ฤทธิ์ต้านออกซิเดชันของสารสกัดจากลำต้นชะลูด *Alyxia reinwardtii*

นางสาวจุไรรัตน์ รัตนพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIOXIDANT ACTIVITY OF EXTRACTS FROM STEMS *Alyxia reinwardtii*

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จุไรรัตน์ รัตนพันธ์: ฤทธิ์ต้านออกซิเดชันของสารสกัดจากลำต้นชะลูด *Alyxia reinwardtii* (ANTIOXIDANT ACTIVITY OF EXTRACTS FROM STEMS *Alyxia Reinwardtii*) อ. ที่ปรึกษา: ผศ.ดร.สันติ ทิพยางค์ อ. ที่ปรึกษาร่วม: อ. ดร. ปรีชา ภูวไพรศิริศาล, 69 หน้า. ISBN 974-17-6705-6

จากการทดสอบฤทธิ์ต้านออกซิเดชันเบื้องต้นในพืชสมุนไพรไทยโดยทดสอบกับ 2,2-diphenyl-1-picrylhydrazyl (DPPH) ซึ่งเป็นอนุมูลอิสระที่มีความเสถียร พบว่าสิ่งสกัดไดคลอโรมีเทนและสิ่งสกัดเอธิลอะซิเตตของลำต้นชะลูด (*Alyxia reinwardtii*) ให้ฤทธิ์ต้านออกซิเดชันที่ดี จากนั้นจึงนำส่วนของสิ่งสกัดเหล่านี้มาศึกษา พบว่าสามารถแยกสารได้ 8 ชนิดได้แก่ coumarin (**1**), 3-hydroxycoumarin (**2**), 6-hydroxycoumarin (**3**), 8-hydroxycoumarin (**4**), scopoletin (**5**), (+)-pinoresinol (**6**), zhebeiresinol (**7**) และ *p*-hydroxybenzoic acid (**8**) การหาสูตรโครงสร้างของสารทั้งหมดหาได้โดยใช้วิธีทางสเปกโทรสโคปีและเปรียบเทียบกับข้อมูลที่ได้มีรายงานไว้แล้ว ในส่วนของสาร **7** ได้ยืนยันสูตรโครงสร้างด้วย X-ray crystallography สำหรับการทดสอบฤทธิ์ต้านออกซิเดชันของสารบริสุทธิ์ที่แยกได้ มีวิธีการทดสอบทั้งหมด 3 วิธี คือ วิธีทดสอบฤทธิ์ต้านอนุมูลอิสระ DPPH วิธีทดสอบฤทธิ์เกี่ยวเนื่องกับเอนไซม์ xanthine oxidase (ฤทธิ์ต้านอนุมูลอิสระ superoxide และฤทธิ์ยับยั้งการทำงานของเอนไซม์ xanthine oxidase) และวิธีการทดสอบการยับยั้งการเกิดออกซิเดชันในไขมัน จากผลการทดสอบฤทธิ์ต้านอนุมูลอิสระ DPPH พบว่าสาร **7** (IC_{50} = 0.19 mM) แสดงฤทธิ์สูงที่สุด ตามด้วยสาร **6** (IC_{50} = 0.31 mM) สาร **2** (IC_{50} = 0.61 mM) สาร **5** (IC_{50} = 3.17 mM) และสาร **4** (IC_{50} = 71.05 mM) ในขณะที่สาร **1**, **3** และ **8** แสดงฤทธิ์ที่ต่ำ (IC_{50} > 100 mM) ส่วนฤทธิ์ต้านอนุมูลอิสระ superoxide พบว่าสาร **2** (IC_{50} = 4.55 mM) สาร **6** (IC_{50} = 4.51 mM) และสาร **7** (IC_{50} = 3.38 mM) แสดงฤทธิ์ที่ดี ในขณะที่สาร **1** และ **8** ไม่แสดงฤทธิ์ (IC_{50} > 100 mM) อย่างไรก็ตามสารทั้งหมดไม่แสดงฤทธิ์ยับยั้งการทำงานของเอนไซม์ xanthine oxidase จากผลการทดสอบการยับยั้งการเกิดออกซิเดชันในไขมัน พบว่าสาร **6** และ **7** แสดงฤทธิ์ที่สูง (IC_{50} = 3.31 และ 2.08 mM ตามลำดับ) ขณะที่สาร **1**, **2**, **3**, และ **4** แสดงฤทธิ์ปานกลางโดยมี IC_{50} = 67.64, 69.07, 67.45, และ 58.13 mM ตามลำดับ

สาขาวิชา.....เทคโนโลยีชีวภาพ.... ลายมือชื่อนิสิต.....

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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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JURAIRAT RATTANAPAN: ANTIOXIDANT ACTIVITY OF EXTRACTS FROM STEMS *Alyxia reinwardtii* THESIS ADVISOR: ASST. PROF. Dr. SANTI TIP-PYANG THESIS CO-ADVISOR: Dr. PREECHA PHUWAPRAISIRISAN, 69 pp. ISBN 974-17-6705-6

In a search for antioxidant compounds from Thai medicinal plants, the dichloromethane and ethyl acetate crude extracts from the stems of *Alyxia reinwardtii* (chalood in Thai) showed potent antioxidant activity, as guided by scavenging effect on the stable radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH). These crude extracts were isolated to afford eight compounds, namely coumarin (**1**), 3-hydroxycoumarin (**2**), 6-hydroxycoumarin (**3**), 8-hydroxycoumarin (**4**), scopoletin (**5**), (+)-pinoresinol (**6**), zhebeiresinol (**7**), and *p*-hydroxybenzoic acid (**8**). The structures of all compounds were characterized by means of NMR, MS, chemical analysis, and comparison with the literature data. The structure of compound **7** was also confirmed by X-ray crystallography. In terms of antioxidant activity, the isolated compounds were evaluated by various *in vitro* model assays, which include the DPPH radical scavenging activity, xanthine oxidase-related activity (Superoxide scavenging activity and inhibitory effect on xanthine oxidase), and lipid peroxidation inhibitory activity.The free radical scavenging activity on DPPH indicated that compound **7** (IC_{50} = 0.19 mM) showed the highest activity, followed by **6** (IC_{50} = 0.31 mM), **2** (IC_{50} = 0.61 mM), **5** (IC_{50} = 3.17 mM), and **4** (IC_{50} = 71.05 mM) respectively, while compounds **1**, **3**, and **8** gave the weakest activity (IC_{50} > 100 mM). In addition, compounds **2** (IC_{50} = 4.55 mM), **6** (IC_{50} = 4.51 mM), **7**(IC_{50} = 3.38 mM) exhibited significant superoxide scavenging activity while compound **1** and **8** were found to be inactive (IC_{50} > 100 mM) However, all compounds displayed no activity on inhibitory activity against xanthine oxidase. In lipid peroxidation inhibitory activity, compounds **6** and **7** showed potent activity (IC_{50} = 3.31 and 2.08 mM, respectively), while compounds **1**, **2**, **3**, and **4** showed moderate activity (IC_{50} = 67.64, 69.07, 67.45, and 58.13 mM, respectively).

Field of study..... Biotechnology……. Student's signature……..

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Co-advisor's signature…….

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List of Abbreviations

^{13}C NMR	carbon 13 nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
° C	degree of Celsius
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CH_2Cl_2	dichloromethane, methylene chloride
$CHCl_3$	chloroform
COSY	correlated spectroscopy
d	doublet (NMR)
dd	doublet of doublet (NMR)
ddd	doublet of doublet of doublet (NMR)
dt	double triplet (NMR)
DPPH	2, 2-diphenyl-1-(2, 4, 6-trinitrophenyl)hydrazyl
EC.1.2.3.2	
	EC enzyme code
	1 Oxidoreductase (enzyme class), oxidation/reduction reaction
	2 Aldehyde or ketone (electron donator)
	3 Oxygen (electron acceptor)
EtOAc	ethyl acetate
EtOH	ethanol
FI	feeding inhibitory
FTC	ferric thiocyanate
g	gram (s)
GCMS	gas chromatography mass spectrometer
HMBC	heteronuclear multiple bond correlation experiment
HSQC	heteronuclear multiple-quantum coherence experement
J	coupling constant
kg	kilogram (s)
wt	weight
NMR	nuclear magnetic resonance
IR	infrared
L	liter (s)

List of abbreviations (continued)

m	multiplet (NMR)
MeOH	methanol
mg	milligram (s)
mL	milliliter (s)
mM	millimolar
m.p.	melting point
MS	mass spectrometry
MW	molecular weight
m/z	mass to charge ratio
M^+	molecular ion
No.	number
ppm	part per million
q	quartet
ROS	reactive oxygen species
R_f	retardation factor
s	singlet (NMR)
SD	standard deviation
SDS	sodium dodeccylsulfate
SiO_2	silica gel
t	triplet (NMR)
TLC	thin layer chromatography
δ	chemical shift
μg	microgram (s)
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight
XOD	xanthine oxidase
λ_m	maximum wavelength

CHAPTER I

INTRODUCTION

Plant constitution are an important source of active natural products which differ widely in terms of structure and biological properties (Argolo *et al.*, 2004). It has been estimated that 80% of the world's population rely predominantly on natural plant products which are sold as herbal/food supplements or drugs and half of the top 50 drugs sold in European chemists/pharmacies are natural products (Zhu *et al.*, 2004). Therefore, the world today need to discover new molecular structures as lead compounds to relief and prevention disease from plant kingdom while avoiding the potential harmful side effects from many great synthetic pharmaceutical drugs.

World Health Organization studies have shown that the state of human health depends largely on nutrition. On the other hand, in human diseases, oxidative stress play a role. The words "free radicals" and "antioxidants" have become well known for health-conscious consumer (Lampart-Szczapa, 2003). The roles active oxygen and free radicals in tissue damage, in different diseases of humans are becoming increasingly recognized (Halliwell, Gutteridge, & Cross, 1992) and various degenerative disorders of human such as cardiovascular disease, aging, cancer and neurodegenerative disease like Alzheimer's disease, (Ames, 1998; Cox and Cohen, 1996; Finkel and Holbrook, 2000; Harman, 1994).

Oxidative stress is the state of imbalance between the level of antioxidant defense system and production of oxygen-derived species (ODS) which are inevitably produced as by-products of normal aerobic metabolism. Increased O_2 concentration and production of oxygen-derived species, *e.g.* superoxide anion ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) radicals can accelerate oxidative stress. The ground-state oxygen (O_2) molecule is gained a single unpaired electron, and becomes $O_2^{\bullet-}$, $O_2^{\bullet-}$ which rapidly reacts with different metabolic enzymes as well as cations (Dhalla, Temsah and Netticadan, 2000). In additional H_2O_2 is product from reduction

of oxygen and it is easily diffusible within and between cells. Since the O-O bond is relatively weak, H_2O_2 readily decomposes to give the hydroxyl radical ($OH^{\bullet}$). $OH^{\bullet}$ is the most reactive species that induces significant damage to the cell by converting guanine into 8-hydroxy guanine (**Figure 1.1**) (Gutteridge and Halliwell, 1994).

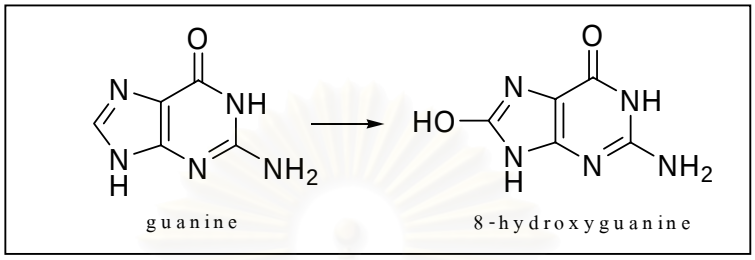


Figure1.1 Conversion of guanine into 8-hydroxyguanine by $OH^{\cdot}$

Free radical and active oxygen species are formed by various intrinsic and extrinsic sources such as light, heat, and metals. They are formed in vivo by various ways at different times and sites. Active oxygen and related species play an important physiological role and, at the same time, they may exert toxic effects as well. The active oxygen species are essential for production of energy, synthesis of biological essential compounds, and phagocytosis, a critical process of our immune system. They also play a vital role in signal transduction, which is important for cell communication and function. However, there is now increasing evidence which shows that these active oxygen species may play a causative role in a variety of diseases as mention before.

Antioxidant are defined as any substances that, when present at low concentrations compared with those of an oxidizable substrate, significantly delay or prevent oxidation of that substrate (Halliwell, 1995). Consequently, the roles of antioxidants, which suppress such oxidative damage are important in aerobic organism (Noguchi and Niki, 1999). Humans have evolved with antioxidant systems to protect against free radicals. These systems include some antioxidants produced in the body (endogenous). The first includes (a) enzymatic defense, such as glutathione peroxidase, catalase, and superoxide dismutase, which metabolize superoxide, hydrogen peroxide, and lipid peroxidase, and (b) nonenzymatic defense, such as glutathione, histidine-peptide, the iron-binding proteins transferin, ferritin, dihydrolipoic acid, reduced CoQ_{10}, metalonin, and others obtained from the diet (exogenous) (Warma, Devamanoharan and Morris, 1995) such as Vitamin A, E, C,

metals, phytochemical, food antioxidant (BHA, BHT) (Papas, 1999). However, when natural defenses are overwhelmed by an excessive generation of free radicals, which can be dealt with external factors (environmental insults, smoking). Hence, dietary intake of antioxidant compounds becomes important to maintain adequate antioxidant status (Halliwell *et al.*, 1995).

The type of antioxidant are classified into two basic categories, namely, synthetic and natural antioxidants. In general, the more popular synthetic antioxidants (**Figure 1.2**) used are phenolic compounds which are always substituted by alkyls to improve their solubility in fats and oils, such as butylate hydroxyanisol (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and esters of gallic acid, e.g. propyl gallate (PG) (Hudson, 1990). After a long period of use synthetic antioxidants, a new toxicological behaviors data impose some caution in their use (Thompson and Moldeus, 1988). In this context, natural products appear as healthier and safer than synthetic antioxidants. (Valenzuela and Nieto, 1996). Natural antioxidants are found in almost all plants, microorganism, fungi, and even in animal tissues (Pokorny, 1999). The natural antioxidants can be phenolic compounds (tocopherol, flavonoids, and phenolic acid), nitrogen compounds (alkaloids, chlorophyl derivatives, amino acids, and amines), or carotenoids as well as ascorbic acid (Larson, 1988).

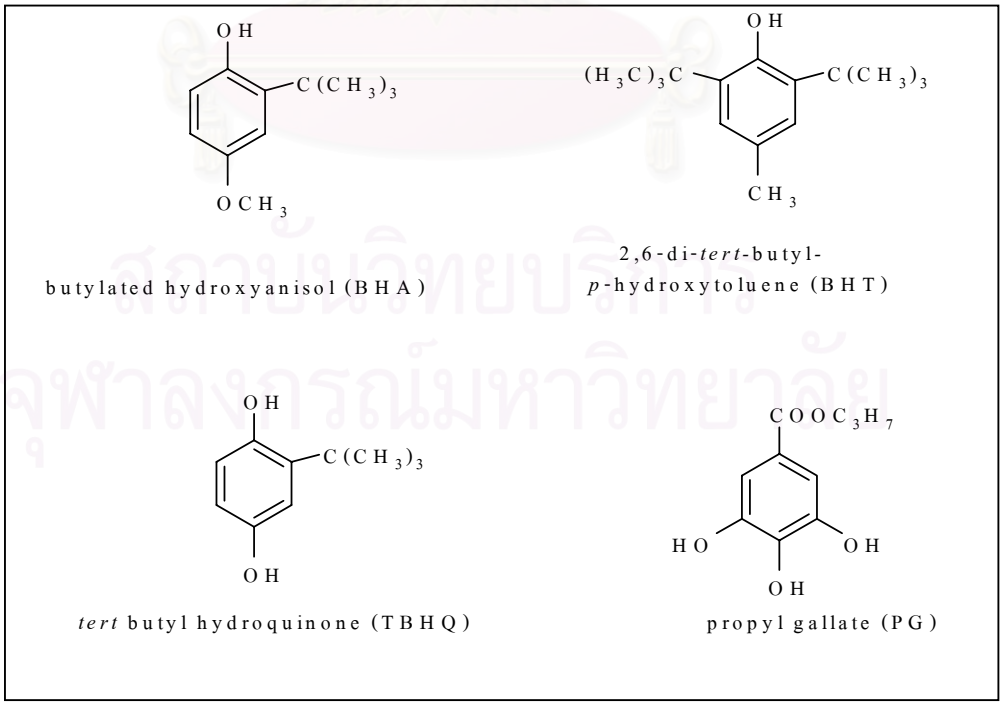


Figure 1.2 Synthetic antioxidants

The antioxidative plant constituents revealed various types of antioxidative phytochemicals (**Figure 1.3**). The different type of flavonoids were isolated from edible plant such as lutelin from rosemary (*Rosmarinus officinalis*) (Okamura *et al*., 1994), eriodictyol from thyme (*Thymus vulgaris*) (Haraguchi *et al*., 1996) and isoflavan derivatives such as glabridin, hispaglabridin A, hispaglabridin B, 4'-*O*-methylglabridin, 3'-hydroxy-4'-*O*-methylglabridin, were isolated from licorice (*Glycyrrhiza glabra*) (Haraguchi *et al*., 1997), which their inhibitory effect on lipid peroxidation in rat liver microsomes. Shisanhenol is the most active lignan, isolated from schisandra plants (*Schisandra rubriflora*) and terpenoids isolated from rosemary such as carnosol, rosmanol, isoromanol and rosmariquinone, which their inhibitory effect on lipid peroxidation in linoleic acid (Lu and Liu, 1992 and Inatani *et al*., 1983).

In this research, the screening for the antioxidant-related activity on medicinal plants has been investigated. Three conventional methods were selected for determining the antioxidant activity of pure compound from plant extracts. Measurement of radical scavenging activity uses discoloration of (2,2-Diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl) (DPPH radical scavenging assay). In addition, for other radical sources, we selected in vitro models, the xanthine oxidase-related activity generated by the enzymatic system and lipid peroxidation determines the efficiency of antioxidants.

DPPH (2,2-Diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl) (**Figure 1.4**) is a class of nitrogen-centered radical and stable with its resonance system and a radical generating substance to monitor the free radical scavenging abilities (the ability of a compound to donate an electron). The DPPH radical has a deep violet color due to its impaired electron, and radical scavenging can be followed spectrophotometrically by the decrease of absorbance at 517 nm, as the pale yellow non-radical form is produced.

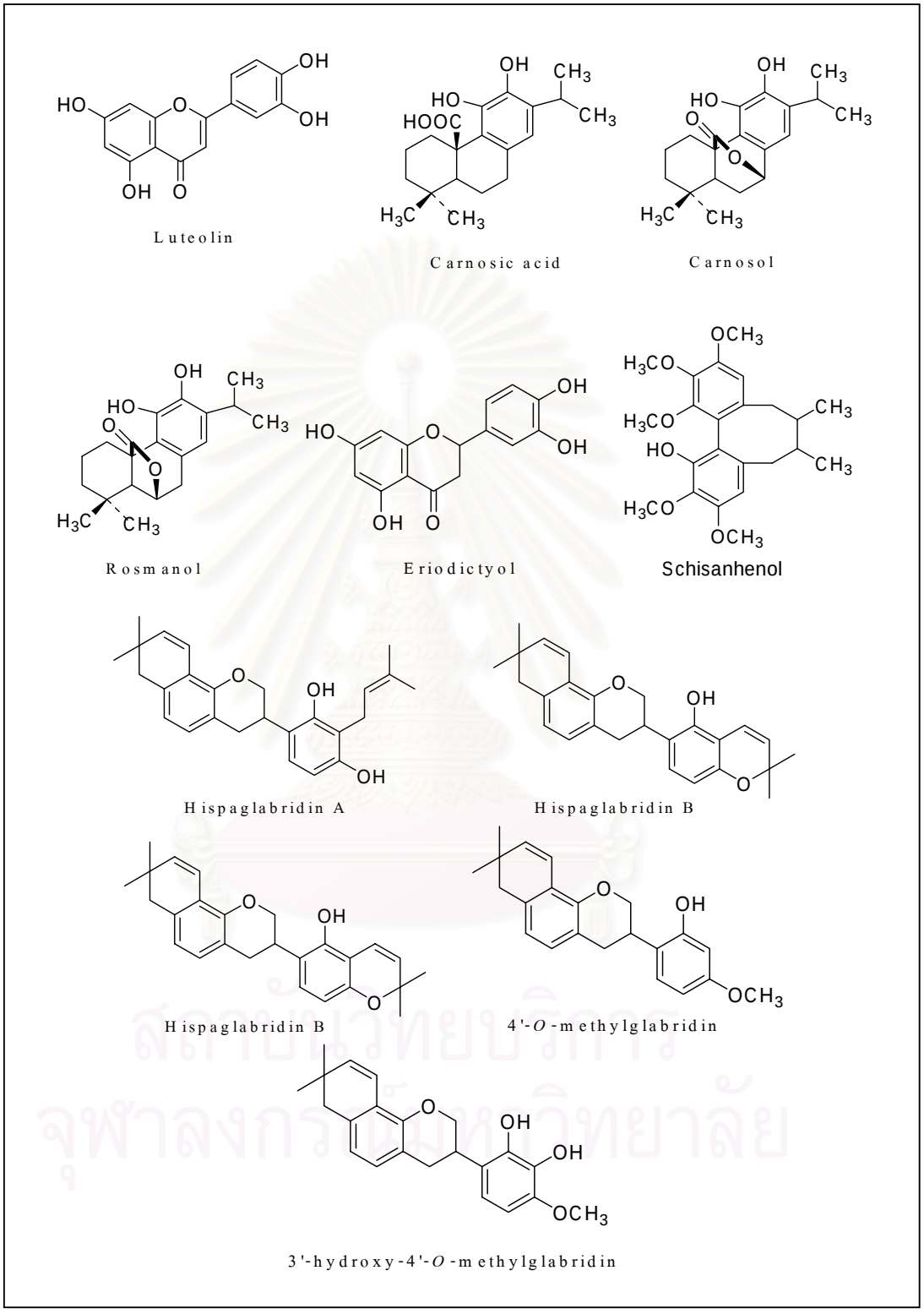


Figure 1.3 Natural antioxidants

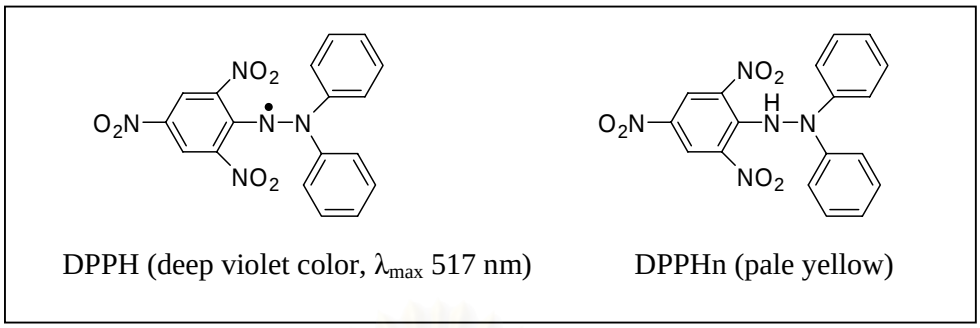
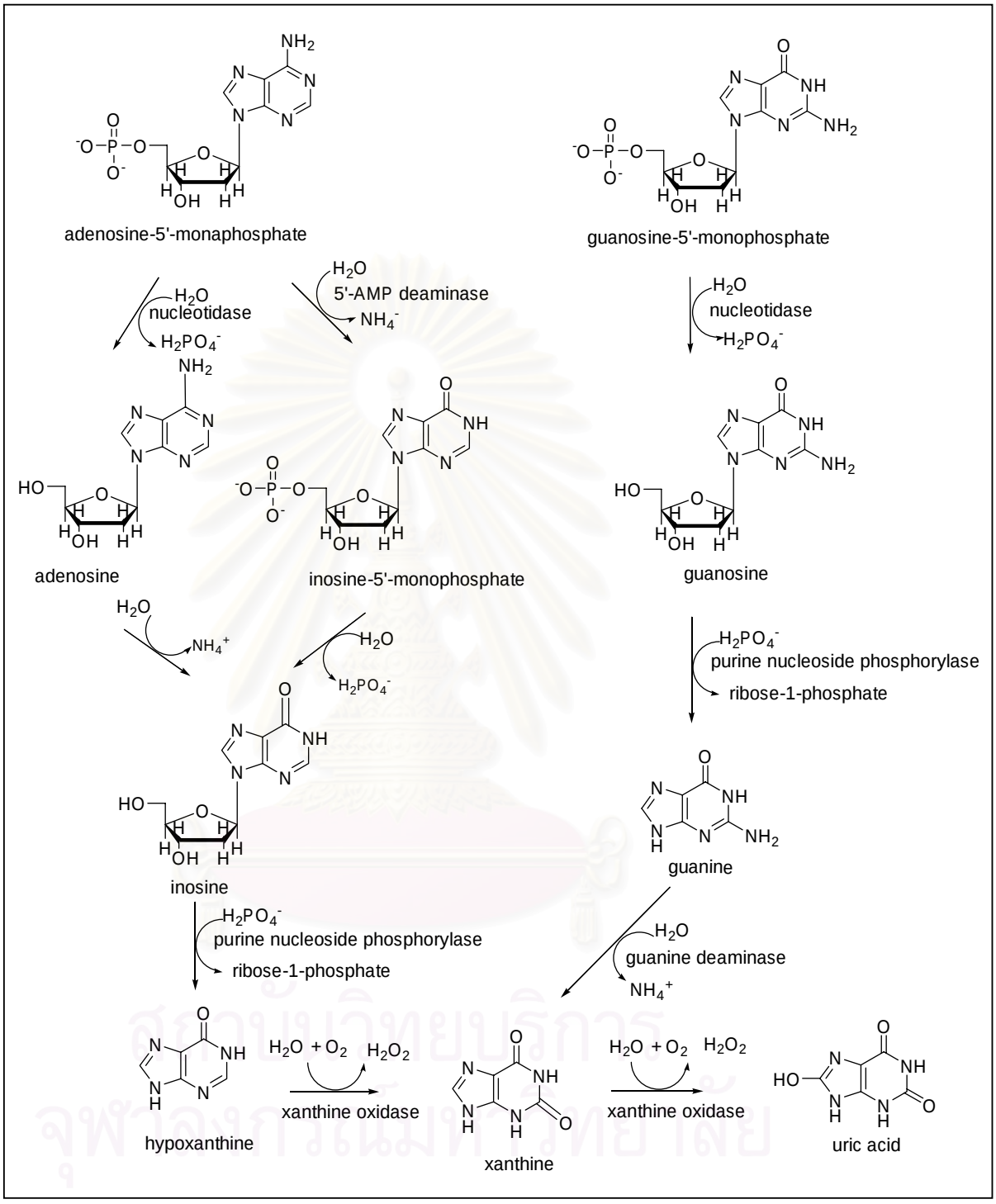


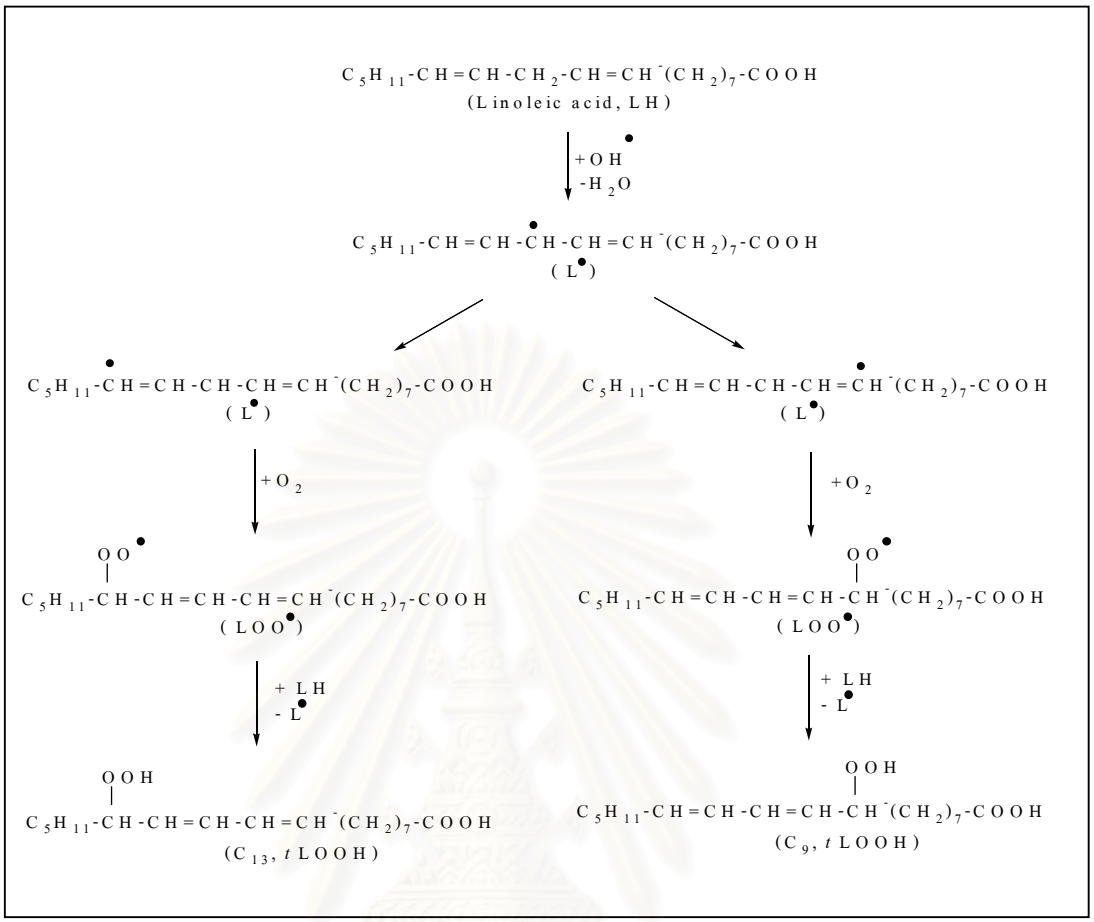
Figure 1.4 Structures of DPPH and DPPHn

Xanthine oxidase (EC 1.2.3.2) is from xanthine dehydrogenase under oxidative conditions. This molybdenum-containing enzyme catalyzes the oxidation of hypoxanthine with oxygen to xanthine and finally to uric acid (**Scheme 1.1**) (Bray, 1975 and Porras, Olson and Palmer, 1981). Superoxide anion and hydrogen peroxide are formed from oxygen. The ratio of superoxide anion generation with hydrogen peroxide formation depends upon the oxidation state of xanthine oxidase. The accumulation of uric acid is known to lead to hyperuricemia and gout while inhibitions of uric acid formation could be useful as therapeutic agents for these diseases. In addition, a large amount of superoxide anion generation by xanthine oxidase leads to peroxidative damages of cells, and inhibitors of the generation and radical scavengers of superoxide anion are prevention of oxidative damages (Fong *et al.*, 1973 and McCord, 1985).

Lipid peroxidation is the role of oxidative injury in pathophysiological disorder (Rice-Evans and Burdon, 1993). Polyunsaturated fatty acids, such as linoleic acid, which has the key unsaturated bonds between carbons C9 and C10, and C12 and C13, are the main components found in cell membranes of biological tissues (Porter *et al.*, 1995 and Reis *et al.*, 2003). Auto-oxidation of linoleic acid initiated by ROS abstracts a hydrogen atom, forming a fatty acid side chain peroxyl radical ($LOO^{\bullet}$), which oxidise a great variety of biological components also including proteins and nucleic acids (**Scheme 1.2**) (Spiteller, 2001).

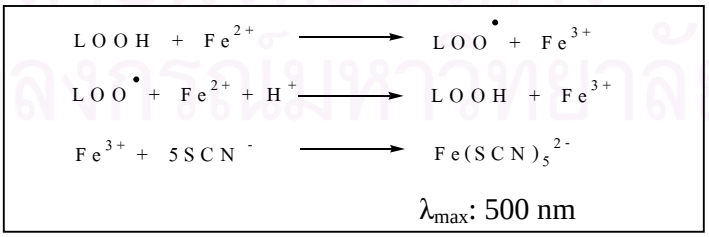


Scheme 1.1 Major pathways for purine nucleotide degradation in humans and other primate (Tropp, 1997).



Scheme 1.2 Partial mechanism of oxidation of linolic acid.

From ferric thiocyanate (FTC) assay, lipid peroxidation is quantified by measuring hydroperoxides, which is the product of this mechanism. Hydroperoxides are highly unstable. Therefore, hydroperoxides were readily reacted with ferrous ions to produce ferric ions. The result of this assay are shown in Scheme 1.3



Scheme 1.3 Reduction/oxidation reaction of ferric thiocyanate assay

As part of our on going studies to identify novel antioxidant agents from medicinal plants, the dichloromethane and ethyl acetate crude extracts from 22 Thai medicinal plants were screened for 2,2-Diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl (DPPH) radical-scavenging activity and the stems of chalood (*Alyxia reinwardtii*) was found to have the promising activity. Hence, this plant was selected for further investigation of the chemical constituents and their antioxidant activity.

1.1 Botanical Aspects and Distribution of *Alyxia reinwardtii*

Alyxia reinwardtii is the plants in Apocynaceae family, genus of *Alyxia*, which have ca. 60-70 species in Eastern Asia, Australia and the Pacific. *A. reinwardtii* is one of 4 species in Thailand and widely distribution. It has been known as 'Chalood' (ชะลูด) (Central, Trat), 'Lut' (ลูด) (Pattani), 'Nut' (นูด) (Trang, Surat Thani) (Santisuk and Larsen, 1999).

Branchlets:	glabrous to densely puberulent.
Leaves:	opposite to whorls of 5; petiole 1-9 mm long; blade coriaceous or subcoriaceous, elliptic to obovate, 1.1-9.3×0.3-4.6 cm, apex acuminate to obtuse, base cuneate to obtuse; secondary veins 12-31 pairs; glabrous or, more rarely puberulent on midrib or all over beneath.
Inflorescence:	axillary and/or terminal cymes, sometimes forming panicles.
Sepals:	without colleters inside.
Corolla:	lobes overlapping to the left in bud; mature corolla salverform.
Stamens:	free from the pistil head; inserted in top haft of corolla tube, completely included in tube; filaments thin and short; anthers ovate, base cordate, fertile entire length.
Disk:	absent.
Ovary:	of 2 separate carpels united into a common style; ovary pubescent all over or only at base; style filiform.

Fruit: a drupe, usually moniliform with one or more subglobose articles, frequently reduced to one in mature fruit; stipulate; outside somewhat succulent when mature; one seed per article.

Seeds: ovoid; with a horny and deeply ruminate endosperm.

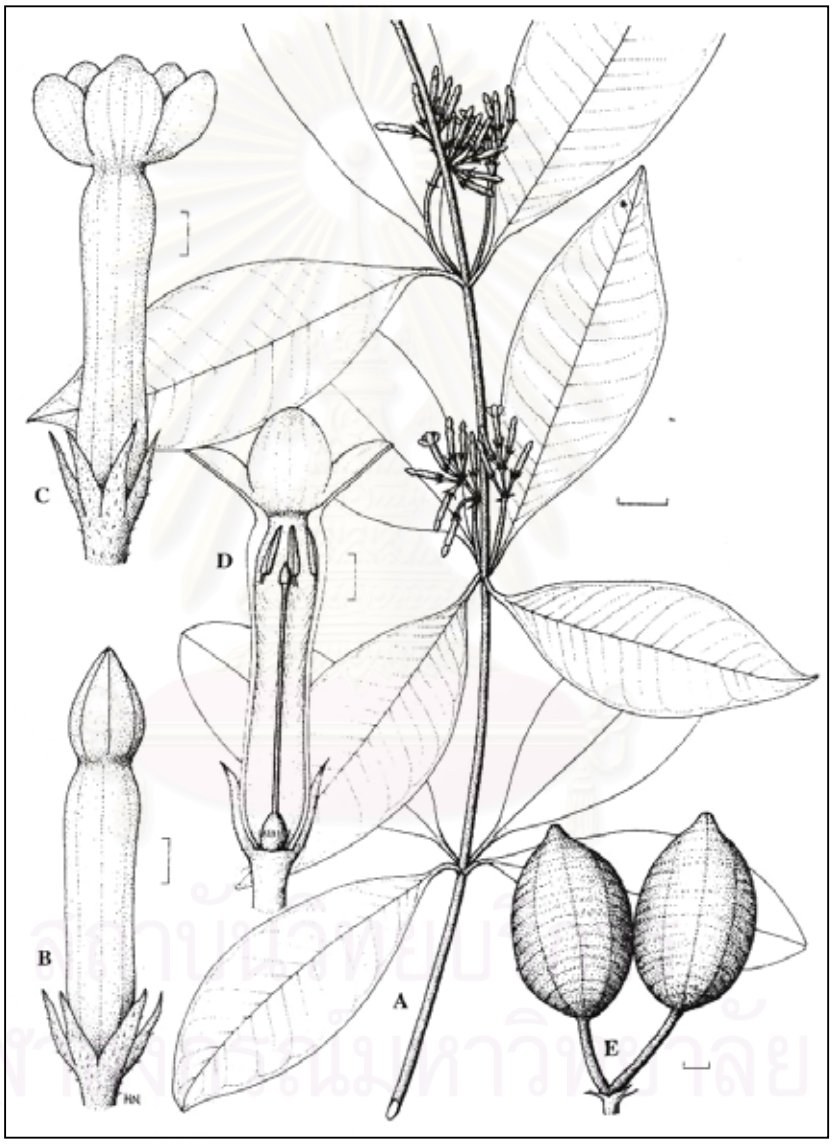


Figure 1.5 *Alyxia reinwardtii* A. habit; B. flower bud; C. flower; D. dissected flower; E. fruit.

Figure 1.6 *Alyxia reinwardtii*

1.2 Ethnobotanical of *Alyxia reinwardtii* and phytochemical investigation of genus *Alyxia*

Alyxia reinwardtii, a traditional Thai medicinal plant, is widely used in Thailand. The various parts of this plant have been used in perfume, flavoring, religious services and medicine. The finely ground stem is used in the manufacture of incense and other aromatic products. Water extracts of the stem is used in traditional or religious rites and to impart aroma to pipe tobacco. According to traditional medicinal practice, the leaves and fruits of this plant can be used to reduce fever; the flowers are effective in treating mental confusion and hallucination associated with high fever, stopping hiccup and correcting unspecified gall bladder ailments. The roots are effective in reducing fever, the stems are used to treat fainting, heart failure and abdominal discomforts due to gaseous distention or other unspecified causes (Boonyaprapatsorn and Chokchaichalernporn; in Thai, 1996).

Plants of genus *Alyxia, A. lucida and A. reinwardtii,* have been reported to contain two classes of secondary metabolites, coumarin and iridoids. Simple hydroxylated and glycosylated coumarins were typically found in barks and stems while iridolactones were isolated from leaves. The chemical constituents of genus *Alyxia* are summarized in Table 1.1, and chemical structures are shown in Figures 1.7 to 1.10. Although chemical constituents of this genus were fairly studied, their biological activity has not been evaluated.

Table 1.1 Chemical constituents in *Alyxia* genus.

Plant names	Part	Isolated compounds	Type
Alyxia lucida (Sadavongvivad and Supavilai, 1977) (**Figure 1.7**)	Stems	Coumarin 3-Hydroxycoumarin 5-Hydroxycoumarin 8-Hydroxycoumarin scopoletin	Coumarin
Alyxia reinwardtii Kitagava *et.al.*,1988) (**Figure 1.8**)	bark	Pulosarioside	Trimeric-iridoid diglucoside
Alyxia reinwardtii Topcu *et.al.*,1990) (**Figure 1.9**)	Leaves	Alyxialactone 4-Epi-alyxialactone	Iridolactone
Alyxia reinwardtii Lin *et.al.*,1993) (**Figure 1.10**)	Inner bark	3-Hydroxycoumarin glycoside **1** 3-Hydroxycoumarin glycoside **2**	Coumarin

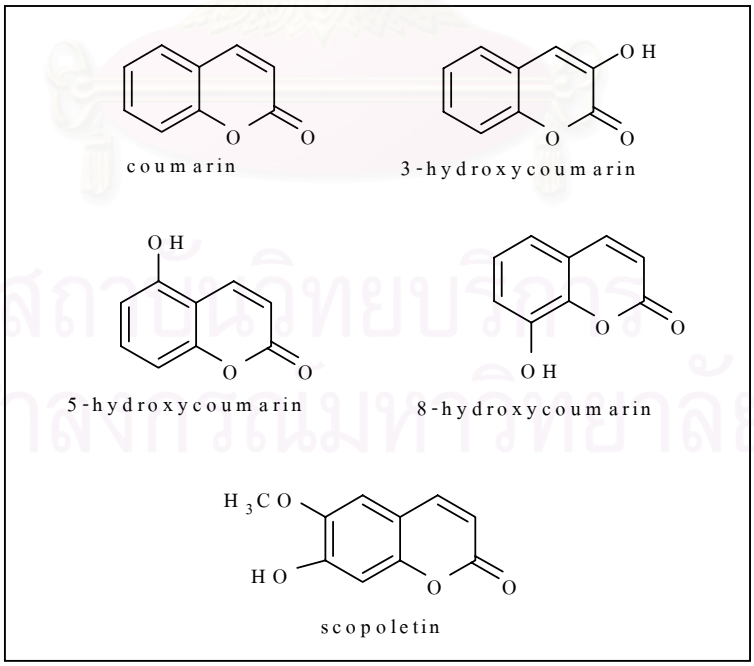


Figure 1.7 Coumarin from *A. lucida*

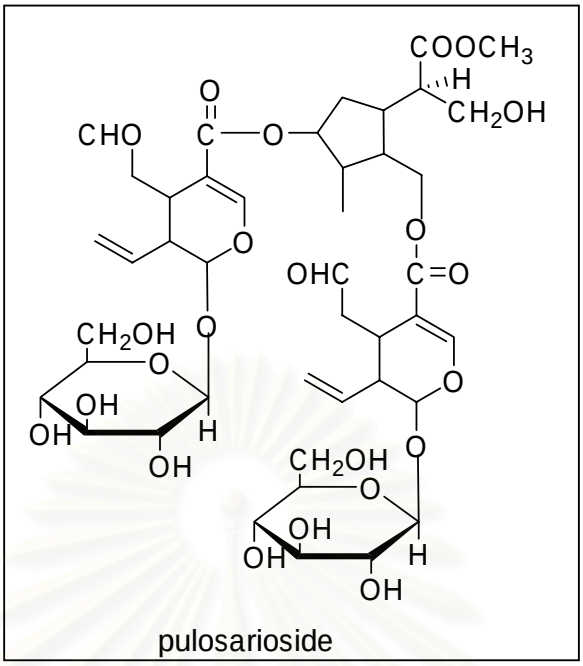


Figure 1.8 Pulosarioside from *A. reinwardtii*

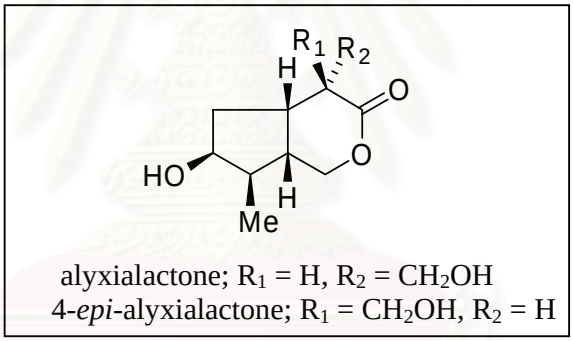


Figure 1.9 Iridolactone from *A. reinwardtii*

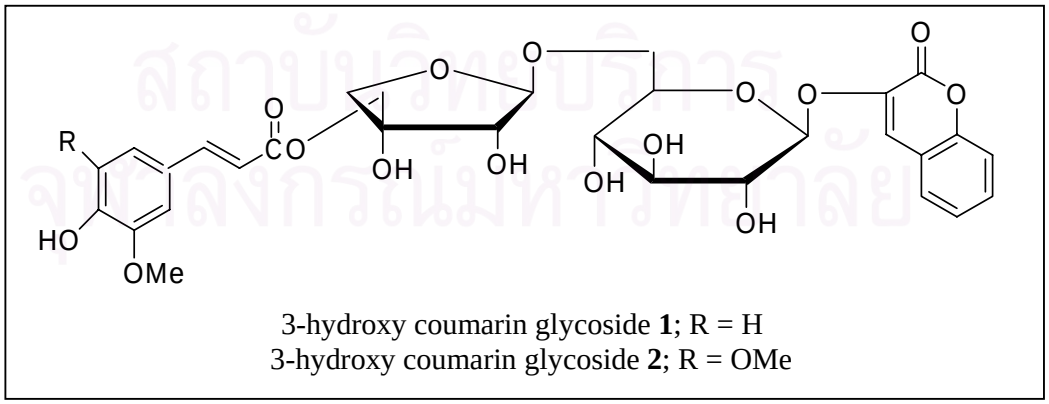


Figure 1.10 Coumarin from *A. reinwardtii*

In a search for antioxidant compounds from Thai medicinal plants, *A. reinwardtii* crude extracts showed potent activity towards DPPH autographic bioassay (see chapter 2). They revealed several white spots on purple background of DPPH, suggesting that their potent activity was caused by these principles. As promising screening results and no previously scientific report on their biological activity of this plant, then this plant was selected for further investigation on chemical constituents and their antioxidant activity.

1.3 The goal of this research

1. To carry out a comprehensive chemical separation and structure determinative of dichloromethane and ethyl acetate crude extracts from the stems of *A. reinwardtii* by chromatography and spectroscopic techniques.

2. To investigate antioxidant activity of the isolated compounds.

CHAPTER II

SCREENING FOR ANTIOXIDANT PROPERTY FROM THAI MEDICINAL PLANTS

The result of screening for the antioxidant activity from 22 Thai medicinal plants (**Table 2.1**), the dichloromethane and ethyl acetate crude extracts from the stems of *Alyxia reinwardtii* (Chalood in Thai) showed potent radical effect towards stable radical, 2,2-Diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl (DPPH), Therefore, this plant was selected for further isolation, purification and test antioxidant activity of pure compound.

2.1 Screening result

Table 2.1 Primary screening test for antioxidant activity from Thai medicinal plants

Plant name	**Solvent**	**DPPH radical scavenging activity**
		TLC autographic
Crytolepis buchanani (Stem) (เถาเอ็นอ่อน)	CH_2Cl_2 EtOAc	+ ++
Cyrtosperma johnstoni (Arial part) (สิงหโมรา)	CH_2Cl_2 EtOAc	+ +
Dioscorea bulbifera (Tuber) (ว่านกลิ้งกลางดง)	CH_2Cl_2 EtOAc	+++ ++
Belamcanda chinensis (Tuber) (ว่านหางช้าง)	CH_2Cl_2 EtOAc	+++ ++

Stephania pierri (Tuber) (สบู่เลือด)	CH_2Cl_2 EtOAc	+++ +++
Anamirta cocculus (Stem) (โคคลาน)	CH_2Cl_2 EtOAc	+ ++
Neuropeltis racemosa (Stem) (ม้ากระทืบโรง)	CH_2Cl_2 EtOAc	+++ ++
Piper sylvaticum (Fruit) (ดีปลี)	CH_2Cl_2 EtOAc	++ ++
Gynura sarmentosa (Arial part) (แป๊ะตำปึง,จักรนารายณ์)	CH_2Cl_2 EtOAc	++ +
Hydnophytum formicarum (Whole plant) (หัวร้อยรู)	CH_2Cl_2 EtOAc	++ +
Pandanus amaryllifolius (Root) (เตยหอม)	CH_2Cl_2 EtOAc	+ +
Alyxia reinwardtii (Stem) (ชะลูด)	CH_2Cl_2 EtOAc	+++ ++
Crinum latifolium (Tuber) (ว่านแร้งคอดำ)	CH_2Cl_2 EtOAc	++ +
Smilax corbularia (Tuber) (ข้าวเย็นเหนือ)	CH_2Cl_2 EtOAc	+ ++
Curcuma aromatica (Tuber) (ว่านนางคำ)	CH_2Cl_2 EtOAc	++ ++
Zingiber ottensii (Tuber) (ว่านไพลดำ)	CH_2Cl_2 EtOAc	+ +
Prema obtusifolia (Arial part) (ช้าเลือด)	CH_2Cl_2 EtOAc	- +
Heritiera littoralis (Arial part) (หงอนไก่ทะเล)	CH_2Cl_2 EtOAc	- +

Abutilon ployandrum (Arial part) (ครอบจักรวาล)	CH_2Cl_2 EtOAc	- +
Albizia procera (Stem) (ทิ้งถ่อน)	CH_2Cl_2 EtOAc	+ -
Cissus uadrangularis (Stem) (เพชรสังฆาต)	CH_2Cl_2 EtOAc	+ +
Ixora lobbii (Root) (เข็ม)	CH_2Cl_2 EtOAc	+ -

Note: +++ = Strong activity
++ = Moderate activity
+ = Weak activity
- = No activity

2.2 Experimental

Three conventional methods was selected for determining the antioxidant activity of pure compounds from plant extracts. Measurement of radical scavenging activity using discoloration of 2,2-Diphenyl-1-(2,4,6,-trinitrophenyl) hydrazyl (DPPH radical scavenging assay) has been widely used due to its stability, simplicity, and reproducibility. Besides, this method requires a small amount of sample and can be altered to apply for both qualitative and quantitative examinations. In addition to other radical sources, we selected in vitro models, the xanthine oxidase-related activity generated by the enzymatic system and lipid peroxidation for determines the efficiency of antioxidants.

2.2.1 DPPH radical scavenging activity

2.2.1.1 TLC autographic assay (Hostettman et al., 1997)

Using 2, 2-Diphenyl-1-(2,4,6,-trinitrophenyl) hydrazyl (DPPH) radical as a TLC spray reagent was confirmed to be well suited for the screening of antioxidants in crude plant extracts. The assay involves spraying TLC plates with a 2 mg/ml DPPH solution in methanol. The plates are considered 30 minutes after spraying. Active compounds occur as yellow spots on a purple background.

2.2.1.2 Spectrophotometric assay (Yen and Hsieh, 1997)

After isolation and purification, activities of pure compounds were quantified in this assay. Various concentrations of samples dissolved in methanolic (0.5 mL) were added to DPPH radical methanolic solution (0.2 mM, 1.0 mL). After 30 minutes incubation at room temperature in the dark, the absorbance was measured at 517 nm with a 96 well microplate. All tests were run in triplicate and calculated sample standard deviation. The scavenging activity was evaluated from the decrease value of 517 nm absorption, which was calculated by the following equation. The activity was shown as IC_{50} values that denote the concentration of sample required scavenging 50% DPPH free radicals.

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

2.2.2 Xanthine oxidase-related activity

2.2.2.1 Assay for scavenging activity of $O_2^{\bullet-}$ by xanthine oxidase (Okamura et al., 1993)

Superoxide anion radical was generated from xanthine-xanthine oxidase method with a slight modification. The reaction mixture consisted of 0.1 M phosphate buffer (pH 8.0) containing 0.4 mM xanthine, 0.24 mM nitroblue tetrazolium, and 0.049 units of xanthine oxidase in a final volume 1.0 mL. Samples at various concentrations in DMSO were added to the mixture (0.15 mL). After being incubated at 37° C for 20 minutes, the reaction was terminated by addition of 0.05 ml of 69 mM sodium dodecyl sulfate. The absorbance of formazen produced was determined at 560 nm, and scavenging activity on $O_2^{\bullet-}$ of each sample was estimated by the same equation as described before. The IC_{50} values were calculated from regression line.

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

2.2.2.2 Assay for inhibitory activity against xanthine oxidase (Kweon et al., 2001)

For studying of xanthine oxidase inhibitory activity, the rise in the absorbance at 290 nm due to uric acid production was measured in the absence of nitroblue tetrazolium. Allopurinol, which is a drug for gout treatment, was used as a standard for this assay. The inhibitory activity was shown as percent inhibition, which

was estimated from the following equation. The IC_{50} values were determined from regression line.

$$\% \text{ Inhibition} = [1 - A_{Sample}/A_{blank*}] \times 100$$

2.2.3 Ferric thiocyanate assay (Wijewickreme et al., 1999)

This assay was slightly modified. The linoleic acid emulsion was prepared by vortex mixing 3.0 mL of linoleic acid with 3.0 mL of sodium dodecyl sulfate (SDS) as emulsifier and 200 mL of 30 % (v/v) ethanol. Each sample at various concentrations in ethanolic solution (0.5 mL) was mixed with 5 mL of emulsion and the final volume of the mixture was adjusted to 12.5 mL. The reaction mixture was incubated in a conical flask at 40°C in the dark. Aliquots of 0.05 mL were taken at eight hours during incubation and tested for lipid peroxidation products. The assay was carried out by adding 2.5 mL of 75 % ethanol, 0.1 mL of ammonium thiocyanate solution (30 % w/v), and 0.1 mL of ferrous chloride (0.1 % w/v) to 0.05 mL of sample. After the mixture was left for 3 minutes, the absorbance of the reaction mixture was measured at 500 nm. The activity was revealed as percent inhibition that was examined from the following equation. The IC_{50} values that denote the concentration of sample required scavenging 50% peroxyl radicals were calculated from regression line.

$$\% \text{ Inhibition} = [1 - A_{Sample}/A_{blank*}] \times 100$$

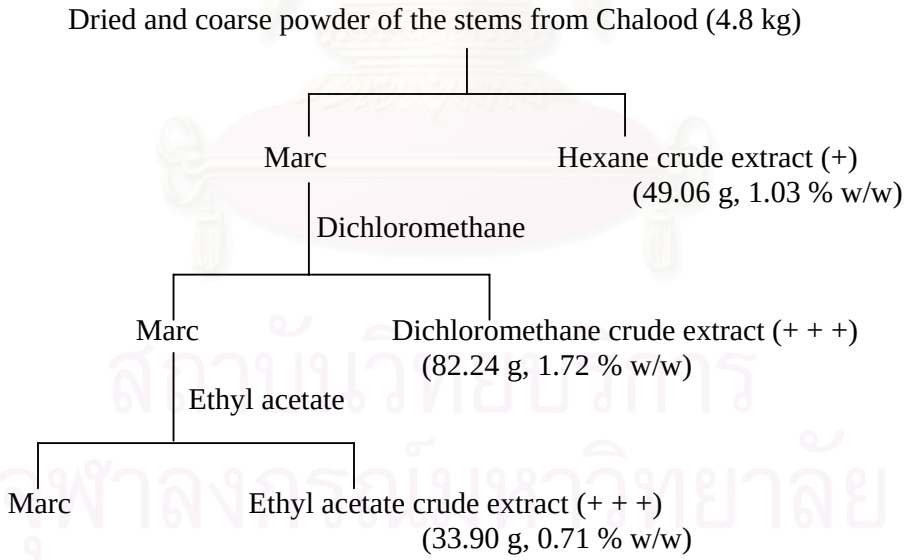
(A_{blank*} = Absorbance of reaction mixtures were prepared without test compounds.)

CHAPTER III

ISOLATION AND CHARACTERIZATION OF ANTIOXIDANT COMPOUNDS FROM *Alyxia reinwardtii*

3.1 Extraction and Isolation

The dried stems (4.8 kg) of chalood (*Alyxia reinwardtii*) were pulverized and then by maceration at room temperature with hexane, dichloromethane, and ethyl acetate thrice for each solvent. The extracts of each solvent were filtrated and evaporated under reduced pressure to afford 49.06 g of hexane crude extract, 82.24 g of dichloromethane crude extract, and 33.9 g of ethyl acetate crude extract, respectively. The procedure of the extraction was summarized in Scheme 3.1.

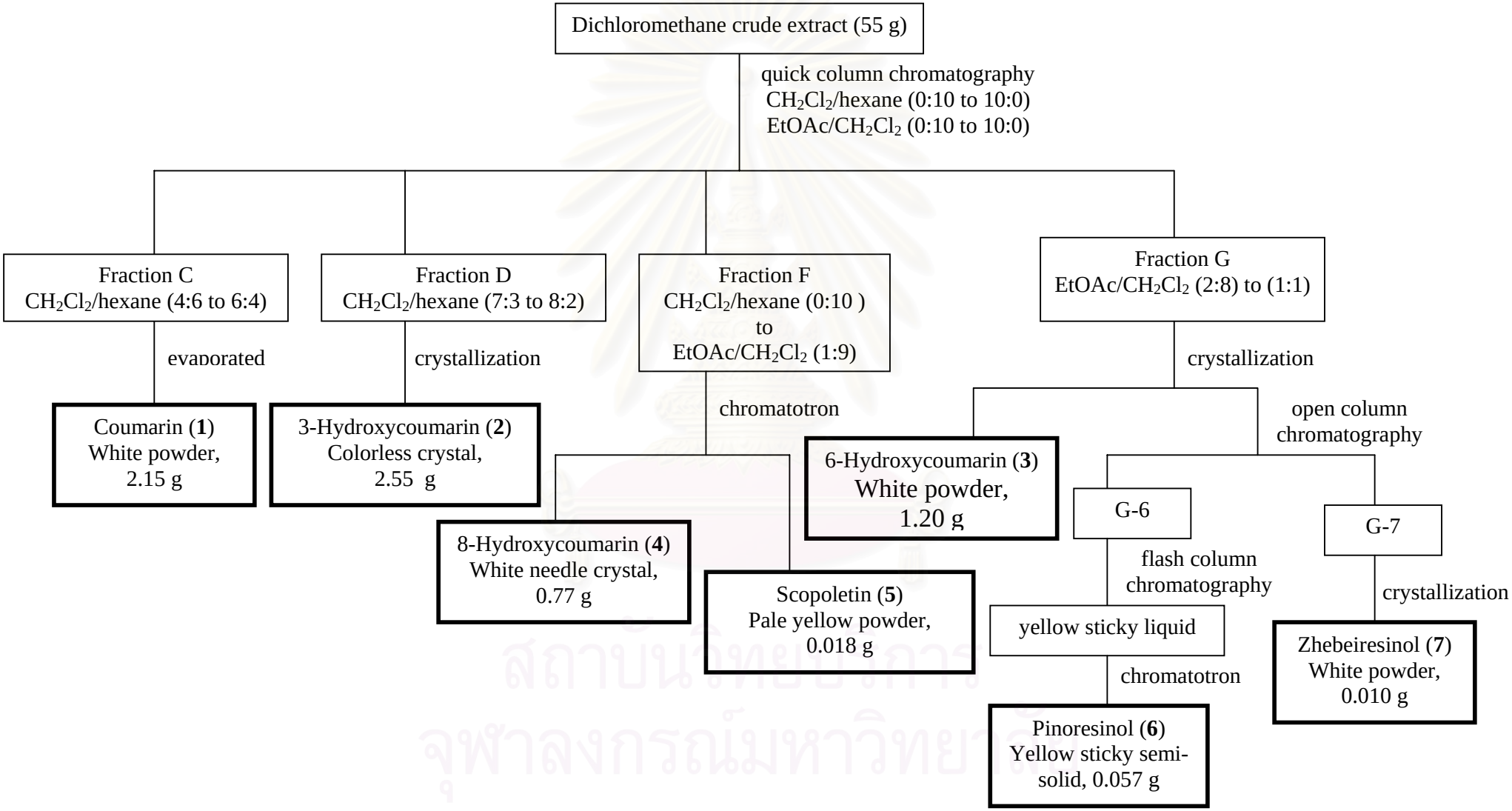


Scheme 3.1 The extraction procedure of the stems of chalood

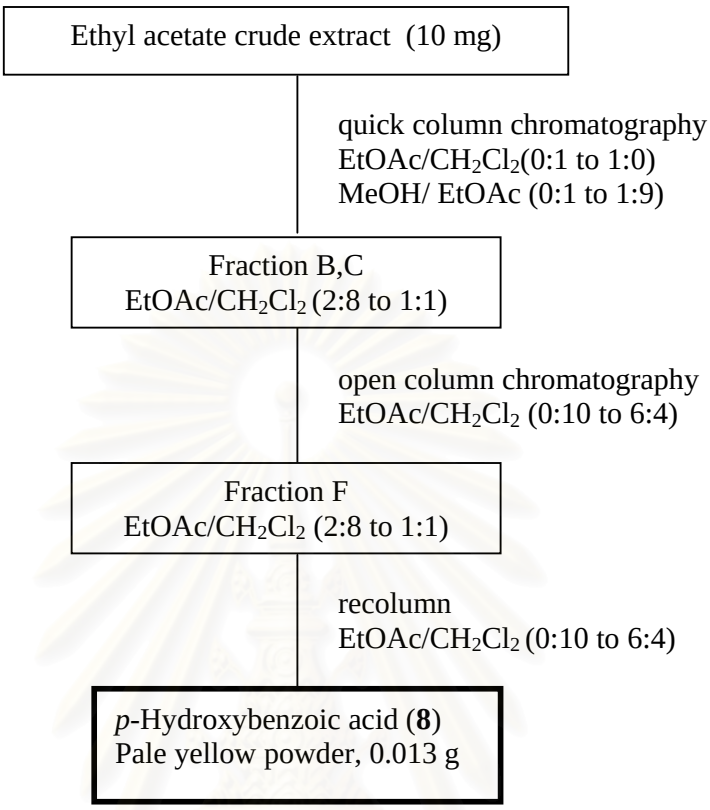
Note: +++ = Strong activity

++ = Moderate activity

\+ = Weak activity



Scheme 3.2 The isolation procedure of dichloromethane crude extract.



Scheme 3.3 The isolation procedure of ethyl acetate crude extract.

From the primary screening test for antioxidant activity of various crude extracts, the dichloromethane and ethyl acetate crude extracts showed potent radical effect towards stable radical, 2,2-Diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl (DPPH) which test result showed potent activity (+ + +). Hence, these crude extracts were selected for further investigation of the chemical constituents. The isolations of the dichloromethane and ethyl acetate crude extracts were briefly summarized in scheme 3.2 and 3.3, respectively.

3.2 Characterization

Coumarin (1)

Compound **1** (2.15 g) a major component from this plant, was a colorless crystal of melting point 59-61°C (67- 68°C; Murray, Mendez and Brown, 1982) and R_f value was 0.80 (SiO_2, 1:4 EtOAc/CH_2Cl_2). The fraction of 30-50% CH_2Cl_2/EtOAc from quick column was evaporated, the colorless crystal of compound **1** was deposited.

The ^1H NMR spectrum showed a pair of doublets at δ_H 7.66 (d, J = 9.4 Hz, 1H) and 6.43 (d, J = 9.4 Hz, 1H) which could be assigned to H-4 and H-3 of an α -pyrone ring system, respectively. The presence of an aromatic proton in the region δ_H *ca* 7.24-7.60 (7.32, m, 2H and 7.55, m, 2H) indicated the degree of unsubstitution on the aromatic nucleus.

The ^{13}C NMR spectrum ($CDCl_3$) of compound **1** demonstrated nine carbons: six methane carbons at δ_C 116.7 (C-8), 116.9 (C-3), 124.4 (C-6), 127.9 (C-5), 131.9 (C-7) and 143.5 (C-4), two quaternary carbons at δ_C 118.9 (C-10) and 159.8(C-9) and finally a carbonyl group at δ_C 160.8. From EIMS, ^1H and ^{13}C NMR data, compound **1** was determined molecular formula as $C_9H_6O_2$.

All spectroscopic results allowed to assign compound **1** as coumarin and a complete structural assignment was carried out by comparing its ^{13}C spectral data with the literature (Breitmaier and Voclter, 1987).

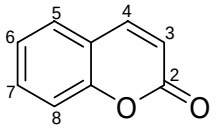


Figure 3.1 The structure of Compound **1** (Coumarin)

Table 3.1 ^{13}C NMR (100 MHz) spectral data of Coumarin and Compound **1** in $CDCl_3$

Position of Carbon	Chemical shift (ppm)	
	Coumarin	Compound **1**
2	160.6	160.8
3	116.7	116.9
4	143.5	143.5
5	128.5	127.9
6	124.4	124.4
7	131.8	131.9
8	116.6	116.7
9	154.1	159.8
10	118.9	118.9

Table 3.2 ^{1}H NMR (400 MHz) and ^{13}C NMR (100MHz) spectral data of **1** in $CDCl_3$

Position	Chemical shift (ppm)	
	^{13}C	^{1}H (multiplicity, *J* in Hz)
2	160.8	
3	116.9	6.43, d, *J*= 9.4 Hz, 1H
4	143.5	7.66, d, *J*= 9.4 Hz, 1H
5	127.9	
6	124.4	7.32, m, 2H
7	131.9	7.55, m, 2H
8	116.7	
9	159.8	
10	118.9	

3-Hydroxycoumarin (2)

Compound **2** (2.55 g) was obtained as a white crystal from dichloromethane crude extracts. Its melting point was 138-139°C (153-155°C; Sadavongvivad and Supavilai, 1977) and R_f value was 0.70 (SiO_2, 1:4 EtOAc:CH_2Cl_2).

The ^{13}C NMR spectrum displayed nine carbons: five methane carbons at δ_C 114.5, 120.5, 125.2, 126.7 and 128.5, three quaternary carbons at δ_C 116.5, 140.4 and 149.2, and, a carbonyl group at δ_C 160.6 (C-2).

From the comparison of compound **2** with coumarin (**Table 3.3**), a pair of doublet of H-3 and H-4 were absence, while exited singlet signal at δ_H 7.17 ppm. This data indicated the substitution on H-3 or H-4. Meanwhile, intregrated multiplet aromatic proton at δ_H *ca* 7.36 (4H, m) afforded 4 protons, which could be assigned to H-5, H-6, H-7, H-8 on aromatic ring of a coumarin nucleus.

The comparison of molecular weight of compound **2** (162) with that of coumarin (146) suggested that compound **2** had one hydroxyl group substituted either H-3 or H-4 position. From this result, the two possible structures of compound **2** were 3-hydroxycoumarin (**2a**) or 4-hydroxycoumarin (**2b**) (**Figure 3.2**).

3-hydroxycoumarin (**2a**) 4-hydroxycoumarin (**2b**)

Figure 3.2 Possible structures of **2**

In terms of all evidences from spectroscopic data we compared with those of previously published, which were differenced from 4-hydroxycoumarin such as chemical shift of carbon and melting point. Consequently, the possible structure of compound **2** should be 3-hydroxycoumarin, which were confirmed structure by comparing its ^{13}C HMR spectrum with coumarin aglycone of 3-hydroxycoumarin glycoside which separation from *A. reinwardtii* (**Table 3.3**) (Lin *et al.*, 1993).

Table 3.3 ^{1}H NMR (400 MHz) spectral data of **1** and Compound **2** in $CDCl_3$

Position of proton	Chemical shift(ppm)	
	Coumarin	Compound **2**
2		
3	6.43, d, *J*= 9.4 Hz, 1H	
4	7.66, d, *J*= 9.4 Hz, 1H	7.17, s, 1H
5		
6	7.32, m, 2H	7.36, m, 4H
7	7.55, m, 2H	
8		

Table 3.4 ^{13}C NMR (100 MHz) spectral data of 3-Hydroxycoumarin glycoside and Compound **2** in $CDCl_3$

Position of carbon	Chemical shift(ppm)	
	3-Hydroxycoumarin glycoside	Compound **2**
2	159.8	161.0
3	142.9	140.4
4	120.8	116.5*
5	128.6	128.5
6	126.2	126.7
7	130.5	125.2
8	117.0	114.5*
9	151.3	149.2
10	120.9	120.5*

* = interchangeable

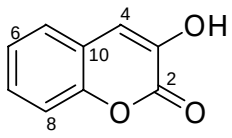


Figure 3.3 The structure of Compound **2** (3-hydroxycoumarin)

6-Hydroxycoumarin (3)

Compound **3** was isolated as white powder with melting point 181-187°C. The EIMS revealed molecular ion peak at *m/z* 162 accounting for the molecular formula $C_9H_6O_3$, requiring seven degrees of unsaturation. The ^{13}C NMR demonstrated six signals of a substituted aromatic system (δ_C 107.4, 108.6, 109.8, 132.5, 154.7, 155.4 ppm) and three signals of unsaturated lactone (δ_C 114.0,138.6 and 160.0 ppm), accounting for coumarin-type compound. The ^1H NMR showed a couple of α, β-unsaturated lactone protons [δ_H 6.34 (1H, d, *J* = 9.4 Hz) and 8.19 (1H, d, *J* = 10.1 Hz)] and aromatic protons [δ_H 6.83 (2H, t, *J* = 8.8, 8.6 Hz) and 7.43 (1H, t, *J* = 8.6, 8.6 Hz)] as well as one exchangeable hydroxyl proton [δ_H 9.18 (1H, br, s)].

The aforementioned data allowed establishing two possible structures for **3**: 6-hydroxycoumarin (**3a**) and 7-hydroxycoumarin (**3b**).

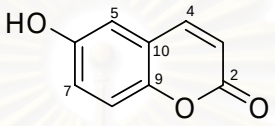
3a **3b**

Figure 3.4 The two possible structures of Compound **3** (**3a** and **3b**) of **3**.

To definitely assign the actual structure of **3**, a methoxy derivative was prepared. Compound **3** was treated with trimethyl silyl diazomethane ($TMSCHN_2$) at room temperature for 2 h. After evaporating the reaction mixture, which was further purified on a plug of silica gel using 20% ethyl acetate in dichloromethane, a methoxy coumarin (**3c**) was obtained. The newly generated methoxy coumarin (**3c**) was readily recognized by the absence of the exchangeable hydroxyl at δ_H 9.87 and the presence of methoxy protons (δ_H 3.94), while the multiplet signals of three contiguous protons of benzene ring (δ_H *ca* 6.83) were better resolved into two sets [δ_H 6.89 (1H) and 6.71 (1H)]. To assign the position of methoxy group, NOE technique was applied. Irradiation of methoxy signal (δ_H 3.94) gave rise to doublet signal of δ_H 7.12, while enhanced peak of H-5 (δ_H 7.45) was not observed. Meanwhile, irradiation of H-5

caused obvious enhancement of H-7 (δ_H 6.71) and H-8 (δ_H 6.89, s). Therefore, the methoxy group of **3c** was unambiguously placed at C-6, and the structure of 6-hydroxycoumarin was conclusively depicted for **3**. The assignment of protons and carbons for **3** was tabulated with the aid of DEPTs and NOE experiments, including comparison with the previously reported (Breitmaier and Voclter, 1987).

Table 3.5 ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) of **3** in CD_3COCD_3



Position	Chemical shift(ppm)	
	^{13}C	^{1}H (mult, *J* in Hz)
2	160.0	
3	114.0	6.34 (1H, d, *J* =9.4 H_z)
4	138.6*	8.19 (1H, d, *J* =10.1 H_z)
5	107.4*	7.43 (1H,t, *J* = 8.6, 8.6 H_z)
6	155.4*	
6-OH		9.87 (br, s)
7	109.8*	6.83 (2H, t, *J* = 8.8, 8.6 H_z)
8	108.6*	
9	154.6*	
10	132.5*	

* = interchangeable

6-Methoxycoumarin (3c): pale yellow amorphous; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10, (1H, d, H-3), 7.45 (1H, m, H-5)*, 6.93 (1H, d, *J* = 8.5 H_z, H-8), 6.72 (1H, d, *J* = 8.3 H_z, H-7)*, 6.35 (1H, d, *J* =9.7 H_z , H-4), 3.94 (3H, s, 6-OMe). * H-7 demonstrated doublet signal at δ_H 6.72 on irradiation of H-5.

8-hydroxycoumarin (4)

Compound **4** was isolated as colorless crystals with melting point 157-160°C, (157-160°C; Sadavongvivad and Supavilai, 1977) molecular ion peak at *m/z* 162 and molecular formula $C_9H_6O_3$. The ^{13}C NMR demonstrated six signals of a substituted aromatic system (δ_C 118.4, 118.8, 119.9, 124.5, 142.6 and 144.6 ppm) and three signals of unsaturated lactone (δ_C 116.3, 144.2 and 159.6 ppm), accounting for coumarin-type compound. The ^1H NMR showed a couple of α, β-unsaturated lactone protons [δ_H 6.44 (1H, d, *J* = 9.4 Hz) and 7.97 (1H , d, *J* = 10.0 Hz)] three contiguous aromatic protons [δ_H *ca* 7.17 (3H, m)] as well as one exchangeable hydroxyl [δ_H 9.18 (1H, br, s)]

The aforementioned data allowed establishing two possible structures for **4**: 5-hydroxy coumarin (**4a**) and 8-hydroxycoumarin (**4b**).

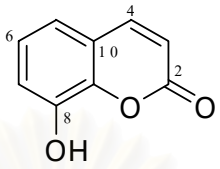
4a **4b**

Figure 3.5 The two possible structures of Compound **4** (**4a** and **4b**)

To definitely assign the actual structure of **4**, a methoxy derivative was prepared. A methoxy coumarin (**4c**) was also prepared in the same manner as compound **3c**. The newly generated methoxy coumarin (**4c**) was readily recognized by the absence of the exchangeable hydroxyl at δ_H 9.18 and the presence of methoxy protons (δ_H 4.00), while the multiplet signals of three contiguous protons of benzene ring (δ_H *ca* 7.17) were better resolved into two sets [δ_H 7.11 (2H) and 7.26 (1H)]. To assign the position of methoxy group, NOE technique was applied. Irradiation of methoxy signal (δ_H 4.00) gave rise to doublet signal of δ_H 7.12, while enhanced peak of H-4 (δ_H 7.72) was not observed. Meanswhile, irradiation of H-4 caused obvious enhancement of H-3 (δ_H 6.47) and H-5(δ_H 7.09, d, *J*=7.6 Hz) alone. Therefore, the methoxy group of **4c** was unambiguously placed at C-8, and the structure of 8-hydroxycoumarin was conclusively depicted for **4**. The assignment of protons and

carbons for **4** was tabulated with the aid of DEPTs and NOE experiments, including comparison with previous reports (Breitmaier and Voclter, 1987).

Table 3.6 ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Spectral Data of **4** in CD_3COCD_3



Position	Chemical shift(ppm)	
	^{13}C	^{1}H (mult, *J* in Hz)
2	159.6	
3	116.3	6.44 (1H, d, *J* = 9.4)
4	144.2	7.97 (1H, d, *J* = 10.0)
5	118.4	*Ca* 7.17 (3H, m)
6	118.8	
7	124.5	
8	144.6*	
8-OH		9.18 (br, s)
9	142.6*	
10	119.8	

* = interchangeable

8-Methoxycoumarin (4c): pale yellow amorphous; ^{1} H NMR (400 MHz, $CDCl_3$), 7.72 (d, *J*=9.5Hz, 1H, H-4) 7.26 (m, 1H, H-6), 7.11 (m, 2H, H-5, H-7)*, 6.47 (1H, d, *J* = 9.5 Hz, H-3), 4.00 (3H, s, 8-OMe). *H-5 demonstrated doublet signal at δ_H 7.09 on irradiation of H-4.

Scopoletin (5)

20 milligrams of compound **5** was gained as a yellow powder of melting point 168-169°C (169-170°C; Phuwapraisirisan, 1998) and R_f 0.44 (SiO_2, 20% EtOAc/CH_2Cl_2). Compounds **5** displayed bright blue fluorescence under UV lamp (365 nm). Molecular peak at m/z 192 corresponded to a molecular formula $C_{10}H_8O_4$.

The 400 MHz ^1H NMR spectrum of **5** showed a pair of doublets at δ_H 6.22 and 7.89 (1H each, *J*= 9.5 Hz) assigned to H-3 and H-4 of an unsaturated lactone ring, respectively. In the aromatic region showed a singlet signal at δ_H 6.84 (1H, s) and δ_H 7.24 (1H, s) ppm. Finally, one methyl protons were also observed at δ_H 3.97(3H, s).

The ^{13}C NMR spectrum displayed four methane carbon at δ_C 103.2, 111.5, 113.4, and 142.3, four quaternary carbons at δ_C 107.5, 143.8 and 149.7 and 150.2. One methoxy carbon at δ_C 56.4 and finally a ketone group at δ_C 161.6 ppm. Other chemical shifts in NMR spectra and all above data corresponded to the structure of coumarin that contained one hydroxyl and one methoxy as substituents. We were able to resolve the structure by comparing ^1H and ^{13}C NMR of this compound with the literature data (**Table 3.7**) (Phuwapraisirisan, 1998). Therefore, Base on all of the above spectroscopic data and comparision with proclaimed compound **5** was assigned as 7-hydroxy-6-methoxy coumarin or scopoletin (**Figure 3.6**).

The assignment of all carbon atoms was done by HMQC and HMBC experiments. From HMBC correlation, δ_H 2.31 correlated with carbon signal at δ_C 179.3 and *ca* 20-40, but their had not correlated at δ_C *ca* 130-160 which was chemical shift of compound **5**. The above NMR data indicated that long chain carboxylic was impurity in compound **5**. In addition, the T.L.C. of compound **5** (R_f = 0.44; 1:4 EtOAc: CH_2Cl_2) was closely related to that of standard scopoletin (R_f = 0.47, Phuwapraisirisan, 1998). Compound **5** was therefore identified as scopoletin.

Table 3.7 ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra data of Scopoletin and Compound **5** in CD_3COCD_3

Position	Chemical shift(ppm)			
	Scopoletin		Compound **5**	
	^{13}C	^{1}H (mult, *J* in Hz)	^{13}C	^{1}H (mult, *J* in Hz)
2	161.2		161.6	
3	110.0	6.17(1H, d, J=9.4 Hz)	113.4	6.22 (1H, d, J=9.5Hz)
4	144.6	7.83(1H, d, J=9.4Hz)	142.3	7.89 (1H, d, J=9.5Hz)
5	113.3	7.19(1H, s)	111.5	7.24 (1H, s)
6	151.8		149.7*	
6-OMe	56.7	3.91(3H, s)	56.4	3.97 (3H, s,)
7	145.9		143.8*	
8	103.7	6.79(1H, s)	103.2	6.84 (1H, s)
9	151.1		150.2	
10	112.1		107.5	

* = interchangeble

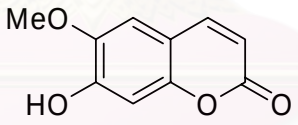


Figure 3.6 The structure of Compound **5** (Scopoletin)

Pinoresinol (6)

Compound **6** (0.057 g) was obtained as a yellow sticky semi-solid from dichloromethane crude extracts. R_f value was 0.45 (SiO_2, 1:4 EtOAc:CH_2Cl_2). The EIMS revealed molecular ion peak at *m/z* 358 accounting for the molecular formula $C_{20}H_{22}O_6$.

The 400 MHz ^{1}H NMR spectrum revealed the typical pattern of a 1, 3, 4,-trisubstituted benzene ring at δ_H 6.84 (m, 1H), 6.90 (br, s, 1H), 6.93 (m, 1H), one OMe at δ_H 3.90 (s, 3H) and one phenolic OH at δ_H 5.95 (br, s, 1H). Oxirane protons appeared as a multiplet at δ_H 3.14 (H-2') and a doublet at 4.77 (*J* = 4.68 Hz, H-3'). The

observed coupling constant of 4.68 Hz suggested a *cis*-disubstituted epoxide. The remaining two signals (*dd*) at δ_H 3.90 and 4.27 were assign as a methylene group (C-1') attached to C-2'.

The ^{13}C NMR data displayed five methine carbon at δ_C 54.1(C-2'), δ_C 85.9 (C-3'), δ_C 108.7(C-6), δ_C 114.4 (C-5), and δ_C 119.0 (C-2), four quaternary carbons at δ_C 132.8 (C-1) , δ_C 145.3 (C-4) and δ_C 146.8 (C-3), one methylene carbon at δ_C 71.7 (C-1') and finally, one methoxy carbon at δ_C 54.1. The aforementioned data allowed establishing possible structures for **6**: coniferyl alcohol (**6a**)

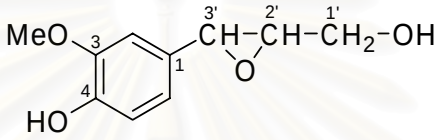


Figure 3.7 Coniferyl alcohol **(6a)**

From M.S. data, the molecular ion peak was *m/z* 358, it was not fit with molecular weight of coniferyl alcohol (*m/z* 180, **6a**). The another possible structure was pinoresinol (**6b**). The reaction test and derivatives of compound **6** were prepared, such as acetylation, methylation and payne rearrangement.

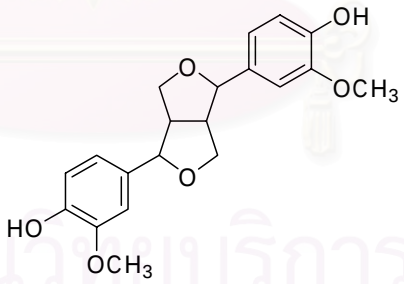


Figure 3.8 pinoresinol (**6b**)

The acetylation and methylation of compound **6** were displayed acetyl protons at δ_H 2.20 and methoxy protons at δ_H 3.90, respectively. The both results were designated as the hydroxyl group on aromatic ring. The payne rearrangement, compound **6** gave no sign of chemical degradation despite of long time exposure to aqueous sodium hydroxide. Consequently, the possible structure of compound **6** was

pinolesinol, which was confirmed this structure by comparing with those of previously published data (Miyazawa *et al.*, 1992).

Table 3.8 The ^{1}H NMR (400MHz) and ^{13}C NMR (100MHz) spectra data of (+)-Pinolesinol and Compound **6** in $CDCl_3$

Position	Chemical shift (ppm)			
	(+)-Pinoresinol		Compound **6**	
	^{13}C	^{1}H (mult, *J* in Hz)	^{13}C	^{1}H (mult, *J* in Hz)
1	54.1	3.10, m	54.1	3.14, s
2	85.8	4.74, d	85.9	4.77, d
8	71.6	3.87, dd	71.7	3.90, s
		4.24, dd		4.27, t
1'	132.9		132.8	
2'	108.6	ca 6.82, m	108.7	6.93, m
3'	146.7		146.8	
4'	145.2		145.3	
5'	114.3	ca 6.88, m	114.4	6.90, br s
6'	118.9		119.0	6.93, m
OMe	55.9	3.90, s	56.0	3.90, s

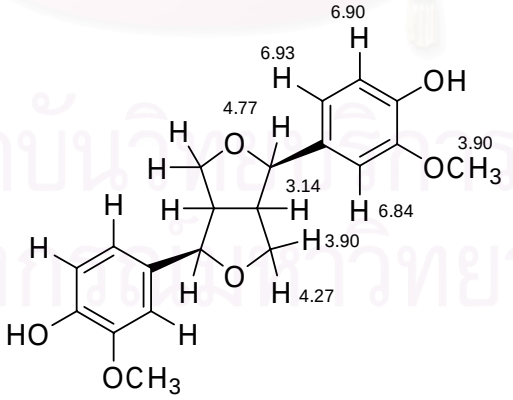


Figure 3.9 The complete assignment of Compound **6** (^{1}H NMR)

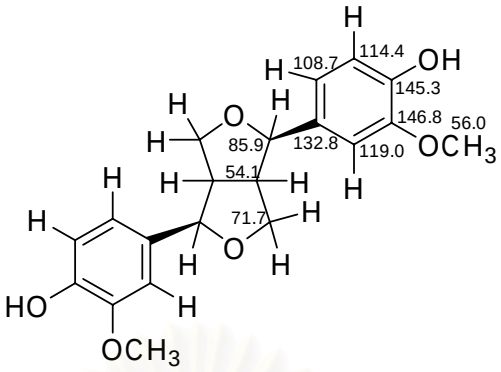


Figure 3.10 The complete assignment of Compound **6** (^{13}C NMR)

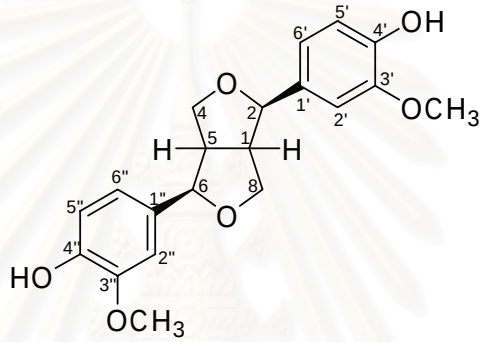


Figure 3.11 The structure of Compound **6** (pinoresinol)

Zhebeiresinol (7)

White powder of compound **7** (0.013 g) was obtain from dichloromethane crude extracts. The molecular formula of **7** was determined to be $C_{14}H_{16}O_6$. Its melting point was 184-186° C and R_f value was 0.4 (SiO_2, 1:4 EtOAc:CH_2Cl_2).

The ^{1}H NMR spectrum showed a singlet aromatic proton signal integrated for two aromatic protons at δ_H 6.61 and two methoxyl signal at δ_H 3.96 and the ^{13}C NMR spectrum indicated the presence of one carbonyl group (δ_C 178.2). The HMQC spectrum revealed the presence of two methylenes (δ_C 69.8 and 70.1) and five methines (δ_C 46.0, 48.5, 86.3, 102.7 and 129.8) in this structure. The ^{1}H-^{1}H COSY spectrum indicated the connection of the methylenes and methines as shown in Figure 3.14. HMBC correlations were observed between a proton signal at δ_H 4.54 (H-9) and a carbonyl carbon signal at δ_C 178.2, between the signals of H-9 and methane carbon at 46.0 (C-8'), as well as between the signals of H-7 and C-9', which led to the partial structure in the aliphatic part of **7**. The HMBC correlations observed

between the signals of H-7 and C-1, H-7 and C-6, enabled the connection of C-7 to C-1 and thus the planar structure of **7** was established as shown Figure 3.15

From the structure of compound **7** had been reported two types, zhebeiresinol and caruilignan D (Ma *et al*., 2001). Its was found that relative stereochem of the two compounds were quite different. Zhebeiresinol was C-1/C-7 α-oriantation and caruilignan D was C-1/C-7 β–oriantation. The structure and stereochemistry of compound **7** were confirmed by a single-crystal X-ray analysis. From the single-crystal X-ray data, the stereochemistry of compound **7** as C-1/C-7 α-oriantation. Therefore, compound 7 was zheberesinol.

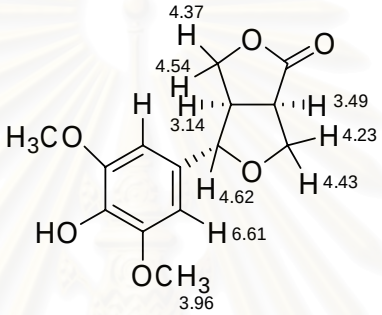


Figure 3.12 The complete assignment of Compound **7 (**^{1}H NMR**)**

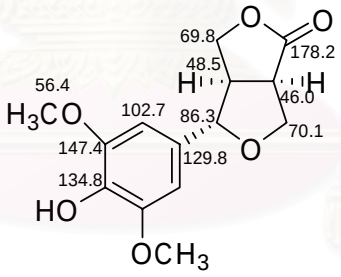


Figure 3.13 The complete assignment of Compound **7 (**^{13}C NMR**)**

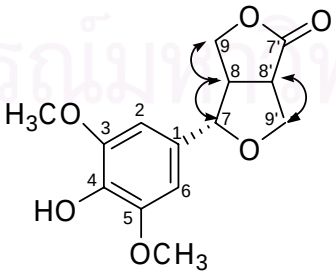


Figure 3.14 Selected COSY of Compound **7**

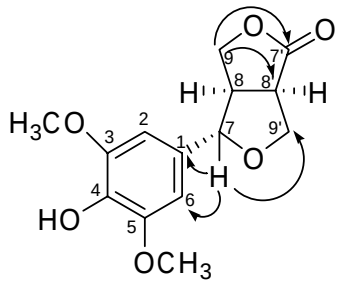


Figure 3.15 Selected HMBC correlation of Compound **7**

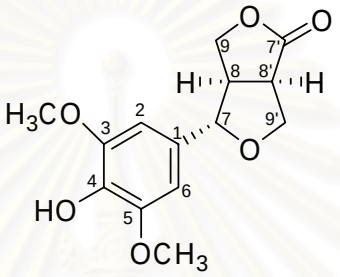


Figure 3.16 The structure of Compound **7** (Zhebeiresinol)

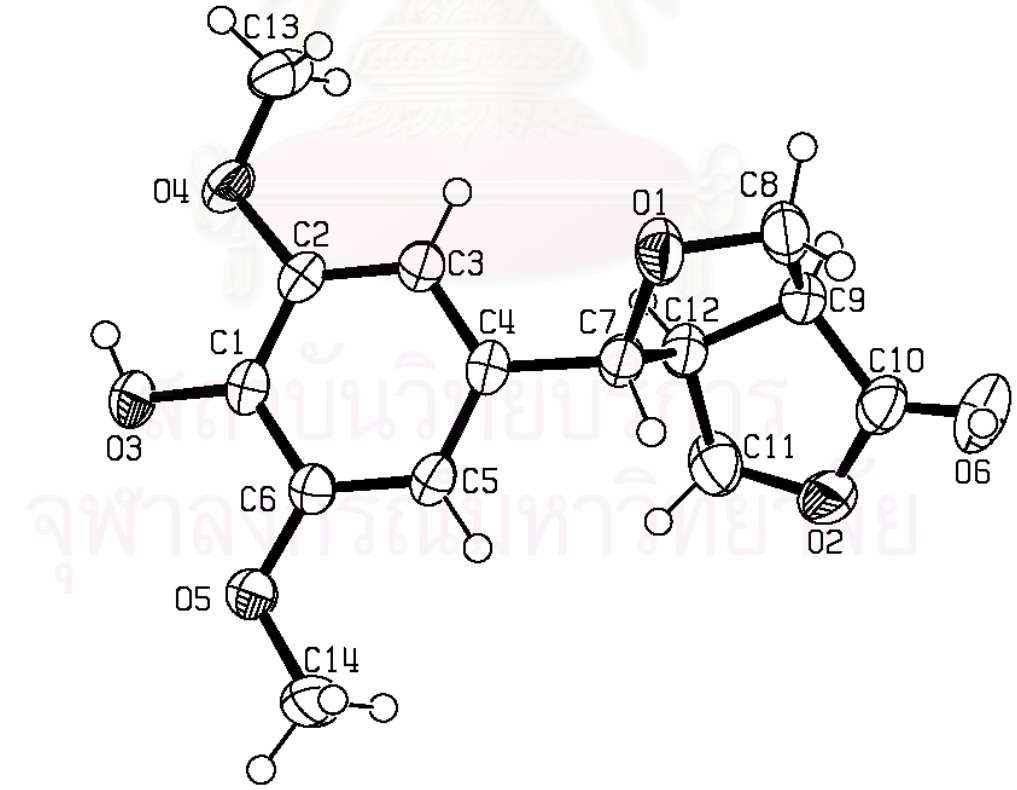


Figure 3.17 ORTEP view of x-ray molecular structure of Compound **7**(Zhebeiresinol)

***p*-Hydroxybenzoic acid (8)**

Compound 8 (0.013 g) was obtained as a white powder from ethyl acetate crude extracts. The ^{1}H NMR spectrum indicated the signal of aromatic protons at δ_H 6.96 (2H, d, J = 8.6Hz) and δ_H 7.95 (2H, d, J = 8.6Hz). The ^{13}C NMR spectrum showed two quaternary carbons at δ_C161.7 (C-4) and δ_C121.9(C-1), two methine carbons at δ_C 115.0(C-2) and δ_C 131.8(C-3). In additional, one carboxylic group at δ_C 166.7 (C-7).

All spectrum data suggested that compound **8** might be *p*-hydroxybenzoic acid. This compound was confirmed with authentic *p*-hydroxybenzoic acid (Fluka). Consequently, The ^{1}H-^{13}C NMR data of compound **8** was compared with those of previously report (Phuwapraisirisan, 1998) which shown in table 3.10.

Table 3.9 ^{1}H NMR and ^{13}C NMR spectral data of *p*-Hydroxybenzoic acid and Compound **8** in CD_3COCD_3

Position	Chemical shift(ppm)			
	p-Hydroxybenzoic acid		Compound **8**	
	^{13}C	^{1}H (mult, J in Hz)	^{13}C	^{1}H (mult, J in Hz)
1	122.7		121.9	
2	115.9	6.91(2H,d, J=8.9Hz)	115.0	6.96 (2H, d, J=8.6 Hz)
3	132.6	7.91(2H, d, J=9.2 Hz)	131.8	7.95 (2H, d, J=8.6 Hz)
4	162.5		161.7	
7	167.5		166.7	

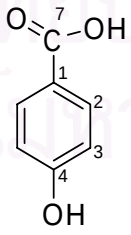


Figure 3.18 The structure of Compound **8** (*p*-hydroxybenzoic acid)

3.3 Antioxidant activity of isolated compounds

From dichloromethane and ethyl acetate crude extracts of *A. reinwardtii* led to the isolation of 8 compounds. All of these and its some derivatives were for antioxidant activity. The antioxidant activity results for compound 1-8 and its some derivatives are summarized in Table 3.11.

Table 3.10 Antioxidant activity of isolated compounds

Isolated Compounds and its some derivatives	IC_{50} (mM)			
	DPPH	Xanthine		Lipid peroxidation
		Superoxide scavenging	Xanthine oxidase inhibition	
Coumarin(**1**)	>100	>100	No activity	67.64 ±1.46
3-Hydroxycoumarin (**2**)	0.61±0.08	4.55±0.05	No activity	69.07 ± 0.81
6-Hydroxycoumarin (**3**)	>100	19.23±0.17	No activity	67.45 ± 0.75
8-Hydroxycoumarin (**4**)	>100	13.35±1.11	No activity	58.13 ± 1.17
Scopoletin (**5**)	3.17±0.31	-	-	-
Pinoresinol (**6**)	0.31±0.02	4.51±0.41	No activity	3.37 ± 0.13
Zhebeiresinol (**7**)	0.19±0.02	3.38 ± 0.29	No activity	2.08 ± 0.06
p-Hydroxybenzoic acid (**8**)	>100	>100	No activity	>100
3-Methoxycoumarin (**2c**)	8.70±1.02	-	-	-
6-Methoxycoumarin (**3c**)	>1	-	-	-
8-Methoxycoumarin (**4c**)	>1	-	-	-
Dimethylate pinolesinol (**6c**)	0.6±0.02	-	-	-
BHA*	0.18±0.03	-	-	0.25 ± 0.01
Gallic acid*	0.50±0.03	0.65±0.02	-	-
Allopurinol*		-	0.0044±0.07	-

* Standard antioxidant

3.4 Disscussion

As part of our ongoing search for antioxidative constituents from *A. reinwardtii*, we reported the isolation and characterization of eight compounds. They were five coumarin-type, Coumarin (**1**), 3-Hydroxycoumarin (**2**), 6-Hydroxycoumarin (**3**), 8-Hydroxycoumarin (**4**), Scopoletin (**5**), two lignan-type, Pinoresinol (**6**), and Zhebeiresinol (**7**), and one aromatic compound: *p*-Hydroxybenzoic acid (**8**). Compound **6** has been isolated previously from *A. reinwardtii*, but compound **1**-**5**, **7** and **8** have not been reported previously from this species. All the isolated compounds (**1**-**8**) were test for antioxidant activity in the DPPH radical scavenging activity, Xanthine oxidase-related activity and Lipid peroxidation inhibitory activity.

DPPH radical scavenging activity

The free radical scavenging activity on DPPH indicated that compound **7 (**IC_{50} = 0.19 mM**)** showed the highest activity and the other compounds followed the order: **6** (IC_{50} = 0.31 mM) > **2** (IC_{50} = 0.61 mM) > **5** (IC_{50} = 3.17 mM) > **4** (IC_{50} = 71.05 mM) respectively.Thus, compound **1**, **3**, and **8** gave the weakest activity (IC_{50} > 100 mM).

In comparision among the coumarin series (compounds **1**-**5**) revealed that compound **3** showed the highest activity which indicated that hydroxyl group at C-3 of 1-2 pyrone ring seem to be essential on scavenging activity than a substituted hydroxyl group on aromatic ring. Furthermore, some methylated coumarin (**2C**, **3C** and **4C**) and lignan (**6C**) showed the lower free radical scavenging activity on DPPH than the parent compounds.

However, it appears that the presence of one hydroxyl group on aromatic ring is not essential on scavenging of free radical. Under the condition compound **7** exhibited a higher scavenging activity on DPPH than the other compounds

Xanthine oxidase-related activity

(Superoxide scavenging activity and inhibitory effect on xanthine oxidase)

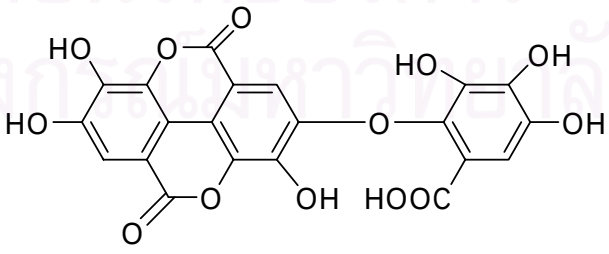
Xanthine oxidase is catalyze the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. The overproduction of uric acid can lead to hyperuricemia. Here, hyperuricemia can be linked to gout, due to the deposition of uric acid in the joints leading to painful inflammation (Harris *et al*., 1999). Accordingly, the use of

the XOD inhibitor that blocks the synthesis of uric acid in the body should be one of the therapeutic approaches for treatment of hyperuricemia (Emmerson, 1996). In addition, during the oxidation of xanthine, generating superoxide radical which it cause to disease. This research provided the evidence that isolated compounds from *A. reinwardtii* exhibits interesting antioxidant properties, expressed either by the capacity to scavenge superoxide radical or to noncompetitively inhibit xanthine oxidase.

From table 3.11, Compound **2**, **3**, **4**, **6** exhibited significant IC_{50} of superoxide scavenging activity of 4.55, 19.23, 13.35 and 4.51 mM, respectively. Therefore, compound **1** and **8** were found to be inactive (IC_{50} > 100 mM). However, their activity were moderately lower than gallic acid (IC_{50} = 0.65 mM).

In addition, all compounds displayed no activity on inhibitory activity against xanthine oxidase (XOD), while allopurinol, a clinical drug used for XOD inhibitor, showed IC_{50} = 0.0044 mM. From the previous report, valoneic acid dilactone was isolated from the leaves of *Lagerstroemia speciosa* which was traditionally used as a folk medicine in the Philippines, showed the strongest xanthine oxidase inhibitory effect (IC_{50} = 0.0025 mM), its is inhibitory activity stronger than allopulinol (IC_{50} = 0.0104 mM). These results may support the dietary use of the aqueous extracts from *Lagerstroemia speciosa* leaves for prevention and treatment of hyperuricemia (Unno, Sugimoto, and Kakuda, 2004).

From the above data, five compounds (compound **2**, **3**, **4**, **6**, and **7**), showed potent superoxide scavenging activity while their exhibited no activity in xanthine oxidase inhibitor. However their were being an alternative treatment for diseases cause from free radicals more than alternative treatment for hyperuricemia.



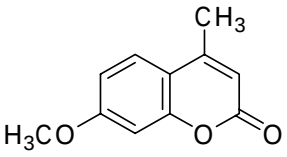
Valoneic acid dilactone

Lipid peroxidation inhibitory activity

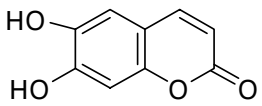
Lipid peroxidation reactions are essential components in many pathobiological process such as atherosclerosis, carcinogenesis or cell death. In this research, we used linoleic acid, is one of the main fatty acids in lipid membranes and low-density (LDL), used as a model in lipid peroxidation inhibitor. Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides. The ferric thiocynate (FTC) assay measures the hydroperoxide directly utilizing the redox reaction with ferrous ion. Hydroperoxides are highly unstable and react readily with ferrous ion to produce ferric ion. The resulting ferric ions are detected using thiocyanate ion as the chromogen. For evaluation of the antioxidant activity of isolated compounds, the inhibition effect on the peroxidation of linoleic acid was investigated.

In lipid peroxidation inhibitory activity, compound **6** and **7** showed potent activity (IC_{50} = 3.31, 2.08 mM, respectively), while compound **1**, **2**, **3**, and **4** showed moderate activity (IC_{50} = 67.64, 69.07, 67.45 and 58.13 mM, respectively). However, compound **8** gave the weakest activity (IC_{50} > 100 mM). On the other hand, gallic acid, as a standard antioxidant, showed IC_{50} = 0.25 mM.

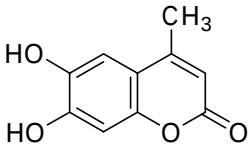
From the previous report, the series of coumarins and phenolic acids were compared for their peroxyl radical-scavenging activity (linoleic acid hydroperoxide-induced cytotoxicity) and DPPH radical scavenging activity (electron spin resonance spectrometry) They were indicated that the radical-scavenging activity of coumarin classified from the quenching of the DPPH radical as follows: esculetin, 4-methylesculetin, fraxetin, caffeic acid >> hydroxycoumarins (3-hydroxycoumarin and 7-hydroxycoumarin), hydroxycinnamic acids > 7-methoxy-4-methylcoumarin, coumarin (Kaneko *et al*., 2003). Coumarins with an *ortho*-catechol moiety in the molecules showed high scavenging activity for free radicals more than other coumarins without an *ortho*-catechol moiety. These molecules are known to be oxidized to from *ortho*-semiquinones, which are to contribute to their oxidizability, that is antioxidant activity. This characteristic may explained why compounds with an *ortho*-catechol moiety show high antioxidant activities and coumarin with only one hydroxyl group had a little activity (Bors, Michel, and Stettmaler, 2000). In other word, in this research, 3-hydroxycoumarin had a highly DPPH and superoxide radical scavenging activity may be 3-hydroxycoumarin was oxidized to from *ortho*-semiquinone.



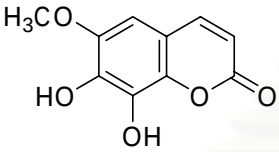
7-methoxy-4-methylcoumarin



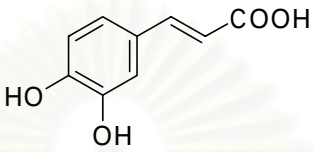
Esculetin



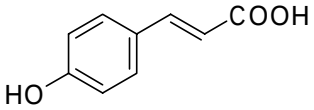
4-Methylesculetin



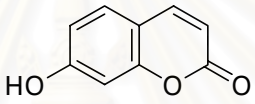
Fraxetin



Caffeic acid



4-Hydroxycinnamic acid



One hydroxyl coumarin
(Umbelliferone)

Compound **5** did not test for xanthine oxidase-related activity and lipid peroxidation inhibitory activity because of very small amount of sample. In addition, in the chemotaxis assay, preliminary results indicated that pure scopoletin is able to induce inhibition of U-937 cell proliferation and differentiation. Therefore, scopoletin might be develope therapeutic role in the management of leukemia (Riveiro *et al.*, 2004).

Compound **6** and **7** were a lignan-type, compound **6** also has been reported for significantly inhibited ecdysis in fourth-instar larvae of *Rhodnius prolixus* (hematophagous insect), (Garcia *et al*., 2000 and Garcia and Azambuja, 2004) and weakest inhibited effect against acetylcholinesterase with a range of 20-35% at higher concentrations (EI-Hassan *et al*., 2003). In addition, this is a frist report for compound **7** from *A. reinwardtii* and it firstly separated from Zhebeimu (*Fritillaria thunberii*) (Jin *et al*., 1993). This compound also showed the highest antioxidant activity among the isolated compounds.

Compound **8** showed lowest antioxidant activity in all assay**.** However, in previous report compound **8** showed an attractive result against brine shrimp with LC_{50} = 33.1 ppm (Phuwapraisirisan, 1998).

3.5 Experimental Section

3.5.1 General Experimental Procedures

3.5.1.1 ^{1}H and ^{13}C-Nuclear Magnetic Resonance Spectrometer

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 proton in deuterated solvent and using TMS as an internal standard in some cases.

3.5.1.2 Mass Spectrometer

The Electron Impact Mass Spectra (EIMS) were obtain on Mass Spectrometer Model VG TRIO 2000.

3.5.1.3 Melting Point Apparatus (m.p.)

Melting points were determined with Fisher-John Melting Point Apparatus.

3.5.1.4 Chromatotron Equiment

Chromatotron Equiment on Harrison Research Model 7924T was occupied for certain separation.

3.5.1.5 Rotary Evaporator

The BUCHI rotary evaporator was utilized to evaporate the large amount of all volatile organic solvents.

3.5.1.6 UV-Visible Spectrophotometer

UV-visible absorption spectra of the active compounds were recorded on UV-Vis Hewlett Packard 8453 diode array spectrophotometer.

3.5.1.7 pH Meter

pH values were determined with 744 pH meter Ω Metrohm analysis.

3.5.1.8 96 well microplate

UV-spectrometre, microtiter plate reader, model sunrise (Tecan Austria GmbH)

3.5.2 Chemical

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 percolated Merck silica gel 60 F_{254} plates (0.25 mm. thick layer).

3.5.3 Plant material

The stems of chalood (*A. rewardtii*) were obtained from traditional drug store in Bangkok. The specimens were identified by Associate Professor Dr. Obchan Thaithong, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. A voucher specimen has been deposited at the Herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

3.5.4 Extraction and Isolation

The dichloromethane crude extract (55 g) was chromatographed over a silica gel quick column with a gradient of CH_2Cl_2-hexane (0:10 to 10:0) and EtOAc-CH_2Cl_2 (0:10 to 10:0). 8fraction, A-H were collected according to TLC analysis. Coumarin (**1**) (2.15 g) was obtained after evaporation from fraction C (CH_2Cl_2/hexane, 1:1). Fraction D gave 3-hydroxycoumarin (**2**) (2.55 g) by recrystallization from CH_2Cl_2. Fraction G gave white powder, purified by recrystallization with EtOAc/CH_2Cl_2(3:7) to provide 6-hydroxycoumarin (**3**) (1.20 g) while fraction F fractionated by radial chromatographic technique (chromatotron) eluting with EtOAc/hexane (4:6 to 7:3) to afford 8-hydroxycoumarin (**4**) (0.77 g) and scopoletin (**5**) (0.018 g). From remaining part of fraction G, was subjected to silica gel open column chromatography eluting with EtOAc-hexane (1:9 to 5:5) to yield two active fraction: G-6 and G-7. Fraction G-6 was further isolate on flash column chromatography eluting with EtOAc-CH_2Cl_2 (4:6 to 7:3) to obtain yellow sticky semi-solid and then purified with chromatotron technique eluting with EtOAc/hexane (3:7) to furnish pinoresinol (**6**) (0.057 g). Zhebeiresinol (**7**) (0.010 g) was obtained from fraction G-7 and recrystallization from methanol.

Ethyl acetate crude extract (10 g) was fractionated by silica gel quick column chromatography eluting with EtOAc/CH_2Cl_2 (0:1 to 1:0) and MeOH/ EtOAc (0:1 to 1:9) to afford 5 fractions, A-E. Fractions B and C were subjected to silica gel open column chromatography eluting with EtOAc/CH_2Cl_2 (0:10 to 6:4) gave 8 fractions, A-H. Fraction F was subjected to further column chromatography on silica gel with EtOAc-CH_2Cl_2 (0:10 to 6:4) system to provide *p*-hydroxybenzoic acid (**8**) (0.013 g).

3.5.5 The derivatives and reaction

*3.5.5.1 Acetylation of **6***

Compound **6** (5 mg) in dried pyridine (300 µl) reacted with acetyl chloride (excess). The reaction mixture was stirred for 10 min and left at room temperature overnight. After solvent removal, the reaction products was dissolved in CH_2Cl_2 (2 ml) and extracted twice with H_2O (2 ml). The CH_2Cl_2 layer were evaporated and separated by Seppack with CH_2Cl_2, 5% MeOH-CH_2Cl_2, 10% MeOH-CH_2Cl_2, 20% MeOH-CH_2Cl_2. Fractions eluted with CH_2Cl_2 were concentrated to afford acetylation product (1 mg)

*3.5.5.2 Methylation of **6***

Compound **6** (5 mg) in CH_2Cl_2 (200 µl) was added $TMSCHN_2$ (6 drops) in the presence of Silica gel (10 mg). The reaction mixture was left for 1 h. at room temperature. The methylated product of compound **6** was obtained by evaporating reaction mixture until dryness.

*3.5.5.3 Payne rearrangement of **6***

Compound **6** (5 mg) in MeOH (5 drops) was added 2M NaOH (10 drops), and the reaction mixture was left for 1 h. at room temperature. The reaction mixture was extracted with CH_2Cl_2 and then followed by EtOAc. The combined organic extracts were evaporated to afford the expected product. The briefly mechanism of this reaction shown in Figure 3.17

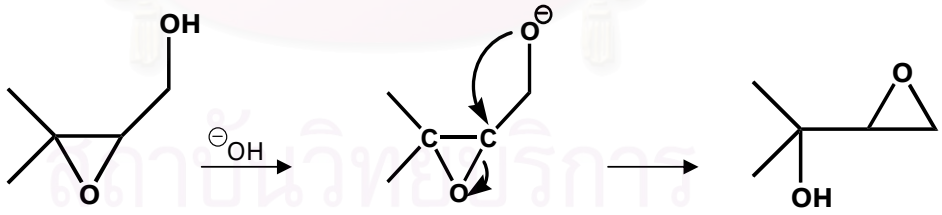


Figure 3.19 Payne rearrangement of Compound **6**

Coumarin (1): white powder; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.43 (1H, d, *J*= 9.4 Hz, H-3), 7.66 (1H, d, *J*= 9.4 Hz, H-4), 7.32 (2H, m, H-5 and H-6), 7.55 (2H, m, H-7 and H-8) ^{13}C NMR (100 MHz, $CDCl_3$) δ 116.7(C-8), 116.9(C-3), 118.9 (C-10), 124.4(C-6), 127.9(C-5), 131.9(C-7), 143.5(C-4), 159.8(C-9), 160.8 (C-2).

3-Hydroxycoumarin (2): colorless crystal**;** ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17(1H, s, H-4), 7.36 (4H, m, H-5, H-6, H-7 and H-8) ^{13}C NMR (100 MHz, $CDCl_3$) δ 114.5*(C-8), 116.5*(C-4), 120.5*(C-10)**,** 125.2 (C-7), 126.7(C-6), 128.5 (C-5), 140.4 (C-3), 149.2(C-9), 161.0 (C-2).

3-Methoxycoumarin (2c): pale yellow amorphous; ^{1}H NMR (400 MHz, $CDCl_3$), δ 7.33 (2H, m), 7.20 (2H, m), 6.78 (1H, s, H-4), 3.86 (3H, s, 3-OMe).

6-Hydroxycoumarin (3): white powder; ^{1}H NMR (400 MHz, CD_3COCD_3) δ 6.34(1H, d, *J* =9.4 H_z, H-3), 7.43 (1H, t, *J* = 8.6, 8.6 H_z, H-5), 8.19(1H, d, *J* =10.1 H_z , H-4), 6.83(2H, t, *J* = 8.8, 8.6 H_z, H-7 and H-8), 9.87 (1H, br s, 6-OH) ^{13}C NMR (100 MHz, CD_3COCD_3) δ 107.4* (C-5), 108.6* (C-8), 109.8* (C-7), 114.0 (C-3), 132.5*(C-10), 138.6* (C-4), 154.6*(C-9), 155.4*(C-6), 160.0(C-2).

6-Methoxycoumarin (3c): pale yellow amorphous; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10, (1H, d, H-3), 7.45 (1H, m, H-5)*, 6.93 (1H, d, *J* = 8.5 H_z, H-8), 6.72 (1H, d, *J* = 8.3 H_z, H-7)*, 6.35 (1H, d, *J* =9.7 H_z , H-4), 3.94 (3H, s, 6-OMe). * H-7 demonstrated doublet signal at δ_H 6.72 on irradiation of H-5.

8-Hydroxycoumarin (4): white needle crystal; ^{1}H NMR (400 MHz, CD_3COCD_3) δ 6.44 (1H, d, *J* = 9.4 Hz, H-3), *Ca* 7.17 (3H, m, H-5, H-6 and H-7), 7.97 (1H, d, *J*=10.0 Hz, H-4), 9.18 (1H, br s, 8-OH) ^{13}C NMR (100 MHz, CD_3COCD_3) 116.3 (C-3), 118.4 (C-5), 118.8 (C-6), 119.8 (C-10), 124.5 (C-7), 144.2 (C-4), 142.6* (C-9), 144.6* (C-8), 159.6 (C-2).

8-Methoxycoumarin (4c): pale yellow amorphous; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (1H, d, *J* = 9.5 Hz, H-4) 7.26 (1H, m, H-6), 7.12 (2H, m, H-5 and H-7)*, 6.47 (1H, d, *J*=9.5Hz, H-3), 4.00 (3H, s, 8-OMe). *H-5 demonstrated doublet signal at δ_H 7.09 on irradiation of H-4.

Scopoletin (5): Pale yellow powder; ^{1}H NMR (400 MHz, CD_3COCD_3) δ 3.97(3H, s, 6-OMe), 6.22 (1H, d, *J* = 9.5 Hz, H-3), 6.84 (1H, s, H-8), 7.24 (1H, s, H-5), 7.89 (1H, d, *J* = 9.5Hz, H-4), ^{13}C NMR (100 MHz, CD_3COCD_3) 56.4 (6-OMe), 103.2 (C-8), 107.5 (C-10), 111.5 (C-5), 113.4 (C-3), 142.3 (C-4), 143.8* (C-6), 149.7* (C-7), 150.2 (C-9), 161.6 (C-2).

Pinoresinol (6): yellow sticky semi-solid; ^{1} H NMR (400 MHz, $CDCl_3$) δ 3.14 (2H, s, H-1 and H-5), 3.90 (2H, s, H-8 and H-4), 3.90 (6H, s, 3'-OMe and 3''-OMe) 4.27 (2H, dd, *J* = 7.02, 7.02 H_z, H-8 and H-4), 4.77 (2H, d, *J* = 4.68 H_z, H-2 and H-6), 6.93 (2H, m, H-2' and H-2″), 6.90 (2H, br s, H-5' and H-5″), 6.93 (2H, m, H-6 and H-6″) ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.1 (C-1 and C-5), 56.0 (3'-OMe and 3''-OMe), 71.7 (C-8 and C-4), 85.9 (C-2 and C-6), 108.7 (C-2' and C-2''), 114.4 (C-5' and C-5''), 119.0 (C-6' and C-6''), 132.8 (C-1' and C-1''), 145.3 (C-4' and C-4''), 146.8 (C-3' and C-3'').

Dimethyl pinoresinol (6c): yellow powder; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.12 (2H, s, H-1 and H-5), 3.89 (2H, d, H-8 and H-4), 3.89 (12H, d, *J* = 9.3 H_z, 3'-OMe, 4'-OMe, 3''-OMe and 4''-OMe) 4.26 (2H, m, H-8 and H-4), 4.75 (2H, m, H-2 and H-6), 6.88 (6H, m, H-2' , H-2″ , H-5' , H-5″ ,H-6' and H-6″).

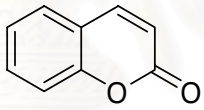
Zhebeiresinol (7): white powder; ^{1} H NMR (400 MHz, $CDCl_3$) δ 3.14 (1H, m, H-5), 3.49 (1H, m, H-1), 3.96 (6H, S, 3'-OMe and 5'-OMe)**,** 4.23 (1H, dd**,** *J* = 3.7, 3.5 H_z**,** H-8), 4.37 (1H, m, H-4), 4.43 (1H, m, H-8), 4.54 (1H, m, H-4), 4.62 (1H, d, *J* = 7.0 H_z, H-6), 6.61 (2H, s, H-2' and H-6') ^{13}C NMR (100 MHz, $CDCl_3$) 46.0 (C-1), 48.5 (C-5), 56.4 (3'-OMe and 5'-OMe), 69.8 (C-4), 70.1 (C-8), 86.3 (C-6), 102.7(C-2' and C-6'), 129.8 (C-1), 134.8 (C-4'), 147.8 (C-3' and C-5'), 178.2 (C-2).

***p*-Hydroxybenzoic acid (8)**: pale yellow powder; ^{1}H NMR (400 MHz, CD_3COCD_3) δ 6.96 (2H, d, *J* = 8.6 Hz, H-3 and H-5), 7.95 (2H, d, *J* = 8.6 Hz, H-2 and H-6) ^{13}C NMR (100 MHz, $CDCl_3$) ^{13}C NMR (50.32 MHz, CD_3COCD_3) 115.0, (C-2 and C-6), 121.9 (C-1), 131.8 (C-3 and C-5), 161.7 (C-4), 166.7 (C-7).

CHAPTER IV

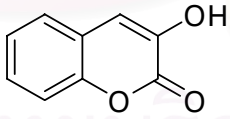
CONCLUSION

Concisely, the isolation of the dichloromethane and ethyl acetate crude extracts obtained eight compounds. They were five coumarin-type, Coumarin (**1**), 3-Hydroxycoumarin (**2**), 6-Hydroxycoumarin (**3**), 8-Hydroxycoumarin (**4**), Scopoletin (**5**), two lignan-type, (+)-Pinoresinol (**6**), and Zhebeiresinol (**7**), and one aromatic compound, *p*-Hydroxybenzoic acid (**8**). The structures of all compounds were characterized by means of NMR, MS, chemical analysis, and comparison with the literature data. The compound **7** was also confirmed by X-ray crystallography. The structures of isolated compounds were summarized as followed.



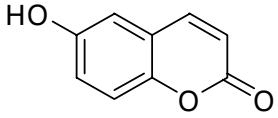
Coumarin (**1**)

Total yield: 2.15 g, 3.909 % w/w



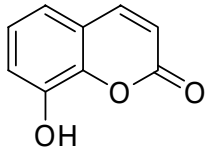
3-Hydroxycoumarin (**2**)

Total yield: 2.55 g, 4.636 % w/w



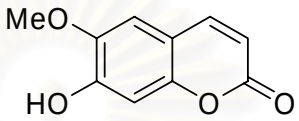
6-Hydroxycoumarin (**3**)

Total yield: 1.20 g, 2.182 % w/w



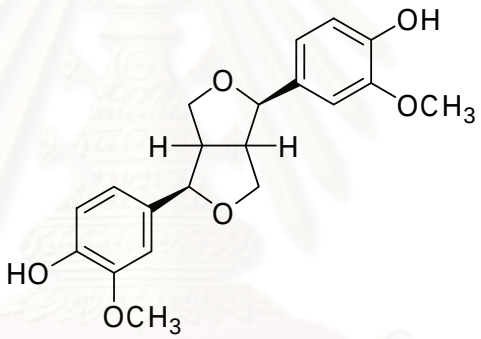
8-Hydroxycoumarin (**4**)

Total yield: 0.77 g, 1.4 % w/w



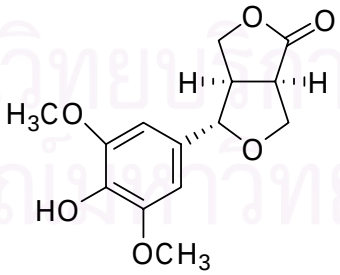
Scopoletin (**5**)

Total yield: 0.018 g, 0.033 % w/w



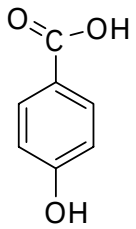
Pinoresinol (**6**)

Total yield: 0.057 g, 0.104 % w/w



Zhebeiresinol (**7**)

Total yield: 0.010 g, 0.018 % w/w



p-Hydroxybenzoic acid (**8**)

Total yield: 0.013 g, 0.024% w/w

The free radical scavenging activity on DPPH indicated that compound **7 (**IC_{50} = 0.19 mM**)** showed the highest activity, followed by **6** (IC_{50} = 0.31 mM), **2** (IC_{50} = 0.61 mM), **5** (IC_{50} = 3.17 mM), **4** (IC_{50} = 71.05 mM) respectively, while compound **1**, **3**, and **8** gave the weakest activity (IC_{50} > 100 mM). In addition, compound **2** (IC_{50} = 4.55 mM), **6** (IC_{50} = 4.51 mM), **7** (IC_{50} = 3.38 mM) exhibited significant superoxide scavenging activity while compound **1** and **8** were found to be inactive (IC_{50} > 100 mM) However, all compounds displayed no activity on inhibitory activity against xanthine oxidase. In lipid peroxidation inhibitory activity, compound **6** and **7** showed potent activity (IC_{50} = 3.31, 2.08 mM, respectively), while compound **1**, **2**, **3**, and **4** showed moderate activity (IC_{50} = 67.64, 69.07, 67.45 and 58.13 mM, respectively). However, compound **8** gave the weakest activity (IC_{50} > 100 mM).

Conclusively, zheberesinol (**7**) was the most powerful antioxidant against all assay models. Meanwhile, pinoresinol (**6**) and 3-hydroxycoumarin (**2**) were also highly active. However, coumarin (**1**) and *p*-hydroxybenzoic acid (**8**) were found to be inactive in antioxidant activity. In addition, the presence of one hydroxyl group on aromatic ring is not essential on scavenging of free radical and some methylated coumarin and lignan showed the lower free radical scavenging activity on DPPH than the parent compounds. Furthermore, coumarins with an *ortho*-catechol moiety in the molecules showed high scavenging activity for free radicals more than other coumarins without an *ortho*-catechol moiety. However, if we had variety of coumarin series in this research, the compete conclusion of antioxidant activity of coumarin will be appear.

Proposal for the Future Work

Coumarin-type compound were separated vary high amount from the stems *A. reinwardtii,* Meanwhile some coumarin gave low antioxidant activity. Thus, coumarin should be further modified structure to enhance antioxidant activity.

Concerning the antioxidant activity, this research determined some *in vitro* assay model. It was suggested that other *in vitro* assay models should be additionally examined. Furthermore, the *in vivo* assay models of the isolated compounds should be determined to fulfill of this research.

REFERENCES

นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร. *สมุนไพรไม้พื้นบ้าน*. เล่มที่1. สำนักงานข้อมูลสมุนไพร คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล 2539, 756-757.

Ames, B. Micronutrients prevent cancer and delay aging. *Toxicology Letters* **1998**, *102*, 5–18.

Argolo, A. C. C.; Sant'Ana, A. E. G.; Pletsch, M.; and Coelho, L. C. B. B. Antioxidant activity of leaf extracts from *Bauhinia monandra*. *Bioresource Technology* **2004**, *95*, 229-233.

Bors, W.; Michel, C.; and Stettmaler, K. Electron paramagnetic resonance studies of radical species of proanthocyanidins and gallate esters. *Arch. Biochem. Biophys.* **2000**, *374*, 347-355.

Bray, R. C. Molybdenum iron-sunfer flavin hydroxylase and related enzymes, in: P.D. Boyer (Ed.). *The Enzymes*, Academic Press, New York **1975**, *12*, 299-419.

Breitmaier, E.; and Voclter, W. Carbon-13 NMR Spectroscopy 3 rd ed. VCH Bublishers, New York, NY, **1987**, 278.

Cox, D. A.; and Cohen, M. L. Effects of oxidized low density lipoproteins on vascular contraction and relaxation. *Pharm. Rev.* **1996**, *48*, 3–9.

Dhalla, N. S.; Temsah, R. M.; and Netticadan, T. *J. Hypertens* **2000**, *18(12)*, 655-673.

El-Hassan, A.; El-Sayed, M.; Hamed, A. I.; Rhee, I. K.; Ahmed, A. A.; Zeller, K. P.; and Verpoorte, R. Bioactive constituents of *Leptadenia arborea*. *Fitoterapia* **2003**, *74*, 184-187.

Emmerson, B. T. The management of gout; The New England. *Journal of Medicine* **1996**, 59, 925-934.

Finkel, T.; and Holbrook, N. J. Oxidants, oxidative stress and the biology of aging. *Nature* **2000**, *408*, 239–247.

Fong, K. L.; McCay, P. B.; Poyer, J. L.; Steele, B. B.; and Misra, H. Evidence that peroxidation of lysosomal membeanes is initiated by hydroxyl free radical produced during flavin enzyme activity. *J. Biol. Chem.* **1973**, *248*, 7792-7797.

Garcia, E. S.; and Azambuja, P. Lignoids in insects: chemical probes for the study of ecdysis, excretion and *Trypanosoma cruzi*–triatomine interactions. *Toxicon* **2004**, *44*, 431-440.

Garcia, E. S.; Cabral, M. M. O.; Schaub, G. A.; Gottlieb, O. R.; and Azambuja, P. Effects of lignoids on a hematophagous bug, *Rhodnius prolixus*: feeding, ecdysis and diuresis. *Phytochemistry* **2000**, *55*, 611-616.

Gutterridge, J. C.; and Halliwell, B. in *Antioxidant in Nutrition, Health, and Disease***.** Oxford University Press, **1994**

Halliwell, B. Antioxidant characterization: Methodology and mechanism. *Biochem. Pharmacol.* **1995**, *49*, 1341-1348.

Halliwell, B.; Gutteridge, J. M. C.; and Cross, C. E. Free radicals antioxidant and human disease: where are we now? *J. Lab. Clin. Med.* **1992**, *119*, 598-620.

Halliwell, B.; Murcic, M. A.; Chirico, S.; and Aruoma, O. I. Free Radicals and Antioxidants in Foods and in vivo: What They Do and How They Work. *Crit. Rev. Food Sci. Nutr.* **1995**, *35*, 7-20.

Haraguchi, H.; Saito, T.; Ishikawa, H.; Date, H.; Tamura, Y.; and Mizutani, K. Antiperoxidative components in *Thymus vulgaris*. *Planta Med.* **1996**, *62*, 217-221.

Haraguchi, H.; Ishkawa, H.; Mizutani, K.Y.; Kinoshita, T.; and Asada, K. Antioxidative components in *Giycyrrhihza* species. Paper presented at the Proceedings of International Symposium on Antioxidant Food Supplements in Human Health, Yamagata, October **1997**, 16-18.

Harman, D. Free radical theory of aging, increasing the functional life span. *Annals of the New York Academy of Science* **1994**, *717*, 1–15.

Harris, M. D.; Siegel, L. B.; and Alloway, J. A. Gout and hyperuriccemia. *American Family Physician* **1999**, *59*, 925-934.

Hostettmann, K.; Terreaux, C.; Marston, A.; and Potterat, O. The role of planar chromatography in the rapid screening and isolation of bioactive compounds from medicinal plants. *J. Planar Chromatography.* **1997**, *10*, 251.

Hudson, J. F. *Food Antioxidants*, London, Elsevier Applied Science **1990**.

Inatani, R.; Nakatani, N.; and Fuwa, H. Carcinogenicity of butyllated hydroxyanisole in F344 rat. *J. Natl. Cancer Inst.* **1983**, *70*, 343-347.

Jin, X. Q.; Xu, D. M.; Xu Y. J.; Cui, D. B.; Xiao, Y. W.; Tian, Z. Y.; Lu, Y.; and Zheng Q. T. *Acta Pharm. Sinica* **1993,** *28*, 212-215.

Kaneko, T.; Baba, N.; and Matsuo, M. Protection of coumarins against linoleic acid hydroperoxide-induce cytotoxicity. *Chemico-Biological Interactions* **2003**, *142*, 239-254.

Kitagawa, I.; Shibuya, H.; Baek, N. I.; Yokokawa, Y.; Nitta, A.; Wiriadinata, H.; and Yoshikawa, M. Pulosarioside, A new bitter trimeric-iridoid diglucoside, From an Indonesian Jamu, The bark of *Alyxia reinwardtii*. *Chem.Pharm.Bull*. **1988**, *36*, 4232-4235.

Kweon, M. H.; Hwang, H. J.; and Sung, H. C. Identification and Antioxidant Activity of Novel Chlorogenic Acid Derivatives from Bamboo (*Phyllostachys edulis*). *J. Agric. Food Chem.* **2001**, *49*, 4646-4655.

Lampart-Szczapa, E.; Korczak, J.; Nogala-Kalucka M.; and Zawirska-Wojtasiak R. Antioxidant properties of lupin seed products. *Food Chemistry* **2003**, *83*, 279-285.

Larson, R. A. The antioxidants of higher plants. *Phytochemistry* **1988**, *27(4)*, 969-978.

Lin, L. J.; Lin, L. Z.; Ruangrungsi, N.; and Cordell, G. A. 3-Hydroxycoumarin glycosides from *Alyxia reinwardtii var. lucida*. *Phytochemistry* **1993**, *34*, 825-830.

Lu, H.; and Liu, G-T. Anti-oxidant activity of dibenzocyclooctane lignans isolated from Schisandraceae. *Planta Med.* **1992**, *58*, 311-313.

Ma, C.-M.; Nakamula, N.; Min, B. S.; and Hattori, M. Triterpemes and Lignans from *Artemisia caruifolia* and their cytotoxic effects on Meth-A and LLC tumer cell lines. *Chem. Pharm. Bull.* **2001**, *49(2)*, 183-187.

McCord, J. M. Oxygens-derived free radicals in postischemic tissue injury. *N. Engl. J. Med.* **1985**, *312*, 159-163.

Miyazawa, M.; Kasahara, H.; and Kameoka, H. Phenolic lignans from flower buds of *Magnolia fargesii*. *Phytochemistry* **1992**, *31(10)*, 3666-3668.

Murray, R.D.H.; Mendez, J.; Brown, S.A. Classification of natural coumarins. *The Natural Coumarin*, **1982**, 323-458.

Noguchi, N.; and Niki, E. in Papas, A. M., Antioxidant Status in Human, *Antioxidant Status, Diet Nutrition and Health*. London, **1999**, 1-19.

Okamura, N.; Haraguchi, H.; Hashimoto, K. and Yagi, A. Flavonoids in *Rosmarinus officinalis* leaves. *Phytochemistry* **1994**, *37*, 1463-1466.

Papas, A. M. Determinants of Antioxidant Status in Humans, *Antioxidant Status, Diet Nutrition and Health*. London, **1999**, 1-19.

Phuwapraisirisan, P. Chemical constituent from the stems of *Arfeuillea arborescens* Pierre. And their biological activity. *Master's Thesis* **1998**, 64-80.

Pokorny, J. Antioxidants in food preservative: in *Handbook of Food Preservation*, Shafiur, R. M. (ed.), New York, Marcel Dekker, **1990**, 309-337.

Porras, A. G.; Olson, J. S.; and Palmer, G. The reaction of reduced xanthine oxidase with oxygen. Kinetics of peroxide and superoxide formation. *J. Biol. Chem.* **1981**, *256*, 9096-9103.

Porter, N. A.; Caldwell, S. E.; and Mills, K. A. Mechanisms of free radical oxidation of unsaturated lipids. *Lipids* **1995**, *30(4)*, 277-290.

Reis, A.; Domingues, M. R. M.; Amado, F. M. L.; Ferre-Correia, A. J. V.; and Domingues, P. Detection and Characterization by Mass Spectrometry of Radical Adducts Produced by Linoleic Acid Oxidation. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 1250-1261.

Rice-Evans, C.; and Burdon, R., Free radical-Lipid Interactions and Their Pathological Consequences. *Prog. Lipid Res.* **1993**, *32*, 71-110.

Riveiro, M. E.; Shayo, C.; Monczor, F.; Fernandez. N.; Baldi, A.; Kimpe, N. D.; Rossi, J.; Debenededetti, S.; and Davio, C. Induction of cell differentiation in human leukemia U-937 cells by 5-oxygenated-6, 7-methylenedioxycoumarins from *Pterocaulon polystachyum*. *Cancer Lett.* **2004**, *210*, 179-188.

Sadavongvivad, C.; and Supavilai, P. Three monohydroxycoumarins from *Alyxia lucida. Phytochemistry* **1977**, *16*, 1451.

Santisuk, T.; and Larsen, K. *Flora of Thailand* **1999**, *7*, 55-57.

Spiteller, G. Lipid peroxidation in aging and age-dependent diseases. *Experimental Gerontology* **2001**, *36*, 1425-1457.

Thompson, D.; and Moldeus, P. Citotoxicity of butylated hydroxyanisole and butylated hydroxytoluene in isolated rat hepatocytes. *Biochem. armacol.* **1988**, *37*, 2201-2207.

Topcu, G.; Che, C.-T.; Cordell, G. A.; and Ruangrungsi, N. Iridolactones from *Alyxia reinwardtii. Phytochemistry* **1990**, *29*, 3197-3199.

Tropp, B. E. Metabolism and Biogenetic. *Biochemistry: concepts and applications*, New York, **1997**.

Unno, T.; Sugimoto, A.; and Kakuda T. Xanthine oxidase inhibitors from the leaves of *Lagerstroemia speciosa* (L.) Pers. *Journal of Ethnopharmacology* **2004**, *93*, 391-395.

Valenzuela, A. B.; and Nieto, S. K. Synthetic and natural antioxidants: food quality protectors. *Grasasy Aceites*, **1996**, *47*, 186-196.

Warma, S. D.; Devamanoharan, P. S.; and Morris, S. M. Prevention of cataracts by nutritional and metabolic antioxidants. *Crit. Rev. Food Sci. Nutr.* **1995**, *35*, 111-129.

Wijewickreme, A. N.; Krejpcio, Z.; and Kitts, D. D. Hydroxy scavenging activity of glucose fructose, and ribose-lysine model maillard products. *Journal of Food Science* **1999**, *64*, 457-461.

Yen, G. C.; and Hsieh, C. L. Antioxidant effects of dopamine and related compounds. *Bioscience, Biotechnology and Biochemistry* **1997**, *61*, 1646-1649.

Zhang, H. Y.; and Wang, L. F. Theoretical elucidation of structure-activity relationship for coumarins to scavenge peroxyl radical. *Theochem* **2004**, *(673)*, 199-202.

Zhu, Y. Z.; Huang, S.H.; Tan, B.K.H.; Sun, J.; Whiteman, M.; and Zhu, Y.-C., Antioxidant in Chinese herbal medicines: a biochemical perspective. *Nat. Prod. Rep.* **2004**, *21*, 478-489.

APPENDICES

Appendix

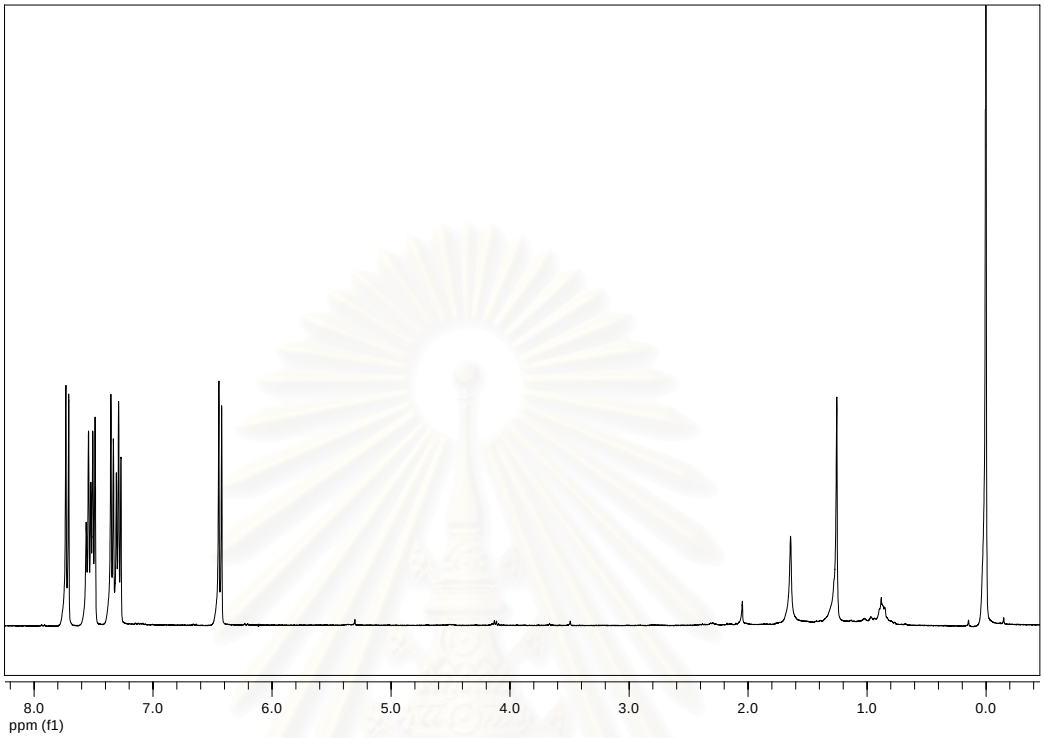


Figure 1 The ^{1}H NMR spectrum ($CDCl_3$) of compound **1**

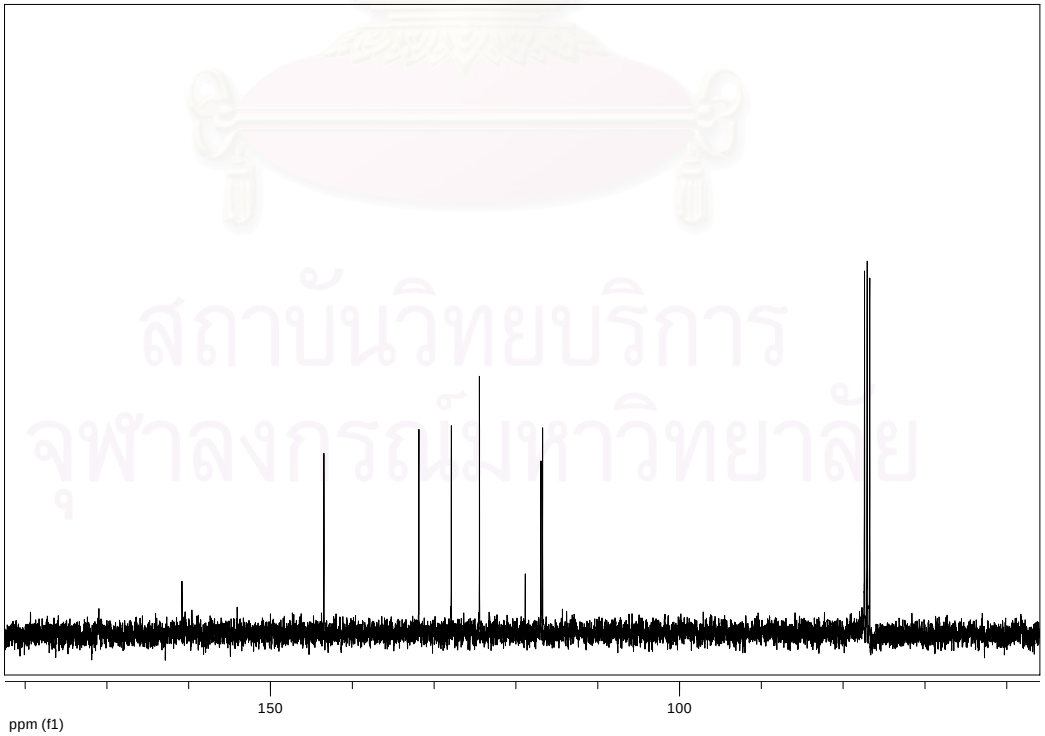


Figure 2 The ^{13}C NMR spectrum ($CDCl_3$) of compound **1**

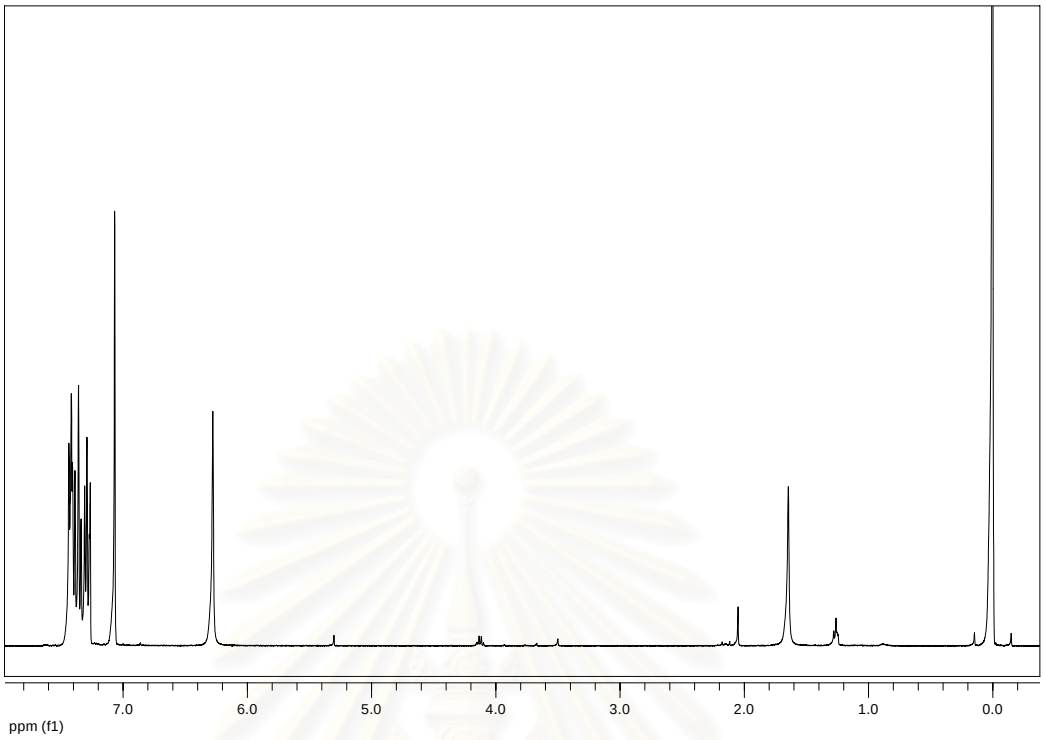


Figure 3 The ^{1}H NMR spectrum ($CDCl_3$) of compound **2**

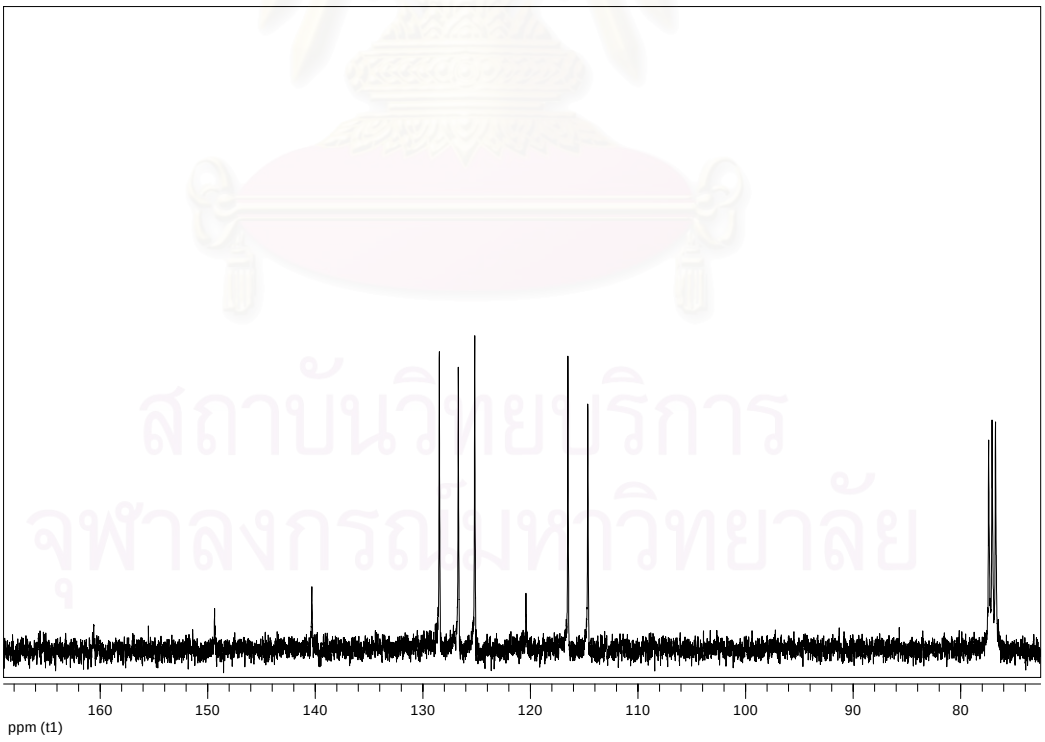


Figure 4 The ^{13}C NMR spectrum ($CDCl_3$) of compound **2**

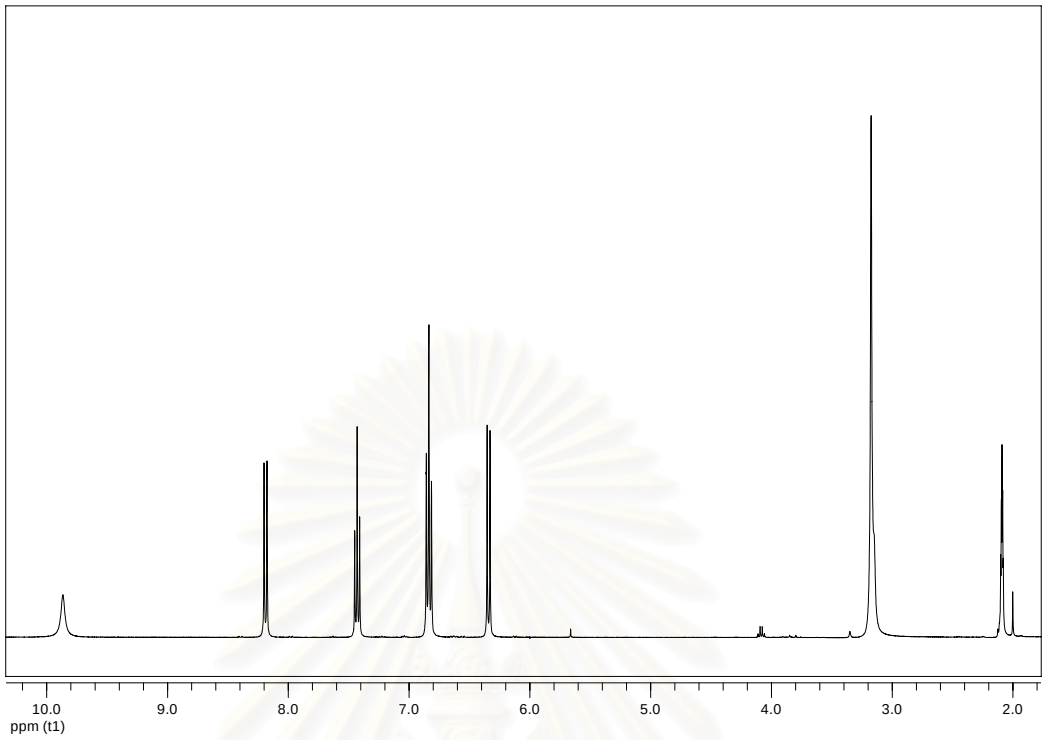


Figure 5 The ^{1}H NMR spectrum (CD_3COCD_3) of compound **3**

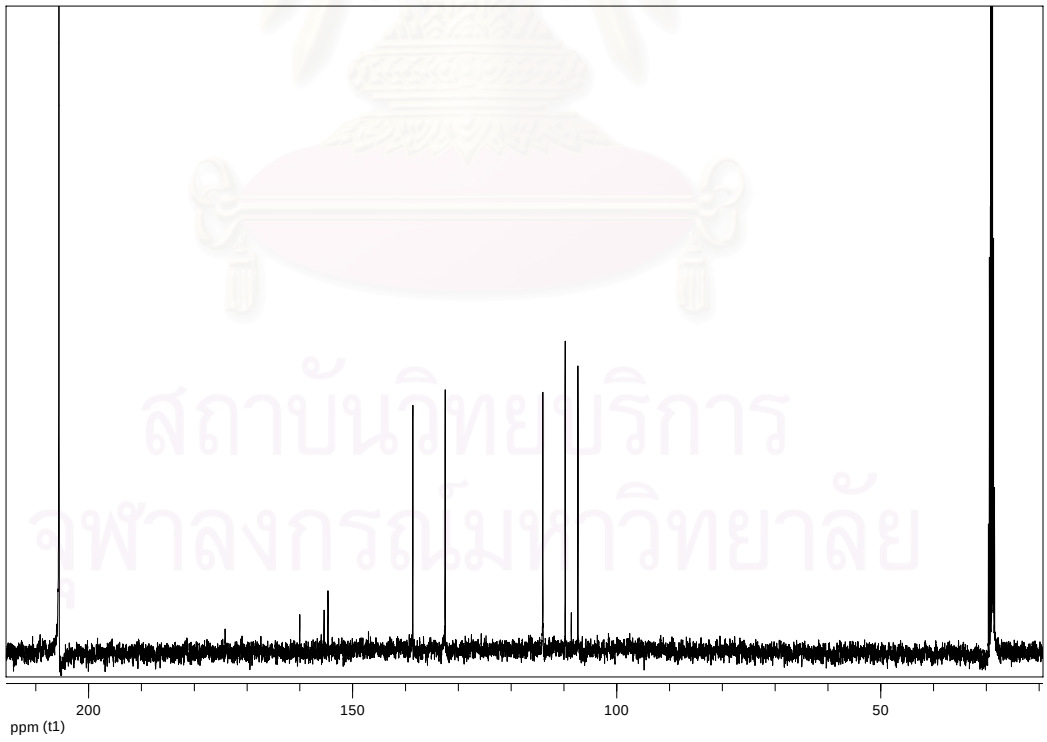


Figure 6 The ^{13}C NMR spectrum (CD_3COCD_3) of compound **3**

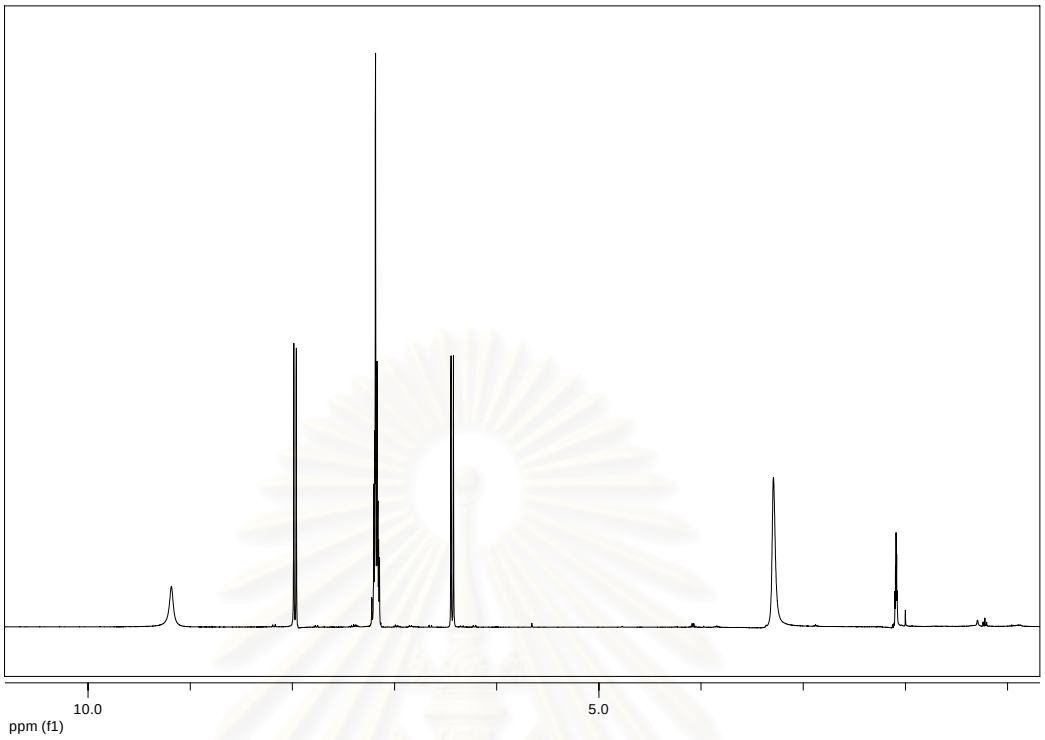


Figure 7 The ^{1}H NMR spectrum (CD_3COCD_3) of compound **4**

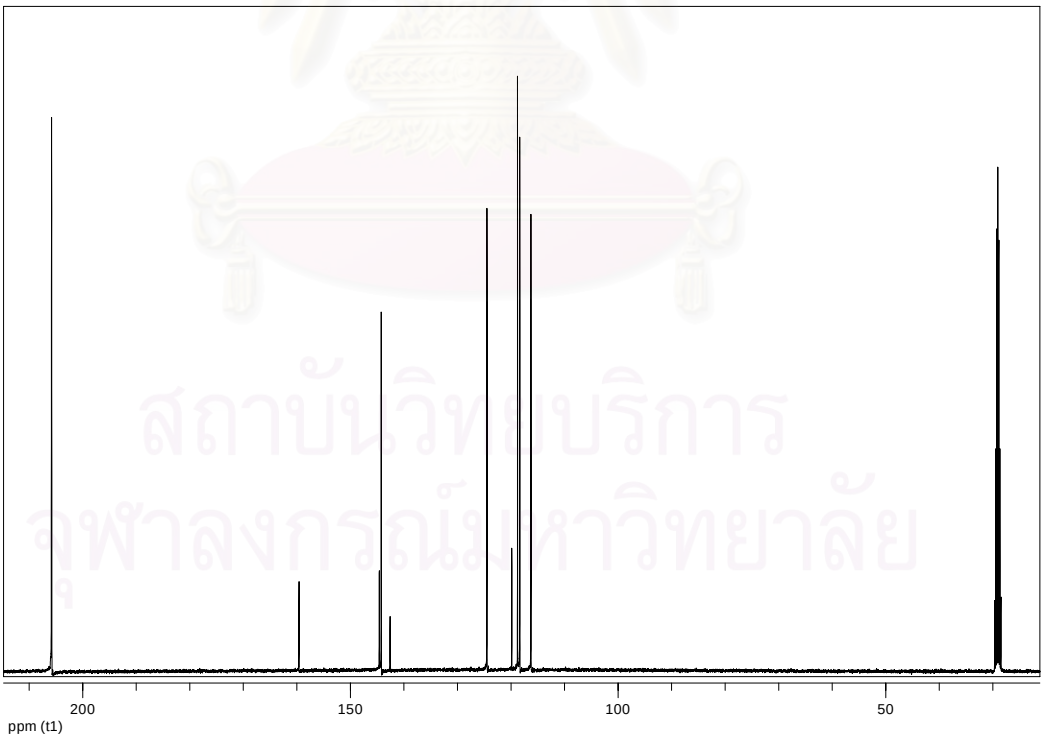


Figure 8 The ^{13}C NMR spectrum (CD_3COCD_3) of compound **4**

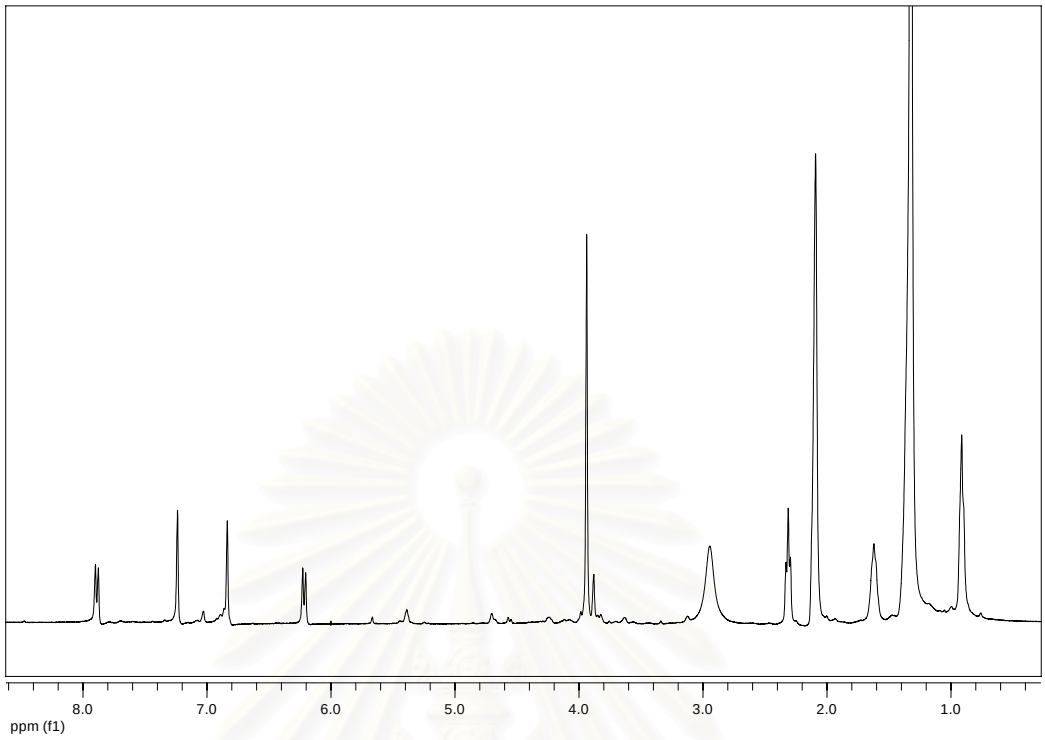


Figure 9 The ^{1}H NMR spectrum (CD_3COCD_3) of compound **5**

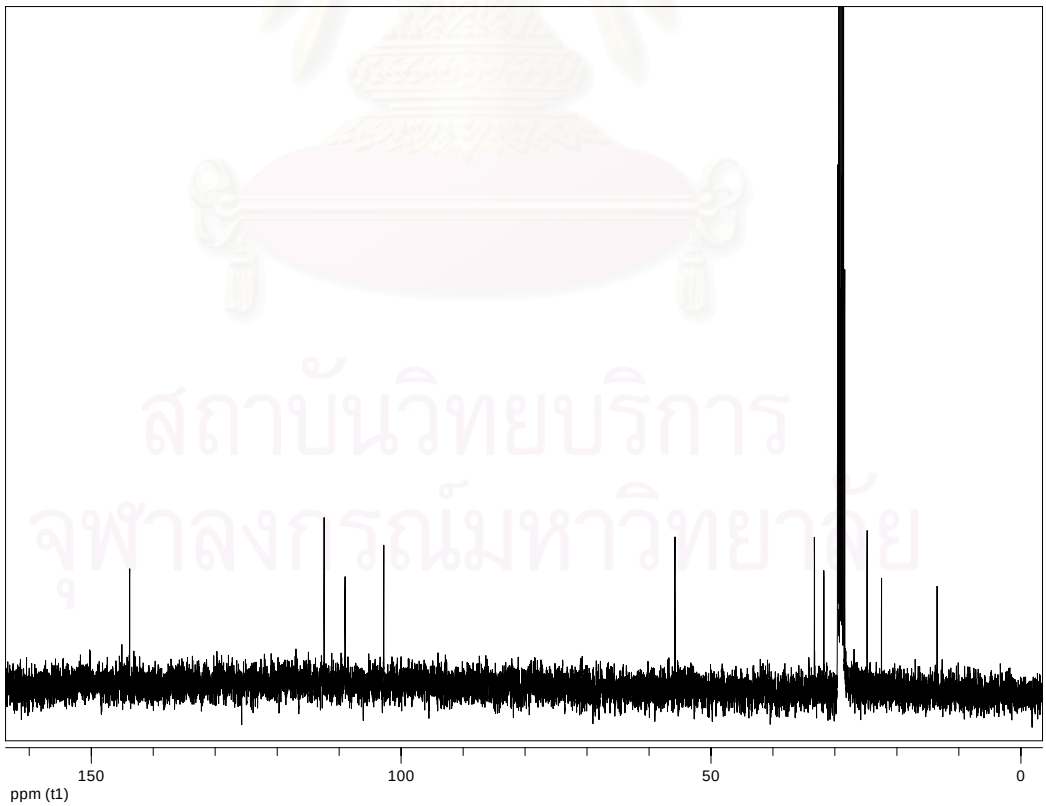


Figure 10 The ^{13}C NMR spectrum (CD_3COCD_3)of compound **5**

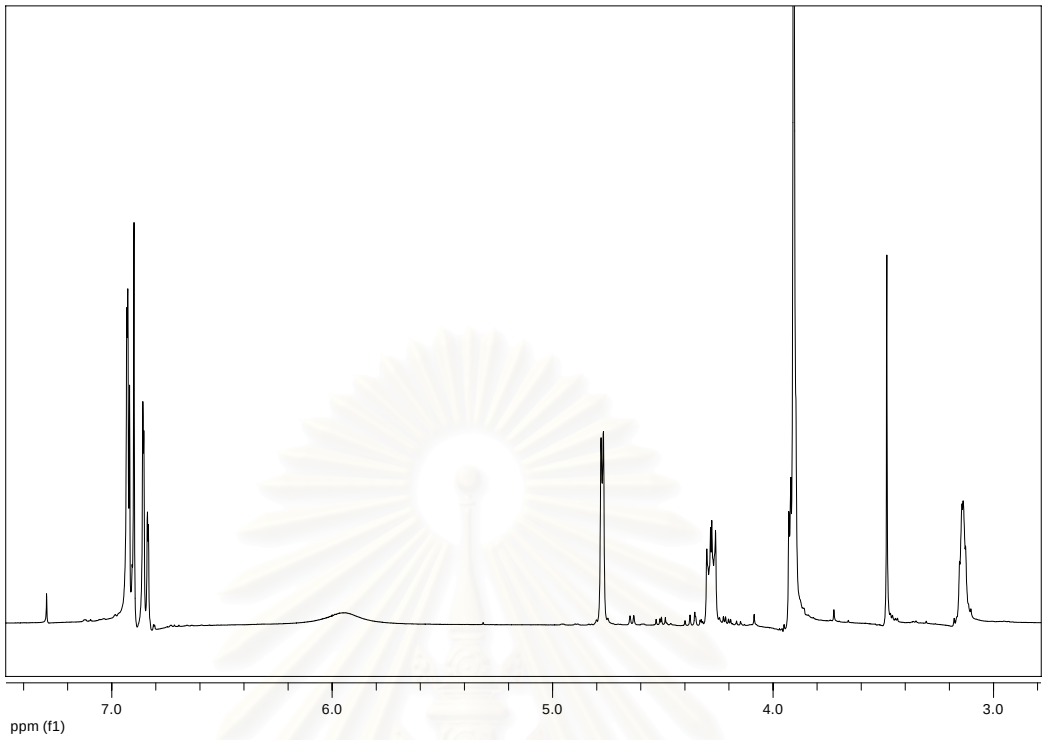


Figure 11 The ^{1}H NMR spectrum ($CDCl_3$) of compound **6**

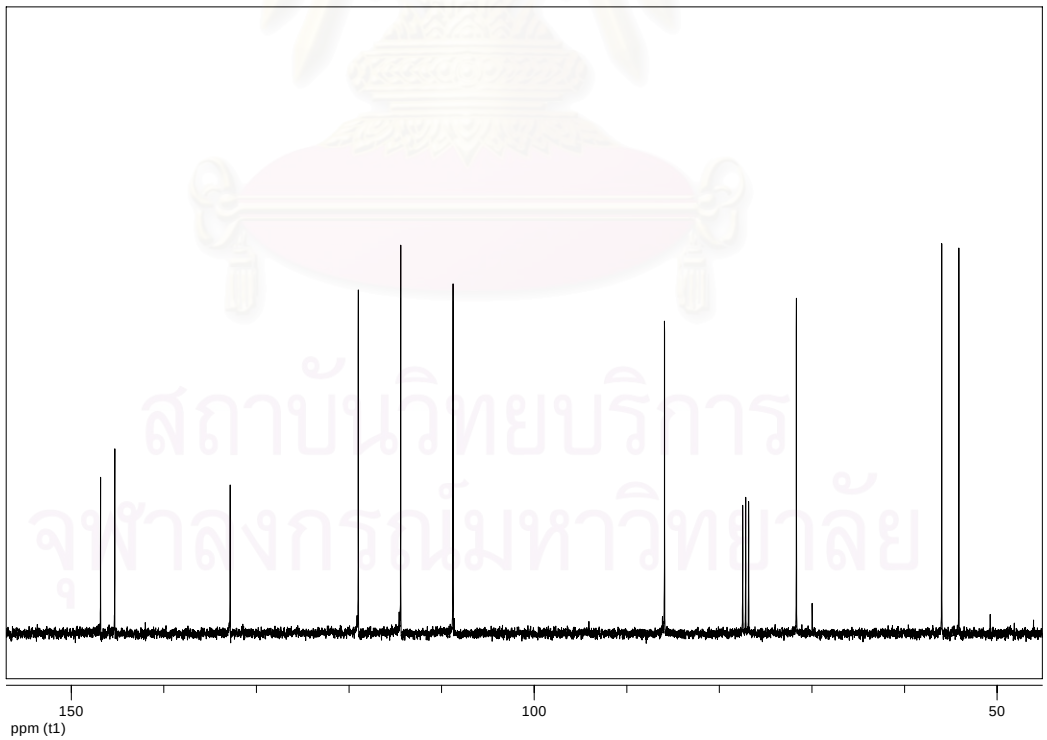


Figure 12 The ^{13}C NMR spectrum ($CDCl_3$) of compound **6**

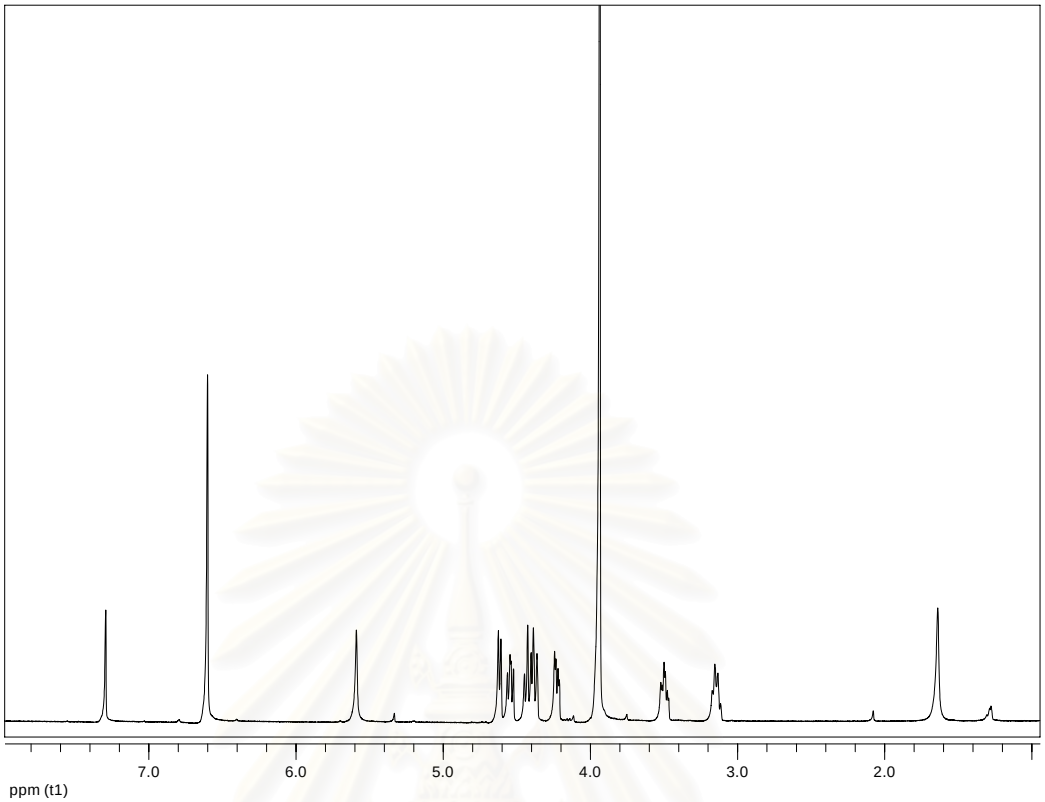


Figure 13 The ^{1}H NMR spectrum ($CDCl_3$) of compound **7**

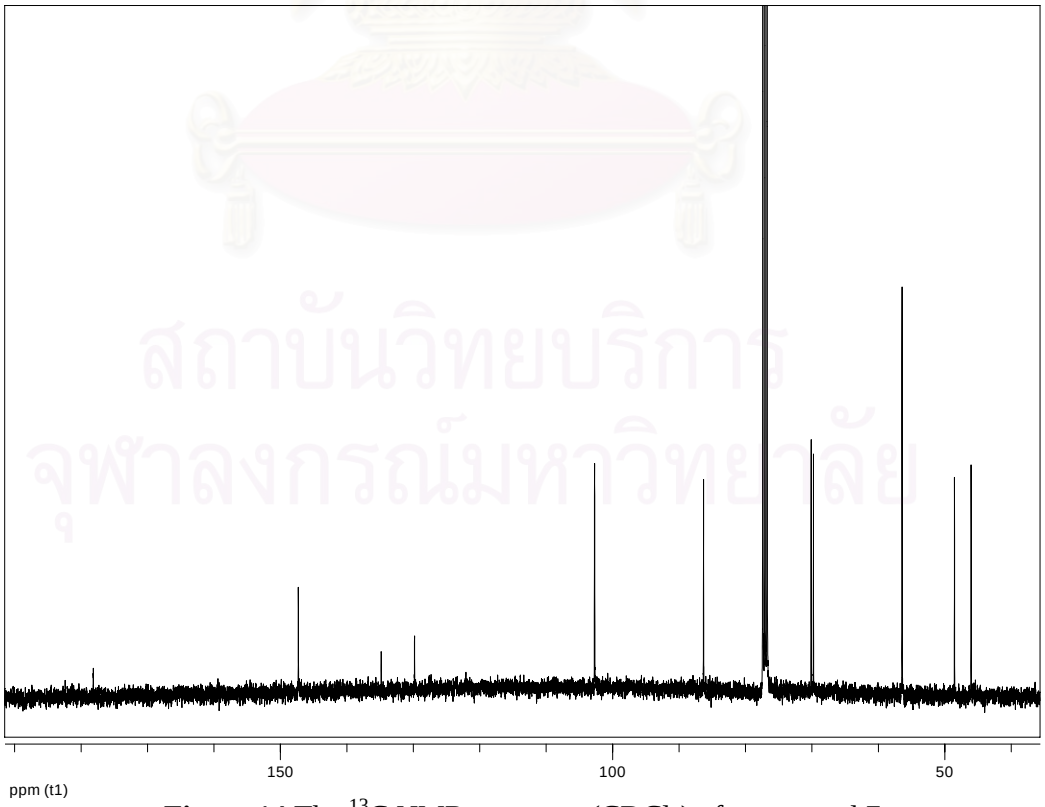


Figure 14 The ^{13}C NMR spectrum ($CDCl_3$) of compound **7**

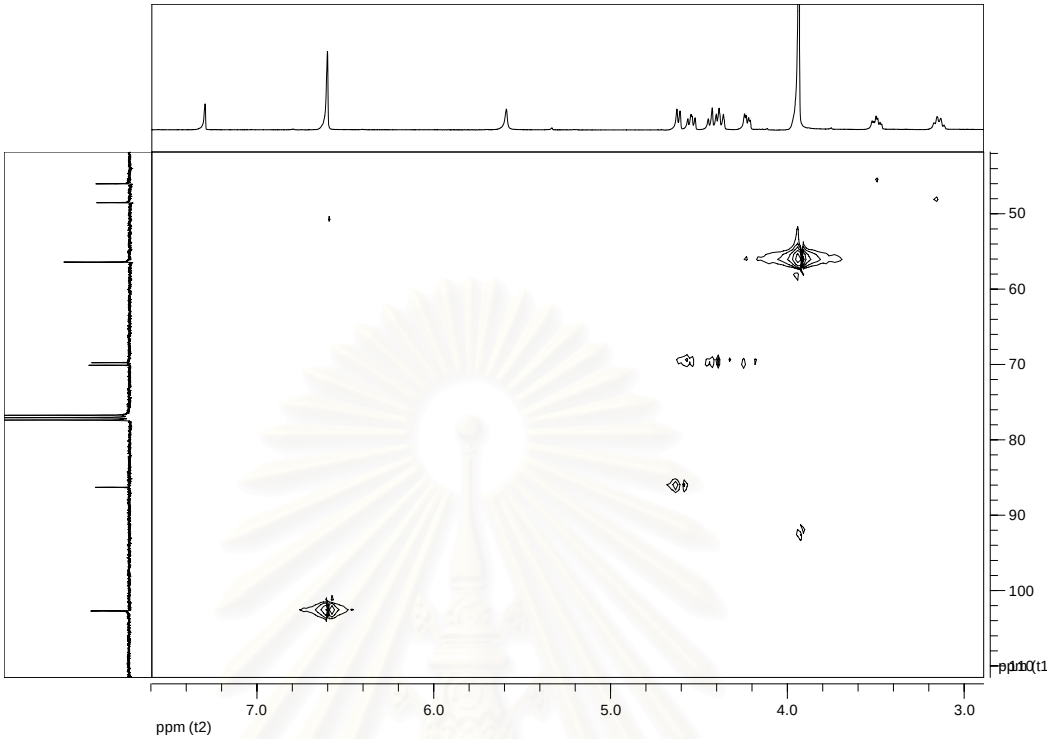


Figure 15 The HMQC spectrum ($CDCl_3$) of compound **7**

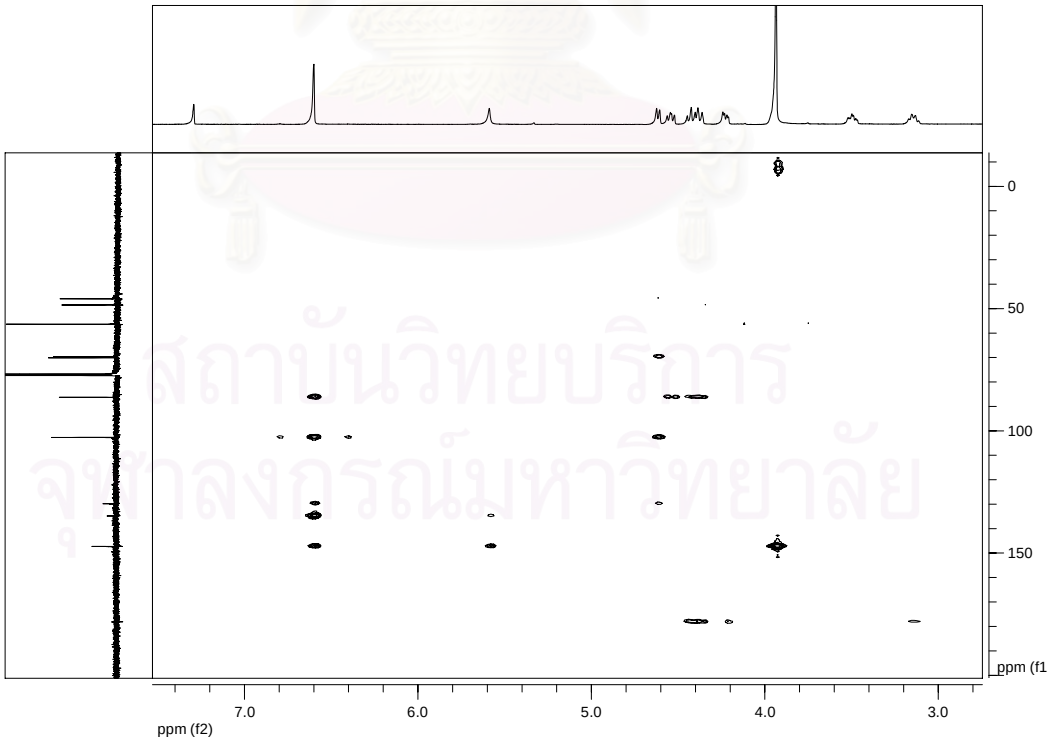


Figure 16 The HMBC spectrum ($CDCl_3$) of compound **7**

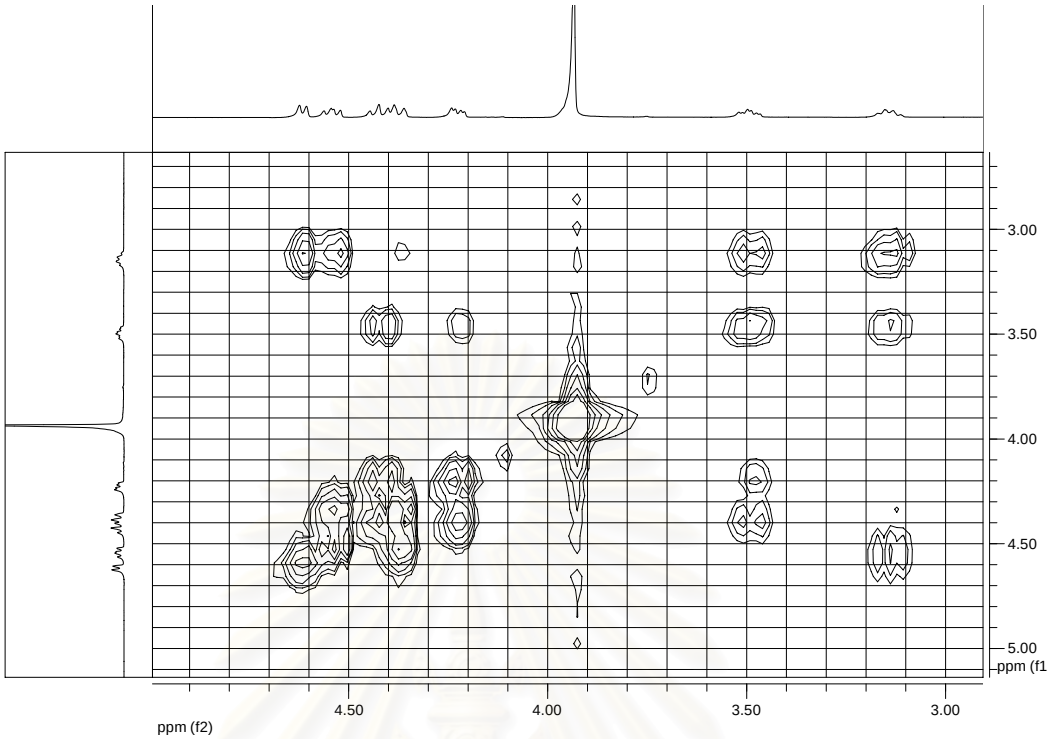


Figure 17 The COSY spectrum ($CDCl_3$) of compound **7**

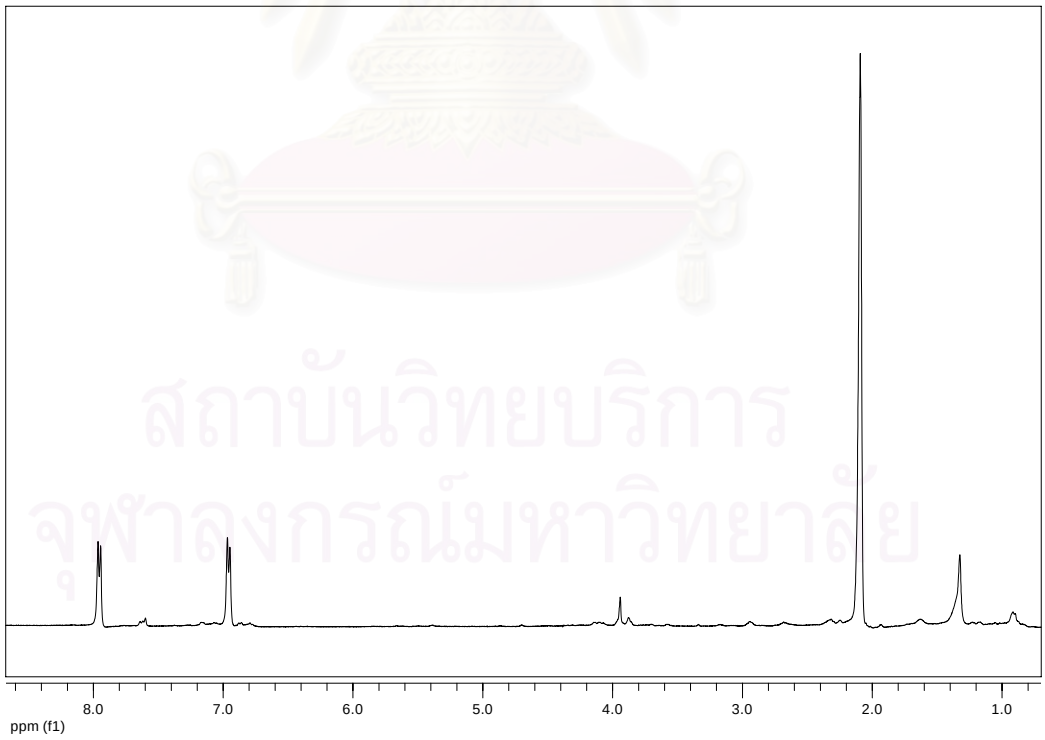


Figure 18 The ^1H NMR spectrum (CD_3COCD_3) of compound **8**

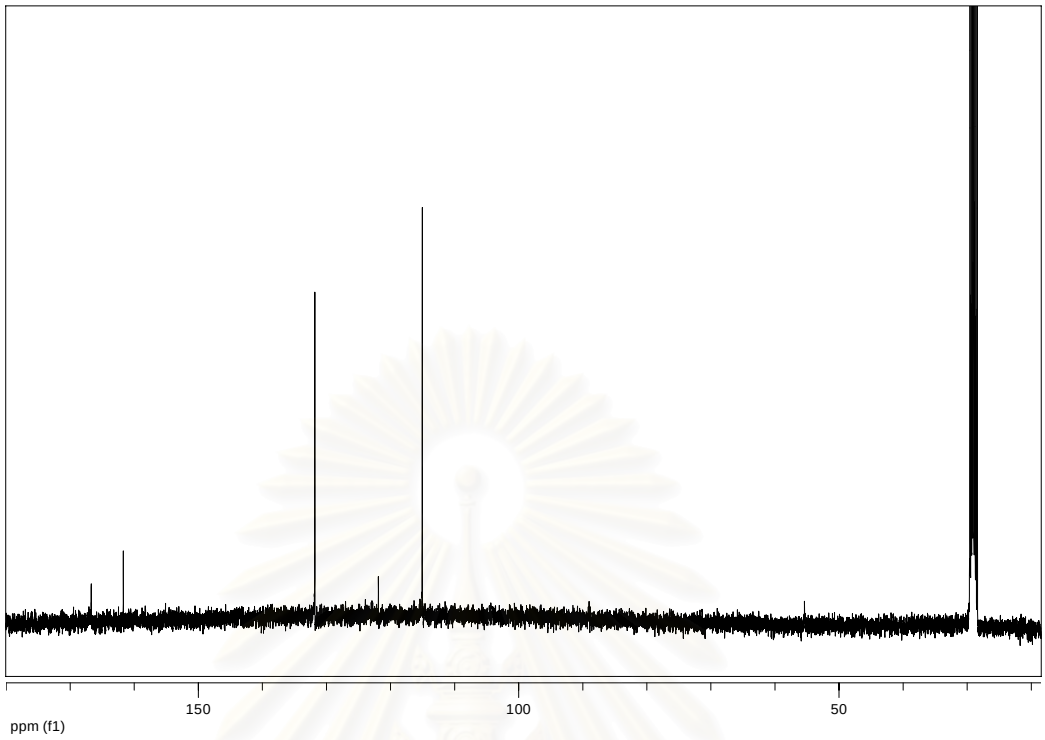


Figure 19 The ^{13}C NMR spectrum (CD_3COCD_3) of compound **8**

VITA

Miss Jurairat Rattanapan was born on September 16, 1980 in Ayutthaya, Thailand. She graduated with Bachelor Degree of Science in Agro-Industrial of Technology from King Mongkut Institued Technology of North Bangkok, Bangkok, Thailand in 2001. During she was studying in Master Degree program, she received financial support from Graduate School Chulalongkorn University.