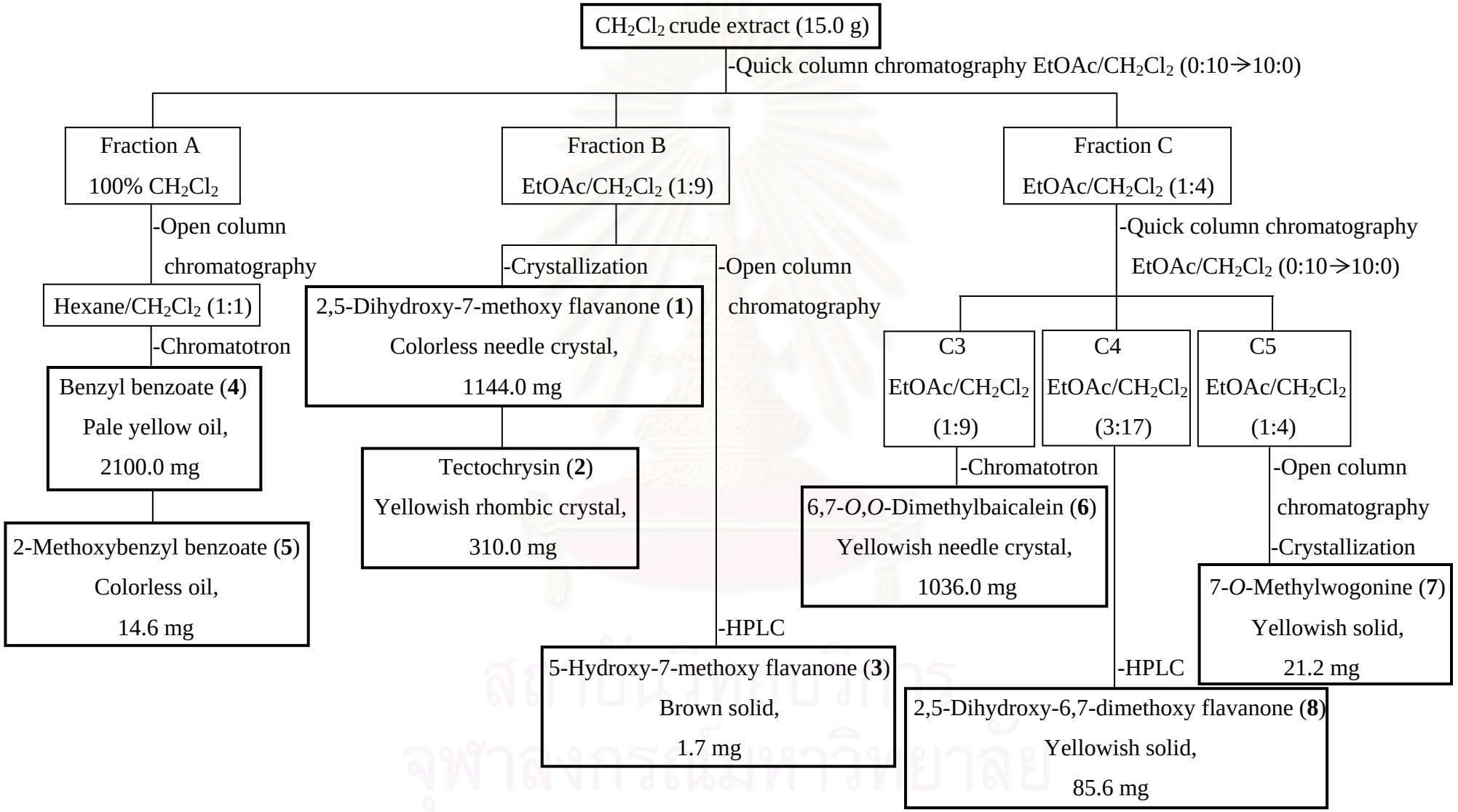
Then, the methanolic crude extract was suspended in water and was partitioned with saturated butanol to afford butanolic crude extract (14.0 g).

The extracts were preliminary tested for radical scavenging activity against DPPH by spectrophotometric method (Table 2.1).

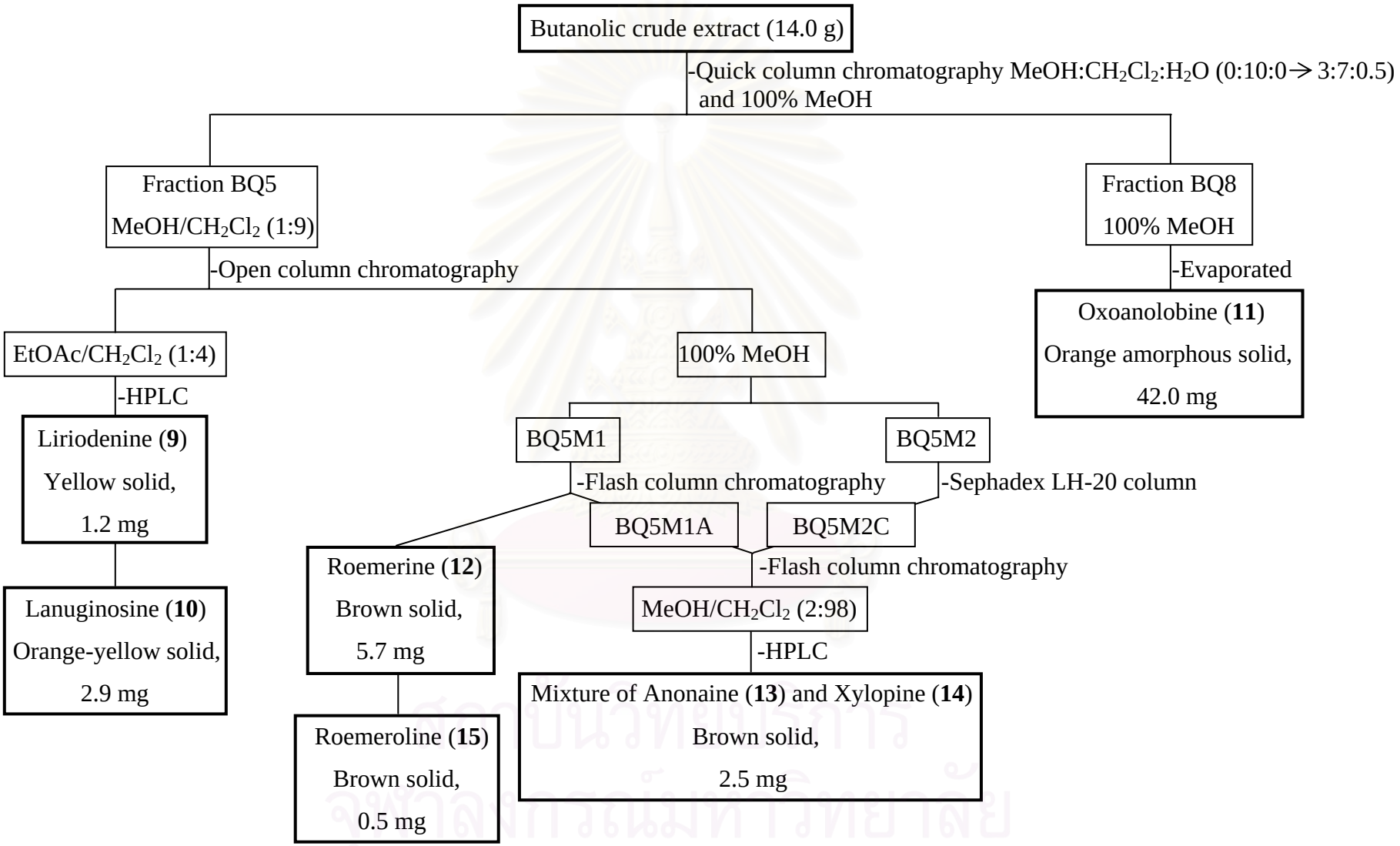
Table 2.1 Antioxidant activity of crude extracts

Crude extract	**IC_{50} (mg/mL)**
Hexane	1.25
CH_2Cl_2	0.48
EtOAc	0.16
Butanolic	0.09
BHA	0.03
Ascorbic acid	0.03

From the above results, the CH_2Cl_2, EtOAc and butanolic crude extracts showed potent radical scavenging towards DPPH. However, from the TLC pattern, the CH_2Cl_2 and EtOAc crude extracts have almost the same spots, and the CH_2Cl_2 and butanolic crude extracts have the higher yield then crude EtOAc. Therefore, the CH_2Cl_2 and butanolic crude extracts were selected for further investigation. The isolation of the CH_2Cl_2 and butanolic crude extracts were briefly summarized in Scheme 2.2 and 2.3, repectively.



Scheme 2.2 The isolation procedure of CH_2Cl_2 crude extract.



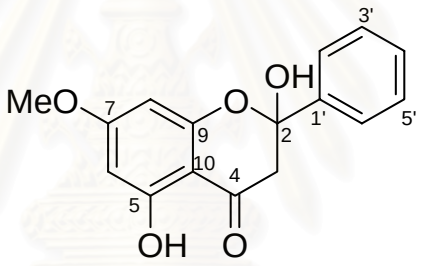
Scheme 2.3 The isolation procedure of butanolic crude extract.

2.2 Structural Characterization of Isolated Compounds

2,5-Dihydroxy-7-methoxy flavanone (**1**)

2,5-Dihydroxy-7-methoxy flavanone (**1**) had a molecular ion peak, [M^+], at *m/z* 286. The ^{1}H NMR spectrum (acetone-d_6) showed signals of methylene proton (δ 2.96, s, 2H, H-3), methoxy group (δ 3.90, s, 3H, 7-OCH_3), two hydroxy protons (δ 3.27, s, 1H, 2-OH and 12.07, s, 1H, 5-OH) and monosubstituted aromatic protons (δ 7.45-7.52, m, 3H and 7.77-7.78, m, 2H). Two aromatic protons of δ 6.12 (brs, 1H) and 6.14 (brs, 1H) indicated their meta substitution. Because of the typical lowfield shift of δ 12.07 at C-5, the methoxy group was inevitably placed at C-7 to account for meta substitution.

The above data suggested a 2-hydroxy flavanone nature of **1**. The identity of **1** was eventually confirmed by comparison of its ^{1}H and ^{13}C NMR data with those previously reported (Chantrapromma *et.al.*, 1989).

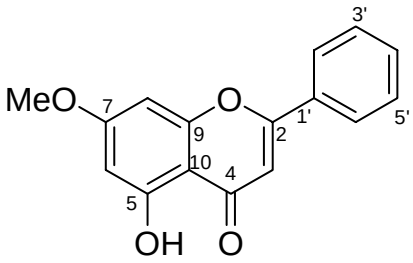


2,5-Dihydroxy-7-methoxy flavanone (**1**)

Tectochrysin (**2**)

Tectochrysin (**2**) had a molecular ion peak, [M^+], at *m/z* 268. The ^{1}H NMR spectrum ($CDCl_3$) showed signals of olefinic proton (δ 6.71, s, 1H, H-3), methoxy group (δ 3.92, s, 3H, 7-OCH_3) and monosubstituted aromatic protons (δ 7.56-7.58, m, 3H and 7.91-7.93, m, 2H). Two aromatic protons of δ 6.42 (d, J = 2.4 Hz, 1H) and 6.54 (d, J = 2.4 Hz, 1H) indicated their meta substitution.

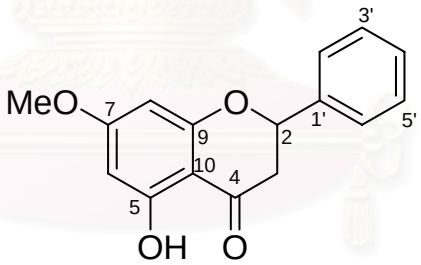
The ^{1}H NMR of **2** resembled that of **1**, except for the presence of olefinic proton H-3, suggesting a flavone nature of **2**. Compound **2** was eventually identified to be tectochrysin by comparison of its ^{1}H and ^{13}C NMR with those reported in the literature (Lojanapiwatna *et.al*, 1981).



Tectochrysin (**2**)

5-Hydroxy-7-methoxy flavanone (**3**)

The ^{1}H NMR spectrum ($CDCl_3$) showed one methoxy signal at δ 3.85 (s, 3H, 7-OCH_3), one hydroxy signal at δ 12.07 (s, 1H, 5-OH), aromatic protons [δ 7.48-7.49 (m, 5H)] and meta substituted proton [δ 6.12 (brs, 1H, H-6) and 6.11 (brs, 1H, H-8)]. Moreover, **3** revealed methylene protons at δ 2.87 (dd, *J* = 2.8, 17.2 Hz, H-3a) and 3.13 (dd, *J* = 13.0, 17.2 Hz, H-3b), and oxygenated methine proton at δ 5.47 (dd, *J* = 3.0, 12.8 Hz, 1H, H-2), suggesting that **3** possessed flavanone skeleton. In addition, the presence of dihydroflavone was indicated by carbon signals at δ 79.3 (C-2) and 43.4 (C-3), including COSY and HMBC analysis. Therefore the structure of 5-hydroxy-7-methoxy flavanone was depicted for **3**.

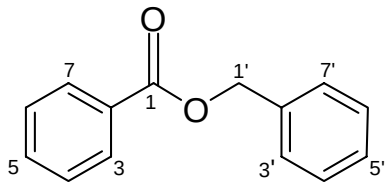


5-Hydroxy-7-methoxy flavanone (**3**)

Benzyl benzoate (**4**)

Benzyl benzoate (**4**) had a molecular ion peak, [M^+], at *m/z* 212. The ^{1}H NMR spectrum ($CDCl_3$) exhibited the presence of one methylene proton at δ 5.43 (s, 2H) and ten aromatic protons at δ 7.40-7.50 (m, 7H), 7.60-7.63 (m, 1H) and 8.15 (d, *J* = 8.0 Hz, 2H). The ^{13}C NMR spectrum ($CDCl_3$) clearly displayed methylene carbon at δ 66.8 and carbonyl carbon at δ 166.5. The remaining signals indicated aromatic carbons at δ 136.2, 133.1, 130.2, 129.8, 128.7, 128.5, 128.4 and 128.3.

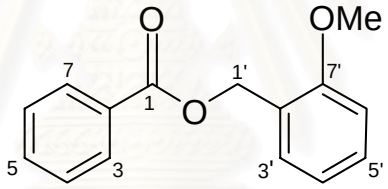
The ^{1}H and ^{13}C NMR data of **4** coinsided well with those of benzyl benzoate (Katritzky *et.al*., 2001). Therefore, **4** was identified as benzyl benzoate.



Benzyl benzoate (**4**)

2-Methoxybenzyl benzoate (**5**)

2-Methoxy benzyl benzoate (**5**) showed NMR spectra ($CDCl_3$) related to those of **4**; methylene proton at δ 5.47 (s, 2H) and aromatic protons at δ 8.13 (d, J = 8.0 Hz, 2H) and 6.95-7.61 (m, 7H). However, it presence of one methoxy group at δ 3.90 (s, 3H) and from HMBC correlation, methylene proton correlated with carbon signal at δ 157.5, suggesting that methoxy group was placed at C-7'. Thus, the structure of **5** was identified as 2-methoxy benzyl benzoate.

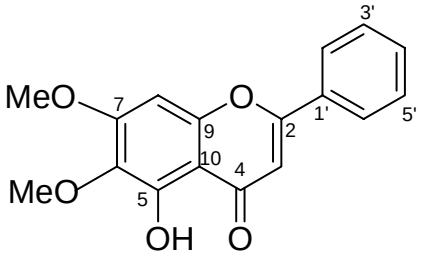


2-Methoxy benzyl benzoate (**5**)

6,7-*O*,*O*-Dimethylbaicalein (**6**)

The ^{1}H NMR spectrum (acetone-d_6) of **6** showed similar patterns to those of **2**; monosubstituted aromatic protons at δ 7.63-7.66 (m, 2H) and 8.10-8.13 (m, 3H), singlet aromatic proton δ 6.86 (s, 1H), and olefinic proton at δ 6.92 (s, 1H). In addition, the spectrum also exhibited the signal of downfield hydroxyl [δ 12.86 (s, 1H)] and two methoxy signals [δ 3.84 (s, 3H) and 4.03 (s, 3H)].

The above data suggested that ring A was substituted by two methoxy groups. The ^{13}C NMR spectrum (acetone-d_6) showed signal of methine carbon at δ 91.2 (C-8). The ^{1}H and ^{13}C NMR data were identical to that of 6,7-*O*,*O*-dimethylbaicalein (Lojanapiwatna *et.al*, 1981). Therefore, the structure of **6** was established as shown.

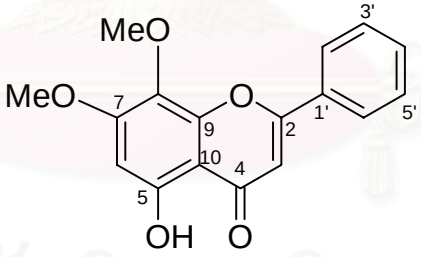


6,7-*O*,*O*-Dimethylbaicalein (**6**)

7-*O*-Methylwogonine (**7**)

The ^{1}H NMR spectrum ($CDCl_3$) of **7** showed similar patterns to those of **6**; monosubstituted aromatic protons at δ 7.98-8.00 (m, 2H) and 7.58-7.60 (m, 3H), singlet aromatic protons at δ 6.48 (s, 1H), olefinic proton at δ 6.72 (s, 1H), and two methoxy groups at δ 3.99 (s, 1H) and 4.00 (s, 1H).

The above data suggested that **7** was structural isomer of **6**, in which C-8 was substituted by a methoxy group. The struture of **7** was supported by its ^{1}H and ^{13}C NMR data, particulary a slightly upfield shift of the singlet aromatic proton (δ 6.48) and signal of methine carbon at δ 95.9 (C-6), which were identical to those of 7-*O*-methylwogonine (Lojanapiwatna *et.al*, 1981). Thus, the structure of **7** was depicted as shown.



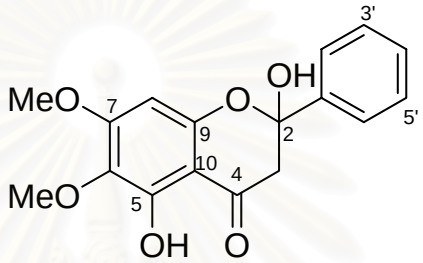
7-*O*-Methylwogonine (**7**)

2,5-Dihydroxy-6,7-dimethoxy flavanone (**8**)

Compound **8** was obtained as a single peak from RPHPLC column. The ^{1}H NMR spectrum indicated that **8** was an inseparable mixture of two components in a ratio of 1:1. The first component was assigned to be **6** as indicated by diagnostic signals (see experimental section). In addition, the other set of diagnostic signals related to those of **6**. They were also observed at δ 12.26 (s, 1H, 5-OH), 8.42-8.44 (m, 2H), 7.95-8.01 (m, 3H), 6.66 (s, 1H, H-8), 4.41 (s, 3H, 7-OCH_3) and 4.37 (s, 3H, 6-OCH_3). The signals of methylene protons at δ 3.56-3.58 (m, 2H) and its

corresponding carbon signal at δ_C 48.4 suggested a flavanone nature of the other component. The ^{13}C NMR also showed signal at δ_C 101.7 (C-2), suggesting a cyclic hemiacetal moiety. This finding was confirmed by HMBC cross peak of H-3 to C-2 (δ 101.7) and C-4 (δ 194.4). Therefore, the structure of 2,5-dihydroxy-6,7-dimethoxy flavanone was depicted for **8**.

From the above data, it was likely that **8** partially underwent dehydration to give flavone **6**. This similar results were also reported by Stevens (1999) and Wang (1999).

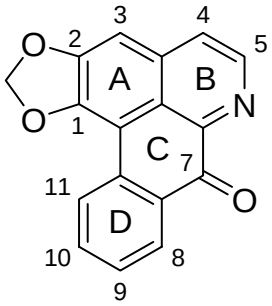


2,5-Dihydroxy-6,7-dimethoxy flavanone (**8**)

Liriodenine (**9**)

The ^{1}H NMR spectrum (DMSO-d_6) exhibited signals for methylenedioxy group at δ 6.51 (s, 2H) and seven aromatic protons in the region of δ 7.59-8.82. The signals at δ 8.05 and 8.81, which appeared as doublets with a small typical coupling constant of δ 5.2 Hz, was ascribable to hetero aromatic protons, while the signals at δ 8.66 (d, J = 8 Hz, 1H), 8.36 (dd, J = 1.0, 7.8 Hz, 1H), 7.89 (dt, J = 1.2, 7.8 Hz, 1H), 7.65 (dt, J = 0.4, 7.3 Hz, 1H) were monosubstituted aromatic protons. The ^{13}C NMR spectrum (DMSO-d_6) of **9**, in combination with HMBC, HSQC and COSY experiments, suggested that **9** was a congener of the oxoaporphine alkaloids.

This finding was futher supported by comparison of ^{1}H and ^{13}C NMR spectra with those previously reported for liriodenine (Khan *et.al*, 2003; Zhang *et.al*, 2002). Finally, the structure of **9** was established as liriodenine.

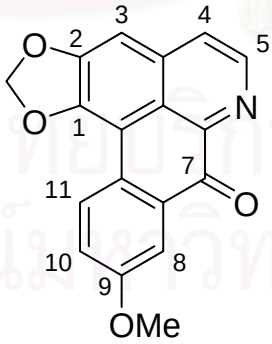


Liriodenine (**9**)

Lanuginosine (**10**)

Compound **10** showed ^{1}H NMR spectrum (DMSO-d_6) related to that of **9**; a methylenedioxy group at δ 6.48 (s, 2H), two hetero aromatic protons at δ 8.04 (d, *J* = 5.2 Hz, 1H) and 8.81 (d, *J* = 5.2 Hz, 1H), and one aromatic proton on ring A at δ 7.53 (s, 1H). However, ring D showed three aromatic protons in the region of δ 7.47-8.82 and one methoxy group at δ 3.93 (s, 3H). This result suggested that ring D was a trisubstituted benzene. From HMBC analysis and spilting pattern of protons, the methoxy group was located at C-9, and signals of proton at δ 7.80 (d, *J* = 2.8 Hz, 1H), 7.48 (dd, *J* = 3.2, 8.8 Hz, 1H) and 8.57 (d, *J* = 8.8 Hz, 1H) were assigned to H-8, H-10 and H-11, respectively. In addition, the signals at δ 7.53 (s, 1H), 8.04 (d, *J* = 5.2 Hz, 1H) and 8.81 (d, *J* = 5.2 Hz, 1H) were assigned to H-3, H-4 and H-5, respectively.

On the basis of this analysis and comparison with published data (Khan *et.al*, 2003; Zhang *et.al*, 2002), the structure of **10** was identified as lanuginosine.



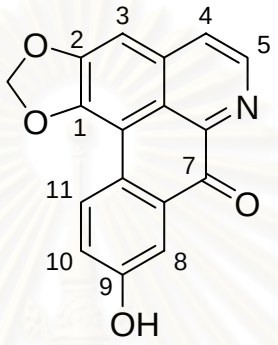
Lanuginosine (**10**)

Oxoanolobine (**11**)

Compound **11** showed ^{1}H NMR spectrum (DMSO-d_6) similar patterns to that of **10**; a methylenedioxy group at δ 6.46 (s, 2H), two hetero aromatic protons at δ

8.03 (d, *J* = 5.2 Hz, 1H) and 8.79 (d, *J* = 4.8 Hz, 1H), one aromatic proton on ring A at δ 7.50 (s, 1H), and three aromatic protons, which the same spilting pattern like **10**, on ring D at δ 7.71 (d, *J* = 2.8 Hz, 1H), 7.30 (dd, *J* = 2.6, 8.6 Hz, 1H) and 8.51 (d, *J* = 8.8 Hz, 1H), except for one hydroxy signal at δ 10.34 (s, 1H).

From the above data, together with comparison with those reported in the literature (Phoebe *et.al*, 1980), **11** was identified as oxoanolobine. This result was also supported by the MS spectrum that showed a molecular ion peak, $[M+H]^+$, at *m/z* 292.

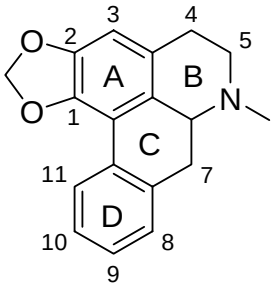


Oxoanolobine (**11**)

Roemerine (**12**)

The ^{1}H NMR (CD_3OD) spectrum showed two signals of nonequivalent dioxymethylene at δ 5.83 (s, 1H) and 5.99 (s, 1H), suggesting the absence of olefinic protons at C-4 and C-5 in aporphines alkaloid (Goodwin *et.al*, 1958). The presence of contiguous aromatic protons at δ 7.96 (d, *J* = 7.6 Hz, 1H) and 7.11-7.20 (m, 3H) indicated that ring D was 1,2-disubstituted benzene. Its ^{1}H NMR also displayed signal of *N*-methyl group at δ 2.49 (s, 3H, *N*-CH_3). The remaining signals of protons in the region of δ 2.48-3.19 were assigned to methine and methylene protons of C-4, C-5, C-6 and C-7, which were confirmed by HSQC data.

All above data of **12** were consistent with those reported for roemerine (You *et.al*, 1995).

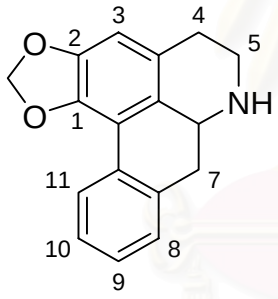


Roemerine (**12**)

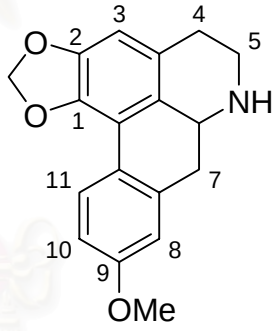
Mixture of Anonaine (**13**) and Xylopine (**14**)

The ^{1}H NMR spectrum of this mixture in $CDCl_3$ showed the presence of **13** and **14** in a ratio of 2:1. Interpretation of NMR data, in combination with HMBC, HSQC and COSY experiments, indicated that structures of **13** and **14** were closely related to that of **12**. The striking resonances were observed for nonequivalent dioxymethylenes at δ 6.09 (s, 1H) and 5.94 (s, 1H) for **13** and at δ 6.05 (s, 1H) and 5.93 (s, 1H) for **14**, suggesting that olefinic proton at C-4 and C-5 were saturated. The methoxy group at δ_H 3.83 (δ_C 55.8) was placed at C-9 of compound **14**, as a result of HMBC and COSY analysis of three aromatic protons at δ 8.01 (d, *J* = 8.4 Hz, 1H), 6.85 (dd, *J* = 1.8, 9.0 Hz, 1H) and 6.79 (brs, 1H). The proposed structures of **13** and **14** were also supported by its MS spectra of a molecular ion peak, $[M+H]^+$ at *m/z* 266 and 296, respectively.

According to all above data and comparison of its spectroscopic data with those previously reported (Hsieh *et.al*, 1999; Guinaudeau *et.al*, 1979; Bhaumik *et.al*, 1979), compounds **13** and **14** were assigned to anonaine and xylopine, respectively.



Anonaine (**13**)

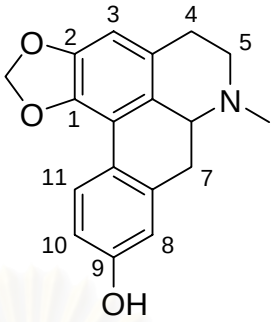


Xylopine (**14**)

Roemeroline (**15**)

Because of the small amount of **15**, the structure was tentatively proposed on the basis of 2D NMR and MS data. The ^{1}H NMR spectrum related to that of **12**; dioxymethylene signals at δ 5.81 (s, 1H) and 5.94 (s, 1H), *N*-methyl protons at δ 2.49 (s, 3H, *N*-CH_3), aromatic proton on ring A at δ 6.42 (s, 1H), and methine and methylene protons at δ 2.42-2.63 and 2.95-3.17. The aromatic protons on ring D at δ 6.61-7.80 (m, 3H) suggested a trisubstituted benzene. The substituted group was proposed to be a hydroxy group as evidence of HMBC correlations of aromatic protons to the oxygenated sp^2 carbon, δ_c 156.6.

From all above data and its MS data of a molecular ion peak, $[M+H]^+$, at *m/z* 296, **15** was tentatively assigned for roemeroline.



Roemeroline (**15**)

2.3 Experimental Section

2.3.1 General Experimental Procedures.

NMR spectra were recorded with a Varian model Mercury+ 400 which operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei. The chemical shift in δ (ppm) was assigned with reference to the signal from the residual protons in deuterated solvents and using TMS as an internal standard in some cases. EIMS and ESIMS data were obtained from Mass Spectrometer Model VG TRIO 2000 and a Micromass LCT mass spectrometer, respectively. HPLC was conducted on a Water® 600 controller equipped with a Water® 2996 photodiode array detector (USA). Cosmosil 5C18-ARII column (10 × 250 mm) was used for seperation purpose. Melting points were determined with Fisher-John Melting Point Apparatus. Centrifugal chromatography was performed on a Harrison Research 7924T chromatotron. Most solvents used in this research were commercial grade and were distilled prior to use. For crystallization, reagent grade solvents were used. Absorbents such as silica gel 60 Merck cat. no. 7734, 7749, and 9815 were used for open column chromatography, chromatotron, and flash column chromatography, respectively. Thin-layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates (0.25 mm thick layer) and visualized by dipping in 10% H_2SO_4-MeOH.

2.3.2 Plant material

The roots of *Uvaria rufa* Blume. (Pee Puan Noi) were collected from Tart Pa Nom, Nakornpanom province, Thailand during July 2004. Authentication was performed by Associate Professor Dr. Obchan Thaithong through comparison with the specimen deposited at the herbarium of Department of Botany, Chulalongkorn University.

2.3.3 Extraction, Isolation and Purification

The CH_2Cl_2 extract (15.0 g) was fractionated by a silica gel quick column chromatography using EtOAc/CH_2Cl_2 gradient (0:10 → 10:0), yielding 8 fractions (A-H). Fraction B, eluted with EtOAc/CH_2Cl_2 (1:9), was recrystallized from EtOAc/CH_2Cl_2 (1:24) to yield colorless needle crystals (compound **1**, 1144.0 mg) and yellowish rhombic crystals (compound **2**, 310.0 mg). The mother liquor of fraction B

was purified by a silica gel column chromatography (EtOAc/hexane and CH_2Cl_2/hexane) and HPLC [Cosmosil 5C18-ARII, 10×250 mm, MeOH/H_2O (4:1)] to obtain compound **3** (1.7 mg). Fraction A, eluted with 100% CH_2Cl_2, was chromatographed on a silica gel column chromatography eluted with hexane/CH_2Cl_2 (1:1) and further purified with chromatotron to obtain compounds **4** (2100.0 mg) and **5** (14.6 mg). Fraction C, eluted with EtOAc/CH_2Cl_2 (1:4), was fractionated by a silica gel quick column chromatography using a gradient of EtOAc/CH_2Cl_2 to afford 7 fractions (fraction C1-C7). Fraction C3, eluted with EtOAc/CH_2Cl_2 (1:9), was separated by chromatotron eluted with a gradient of EtOAc/hexane to afford compound **6** (1036.0 mg). Fraction C5, eluted with EtOAc/CH_2Cl_2 (1:4), was chromatographed on a silica gel column chromatography eluted with a gradient of CH_2Cl_2/hexane. Fractions eluted with CH_2Cl_2/hexane (3:7) were crystallized to yield compound **7** (21.2 mg). Fraction C4, eluted with EtOAc/CH_2Cl_2 (3:17), was futher purified by several step (a siliga gel column chromatography, a siliga gel flash column chromatography, chromatotron, preparative TLC) and finally using HPLC [Cosmosil 5C18-ARII, 10×250 mm, MeOH/H_2O (7:3)] to obtain an inseparable mixture of compounds **8** and **6** (85.6 mg).

The butanolic extract (14.0 g) was chromatographed on a silica gel quick column chromatography using MeOH/CH_2Cl_2 gradient (0:10 → 1:4), MeOH:CH_2Cl_2:H_2O (1:4:0.5 and 3:7:0.5) and 100% MeOH, yielding 8 fractions (BQ1-BQ8). Fraction BQ8, eluted with 100% MeOH, gave orange powders (compound **11**, 42.0 mg). Fraction BQ5, eluted with MeOH/CH_2Cl_2 (1:9), was futher isolated by a silica gel column chromatography eluted with gradient of EtOAc/CH_2Cl_2 followed by MeOH (BQ5M1 and BQ5M2). The fraction eluted with EtOAc/CH_2Cl_2 (1:4) was futher purified by HPLC [Cosmosil 5C18-ARII, 10×250 mm, MeOH/H_2O (3:2)] to afford compounds **9** (1.2 mg) and **10** (2.9 mg). Fraction BQ5M1 was futher purified by a silica gel flash column chromatography to furnish compound **12** (5.7 mg), compound **15** (0.5 mg), and fraction BQ5M1A. Fraction BQ5M2 was further purified by sephadex LH-20 colunm eluted with MeOH/CH_2Cl_2 (1:1), yielding 5 fractions (BQ5M2A-BQ5M2E). From TLC analysis, the combined fraction BQ5M2C and BQ5M1A was further purified by a silica gel flash column chromatography using gradients of MeOH/CH_2Cl_2 from 0:1→1:0 to obtain 11 fractions. Fraction eluted with

MeOH/CH_2Cl_2 (2:98) was further purified by HPLC [Cosmosil 5C18-ARII, 10×250 mm, 100% MeOH], giving a mixture of compounds **13** and **14** (2.5 mg).

2,5-Dihydroxy-7-methoxy flavanone (1): colorless needle crystal; EIMS *m/z* 286 (M^+), 269, 268, 209, 167, 166, 140, 138, 105 and 77; mp 173-175 °C; R_f 0.36 (SiO_2, CH_2Cl_2); UV λ_{max} (MeOH) 288, 219 nm; ^{1}H NMR (400 MHz, acetone-d_6) δ 12.07 (s, 1H, OH-5), 7.77-7.78 (m, 2H, H-2' and H-6'), 7.45-7.52 (m, 3H, H-3', H-4' and H-5'), 6.14 (brs, 1H, H-6 or H-8), 6.12 (brs, 1H, H-6 or H-8), 3.90 (s, 3H, 7-OCH_3), 3.27 (s, 1H, OH-2), 2.96 (s, 2H, H-3); ^{13}C NMR (100 MHz, acetone-d_6) δ 195.5 (C-4), 167.9 (C-7), 163.8 (C-5), 160.3 (C-9), 142.6 (C-1'), 128.7 (C-4'), 128.3 (C-2' and C-6'), 125.5 (C-3' and C-5'), 102.6 (C-2), 102.0 (C-10), 94.9 (C-6), 94.5 (C-8), 55.4 (7-OCH_3), 48.7 (C-3).

Tectochrysin (2): yellowish rhombic crystal; EIMS *m/z* 268 (M^+), 238, 225, 138, 105, 95 and 69; mp 163-164 °C; R_f 0.60 (SiO_2, CH_2Cl_2); UV λ_{max} (MeOH) 309, 269, 219 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91-7.93 (m, 2H, H-2' and H-6'), 7.56-7.58 (m, 3H, H-3', H-4' and H-5'), 6.71 (s, 1H, H-3), 6.54 (d, *J* = 2.4 Hz, 1H, H-6 or H-8), 6.42 (d, *J* = 2.4 Hz, 1H, H-6 or H-8), 3.92 (s, 3H, 7-OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.5 (C-4), 165.6 (C-7), 164.0 (C-2), 162.2 (C-9), 157.8 (C-5), 131.9 (C-4'), 131.3 (C-1'), 129.1 (C-3' and C-5'), 126.3 (C-2' and C-6'), 105.8 (C-3), 105.8 (C-10), 98.2 (C-6), 92.7 (C-8), 55.8 (7-OCH_3).

5-Hydroxy-7-methoxy flavanone (3): brown solid; R_f 0.62 (SiO_2, CH_2Cl_2); UV λ_{max} (MeOH) 288, 219 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.07 (s, 1H, 5-OH), 7.48-7.49 (m, 5H, H-2', H-3', H-4', H-5'and H-6'), 6.12 (brs, 1H, H-6 or H-8), 6.11 (brs, 1H, H-6 or H-8), 5.47 (dd, *J* = 3.0, 12.8 Hz, 1H, H-2), 3.85 (s, 3H, 7-OCH_3), 3.13 (dd, *J* = 13.0, 17.2 Hz, H-3b), 2.87 (dd, *J* = 2.8, 17.2 Hz, H-3a); ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.0 (C-4), 167.8 (C-7), 164.1 (C-5), 162.7 (C-9), 138.3 (C-1'), 128.9 (C-3', C-4' and C-5'), 126.2 (C-2' and C-6'), 103.1 (C-10), 95.2 (C-6), 94.3 (C-8), 79.3 (C-2), 55.7 (7-OCH_3), 43.4 (C-3).

Benzyl benzoate (4): pale yellow oil; EIMS *m/z* 212 (M^+); R_f 0.50 (SiO_2, 1:4 EtOAc/Hexane); UV λ_{max} (MeOH) 229 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, *J* = 8.0 Hz, 2H, H-3 and H-7), 7.60-7.63 (m, 1H, H-5), 7.40-7.50 (m, 7H, H-4, H-6, H-3', H-4', H-5', H-6' and H-7'), 5.43 (s, 2H, H-1'); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.5 (C-1), 136.2 (C-2'), 133.1 (C-5), 130.2 (C-2), 129.8 (C-3 and C-7), 128.7 (C-5'), 128.5 (C-4' and C-6'), 128.4 (C-3' and C-7'), 128.3 (C-4 and C-6), 66.8 (C-1').

2-Methoxybenzyl benzoate (5): colorless oil; R_f 0.44 (SiO_2, 1:4 EtOAc/Hexane); UV λ_{max} (MeOH) 273, 226 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, *J* = 8.0 Hz, 2H, H-3 and H-7), 6.95-7.61 (m, 7H, H-4, H-5, H-6, H-3', H-4', H-5' and H-6'), 5.47 (s, 2H, H-1'), 3.9 (s, 3H, 7'-OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.6 (C-1), 157.5 (C-7'), 132.9 (C-5), 130.4 (C-2), 129.7 (C-3 and C-7), 129.5 (C-3' or C-5'), 129.4 (C-3' or C-5'), 128.4 (C-4 and C-6), 124.4 (C-2'), 120.5 (C-4'), 110.5 (C-6'), 62.2 (C-1'), 55.5 (7'-OCH_3).

6,7-*O*,*O*-Dimethylbaicalein (6): yellowish needle crystal; mp 157-158 °C; R_f 0.24 (SiO_2, CH_2Cl_2); UV λ_{max} (MeOH) 312, 269, 219 nm; ^{1}H NMR (400 MHz, acetone-d_6) δ 12.86 (s, 1H, OH-5), 8.10-8.13 (m, 2H, H- H-2' and H-6'), 7.63-7.66 (m, 3H, H-3', H-4' and H-5'), 6.92 (s, 1H, H-3), 6.86 (s, 1H, H-8), 4.03 (s, 3H, 6-OCH_3 or 7-OCH_3), 3.84 (s, 3H, 6-OCH_3 or 7-OCH_3); ^{13}C NMR (100 MHz, acetone-d_6) δ 182.7 (C-4), 163.9 (C-2), 159.4 (C-7), 153.3 (C-5), 153.0 (C-9), 131.9 (C-6), 131.3 (C-1' and C-4'), 129.2 (C-3' and C-5'), 126.4 (C-2' and C-6'), 105.1 (C-10), 105.0 (C-3), 91.2 (C-8), 59.7 (7-OCH_3), 55.9 (6-OCH_3).

7-*O*-Methylwogonine (7): yellowish solid; mp 181-182 °C; R_f 0.40 (SiO_2, CH_2Cl_2); UV λ_{max} (MeOH) 274, 219 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98-8.00 (m, 2H, H-2' and H-6'), 7.58-7.60 (m, 3H, H-3', H-4' and H-5'), 6.72 (s, 1H, H-3), 6.48 (s, 1H, H-6), 4.00 (s, 3H, 7-OCH_3 or 8-OCH_3), 3.99 (s, 3H, 7-OCH_3 or 8-OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.8 (C-4), 164.0 (C-2), 158.8 (C-7), 157.6 (C-5 and C-9), 132.0 (C-1'), 131.3 (C-4'), 129.2 (C-3' and C-5'), 126.4 (C-2' or C-6'), 126.3 (C-2' or H-6'), 105.4 (C-3), 105.0 (C-9), 95.9 (C-6), 61.7 (7-OCH_3), 56.4 (8-OCH_3).

2,5-Dihydroxy-6,7-dimethoxy flavanone (8): yellowish solid; R_f 0.14 (SiO_2, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.26 (s, 1H, 5-OH), 8.42-8.44 (m, 2H, H-2' and H-6'), 7.95-8.01 (m, 3H, H-3', H-4' and H-5'), 6.66 (s, 1H, H-8), 3.56-3.58 (m, 2H, H-3), 4.41 (s, 3H, 7-OCH_3), 4.37 (s, 3H, 6-OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.4 (C-4), 160.8 (C-7), 155.0 (C-9), 154.7 (C-5), 130.8 (C-6), 129.4 (C-3', C-4' and C-5'), 128.7 (C-2' and C-6'), 102.4 (C-10), 101.7 (C-2), 92.6 (C-8), 61.1 (6-OCH_3), 56.1 (7-OCH_3), 48.4 (C-3).

Liriodenine (9): yellow solid; R_f 0.69 (SiO_2, 1:9 MeOH/CH_2Cl_2); UV λ_{max} (MeOH) 304, 269, 248, 218 nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81 (d, *J* = 5.2 Hz, 1H, H-5), 8.66 (d, *J* = 8.0 Hz, 1H, H-11), 8.36 (dd, *J* = 1.0, 7.8 Hz, 1H, H-8), 8.05 (d, *J* = 5.2 Hz, 1H, H-4), 7.89 (dt, *J* = 1.2, 7.8 Hz, 1H, H-10), 7.65 (dt, *J* = 0.4, 7.3 Hz, 1H, H-9), 7.59 (s, 1H, H-3), 6.51 (s, 2H, 1,2-OCH_2O); ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.5 (C-7), 151.9 (C-2), 149.1 (C-1), 144.9 (C-5), 144.4 (C-3a), 135.9 (C-6a), 134.6 (C-10), 132.7 (C-11a), 130.8 (C-7a), 128.9 (C-9), 128.0 (C-8), 127.5 (C-11), 124.7 (C-4), 122.8 (C-1b), 106.4 (C-1a), 103.6 (C-3 and 1,2-OCH_2O-).

Lanuginosine (10): orange-yellow solid; R_f 0.69 (SiO_2, 1:9 MeOH/CH_2Cl_2); UV λ_{max} (MeOH) 269, 246, 219 nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81 (d, *J* = 5.2 Hz, 1H, H-5), 8.57 (d, *J* = 8.8 Hz, 1H, H-11), 8.04 (d, *J* = 5.2 Hz, 1H, H-4), 7.80 (d, *J* = 2.8 Hz, 1H, H-8), 7.48 (dd, *J* = 3.2, 8.8 Hz, 1H, H-10), 7.53 (s, 1H, H-3), 6.48 (s, 2H, 1,2-OCH_2O), 3.93 (9-OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.4 (9-OCH_3), 151.9 (C-2), 147.7 (C-1), 144.4 (C-3a), 136.0 (C-6a), 132.7 (C-7a), 126.1 (C-11a), 125.2 (C-4), 122.4 (C-1b and C-10), 106.4 (C-1a), 102.7 (C-3).

Oxoanolobine (11): orange amorphous solid; ESIMS *m/z* 292 $[M+H]^+$; UV λ_{max} (MeOH) 378, 314, 269, 247, 218 nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H, 9-OH), 8.79 (d, *J* = 4.8 Hz, 1H, H-5), 8.51 (d, *J* = 8.8 Hz, 1H, H-11), 8.03 (d, *J* = 5.2 Hz, 1H, H-4), 7.71 (d, *J* = 2.8 Hz, 1H, H-8), 7.30 (dd, *J* = 2.6, 8.6 Hz, 1H, H-10), 7.50 (s, 1H, H-3), 6.46 (s, 2H, 1,2-OCH_2O); ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.2 (9-OH), 152.0 (C-2), 147.2 (C-1), 144.9 (C-3a), 136.0 (C-6a), 133.0 (C-7a), 124.5 (C-11a), 113.0 (C-8), 129.4 (C-11), 124.7 (C-4), 122.8 (C-10), 122.0 (C-1b), 106.9 (C-1a), 102.6 (C-3).

Roemerine (12): brown solid; R_f 0.30 (SiO_2, 1:99 MeOH/CH_2Cl_2); UV λ_{max} (MeOH) 370, 316, 269, 235, 218 nm; ^{1}H NMR (400 MHz, CD_3OD) δ 7.96 (d, *J* = 7.6 Hz, 1H, H-11), 7.11-7.20 (m, 3H, H-8, H-9 and H-10), 6.50 (s, 1H, H-3), 5.99 and 5.83 (s, 2H, 1,2-OCH_2O), 3.12-3.19 (m, 1H, H-7a), 3.08-3.14 (m, 1H, H-6a), 3.00-3.12 (m, 1H, H-4a), 2.99-3.05 (m, 1H, H-5a), 2.56-2.63 (m, 1H, H-4b), 2.51-2.57 (m, 1H, H-7b), 2.48-2.54 (m, 1H, H-5b), 2.49 (s, 3H, *N*-CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 146.8 (C-2), 142.5 (C-1), 134.6 (C-7a), 130.3 (C-11a), 127.8 (C-8), 127.3 (C-10), 126.7 (C-9), 126.6 (C-11), 125.6 (C-3a and C-1b), 115.8 (C-1a), 107.0 (C-3), 100.8 (1,2-OCH_2O-), 62.1 (C-6a), 53.1 (C-5), 42.3 (*N*-CH_3), 33.6 (C-7), 28.0 (C-4).

Anonaine (13): brown solid; ESIMS *m/z* 266 $[M+H]^+$; R_f 0.08 (SiO_2, 1:99 MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (d, *J* = 7.6 Hz, 1H, H-11), 7.20-7.33 (m, 3H, H-8, H-9 and H-10), 6.57 (s, 1H, H-3), 6.09 and 5.94 (s, 2H, 1,2-OCH_2O), 3.94-3.98 (d, *J* = 14, 1H, H-6a), 3.37-3.38 (d, *J* = 6.4, 1H, H-5a), 3.01-3.03 (m, 1H, H-4a), 2.98-3.06 (m, 1H, H-5b), 2.90-2.95 (dd, *J* = 4.8, 14.4 Hz, 1H, H-7a), 2.79-2.83 (d, *J* = 14, 1H, H-7b), 2.64-2.67 (m, 1H, H-4b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 146.8 (C-2), 142.1 (C-1), 135.2 (C-7a), 131.2 (C-11a), 128.1 (C-8), 128.0 (C-3a or C-1b), 127.5 (C-9), 127.0 (C-10 and C-11), 126.7 (C-3a or C-1b), 116.1 (C-1a), 108.0 (C-3), 100.7 (1,2-OCH_2O-), 53.5 (C-6a), 43.4 (C-5), 37.1 (C-7), 29.3 (C-4).

Xylopine (14): brown solid; ESIMS *m/z* 296 $[M+H]^+$; R_f 0.08 (SiO_2, 1:99 MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, *J* = 8.4 Hz, 1H, H-11), 6.85 (dd, *J* = 1.8, 9.0 Hz, 1H, H-10), 6.79 (s, 1H, H-8), 6.52 (s, 1H, H-3), 6.05 and 5.93 (s, 2H, 1,2-OCH_2O), 3.95-3.99 (d, *J* = 13.6, 1H, H-6a), 3.83 (s, 1H, 9-OCH_3), 3.37-3.38 (d, *J* = 6.4, 1H, H-5a), 3.01-3.03 (m, 1H, H-4a), 2.98-3.06 (m, 1H, H-5b), 2.86-2.91 (dd, *J* = 4.8, 14.8 Hz, 1H, H-7a), 2.76-2.79 (d, *J* = 14, 1H, H-7b), 2.64-2.67 (m, 1H, H-4b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.8 (C-9), 146.8 (C-2), 141.1 (C-1), 136.9 (C-7a), 128.3 (C-11), 127.0 (C-3a or C-1b), 126.1 (C-3a or C-1b), 123.8 (C-11a), 116.1 (C-1a), 113.6 (C-8), 112.2 (C-10), 107.1 (C-3), 100.5 (1,2-OCH_2O-), 55.3 (9-OCH_3), 53.5 (C-6a), 43.4 (C-5), 37.5 (C-7), 29.3 (C-4).

Roemeroline (15): brown solid; ESIMS *m/z* 296 $[M+H]^+$; R_f 0.08 (SiO_2, 1:99 MeOH/CH_2Cl_2); ^1H NMR (400 MHz, CD_3OD) δ 7.80 (d, *J* = 8.0 Hz, 1H, H-11), 6.63 (s, 1H, H-8), 6.61 (m, 1H, H-10), 6.42 (s, 1H, H-3), 5.94 and 5.81 (s, 2H, 1,2-OCH_2O), 2.49 (s, 3H, *N*-CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 156.6 (9-OH), 147.2 (C-2), 141.6 (C-1), 135.6 (C-7a), 125.6 (C-3a or C-1b), 124.7 (C-3a or C-1b), 121.9 (C-11a), 114.2 (C-8), 112.9 (C-10), 61.8 (C-6a), 52.9 (C-5), 42.1 (*N*-CH_3), 33.7 (C-7), 27.6 (C-4).

CHAPTER III

ANTIOXIDANT ACTIVITY OF ISOLATED COMPOUNDS FROM *Uvaria rufa*

3.1 Antioxidant Activity of Isolated Compounds

The fractionation and purification of CH_2Cl_2 and butanolic crude extracts of *U. rufa* led to the isolation of 15 compounds. They were six flavonoids (compounds **1**, **2**, **3**, **6**, **7** and **8**), two aromatic compounds (compounds **4** and **5**) and seven alkaloids (compounds **9-15**). Compounds **1**, **2**, **6** and **7** have been isolated previously from *U. rufa*, but the others compounds are the first report from this species. The antioxidant activity of all compounds were determined in terms of IC_{50} by using in vitro spectrophotometric assay. The free radical scavenging activity were evaluated against DPPH radicals and superoxide radicals, which are generated in xanthine/xanthine oxidase system (Table 3.1). In addition, all compounds were also evaluated antioxidant activity by inhibitory activity against xanthine oxidase (data not shown in Table 3.1).

Table 3.1 Antioxidant activity of isolated compounds

Isolated Compounds	IC_{50} (mg/mL)	
	DPPH scavenging	**Superoxide scavenging**
2,5-Dihydroxy-7-methoxy flavanone (**1**)	0.34	>4
Tectochrysin (**2**)	>2	>4
5-Hydroxy-7-methoxy flavanone (**3**)	>2	-
Benzyl benzoate (**4**)	>2	-
2-Methoxy benzyl benzoate (**5**)	>2	-
6,7-*O*,*O*-Dimethylbaicalein (**6**)	>2	>4
7-*O*-Methylwogonine (**7**)	>2	>4
2,5-Dihydroxy-6,7-dimethoxy flavanone (**8**)	0.16	1.03
Mixture of Liriodenine (**9**) and Lanuginosine (**10**)	>2	3.04
Oxoanolobine (**11**)	>2	-
Roemerine (**12**)	>2	-
Mixture of Anonaine (**13**) and Xylopine (**14**)	0.35	-
Roemeroline (**15**)	1.63	-
Ascorbic acid*	0.03	-
BHA*	0.03	-
Quercetin*	-	0.97
Gallic acid*	-	0.30

* Standard antioxidant

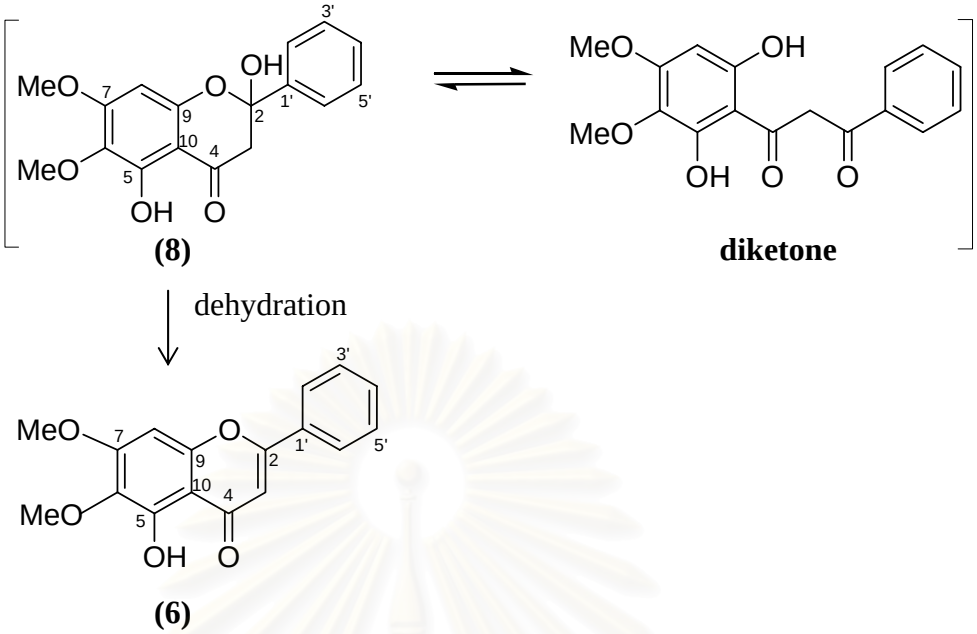
- Not determined

3.2 Discussion

DPPH radicals scavenging activity

As shown in Table 3.1, compound **8** showed the highest activity (IC_{50} = 0.16 mg/mL) and the activity decreased in the following order: **1** (IC_{50} = 0.34 mg/mL) > mixture of **13** and **14** (IC_{50} = 0.35 mg/mL) > **15** (IC_{50} = 1.63 mg/mL), while compounds **2**, **3**, **4**, **5**, **6**, **7**, mixture of **9** and **10**, **11** showed no activity (IC_{50} >2 mg/mL). However, they exhibited moderate to weak antioxidant activity when compared with the standard antioxidant, ascorbic acid (IC_{50} = 0.03 mg/mL) and BHA (IC_{50} = 0.03 mg/mL).

Generally, flavonoids with multiple hydroxyl groups, especially on the ring B, enhance their antioxidant activity (Fazilatun *et al.*, 2004). Previously report provided clear evidence that the radical scavenging activity of flavonoid depends on the structure and the substituents of rings C and B (Bors *et al.*, 1990). More specifically, the major determinants for radical scavenging capability are the presence of a catechol group in ring B, which has the better electron-donating properties and is a radical target, and a 2,3-double bond conjugated with the 4-oxo group, which is responsible for electron delocalization, while additional hydroxyl or methoxyl groups at positions 3, 5 and 7 of rings A and C seem to be less important (Pietta, 2000). Thus, **2**, **3**, **6** and **7** show no activity because their structures are not essential on scavenging of free radical. In addition, when compared under the condition of compounds **1**, **2**, **6** and **8**, compounds **1** and **8** that are both 2,5-dihydroxy-substituted showed potent antioxidant activity against DPPH radicals, whereas **2** and **6** that lack hydroxyl groups at C-2 were less active than their 2-hydroxylated analogues suggesting that 2-hydroxylation was beneficial for scavenging activity against DPPH radicals in this series of compounds. Moreover, **8** has been previously reported for scavenging activity on DPPH. Its activity was shown to be weaker than that of quercetin but higher then BHT (Wang *et.al*, 1999). Potent antioxidation activity of compounds **1** and **8** over other flavonoids might be associated with equilibrium composition between cyclic hemiacetal and diketone in solution (Stevens *et.al*, 1999; Mustafa *et.al*, 2003).



The presence of additional phenolic group in diketone structure enhances radical scavenging activity. The higher activity of **8** over **1** might be explained by resonance effect of additional 6-OMe, which stabilizes electron density of aromatic ring.

In comparison among the alkaloid series, this result indicated that the aporphine alkaloid series (**12-15**) displayed higher free radical scavenging activity on DPPH than the oxoaporphine alkaliod series (**9-11**).

Superoxide radicals scavenging activity

As shown in Table 3.1, compound **8** also exhibited the highest activity with IC_{50} value 1.03 mg/mL. It showed an activity to scavenge superoxide radicals close by quercetin (IC_{50} = 0.97 mg/mL). While **1** exhibited more weaker activity (IC_{50} >4 mg/mL) when compared to its DPPH scavenging activity. This result may possibly appeared because of partial solubility in aqueous test media and its interference with the spectroscopic measurements. Mixture of **9** and **10** displayed moderate activity with IC_{50} value 3.04 mg/mL. The other compounds could not be determined because of their limited amount and solubility.

Inhibition of xanthine oxidase activity

Inhibition of xanthine oxidase activity was evaluated by the spectrophotometric measurement of production of uric acid from xanthine at 291 nm. Allopurinol, a powerful inhibitor of the xanthine oxidase and used as a medication, was used as a positive control.

The activity of all compounds were found to be more lower than allopurinol (IC_{50} = 0.01 mg/mL). Compound **1** displayed the inhibition effect with IC_{50} value 1.05 mg/mL followed by **8** (IC_{50} = 1.54 mg/mL), while **2**, **6** and **7** showed no activity at concentration of 0.3 mg/mL and could not increase their concentration to evaluate IC_{50} because they had shown suspension phenomenon that interrupted the capacity of them to interact with the xanthine oxidase and caused a shift absorbance when the absorption was detected. The other compounds have limited amount and also showed weak activity (IC_{50} >2 mg/mL), so IC_{50} could not be calculated.

According to the result indicated that all compounds did not show xanthine oxidase inhibitor.

From previous report on capability of flavonoids as a xanthine oxidase inhibitor revealed that the olefins at C-2 and C-3 that maintain a planar structure of flavonoids are essential for potent inhibitory activity on xanthine oxidase. In addition, hydroxyl moiety at C-7 and C-5, and the carbonyl group at C-4 contribute favorable hydrogen bonds and electrostatic interactions between inhibitors and the active site of enzyme. Thus, flavanones and flavones that lack of hydroxyl moiety at C-7 or C-5 showed no xanthine oxidase inhibition activity (IC_{50} > 40 μM) (Van Hoorn *et.al*, 2002; Lin *et.al*, 2002; da Silva *et.al*, 2004).

On the basis of above results, 2-hydroxyflavanone (**1** and **8**), which are extremely rare natural products, exhibited some antioxidant activity although it lack of multiple hydroxyl groups on benzene ring. However, there are so far only few studies that regraded about antioxidant activity of 2-hydroxyflavanone. It would thus be of interest to learn what mechanism that they scavenge free radical. In addition, **1** and **8** have been reported for cytotoxic activity against breast cancer cell lines and weakly toxicity against *Artemia salina*, while their analogues, **2** and **6,** showed less active (Khamis *et.al*, 2004).

Compound **2**, tectochrysin, which showed no antioxidant activity on scavenging free radicals and inhibitory on xanthine oxidase, has been reported as potent and specific inhibitors of breast cancer resistance protein ABCG2 and found to

have a promising inhibitors for the reversal of ABCG2-mediated drug transport (Ahmed-Belkacem *et.al*, 2005). Futhermore, tectochrysin possesses considerable antimutagenic properties against ofloxacin-induced bleaching of *E. gracilis* (Križková *et.al*, 1998).

Compound **4**, benzyl benzoate, which showed no antioxidant activity in all assay, have been reported for insecticidal activity towards neonate larvae of *S. littoralis* only when applied topically via the larval integument with LC_{50} value 5.6 $\mu g/cm^2$ in the glass vial assay (Nugroho *et.al*, 1996) and it posseses a strong acaricidal activity against *Dermatophagoides pteronyssinus* strain, the European house dust mite with EC_{50} value 0.04 g/m^2 (Akendengue *et.al*, 2002). Moreover, it presently is the reference agent for pediculocidal, scabicidal and acaricidal activity

Compound **9**, liriodenine, did not test as pure compound because of small amount of sample and difficult purification. From all above data, it did not show a strong antioxidant activity but in many studies, numerous positive effect of liriodenine have been described, such as antibacterial activity, antifungal activity (Nissanka *et.al*, 2001; Khan *et.al*, 2002; Zhang *et.al*, 2002; Khan *et.al*, 2003; Rahman *et.al*, 2005), antiplasmodial agents (Mbah *et.al*, 2004), antiplatelet aggregation (Chen *et.al*, 1997; Pyo *et.al*, 2003), cytotoxicity (Ogura *et.al*, 1997; Goren *et.al*, 2003; Rahman *et.al*, 2005), topoisomerase II Inhibitor (Woo *et.al*, 1997; Woo *et.al*, 1999) and other (Chang *et.al*, 2004; Hsieh *et.al*, 2005). Whereas compound **10**, lanuginosine has been reported only antimicrobial activity (Khan *et.al*, 2003) and cytotoxicity (de Siqueira *et.al*, 2001; Khamis *et.al*, 2004).

Compound **12**, roemerine, showed no antioxidant activity, however, in previous report it exhibited relaxant activity (Chulia *et.al*, 1995) and vascular activity (Valiente *et.al*, 2004).

Compound **13** and **14**, anonaine and xylopine, did not test as pure compound because of small amount of sample and difficult purification. From all above data, they showed modest antioxidant activity on scavenging DPPH radicals. Moreover in many previous report, numerous positive effect of anonaine and xylopine have been described. Anoniane has been reported as the CD45 protein tyrosine phosphatase inhibitor, from *Rollinia ulei* (Miski *et.al*, 1995). In additon, it showed various activity such as relaxant activity (Chulia *et.al*, 1995), antidepressant activity (Protais *et.al*, 1995), fungicidal activity (Bettarini *et.al*, 1993) and antibacterial activity (Pauloa *et.al*, 1992). For xylopine, it exhibited antimicrobial activity (Hossain *et.al*, 1993) and

leischmanicidal activity (Montenegro *et.al*, 2000). In addition, xylopine showed strong inhibition of platelet-activating factor (Chen *et.al*, 1996).

3.3 Experimental Section

3.3.1 General Experimental Procedures.

pH values were determined with MP220 pH meter Mettler Toledo. UV-visible absorption spectra were recorded on UV-2500PC UV-Vis spectrometer (Shimadzu, Kyoto, Japan) and UV-spectrometer, microtiter plate reader, model sunrise (Tecan Austria GmbH).

3.3.2 DPPH radicals scavenging activity.

TLC autographic assay**,** using 2,2-diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl (DPPH) radical as spray reagent was employed for rapid detection of antioxidants in crude extracts. The assay involves spraying TLC plates with 0.3 mM DPPH in methanol. Active compounds occur as yellow spots on a purple background (Maver *et.al*, 2005). After isolation and purification, activities of pure compounds were quantified in a modified spectrophotometric assay (Yen and Hsieh, 1997; Westenburg *et.al*, 2000). Briefly, sample dissolved in methanol was added with 0.3 mM DPPH solution. After 30 min incubation at room temperature in the dark, the absorbance was measured at 517 nm with a spectrophotometer. All tests were run in triplicate and averaged. The scavenging activity was evaluated from the decrease value of 517 nm absorption, which was calculated by the following equation and the activity was expressed as IC_{50} value.

$$\%\text{Scavenging activity} = [1-A_{sample}/A_{blank}] \times 100$$

3.3.3 Superoxide radicals scavenging activity.

Superoxide radicals were generated by Okamura's method (1993) with slight modification. Test compound in 50%DMSO (100 µL) was added to the mixture (1 mL) consisting of 0.4 mM xanthine and 0.24 mM nitroblue tetrazolium (NBT) in 0.1 M phosphate buffer (pH 8.0). Xanthine oxidase (0.05 unit/mL, 100 µL) was added to the reaction mixture followed by incubation at 37°C for 30 min. The reaction was stopped by adding 69 mM sodium dodecyl sulfate (SDS) 50 µL. The absorbance was measured at 650 nm.

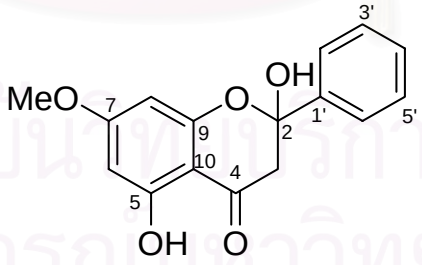
3.3.4 Inhibition of xanthine oxidase activity.

The method described by Schuldt (2004), using xanthine as the substrate, was applied with slight modification. The reaction mixture consisted of test compound in 50%DMSO 100 µL, 0.1 M phosphate buffer (pH 8.0), and xanthine oxidase (0.05 unit/mL) solution 100 µL. The reaction was intiated by adding 0.1 mM solution of xanthine and incubated at 37°C for 30 min. After the reaction was terminated by adding 69 mM sodium dodecyl sulfate (SDS), formation of uric acid was detected by measuring absorbance at 291 nm.

CHAPTER IV

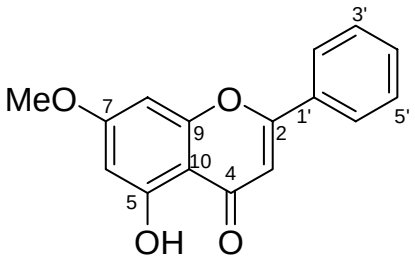
CONCLUSION

In conclusion, six flavonoid compounds, 2,5-dihydroxy-7-methoxy flavanone (**1**), tectochrysin (**2**), 5-hydroxy-7-methoxy flavanone (**3**), 6,7-*O*,*O*-dimethylbaicalein (**6**), 7-*O*-methylwogonine (**7**) and 2,5-dihydroxy-6,7-dimethoxy flavanone (**8**), two aromatic compounds, benzyl benzoate (**4**) and 2-methoxybenzyl benzoate (**5**), and seven alkaloid compounds, liriodenine (**9**), lanuginosine (**10**), oxoanolobine (**11**), roemerine (**12**), anonaine (**13**), xylopine (**14**) and roemeroline (**15**), were isolated from the CH_2Cl_2 and butanolic crude extracts of *Uvaria rufa* roots. The occurence of 5-hydroxy-7-methoxy flavanone (**3**), 2,5-dihydroxy-6,7-dimethoxy flavanone (**8**), benzyl benzoate (**4**), 2-methoxybenzyl benzoate (**5**), liriodenine (**9**), lanuginosine (**10**), oxoanolobine (**11**), roemerine (**12**), anonaine (**13**), xylopine (**14**) and roemeroline (**15**) in *Uvaria rufa* were reported for the first time. NMR, MS experiments and comparison with previously reported were used to characterize the structures of all compounds. The structures of these isolated compounds are shown as followed



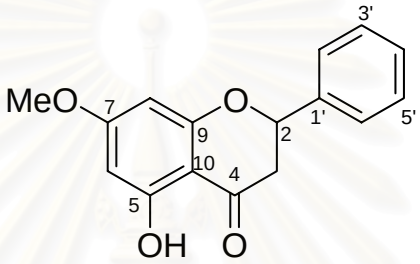
2,5-Dihydroxy-7-methoxy flavanone (**1**)

Total yield: 1144.0 mg, 7.63 % w/w



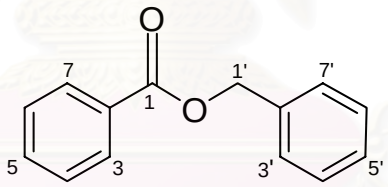
Tectochrysin (**2**)

Total yield: 310.0 mg, 2.07 % w/w



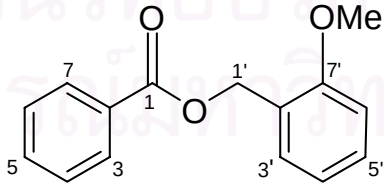
5-Hydroxy-7-methoxy flavanone (**3**)

Total yield: 1.7 mg, 0.01 % w/w



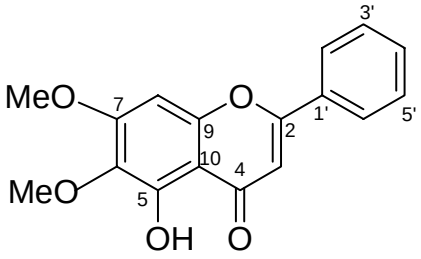
Benzyl benzoate (**4**)

Total yield: 2100.0 mg, 14.00 % w/w



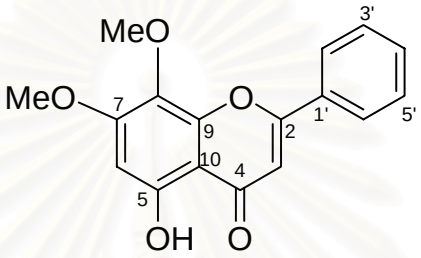
2-Methoxybenzyl benzoate (**5**)

Total yield: 14.6 mg, 0.10 % w/w



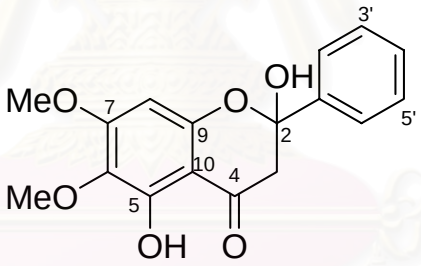
6,7-*O*,*O*-Dimethylbaicalein (**6**)

Total yield: 1036.0 mg, 6.91 % w/w



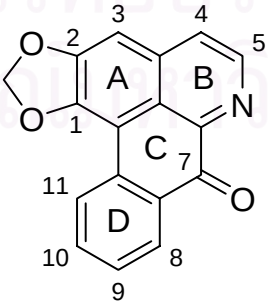
7-*O*-Methylwogonine (**7**)

Total yield: 21.2 mg, 0.14 % w/w



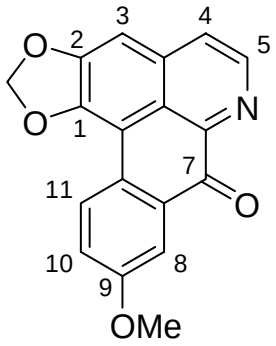
2,5-Dihydroxy-6,7-dimethoxy flavanone (**8**)

Total yield: 85.6 mg, 0.57 % w/w



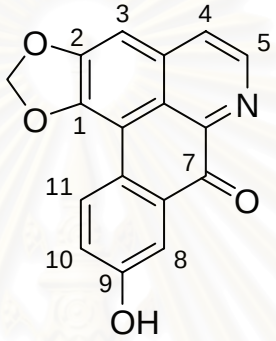
Liriodenine (**9**)

Total yield: 1.2 mg, 0.009 % w/w



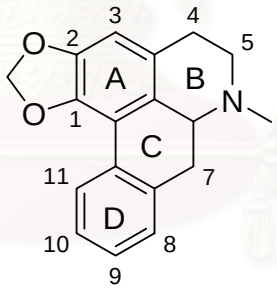
Lanuginosine (**10**)

Total yield: 2.9 mg, 0.02 % w/w



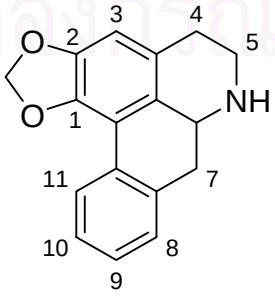
Oxoanolobine (**11**)

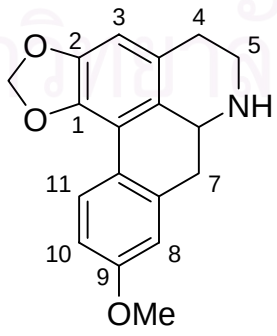
Total yield: 42.0 mg, 0.30 % w/w



Roemerine (**12**)

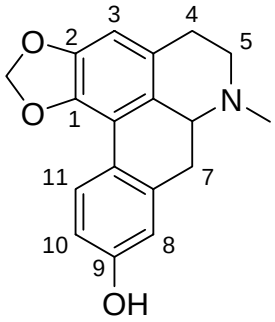
Total yield: 5.7 mg, 0.04 % w/w





Anonaine (**13**) + Xylopine (**14**)

Total yield: 2.5 mg, 0.02 % w/w



Roemeroline (**15**)

Total yield: 0.5 mg, 0.004 % w/w

The free radical scavenging activity against DPPH radicals revealed that compound **8** exhibited the highest activity (IC_{50} = 0.16 mg/mL), followed by **1** (IC_{50} = 0.34 mg/mL), mixture of **13** and **14** (IC_{50} = 0.35 mg/mL), **15** (IC_{50} = 1.63 mg/mL), the other compounds showed weak activity (IC_{50} > 2 mg/mL). In addition, compound **8** also displayed the highest scavenging activity against superoxide radicals (IC_{50} = 1.03 mg/mL), followed by mixture of **9** and **10** (IC_{50} = 3.04 mg/mL). In inhibitory effect on xanthine oxidase, all isolated compounds showed weak activity (IC_{50} > 0.30 mg/mL) when compared with reference, allopurinol (IC_{50} = 0.01 mg/mL).

In summary, the results presented here, revealed that compound **8** was the most potent antioxidant agent for scavenging free radicals. On the other hand, the other flavonoid compounds showed moderate to low activity. This result suggested that the presence of hydroxyl group on C-2 of flavonoid enhanced scavenging activity. Potent antioxidation activity might be associated with equilibrium composition between cyclic hemiacetal and diketone in solution. However, futher study is needed. Alkaloid in aporphine group might be better antioxidant agents than oxoaporphine group. The presence of compound **13**, together with **14**, showed the highest scavenging activity against DPPH when compared among alkaloid type. However, the absolute conclusion of antioxidant activity of this alkaloid series should be futher investigated and needed to use a more variety compounds. Two aromatic compounds, **4** and **5**, were devoid antioxidant activity. For the role as a xanthine oxidase inhibitor, all isolated compounds showed to be a weak xanthine oxidase inhibitor.

Proposal for the Future Work

2-hydroxyflavanone, which appears to be rare in nature and has few studies to date, showed potent antioxidant activity. Thus, its should be further studied on its mechanism and might be modified structure to enhance antioxidant activity.

Moreover, alkaloid in aporphine and oxoaporphine group have also few studies about their antioxidant activity. Therefore, they should be further investigated.

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APPENDIX

Appendix

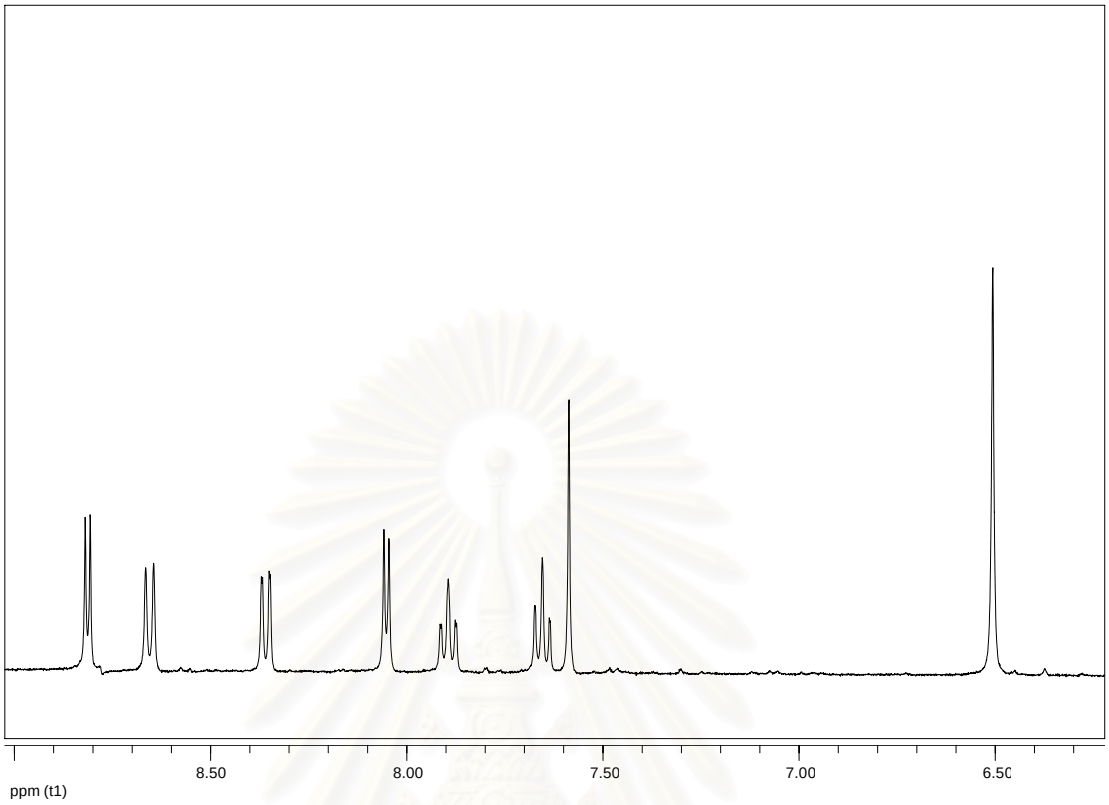

Figure 1 The ^{1}H NMR spectrum (DMSO-d_6) of compound **9**

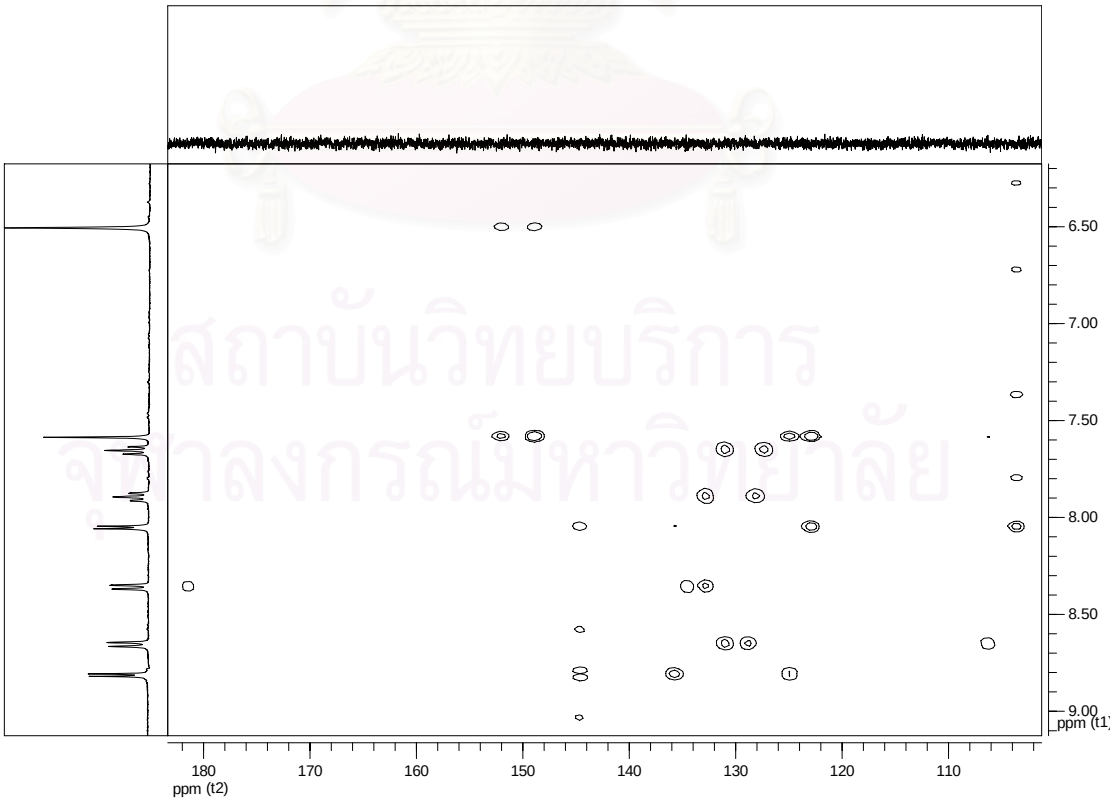

Figure 2 The HMBC spectrum (DMSO-d_6) of compound **9**

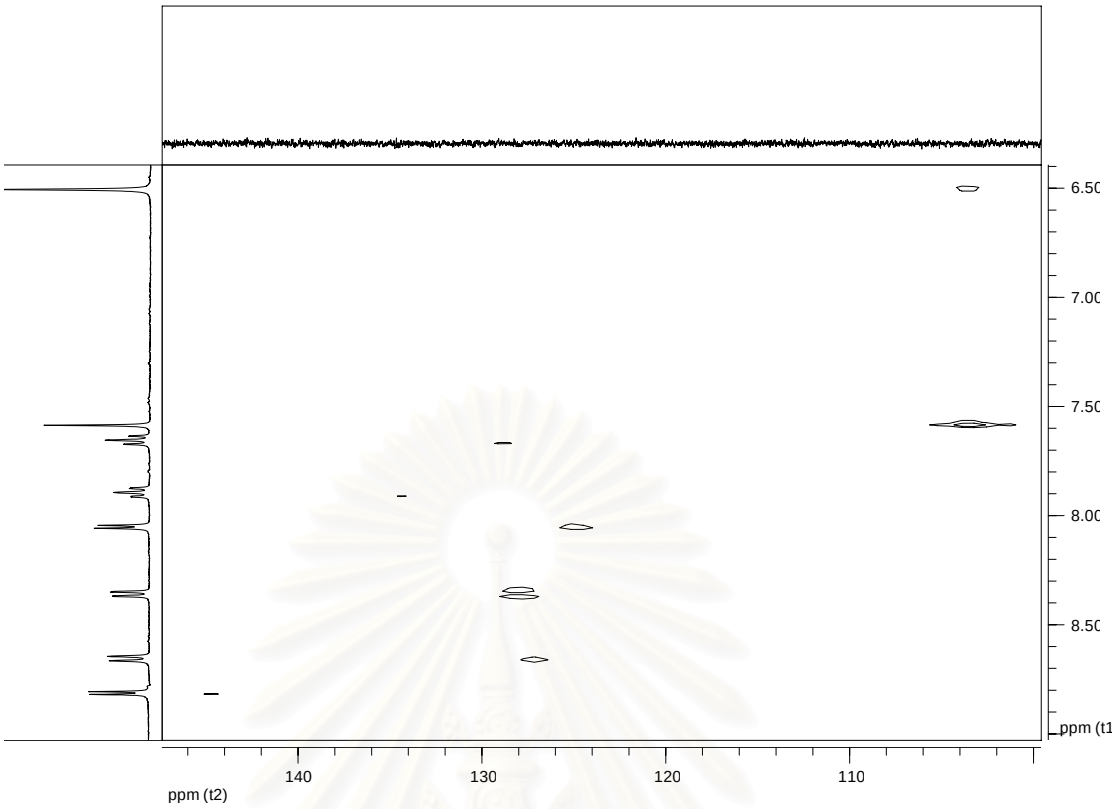

Figure 3 The HSQC spectrum (DMSO-d_6) of compound **9**

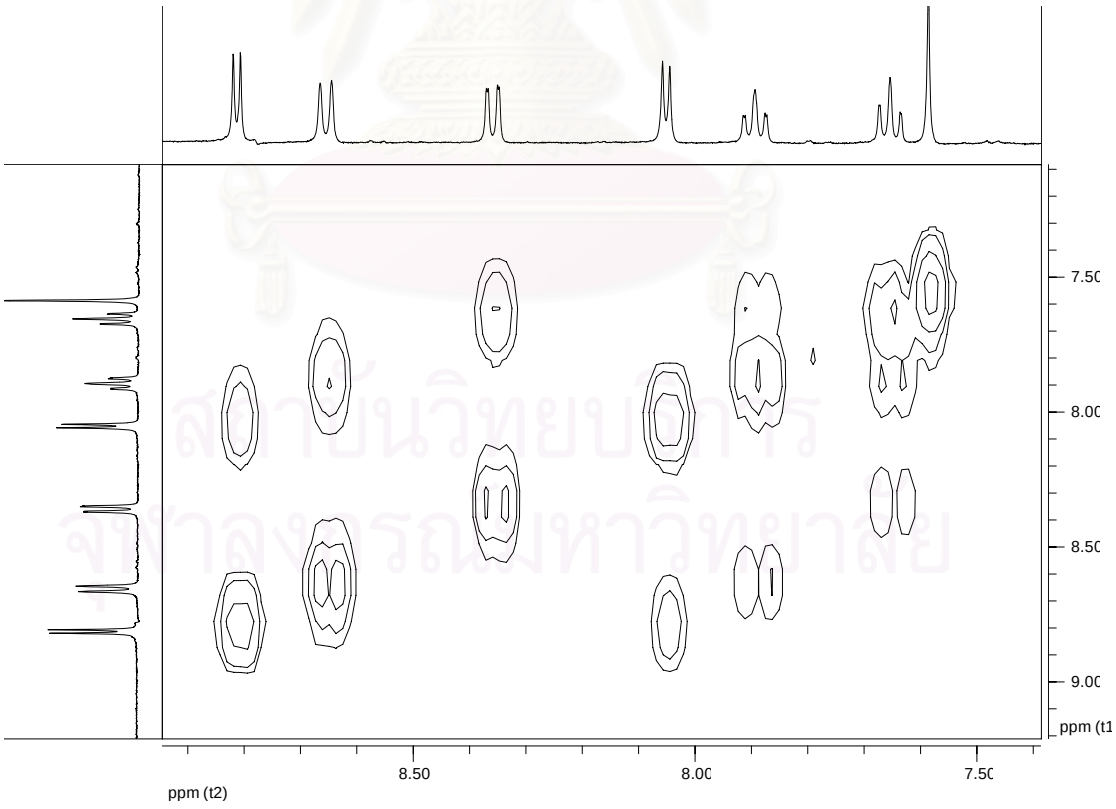

Figure 4 The COSY spectrum (DMSO-d_6) of compound **9**

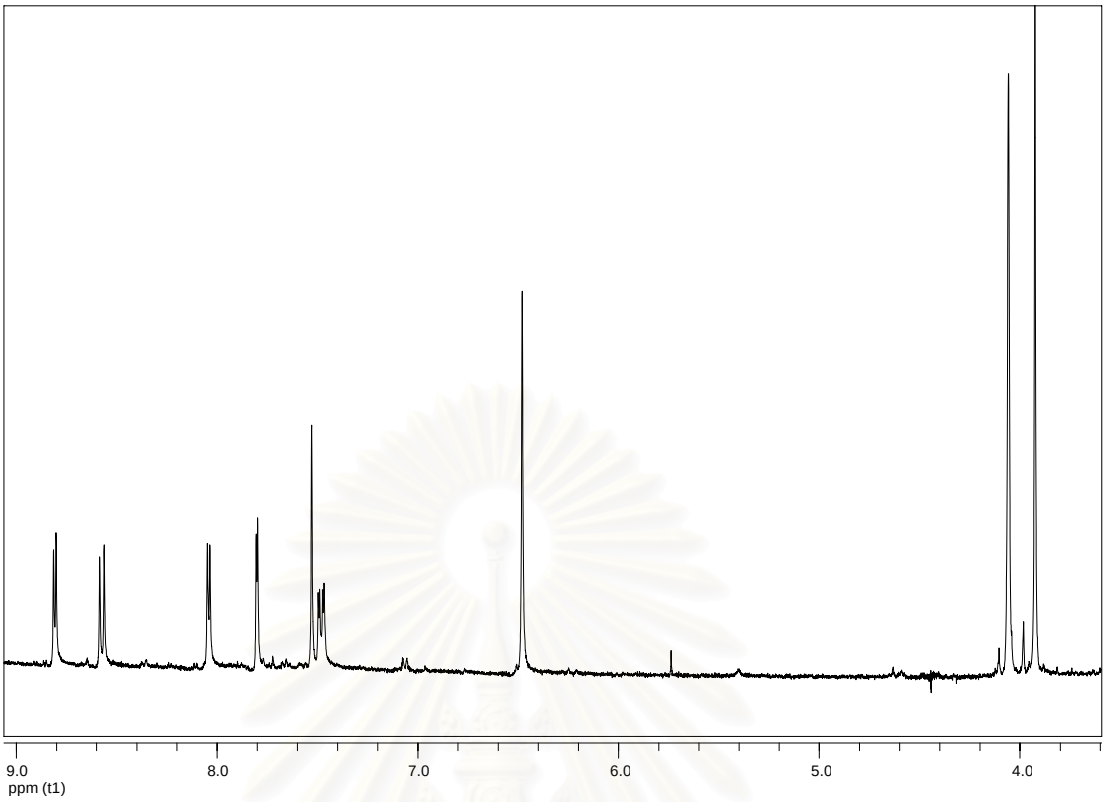

Figure 5 The ^{1}H NMR spectrum (DMSO-d_6) of compound **10**

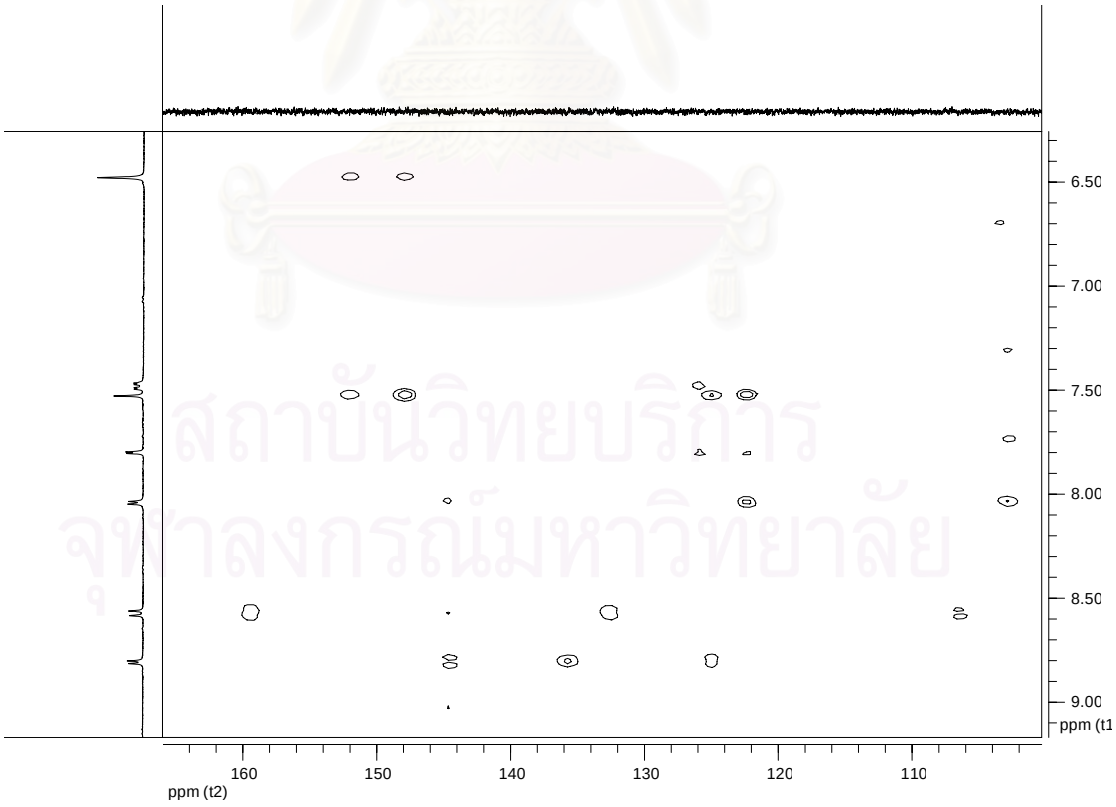

Figure 6 The HMBC spectrum (DMSO-d_6) of compound **10**

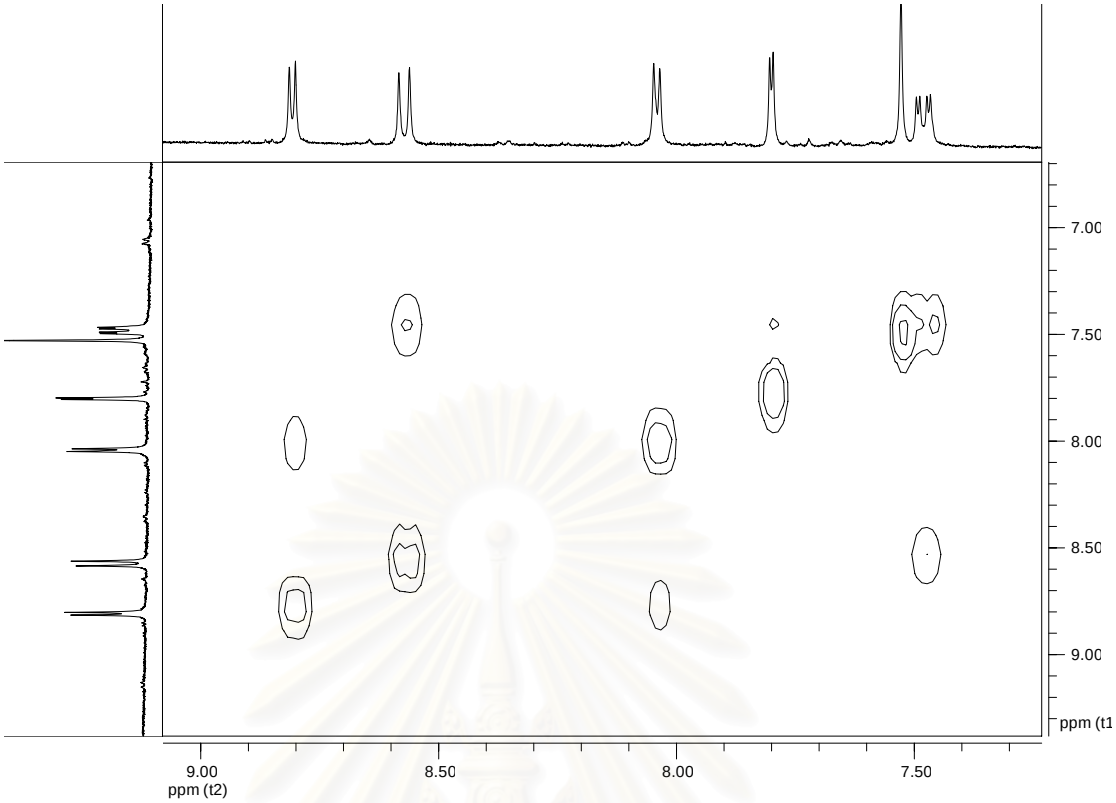

Figure 7 The COSY spectrum (DMSO-d_6) of compound **10**

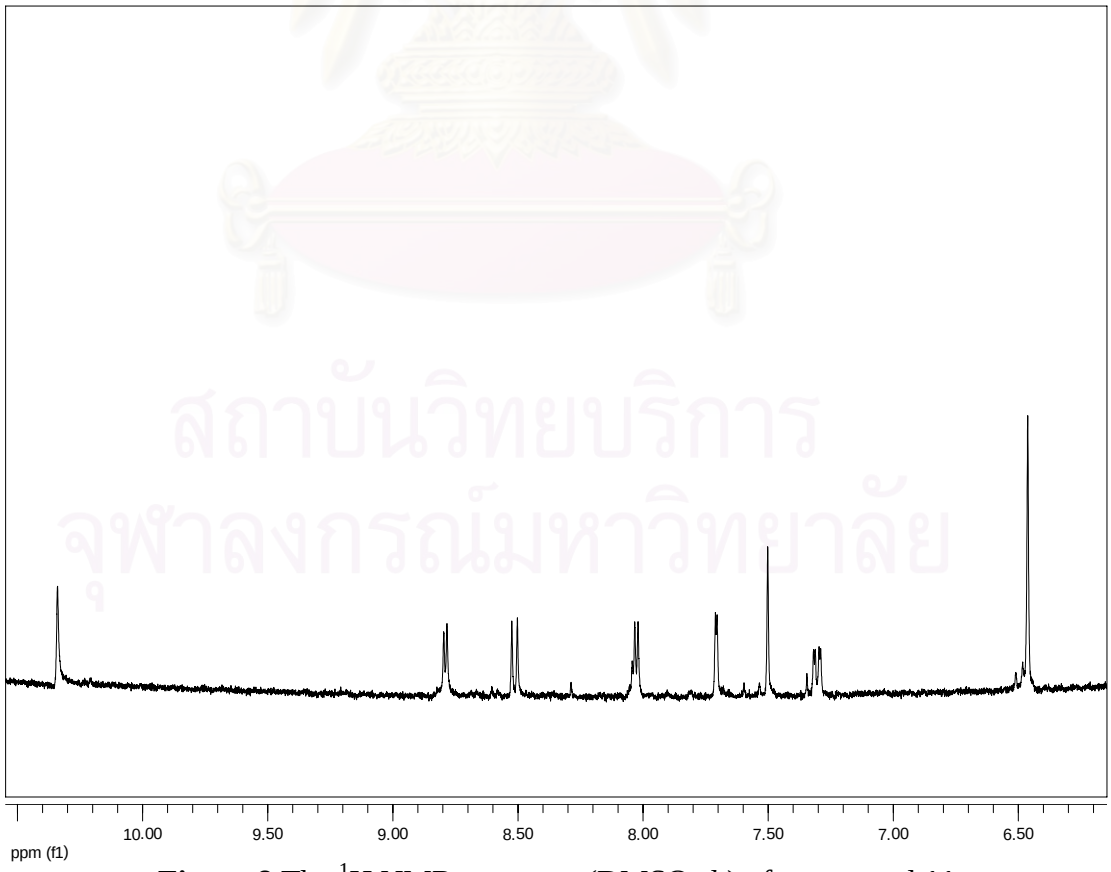

Figure 8 The ^{1}H NMR spectrum (DMSO-d_6) of compound **11**

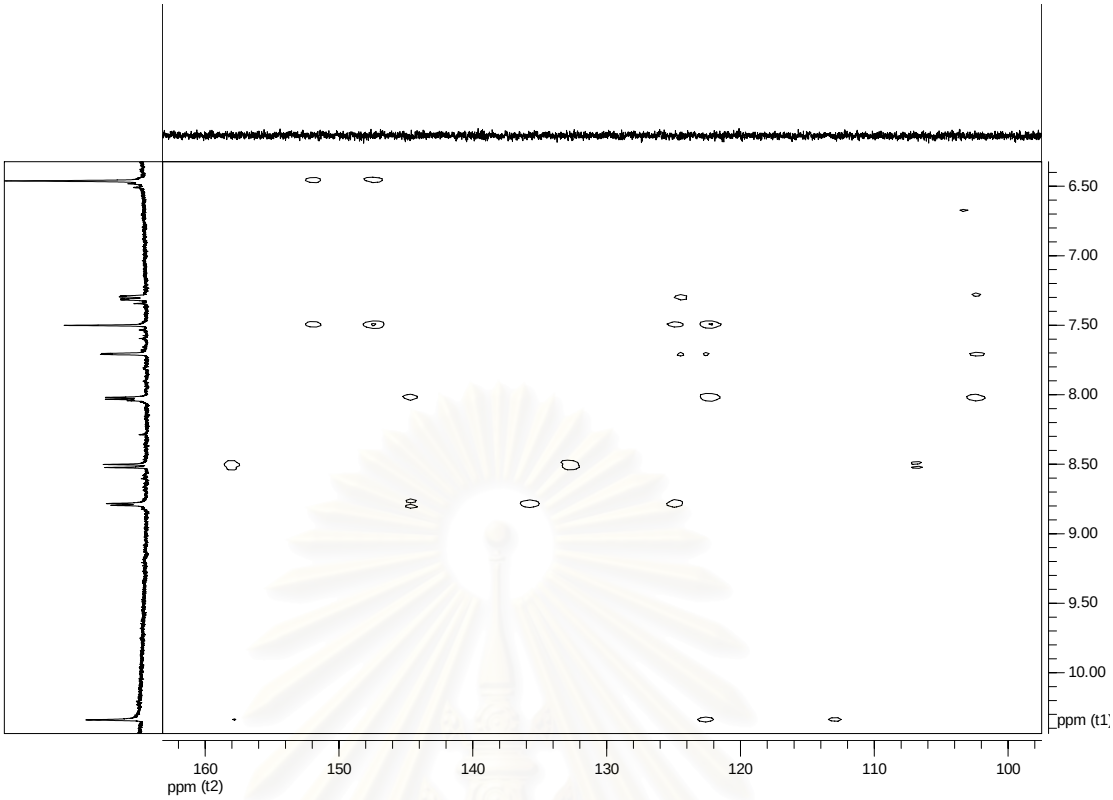

Figure 9 The HMBC spectrum (DMSO-d_6) of compound **11**

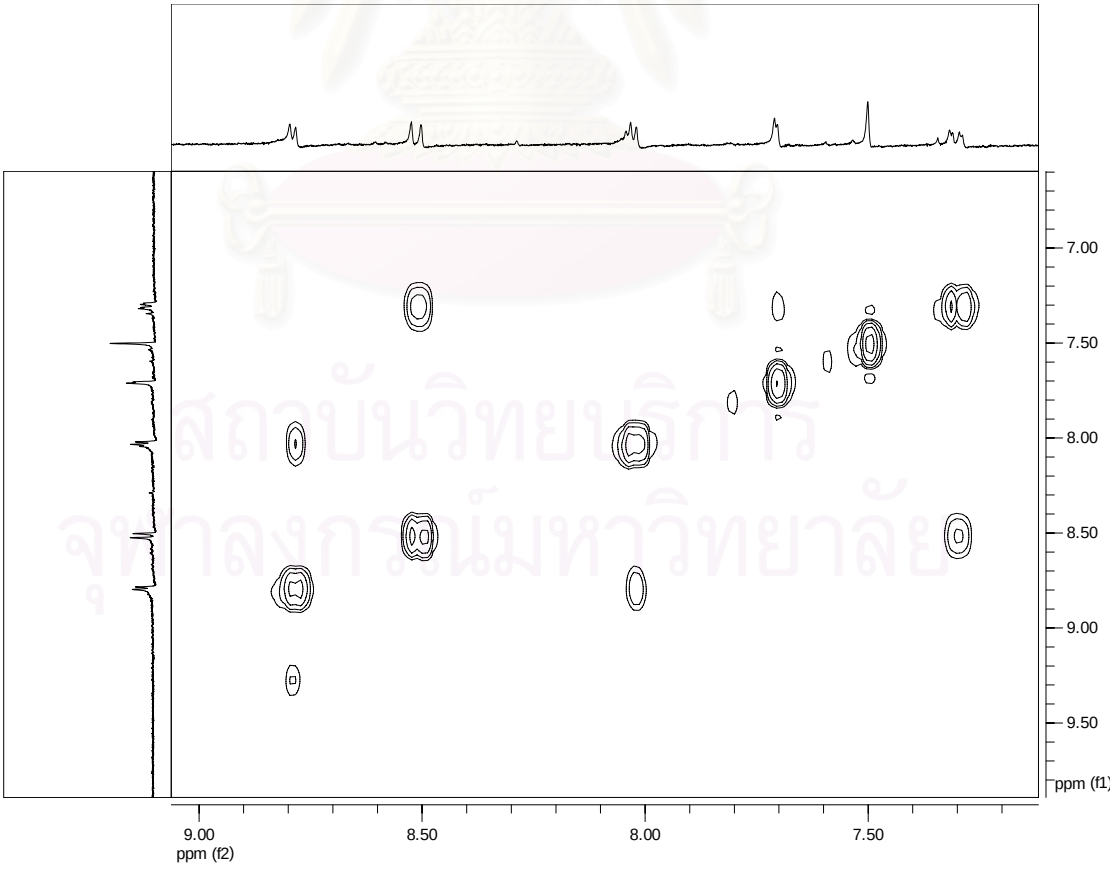

Figure 10 The COSY spectrum (DMSO-d_6) of compound **11**

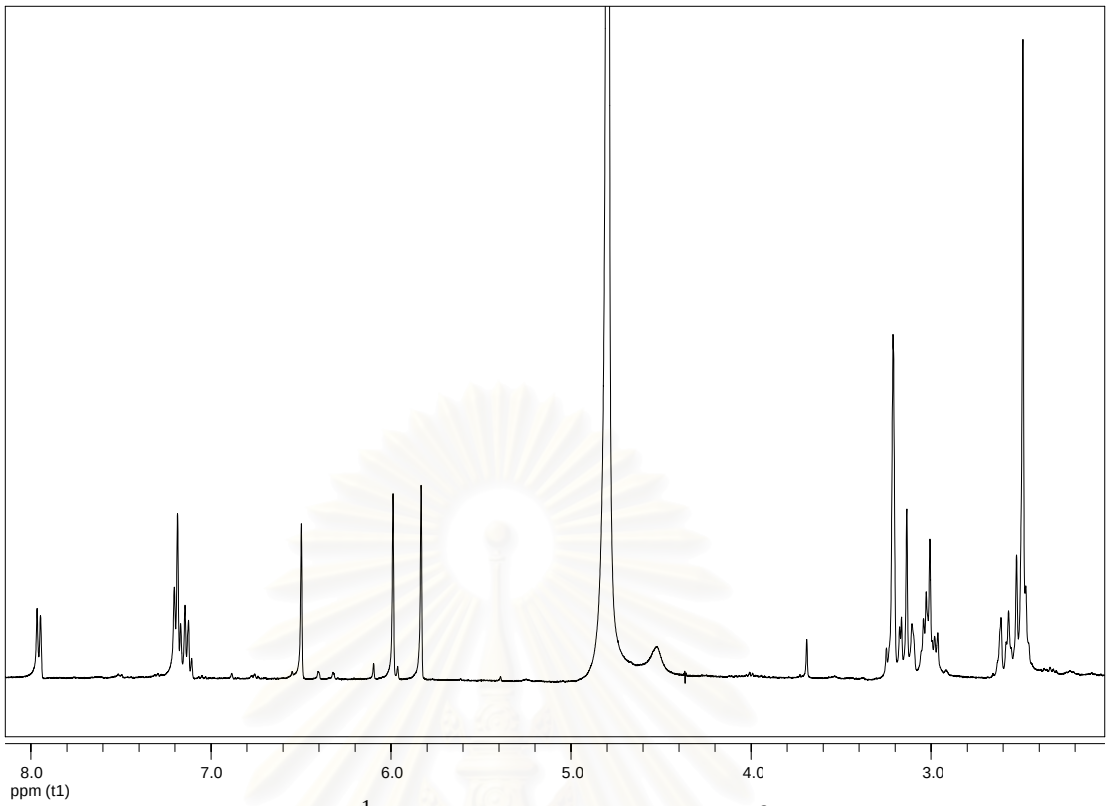

Figure 11 The ^{1}H NMR spectrum (CD_3OD) of compound **12**

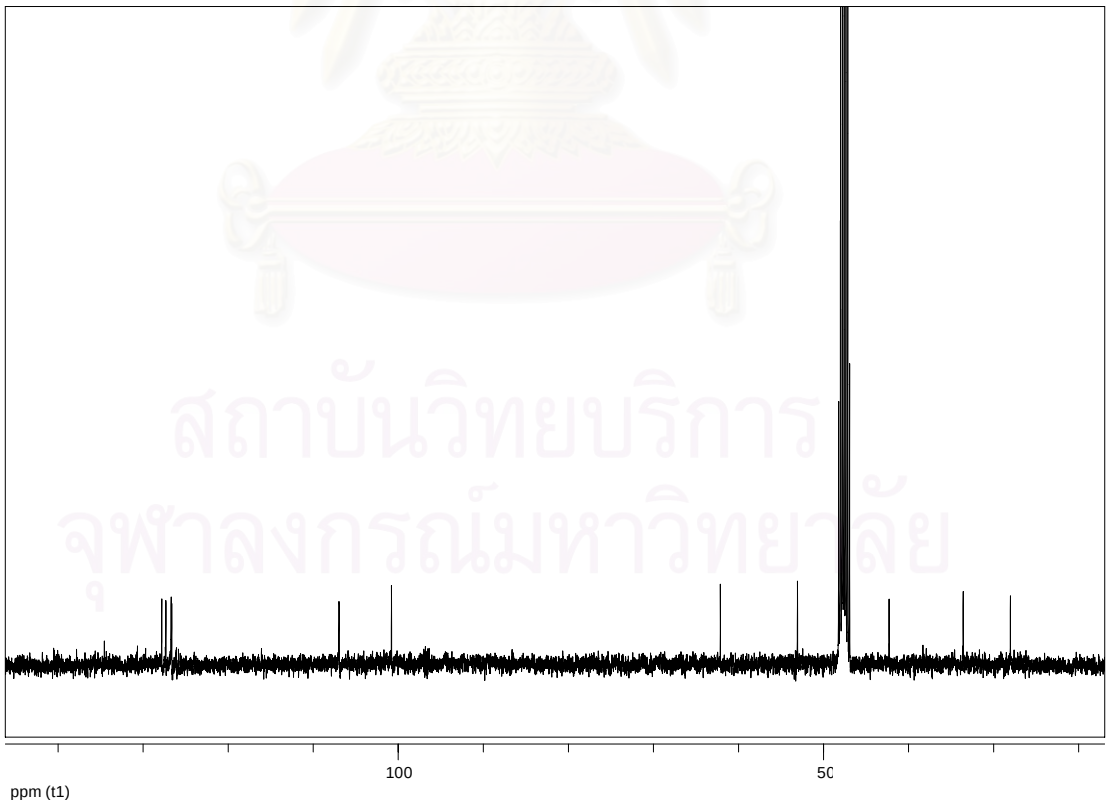

Figure 12 The ^{13}C NMR spectrum (CD_3OD) of compound **12**

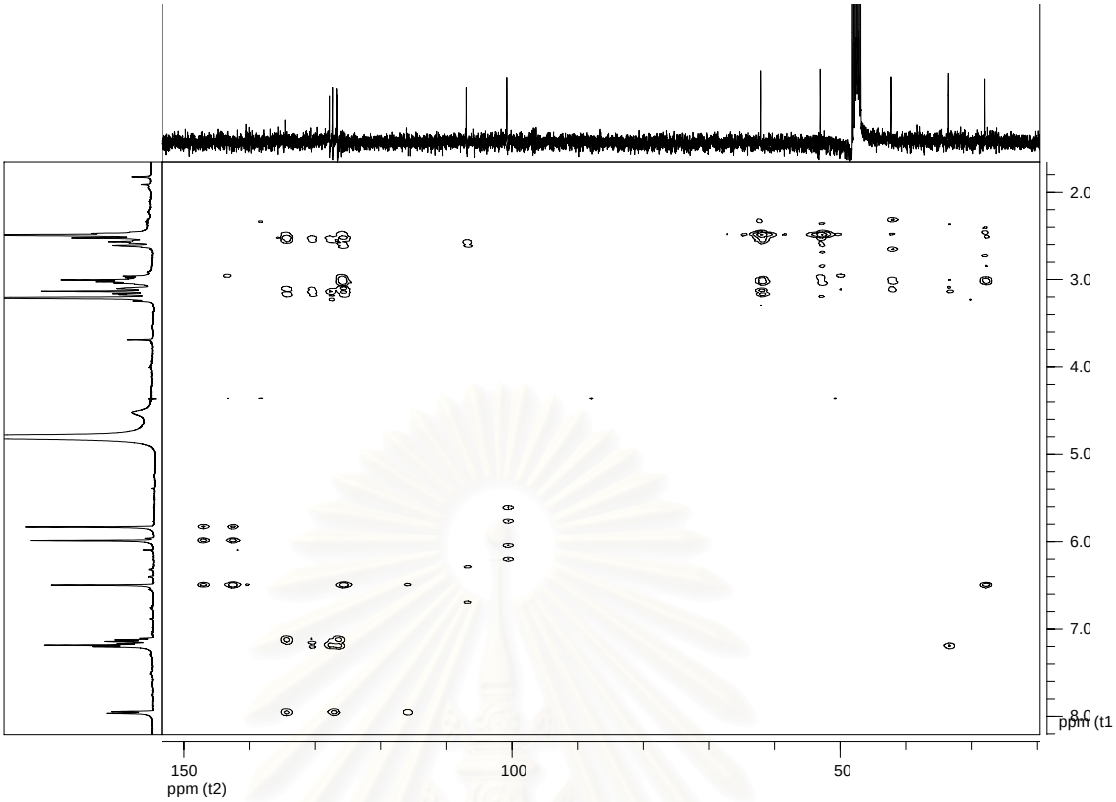

Figure 13 The HMBC spectrum (CD_3OD) of compound **12**

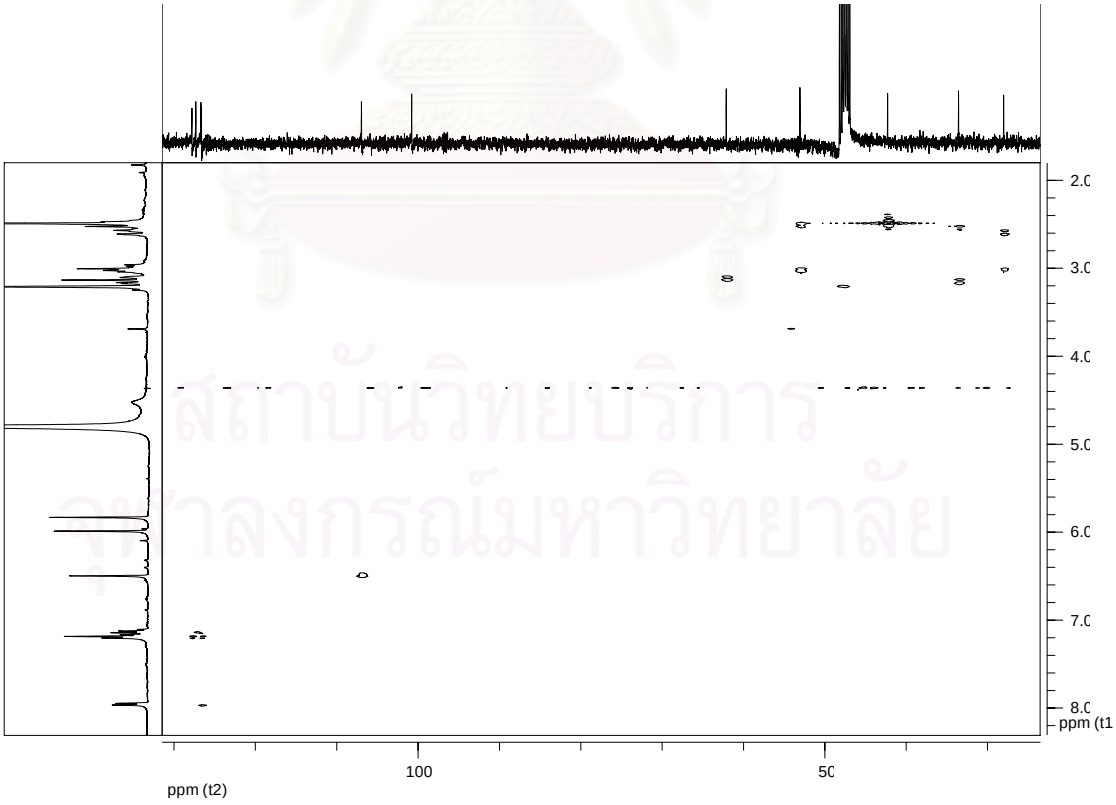

Figure 14 The HSQC spectrum (CD_3OD) of compound **12**

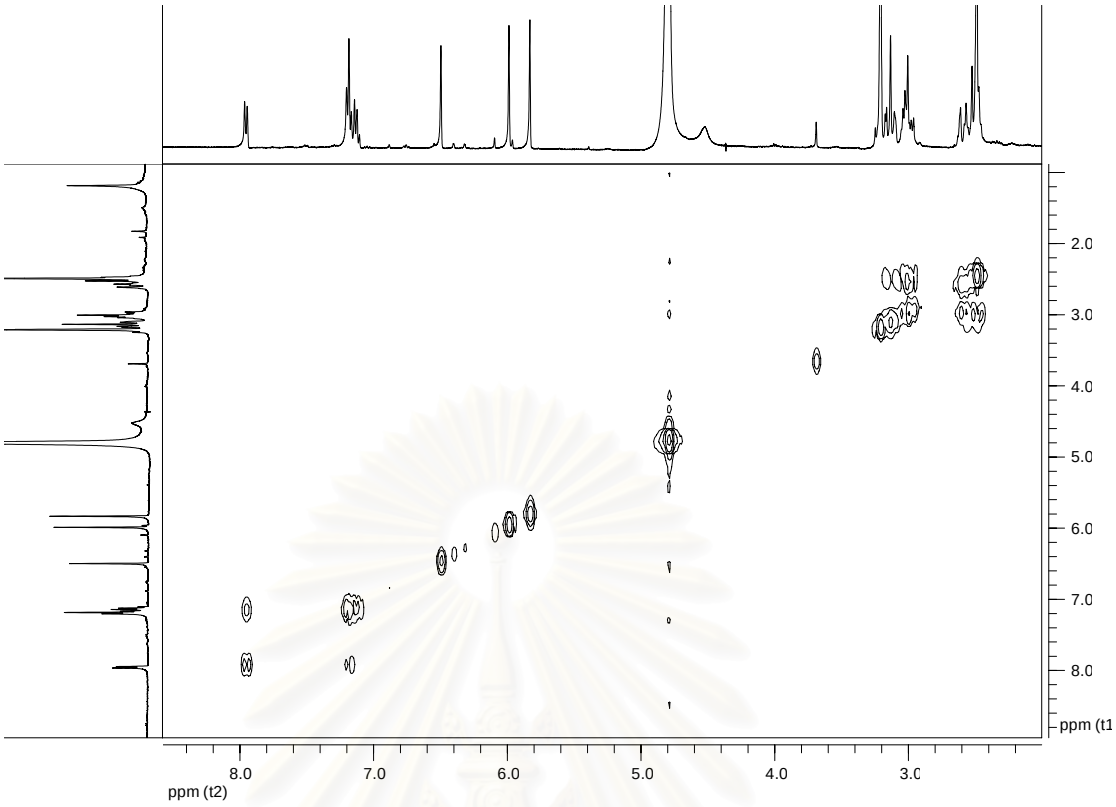

Figure 15 The COSY spectrum (CD_3OD) of compound **12**

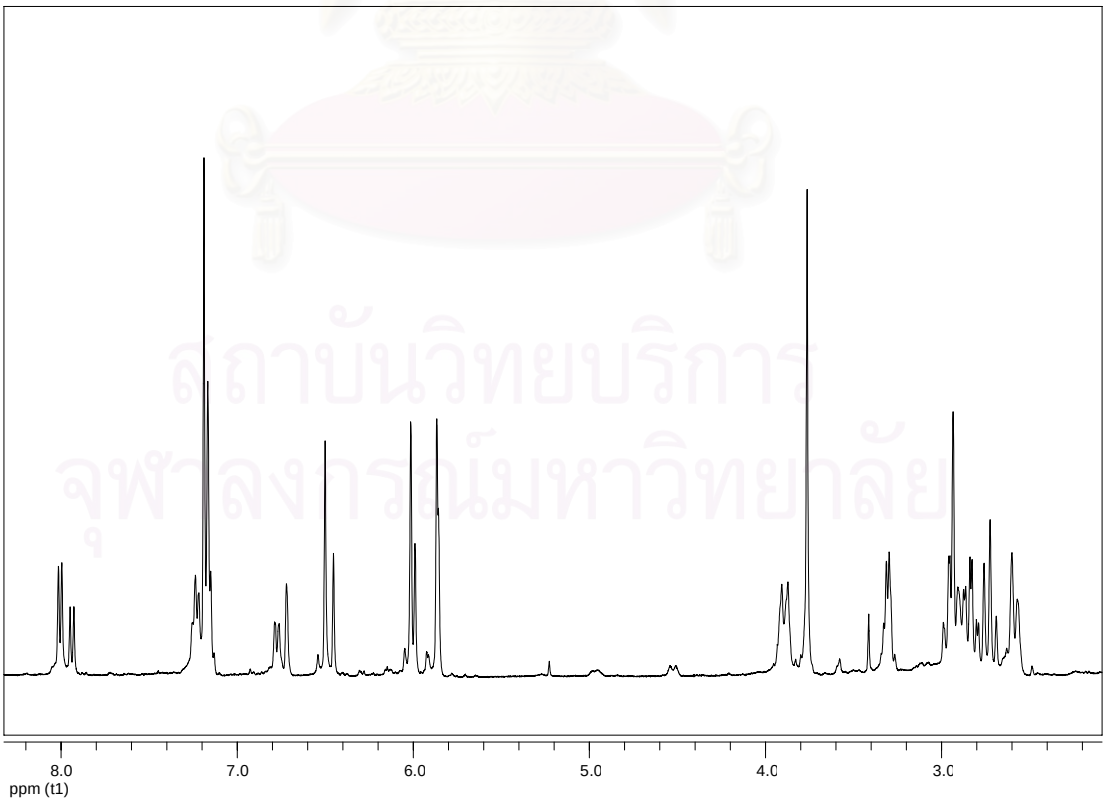

Figure 16 The ^1H NMR spectrum ($CDCl_3$) of mixture of compounds **13** and **14**

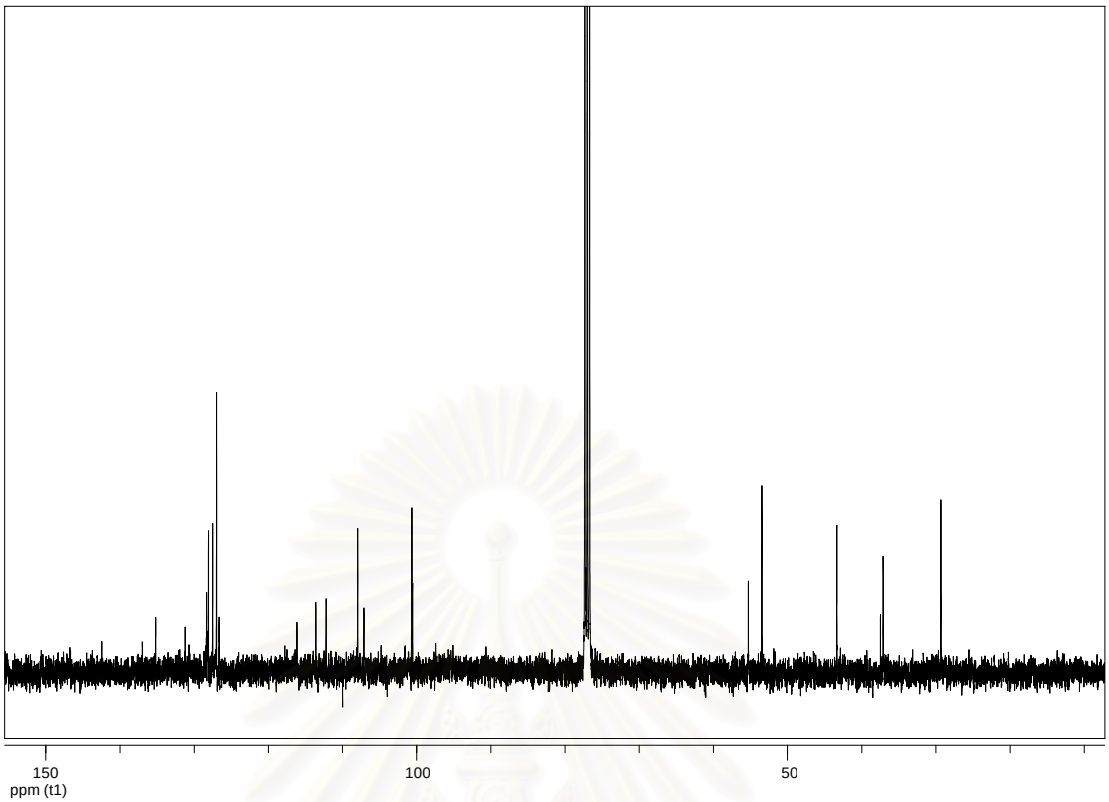

Figure 17 The ^{13}C NMR spectrum ($CDCl_3$) of mixture of compounds **13** and **14**

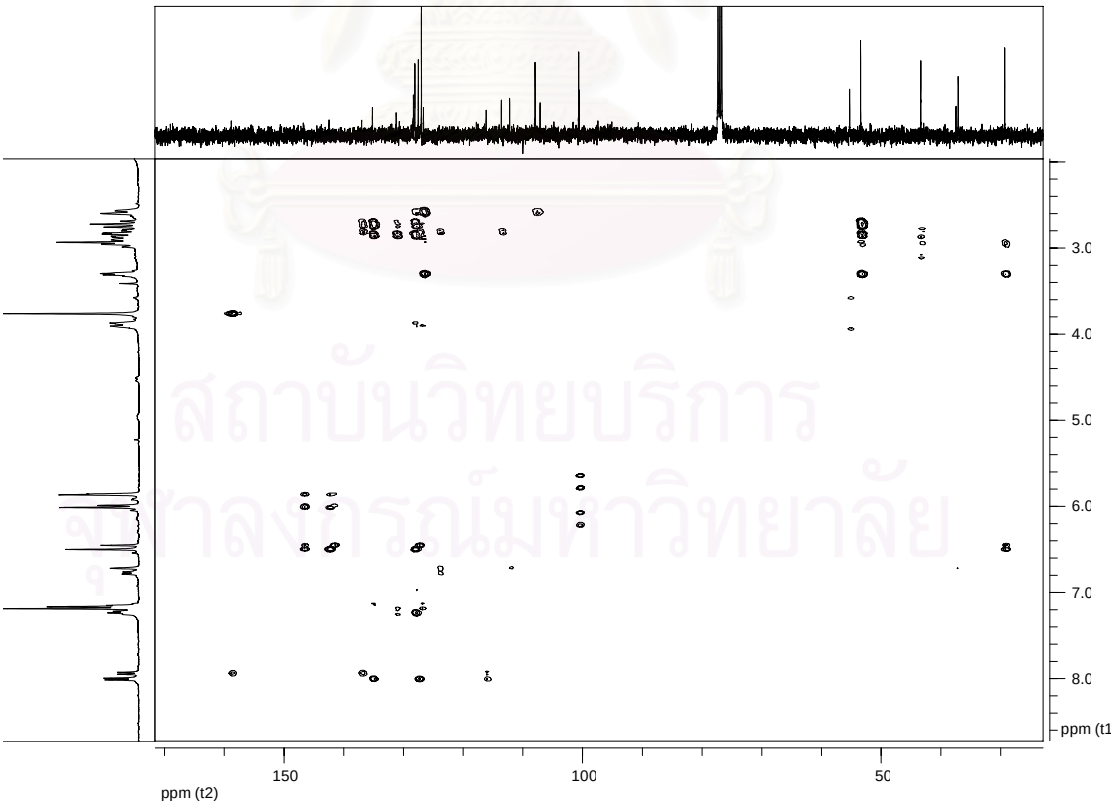

Figure 18 The HMBC spectrum ($CDCl_3$) of mixture of compounds **13** and **14**

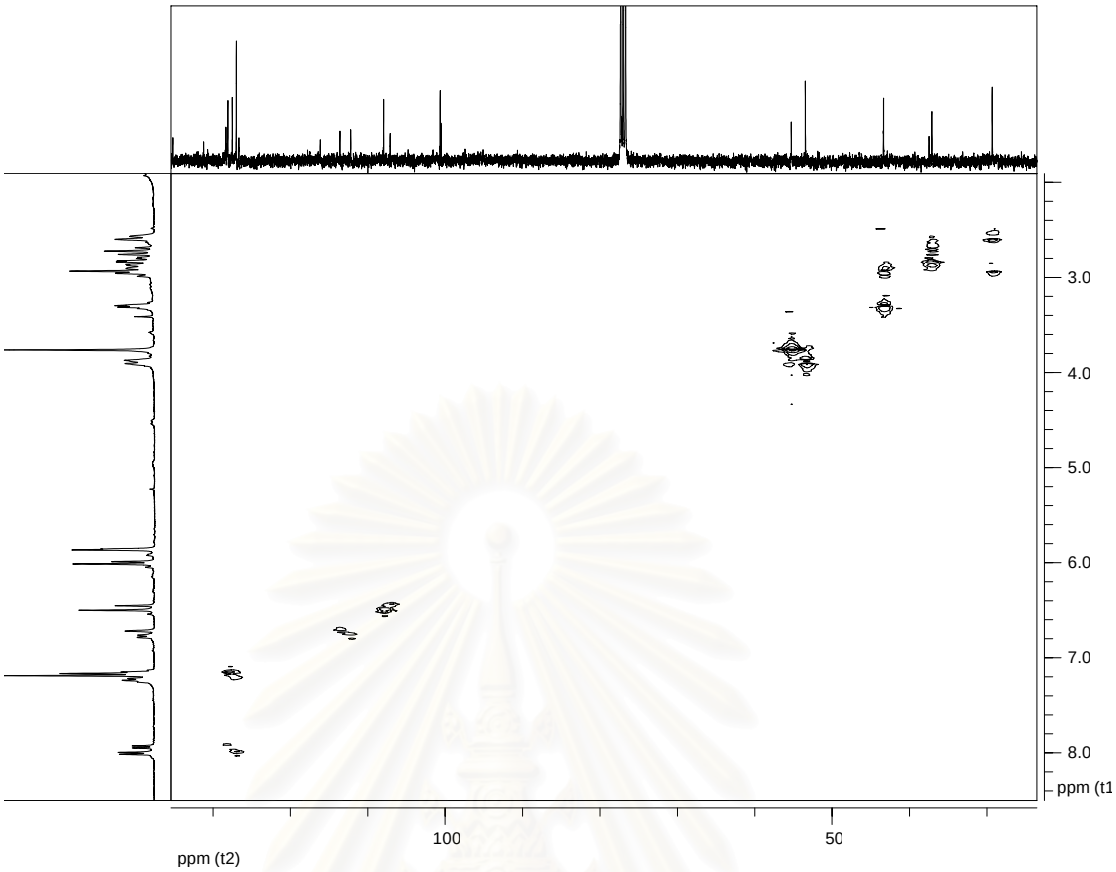

Figure 19 The HSQC spectrum ($CDCl_3$) of mixture of compounds **13** and **14**

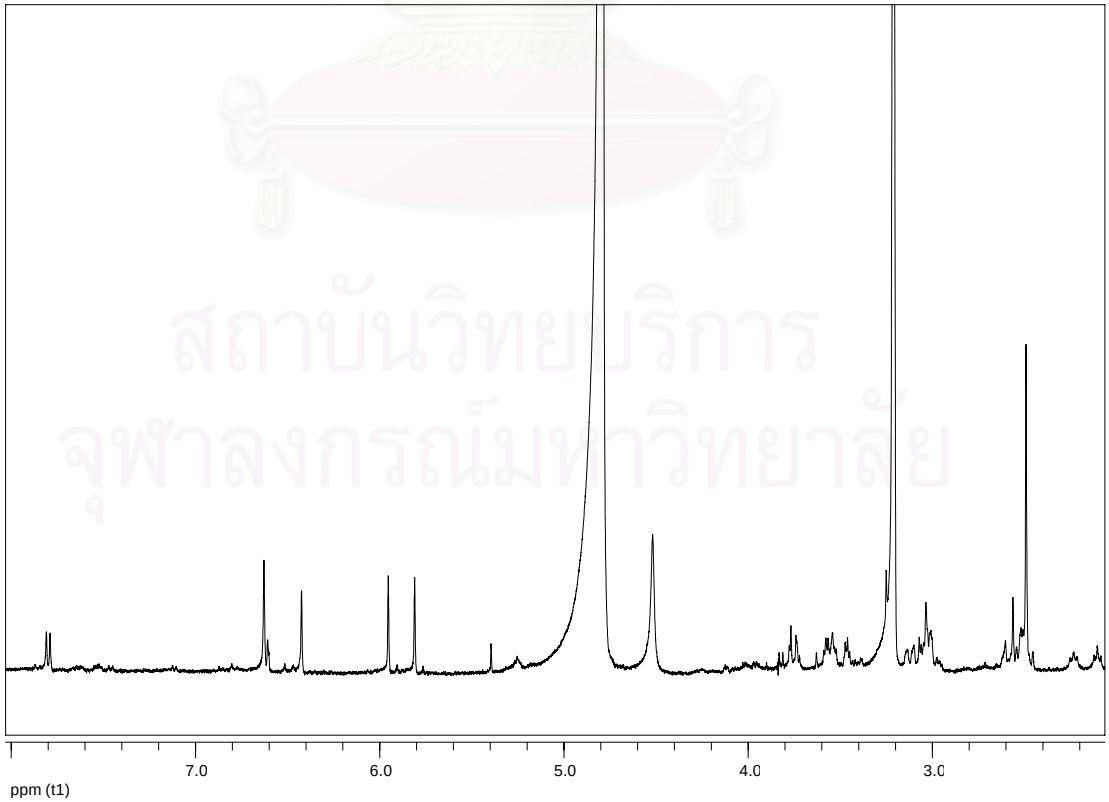

Figure 20 The ^1H NMR spectrum (CD_3OD) of compound **15**

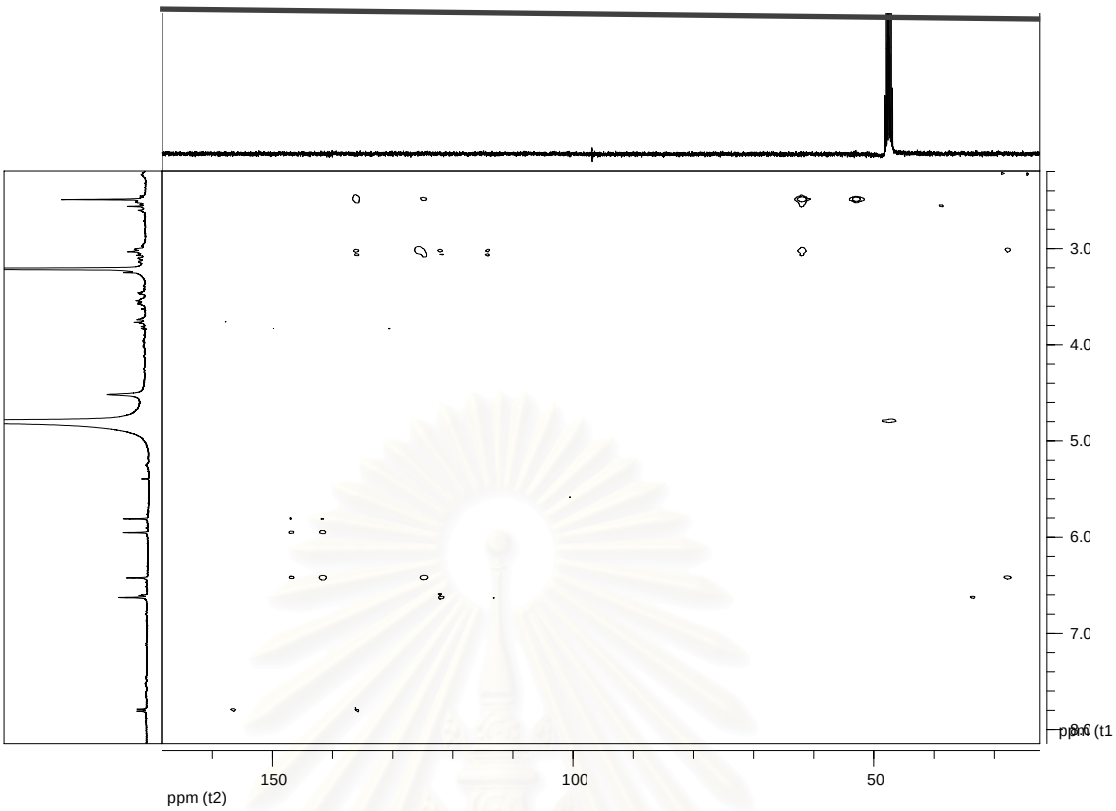

Figure 21 The HMBC spectrum (CD_3OD) of compound **15**

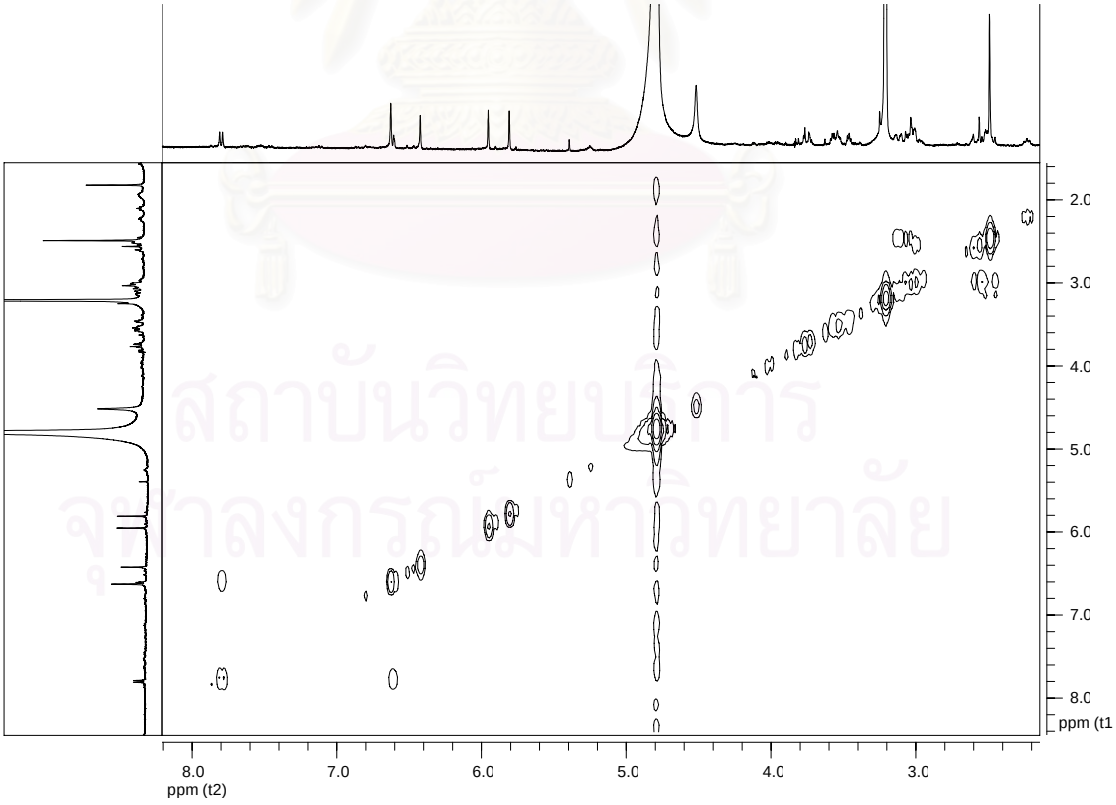

Figure 22 The COSY spectrum (CD_3OD) of compound **15**

VITA

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