ฤทธิ์ต้านออกซิเดชันของแซนโทนจากลำต้นติ้วเกลี้ยง *Cratoxylum cochinchinense*

นาย สุธี อุดมโชติพฤทธิ์

สถาบันวิทยบริการ
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ANTIOXIDANT ACTIVITY OF XANTHONES FROM STEMS OF *Cratoxylum cochinchinense*

Mr. Sutee Udomchotphruet

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จุฬาลงกรณ์มหาวิทยาลัย

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สุธี อุดมโชติพฤทธิ์: ฤทธิ์ต้านออกซิเดชันของแซนโทนจากลำต้นติ้วเกลี้ยง *Cratoxylum cochinchinense* (ANTIOXIDANTS ACTIVITY OF XANTHONES FROM STEMS OF *Cratoxylum cochinchinense*) อ. ที่ปรึกษา: รศ.ดร. สันติ ทิพยางค์ อ. ที่ปรึกษาร่วม: ผศ.ดร. ปรีชา ภูวไพรศิริศาล, 78 หน้า.

ในการเสาะหาสารออกฤทธิ์ต้านการเกิดออกซิเดชันของสมุนไพรไทย พืชในวงศ์ Guttifereae ถูกนำมาทดสอบฤทธิ์เบื้องต้นในการต้านอนุมูลอิสระ 2,2-Diphenyl-1-picryhydrazyl (DPPH) ซึ่งเป็นอนุมูลอิสระที่เสถียร พบว่าสิ่งสกัดเฮกเซนและไดคลอโรมีเทนของลำต้นติ้วเกลี้ยงแสดงฤทธิ์ที่ดีจึงนำสิ่งสกัดทั้งสองมาแยกด้วยวิธีทางโครมาโทกราฟี สามารถแยกแซนโทนใหม่ได้ 6 ชนิด คือ cratoxylumxanthones A-F (**1**, **9-11**, **13-14**) พร้อมกับแซนโทนมีรายงานมาแล้ว 8 ชนิด คือ dulcisxanthone B (**2**), 2-geranyl-1,3,7-trihydroxy-4-(3-methyl-but-2-enyl)xanthone (**3**), α-mangostin (**4**), β-mangostin (**5**), cochinchinone A (**6**) garcinone A (**7**), cochinchinone B (**8**) และ cudratricusxanthone E (**12**) การพิสูจน์ทราบโครงสร้างของแซนโทนที่แยกได้ทั้งหมดด้วยวิธีการทางสเปกโทรสโกปีและการเปรียบเทียบกับข้อมูลที่มีรายงานไว้แล้ว การศึกษาความสามารถต้านการเกิดออกซิเดชันได้ใช้ 3 วิธี คือ การทดสอบฤทธิ์ต้านอนุมูลอิสระ DPPH การทดสอบการยับยั้งการเกิดออกซิเดชันในไขมันและการทดสอบฤทธิ์เกี่ยวเนื่องกับเอนไซม์ xanthine oxidase (ฤทธิ์ต้านอนุมูลอิสระ superoxide และฤทธิ์ยับยั้งการทำงานของเอนไซม์ xanthine oxidase) โดยการเปรียบเทียบกับ ascorbic acid, curcumin, gallic acid และ allopurinol แซนโทนใหม่ 3 ชนิด และแซนโทนที่มีรายงานมาแล้ว 3 ชนิด แสดงฤทธิ์ที่ดีในการต้านอนุมูลอิสระ DPPH และการยับยั้งการเกิดออกซิเดชันในไขมัน โดยเฉพาะสาร **10** (IC_{50} = 0.030 และ 0.013 mM) สาร **11** (IC_{50} = 0.100 และ 0.180 mM) สาร **8** (IC_{50} = 0.120 และ 0.130 mM) สาร **13** (IC_{50} = 0.120 และ 0.190 mM) สาร **2** (IC_{50} = 0.140 และ 0.040 mM) และสาร **12** (IC_{50} = 0.190 และ 0.030 mM) ตามลำดับ อย่างไรก็ตาม แซนโทนทั้งหมดไม่แสดงฤทธิ์ต้านอนุมูลอิสระ superoxide และฤทธิ์ยับยั้งการทำงานของเอนไซม์ xanthine oxidase

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

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SUTEE UDOMCHOTPHRUET: ANTIOXIDANTS ACTIVITY OF XANTHONES FROM STEMS OF *Cratoxylum cochinchinense* THESIS ADVISOR: ASSOC.PROF. SANTI TIP-PYANG, Ph.D. THESIS CO-ADVISOR: ASST.PROF. PREECHA PHUWAPRAISIRISAN, Ph.D., 78 pp.

In a search for antioxidant from Thai medicinal herbs, plants in the family Guttifereae were preliminary evaluated using TLC autographic assay with 2,2-Diphenyl-1-picryhydrazyl (DPPH) as a stable radical. The hexane and dichloromethane crude extracts of *Cratoxylum cochinchinense* were found to have a promising activity. The chromatographic separation of these crude extracts led to the isolation of six new xanthones, cratoxylumxanthone A-F (**1**, **9-11**, **13-14**), along with eight know xanthones, dulcisxanthone B (**2**), 2-geranyl-1,3,7-trihydroxy-4-(3-methyl-but-2-enyl)xanthone (**3**), α-mangostin (**4**), β-mangostin (**5**), cochinchinone A (**6**), garcinone A (**7**), cochinchinone B (**8**) and cudratricusxanthone E (**12**). The structures of all isolated xanthones were characterized by spectroscopic methods as well as comparison with the previous literature data. The antioxidant capability was determined using three complementary *in vitro* assays, which included the DPPH radical scavenging activity, lipid peroxidation inhibition and xanthine oxidase related activity (superoxide radicals scavenging activity and inhibitory of xanthine oxidase), by comparison with ascorbic acid, curcumin, gallic acid and allopurinol. The three new and three known of isolated xanthones exhibited significant in both DPPH radical scavenging and lipid peroxidation, particularly compound **10** (IC_{50} = 0.030 and 0.013 mM), followed by compound **11** (IC_{50} = 0.100 and 0.180 mM), **8** (IC_{50} = 0.120 and 0.130 mM), **13** (IC_{50} = 0.120 and 0.190 mM), **2** (IC_{50} = 0.140 and 0.040 mM) and **12** (IC_{50} = 0.190 and 0.030 mM), respectively. On the other hand, all isolated xanthones had no effect on superoxide scavenging activity and inhibition of xanthine oxidase activity.

Field of study...... Biotechnology.......Student's signature...Sutee Udomchotphruet...

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List of Abbreviations

$[M+H]^+$	molecular ion plus hydrogen
$[M-H]^-$	molecular ion minus hydrogen
$[M+Na]^+$	molecular ion plus sodium
^{13}C NMR	carbon 13 nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
A	absorbance
ATP	adenosine triphosphate
AMP	adenosine monophosphate
ºC	degree celsius
$CDCl_3$	deuterated chloroform
CH_2Cl_2	dichloromethane, methylene chloride
COSY	homonuclear correlated spectroscopy
d	doublet (NMR)
dd	doublet of doublet (NMR)
δ	chemical shift
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryhydrazyl
ESIMS	electrospray ionization mass spectrometry
EtOAc	ethyl acetate
g	gram
H_2SO_4	sulfuric acid
HRESIMS	high resolution electrospray ionization mass spectrometry
HMBC	heteronuclear multiple bond correlation
HSQC	heteronuclear single quantum correlation
Hz	Hertz
IC_{50}	inhibition concentration at 50 %
J	coupling constsnt
kg	kilogram
L	liter
λ_{max}	maximum wavelength
m	meter

m	multiplet (NMR)
M	molar
mp	melting point
m/z	mass to charge ratio
M^+	molecular ion
MeOH	methanol
mg	milligram
MHz	megahertz
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
µM	micromolar
MS	mass spectrometry
NADP	nicotinamide adenosine dinucleotide phosphate
DADPH	nicotinamide adenosine dinucleotide phosphate hydrogen
NBT	nitroblue tetrazolium
nm	nanometer
NMR	nuclear magnetic resonance
ppm	part per million
rpm	round per minute
ROS	reactive oxygen species
s	singlet (NMR)
SDS	sodium dodecyl sulfate
SiO_2	silica gel
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultraviolet
XOD	xanthine oxidase

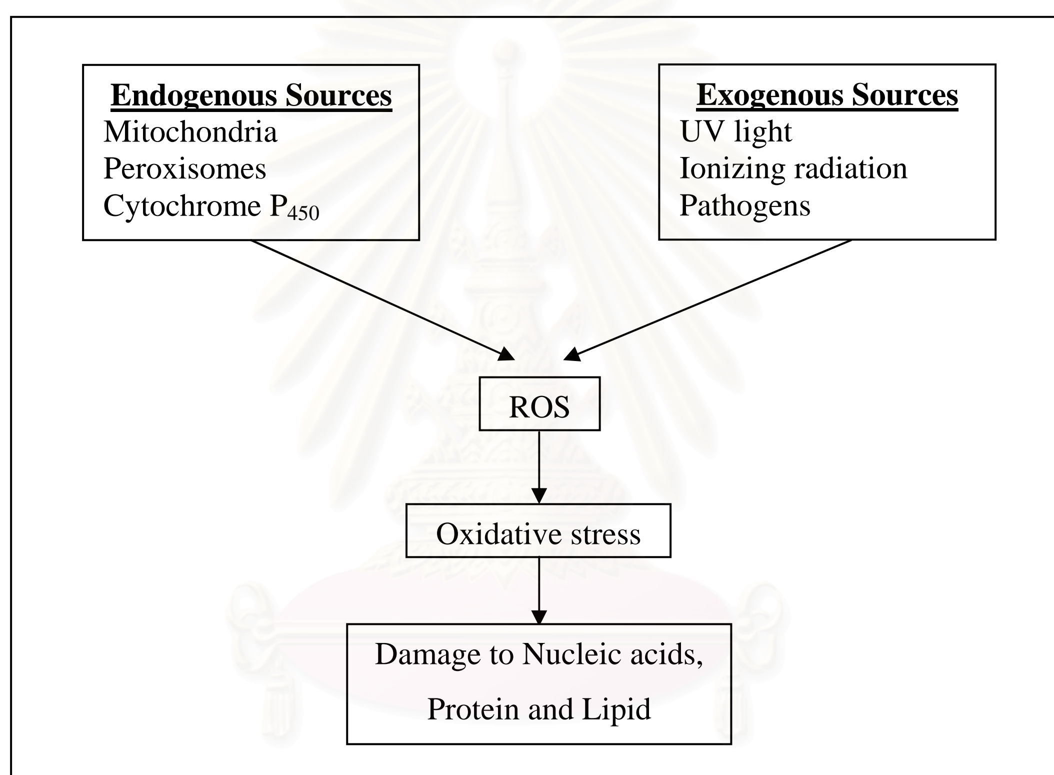
CHAPTER I

INTRODUCTION

It is now commonly recognized that free radicals are involved in a variety of physiological and pathological processes, including cellular signal transduction, cell proliferation and differentiation, apoptosis as well as ischemia reperfusion injury, inflammation and many degenerative disease (Halliwell and Gutteridge, 1999; Sen and Packer, 1996; Kroemer *et al*., 1995; Abuja and Albertini, 2001). Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules. There are numerous types of free radicals that can be formed within the body. Any free radicals involving oxygen can be referred to as “reactive oxygen species” (ROS). The most common ROS include: superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$) and nitric oxide radical ($NO^{\bullet}$) (Franke *et al*., 2004; Zou *et al*., 2004b). ROS can cause tissue damage by reacting with lipids in cellular membranes, nucleotides in DNA, sulphydryl groups in proteins and cross-link/fragmentation of ribonucleoproteins (Waris and Ahsan, 2006). Especially, ROS mediated lipid peroxidation and DNA damage is associated with a variety of chronic health problems, such as cancer, aging and arteriosclerosis. The ROS are generated by two ways: from the body and the environment (**Scheme 1.1**).

In the body, the compartmentalization of phagocytic microbicide of macrophage within the phagosome is one example which gives products as superoxide and nitric oxide radical ($NO^{\bullet}$). Another is provided by the mechanism of energy (ATP) generation by oxidative phosphorylation such as mitochondrial respiration, which release superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$). Not only is this mechanism compartmentalized within the mitochondria, it is effected by each of the component molecules (cytochromes P_{450} enzymes) being arrayed on the mitochondrial membrane, directly adjacent to one another to form the respiratory chain.

The environment is the sum of numerous influences on organic life, some of which are capable of promoting or generating oxidative stress. Such influences may be of natural origin but artificial, anthropogenic sources are gaining more and more importance. Their physical and chemical properties determine their mode of action and their degree of risk. The following environmental factors are known sources of environmental oxidative stress: industrial pollution, traffic exhaust, cigarette smoke, nutrition, radiotherapy, cosmetic devices and solar radiation (Schröder and Krutmann, 2005).



Scheme 1.1 Pathways illustrating the sources of reactive oxygen species (Waris and Ahsan, 2006)

Normally, free radicals are also an important component of the body's defense systems, which created in metabolism process during times of increased oxygen flux. Under normal conditions the antioxidant defense system within the body can easily handle free radicals that are produced. The changes wrought on adjacent molecular targets of free radicals can vary in magnitude, but because many of the components of the living cell are particularly susceptible to free radical injury, the molecular chain reactions can have substantial effects on the

structure and function of living tissue. As a consequence, natural selection has driven the evolution of a number of intracellular defense mechanisms to neutralize or control the potentially destructive reactivity of ROS. These include molecules that react preferentially with ROS without passing that reactivity along. Some of these are simple molecules like vitamins C and E, while some are enzymes like superoxide dismutase, glutathione peroxidase and catalase, which catalyze such electron quenching reactions (**Table 1.1**). Although there are several enzyme systems within the body that scavenging free radicals. Additionally, selenium, a trace metal that is required for proper function of body's antioxidant system. However, exogenous antioxidant are essential for diminishing the cumulative effects of oxidative damage over the life span (Pietta, 2000). They include natural and phytochemicals from plants with antioxidant activity and synthetic (Papas, 1999).

Table 1.1 Reactive oxygen species and relevant antioxidants (Mello, 2007)

ROS	Nonenzyme antioxidants (LMWA)	Enzymatic antioxidants
Singlet O_2	NADPH and NADH	Catalase (CAT)[a]
$O_2^{\bullet -}$ (superoxide radical)	Glutathione (GSH) and thiols	Glutathione peroxidase (GSH-Px)[a]
H_2O_2 (hydrogen peroxide)	Ubiquinol (coenzyme Q)	Superoxide dimutase (SOD)[a]
$^{\bullet}OH$ (hydroxyl radical)	Uric acid	Ceruloplasmin (Cu)[b]
$NO^{\bullet}$ (nitric oxide)	Carotenoids (most commonly *β*-carotein)[c]	Albumin (Cu)[b]
Lipid peroxide	Vitamin C (ascorbic acid)[c]	Transferin (Fe)[b]
$RO^{\bullet}$ (alkoxyl radical)	Vitamin E (tocopherols)[c]	Ferrintin (Fe)[b]
$ROO^{\bullet}$ (peroxyl radical)	Phytochemicals[c]	Myoglobin (Fe)[b]

[a] Free radical scavenging enzyme.

[b] Metal binding protein.

[c] Dietary antioxidants.

The synthetic antioxidants (**Figure 1.1**), such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and dodecyl gallate (DG) are the antioxidant most frequently used as additives in lipid containing food (Primo, 1997; Delgado-Zamarreno *et al.*, 2007). Therefore, the importance of search for natural antioxidants, especially of plant origin has greatly increased in recent years (Jayaprakasha and Jaganmohan, 2000).

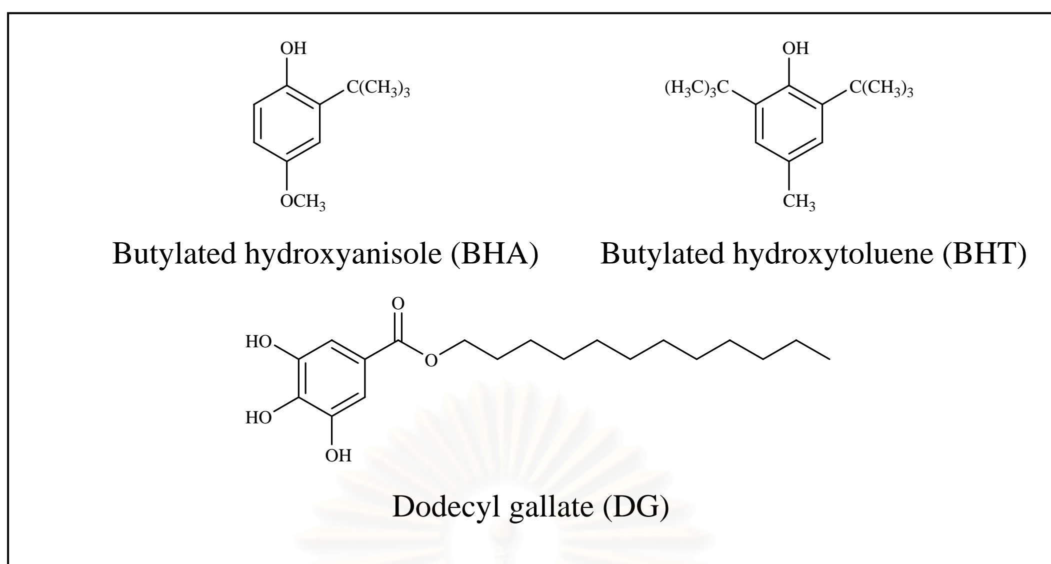


Figure 1.1 Synthetic antioxidants

Many mutagens and carcinogens may act through the generation of ROS. The role of ROS in various human diseases is becoming increasingly recognized (Halliwell *et al*., 1992; Martinez-Cayuela, 1995). ROS may also play a major role as endogenous initiators of degenerative processes, such as DNA damage and mutation (promotion) that may be related to cancer, heart disease and aging (Ames, 1993). Beside the endogenous defenses, plants constituent are an important source of active natural products which differ widely in term of structure and biological propertied. They have played a remarkable role in the traditional medicine of various countries. The prevention of cancer and cardiovascular disease has been associated with the ingestion of fresh fruit, vegetables or teas rich in natural antioxidant (Virgili *et al*., 2001; Johnson, 2001). The protective effects of plant products are due to the presence of several components which have distinct mechanism of action; some are enzymes and proteins and others such as vitamins (Halliwell, 1996; Head, 1998), carotenoids (Edge *et al*., 1997), flavonoids (Zhang and Wang, 2002), anthocyanins, *α*-tocopherol, L-ascorbic acid and other phenolic compounds (Sanchez-Moreno *et al*, 1998; Tadhani, 2007) (**Figure 1.2**).

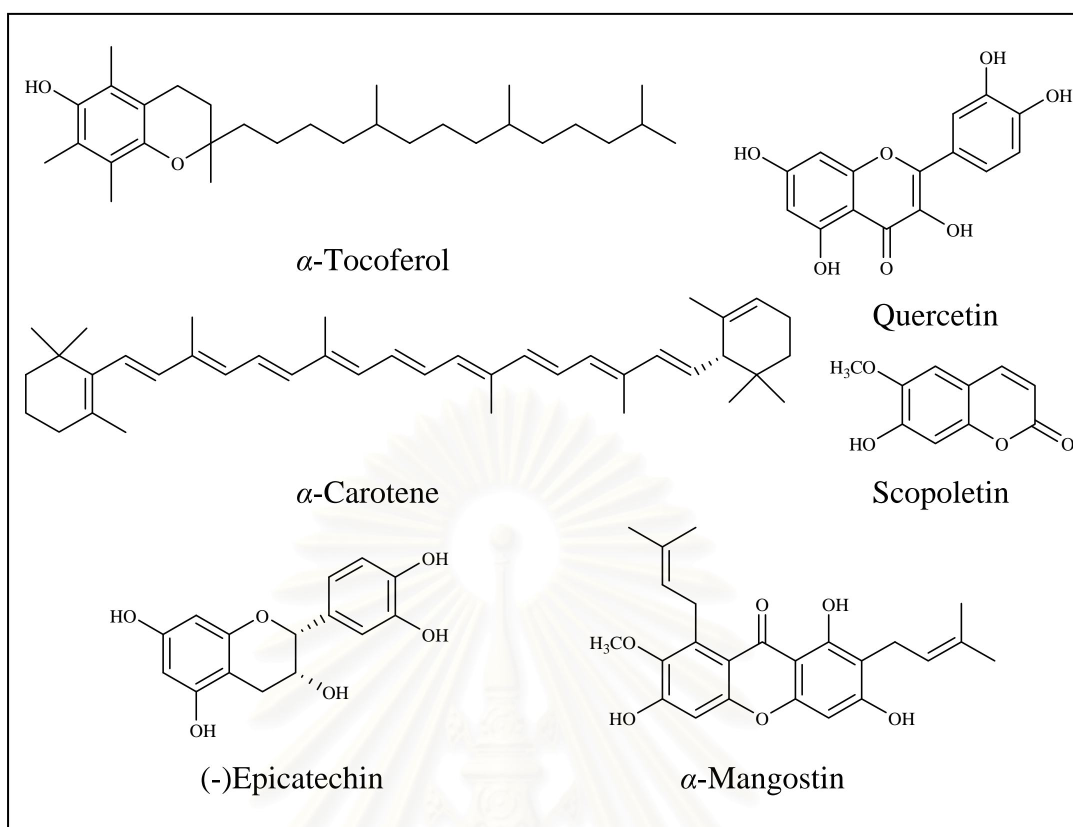


Figure 1.2 Natural antioxidants

The medicinal propertied of plants have been investigated, in the light of recent scientific developments, through out the world due to their potent pharmacological activities and economic viability. A great number of aromatic, spicy, medicinal and other plants contain chemical compounds, exhibiting antioxidant properties. Source of natural antioxidants are primarily, plant phenolics that may occur in all parts of plants such as fruits, vegetables, nuts, seed, leaves, roots and bark (Pratt and Hudson, 1990). Many of these antioxidant compounds possess antiinflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activity to a greater or lesser extent (Halliwell, 1994; Mitscher *et al*., 1996; Owen *et al*., 2000; Sala *et al*., 2002; Mathew and Abraham, 2005). Many plants polyphenols, such as ellagic acid, catechin, chlorogenic, caffeic and ferulic acids, as well as their dietary source such as tea, have been shown to act as potent antimutagenic and anticarcinogenic agents (Ayrton *et al*., 1992; Bu-Abbas *et al*., 1994; Tanaka *et al*., 1993; Yen and Chen, 1994). Various tea extracts have been reported to be both antioxidant and antimutagenic (Yen and Chen, 1995). The extracts from the root

bark of *Cudrania tricuspidata* showed antioxidant activity (DPPH, superoxide and hydroxyl radical) (Lee *et al*., 2005). The *Garcinia dulcis* extracts possessed radical scavenging and antibacterial activities (Deachatthai *et al*., 2005). Both methanolic leave extracts of kadok (*Piper sarmentosum*) and mengkudu (*Morinda elliptica*) showed high superoxide scavenging antioxidant (Subramaniam *et al*., 2003).

The antioxidant activities were determined by using four complementary *in vitro* assays, DPPH (2,2-Diphenyl-1-picryhydrazyl) radicals scavenging activity, lipid peroxidation inhibition, superoxide radicals scavenging activity and xanthine oxidase activity.

The DPPH radical scanvenging is a rapid and low cost assay so, this assay are widely test the radicals scavenging ability of various natural products (Fazilatun *et al*., 2004; Aligiannis *et al*., 2003). DPPH is classified as nitrogen centered radical and stable at room temperature because it has virtual of the delocalization of the spare electron over the molecule. The radical scavenging of plant extracts against stable DPPH was determined by spectrophotometrically. When DPPH radical react with antioxidant compound which can donate hydrogen. Antioxidants scavenging DPPH radical by converting DPPH to DPPHn (2,2-Diphenyl-1-picryhydrazine) (**Figure 1.3**). The changing of color (from deep violet to light yellow) was measured at 517 nm on a visible light spectrophotometer. Radical scavenging activity show in term of IC_{50} (Inhibition Concentration at 50 %) (Miliauskas *et al*., 2003).

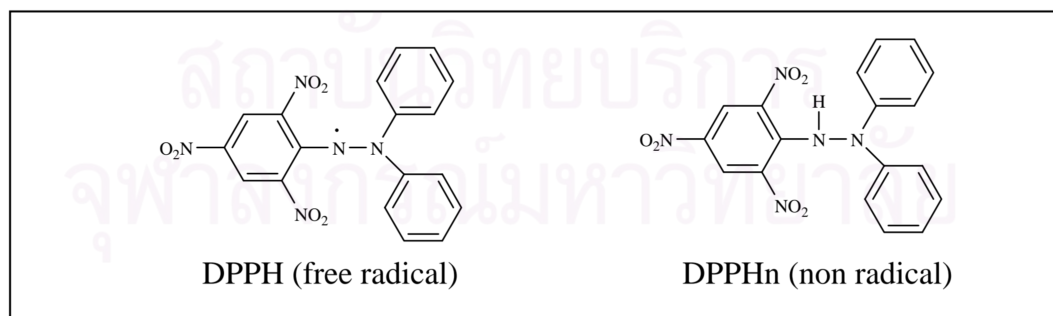


Figure 1.3 Structures of DPPH and DPPHn

An important component of the signaling process in apoptosis induced by ionizing radiation or oxidative damage appears to be the early generation of free radicals in the plasma membrane. The plasma membrane is especially susceptible to oxidative damage because it contains significant quantities of easily peroxidizable lipids. These reactions can be self-propagating, by a process known as lipid peroxidation chain reaction (Stark, 1991). The resulting radicals and toxic metabolites are thought to be the main cause of damage in the cell (Halliwell and Gutteridge, 1999; Kalinich *et al*., 2000).

Lipid peroxidation (LPO) (**Figure 1.4**) is a free radical-related process that in biological system may occur under enzyme control, e.g., for the generation of lipid-derived inflammatory mediator, or nonenzymatically. This latter form is associated mostly with cellular damage as a result of oxidative stress, and a great variety of aldehydes is formed when lipid hydroperoxides break in biological system, among them, malondialdehydes (MDA) and 4-hydroxynonenal (HNE). As MDA, HNE is able to form adducts with free amino acids and many more with proteins. MDA introduces cross-links in proteins which may induce profound alteration in their biological properties. It also been proposed that MDA could react physiologically with several nucleoside (deoxy-guanosine, cytidine). HNE is the major aldehydic product resulting from lipid peroxidation and has been implicated as involved in several pathological conditions, such as atherosclerosis, alcohol-induced liver disease and neurodegenerative disorders. (Kalinich *et al*., 2000; Esterbauer *et al*., 1991; Francisco *et al*., 1998), both of which can be detected *in vitro* (Zaleska and Floyd, 1985) and *in vivo* (Kogure *et al*., 1982). This lipid peroxidation product is in tune toxic, causing disruption of cellular enzyme, membrane receptors and transport process (Braughler, 1985; Picklo *et al*., 1999; Brown-Galatola and Hall, 1992). The antioxidant potential of lipid peroxidation inhibition is using system *in vitro* by spectrophotometric measurement. In this method, the peroxyl radical generated by metal cation (Fe^{2+}) react with phospholipids to MDA and HNE. MDA can be form with thiobarbituric acid (TBA) to produce the MDA-TBA formation, which change from yellow dye to pink dye and detected by spectrophotometrically at 532 nm.

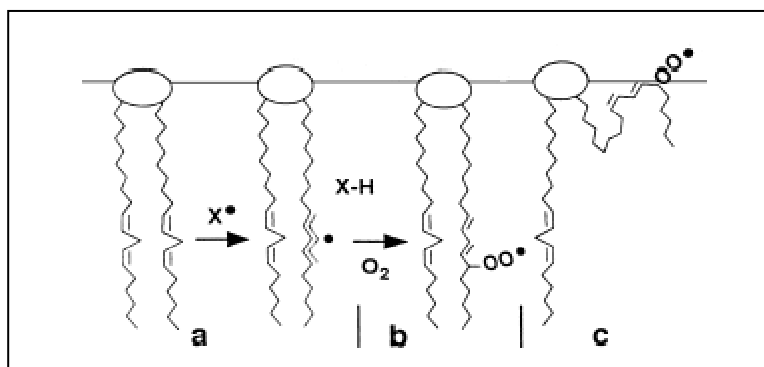
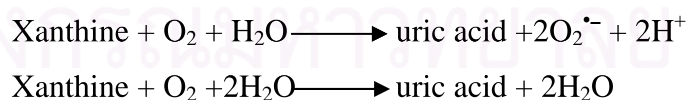


Figure 1.4 Membrane peroxidation (a) Initiation of the peroxidation precess by an oxidizing radical X, by abstraction of a hydrogen atom, thereby forming a pentadienyl radical. (b) Oxygenation to form a peroxyl radical and a conjugated diene. (c) Peroxyl radical moiety partitions to the water-membrane interface

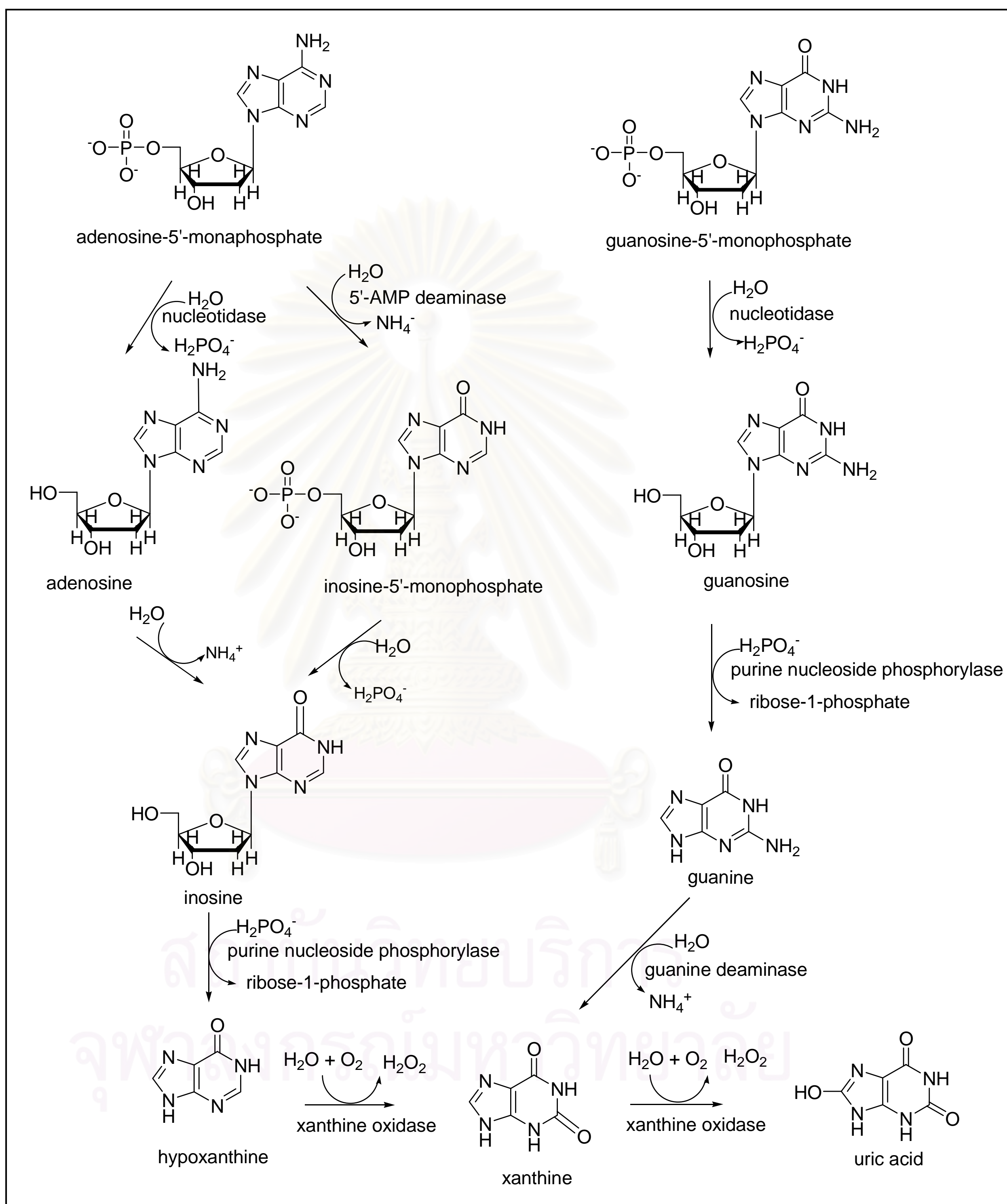
The xanthine oxidase (EC 1.1.3.22) is from xanthine dehydrogenase under oxidative condition. This molybdenum-containing enzyme catalyzes the oxidation of hypoxanthine with oxygen to xanthine and finally to uric acid (**Scheme 1.2**). The over activity of this enzyme results in a condition, causes hyperuricacidemia associated with gout and is also responsible for oxidative damage to living tissues (Noro *et al*., 1983; Hayashi *et al*., 1989; González *et al*., 1995). The deposition of needle shaped monosodium urate crystals in the synovial fluid of the major joints produces an extremely painful acute arthritis with repeated attacks of gout (Rang *et al*., 2001; Umamaheswari *et al*., 2007). In addition, the catalyze of xanthine oxidase, producing superoxide anion radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) according to the following equations.



$$\text{Xanthine} + O_2 + H_2O \longrightarrow \text{uric acid} + 2O_2^{\bullet -} + 2H^+$$
$$\text{Xanthine} + O_2 + 2H_2O \longrightarrow \text{uric acid} + 2H_2O$$

Superoxide anion radical ($O_2^{\bullet -}$), it is produced from a variety of sources including γ-irradiation (Cerutti, 1985), enzyme-substrate reactions such as xanthine-xanthine oxidase (Brown and Fridouich, 1981), chemical such as paraquat (Moody and Hassan, 1982; Bagley *et al*., 1986), phorbol esters

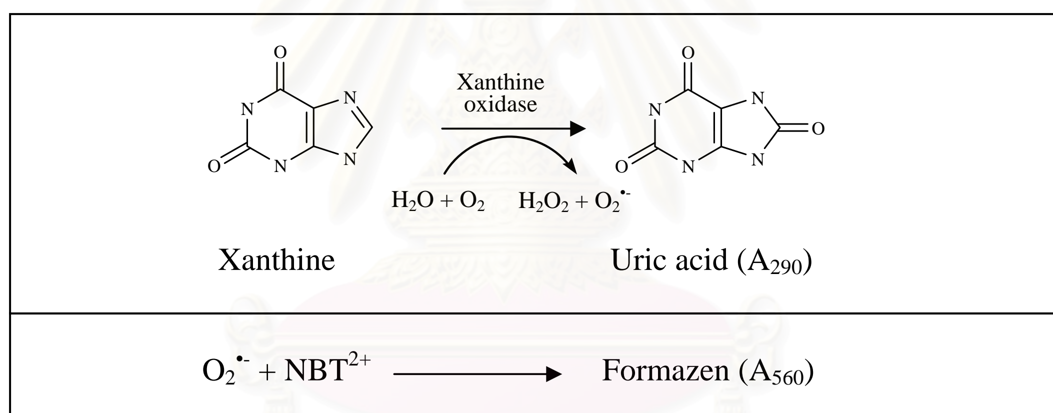
(Birnboim, 1982), bleomycin (Burger *et al*., 1981), ultraviolet and solar radiation (Cunningham *et al*., 1985a; b; Peak and Foot, 1986).



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

The prototypical xanthine oxidase (XO) inhibitor has been the cornerstone of the clinical management of gout and conditions associated with hyperuricemia for several decades. More recent data indicated that XO also plays an important role in various form of ischemic and other types of tissue and vascular injuries, inflammatory diseases and chronic heart failure (Pacher *et al.*, 2006).

In this method, the antioxidant potential is appraised using the xanthine-xanthine oxidase system (**Scheme 1.3**) *in vitro* by spectrophotometric measurement. For estimation of scavenging effect on the superoxide anion radical, nitrobluetetrazolium (NBT) reduction method is used. Superoxide anion radical reduces the yellow dye (NBT^{+}) to produce the blue formazen**,** which measured spectrophotometrically at 560 nm (Parejo *et al.*, 2004). For evaluation of the inhibitory effects on xanthine oxidase, a decreased production of uric acid is measured by UV absorption method at absorbance 290 nm.



Scheme 1.3 The xanthine-xanthine oxidase system

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Botanical aspect and distribution of *Cratoxylum cochinchinense*

Cratoxylum cochinchinense (Lour.) Blume (**Figure1.5**) is a large to shrub tree belonging to the family Guttiferae. This species is called, Tiu Kliang "ติ้วเกลี้ยง", Kheetiu "ขี้ติ้ว", Kuichong-Baag "กุ่ยฉ่องบ้าง" (Karen-lampang), Tiu Bai Lueam "ติ้วใบเลือม" (Northern), (เต็ม สมิตินันท์, 2523) which distributed in several Southeast Asia countries (Robson, 1974). Six species are found in Thailand (Smitinad, 2001); *Cratoxylum arboresens*, *Cratoxylum cochinchinense*, *Cratoxylum maingayi*, *Cratoxylum sumatanum* ssp. *neriifolium*, *Cratoxylum formosum* ssp. *formosum* (Jack) Dyer and *Cratoxylum formosum* (Jack) Dyer ssp. *pruniflorum* (Kruz) Gogel (Boonnak *et al*., 2006).

This tree is a large to shrub and tall 3-8 m with slender branches. Its bark is gray, crack and flaking in small irregular pieces. Its leaves have an elliptical shape with 2-4 cm width and 4-10 cm long. Its stalks have long lower than 3 cm. Their flowers have crimson or dark red, which have five sepals and petals at end of twig and in axils of mature leaves. Its fruit is elliptical shape, which 0.8-1.2 cm width, about 2/3 of fruit covered by the persistent sepals (Gardner *et al*., 2000).

Figure 1.5 *Cratoxylum cochinchinense* (Lour.) Blume

Ethanobotanical and phytochemical investigation of *Cratoxylum cochinchinense*

Cratoxylum cochinchinense, a traditional medicine, this plant has been used to treat fevers, coughs, diarrhea, itches, ulcers and abdominal complaints (Vo, 1997). Previous chemical investigations of this plant have revealed the presence of triterpenoids (**Figure 1.6**), xanthones (**Figure 1.7**) and tocotrienols (Bennett *et al*., 1993; Sia *et al*., 1995; Nguyen and Harrison, 1998) (**Table 1.2**).

Table 1.2 Chemical constituents in *Cratoxylum cochinchinense*

Parts of plant	Isolated compounds	Types	Ref.
Bark	polypoda-8(26),13,17,21-tetraen-3*β*-ol, (13*E*,17*E*)-polypoda-7,13,17,21-tetraen-3*β*-ol	Triterpenoid	Graham *et al*., 1993; Nguyen *et al*., 1998
	mangostin, *β*-mangostin, garcinone D, tovophyllin A, 11-hydroxy-1-isomangostin, xanthonolignoid (5′-demethoxycadensin G), 1,3,5,6-tetrahydroxyxanthone, xanthone *E*-7-geranyloxy-1,3-diihydroxyxanthone	Xanthone	Graham *et al*., 1993; Sia *et al*., 1995; Nguyen *et al*., 1998
Roots	cochinchinenses A-D, 5-*O*-methylcelebixanthone	Xanthone	Mahabusarakam, *et al*., 2006; Laphookhieo, *et al*., 2006

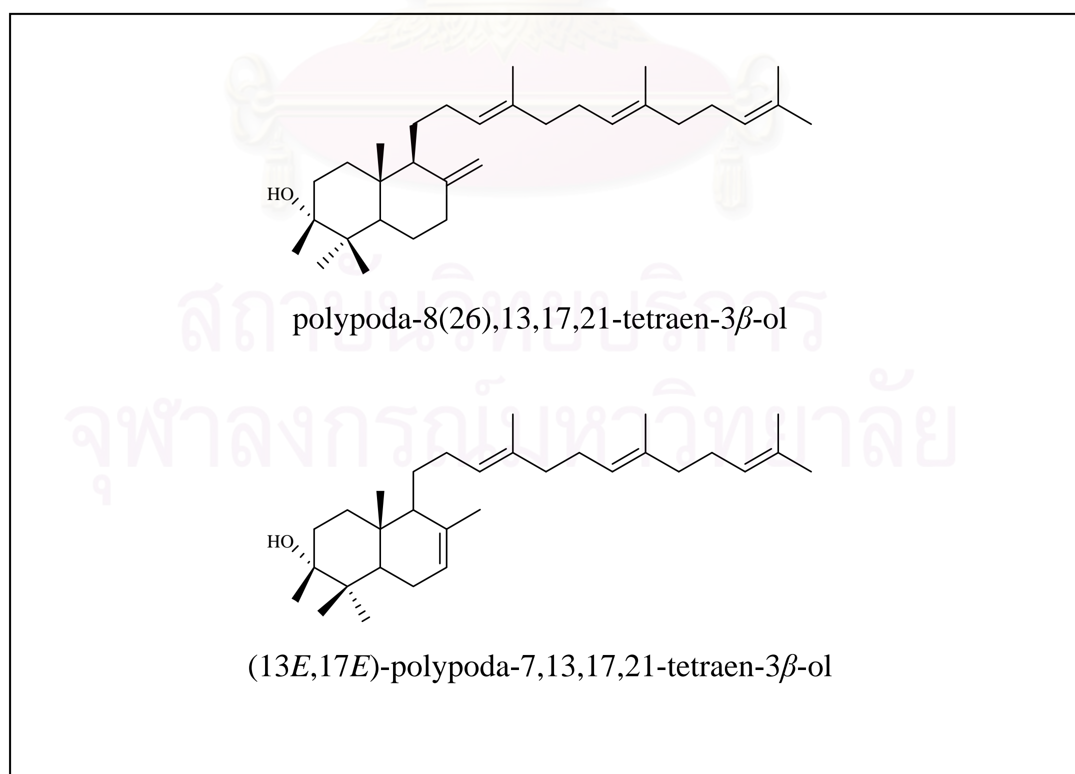

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Figure 1.6 Triterpenoids from *C. cochinchinense*

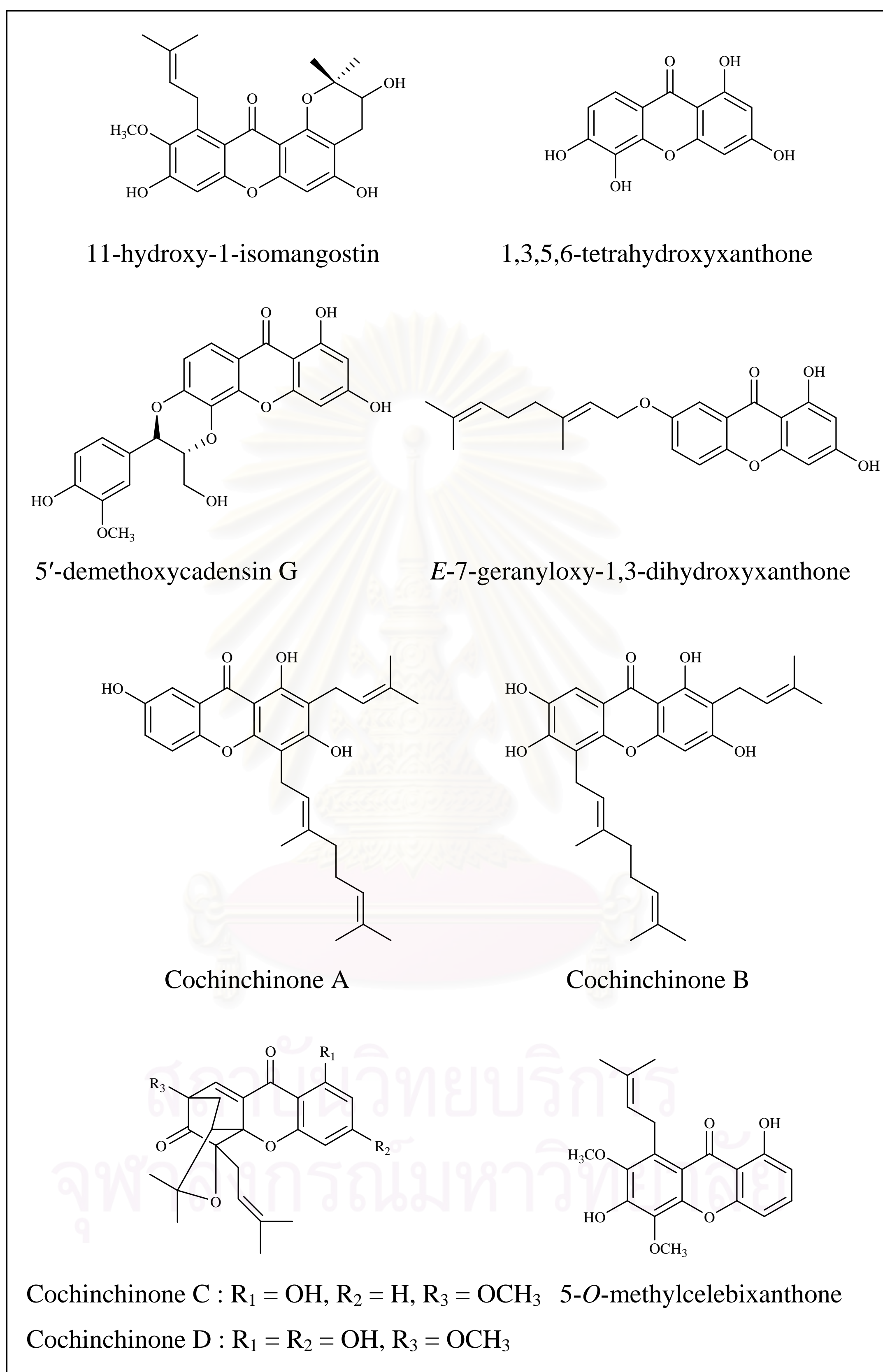

Figure 1.7 Xanthones from *C. cochinchinense*

From the attractive results of primary screening test based on DPPH radical scavenging activity and a few information on the chemical constituents and their biological activities, *C. cochinchinense* (Tiu Kliang) was selected for further investigation.

The goal of this research:

1. To carry out a comprehensive chemical separation and structure determination of xanthones from the stems of *Cratoxylum cochinchinense* by chromatographic and spectroscopic techniques.
2. To investigate the antioxidant activities of the isolated xanthones.

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CHAPTER II

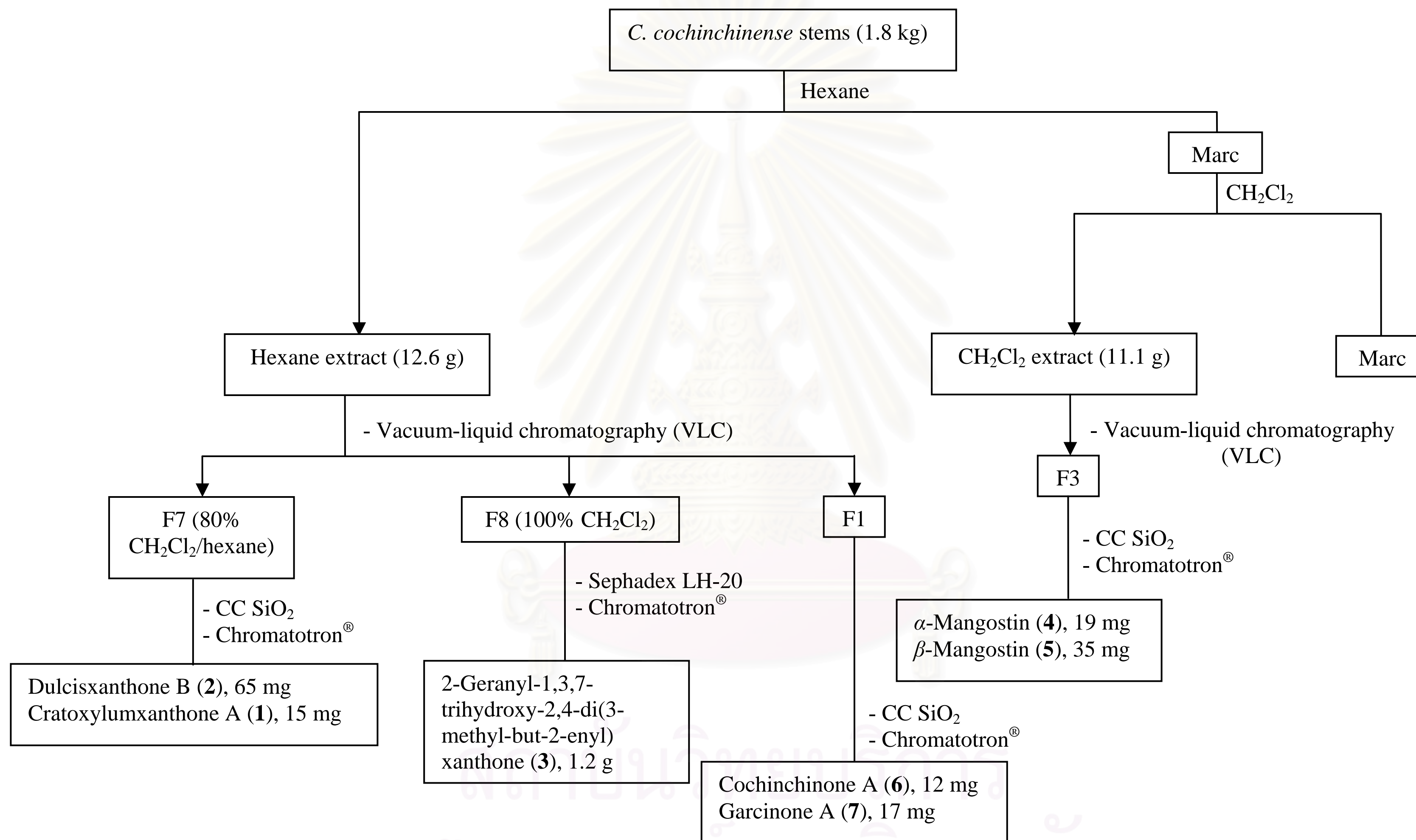
ISOLATION AND CHARACTERIZATION OF XANTHONES FROM *Cratoxylum cochinchinense*

2.1 Extraction and Isolation

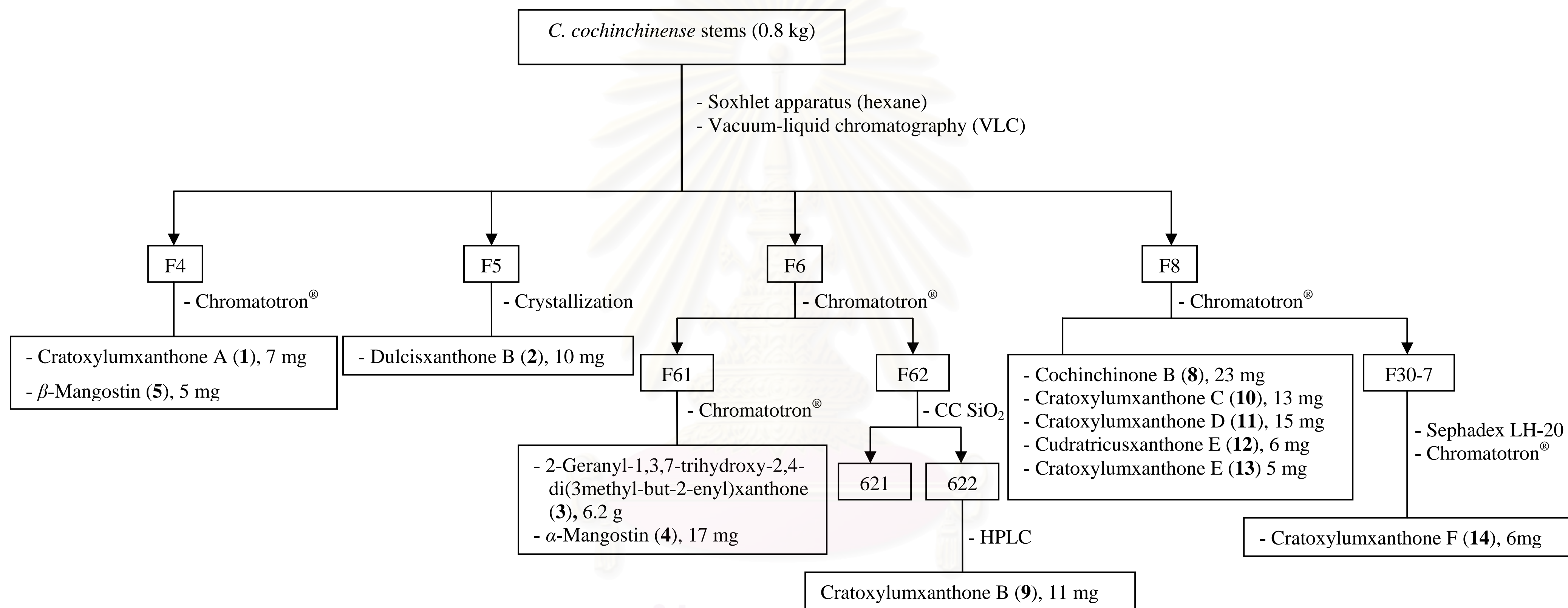
The air-dried stems of *Cratoxylum cochinchinense* (1.8 kg) were pulverized and then macerated with hexane and CH_2Cl_2, respectively. The hexane extract was fractionated on vacuum liquid chromatography (VLC) to yield 8 fractions. Fractions 7 and 8 were purified by silica gel CC and then radial chromatography on silica gel (Chromatotron®) to give cratoxylumxanthone A (**1**), dulcisxanthone B (**2**), 2-geranyl-1,3,7-trihydroxy-4-(3-methyl-but-2-enyl) xanthone (**3**), cochinchinone A (**6**) and garcinone A (**7**). The CH_2Cl_2 extract was fractionated on VLC to give 10 fractions. Fractions 3 and 5 were purified by silica gel CC and then Chromatotron® to yield α-mangostin (**4**) and β-mangostin (**5**). (**Scheme 2.1**, **Figure 2.1**).

In addition, the stems of *C. cochinchinense* (0.8 kg) were also extracted with hexane by soxhlet apparatus. This extract was further chromatographed on VLC to give 10 fractions. Fractions 4, 5, 6 and 8 were further purified by combination of silica gel CC, Sephadex LH-20 (size-exclusion), Chromatotron® and high performance liquid chromatography (HPLC) to yield cochinchinone B (**8**), cratoxylumxanthone B (**9**), cratoxylumxanthone C (**10**), cratoxylumxanthone D (**11**), cudratricusxanthone E (**12**), cratoxylumxanthone E (**13**) and cratoxylumxanthone F (**14**) (**Scheme 2.2**, **Figure 2.1**).

From maceration and sohxlet extraction of *Cratoxylum cochinchinese* stems led to the isolation of six new xanthones (**1**, **9-11**, and **13-14**), along with eight known xanthones.



Scheme 2.1 The maceration and isolation procedure of *C. cochinchinense* stems.



Scheme 2.2 The soxhlet extraction and isolation procedure of *C. cochinchinense* stems.

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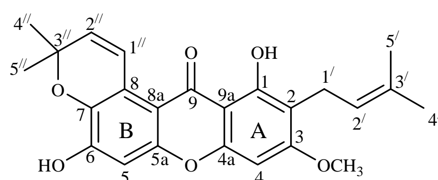

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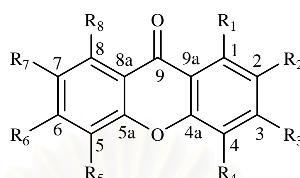
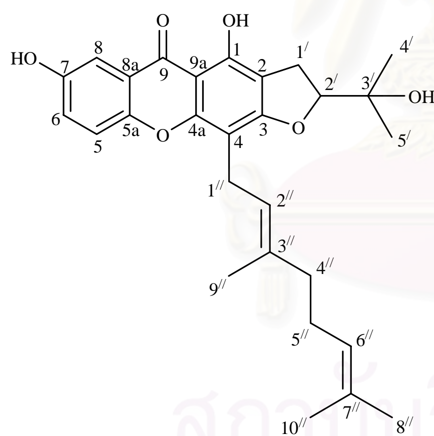
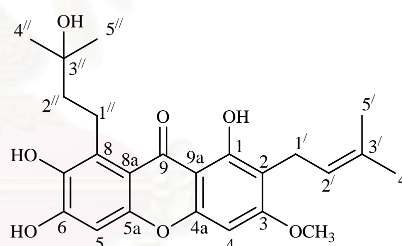


2 R_1, R_6, R_7 = OH, R_2, R_8 = prenyl, R_3 = OCH_3, R_4, R_5 = H
3 R_1, R_3, R_7 = OH, R_2 = geranyl, R_4 = prenyl, R_5, R_6 = H
4 R_1, R_3, R_6 = OH, R_2, R_8 = prenyl, R_4, R_5 =H, R_7 = OCH_3
5 R_1, R_6 = OH, R_2, R_8 = prenyl, R_3, R_7 = OCH_3, R_4, R_5 = H
6 R_1, R_3, R_7 = OH, R_2, = prenyl, R_4 = geranyl, R_5, R_6, R_8 = H
7 R_1, R_3, R_7 = OH, R_2, R_4 = prenyl, R_5, R_6, R_8 = H
8 R_1, R_3, R_6, R_7 = OH, R_2, = prenyl, R_4, R_8 = H, R_5 = geranyl
10 R_1, R_3, R_6, R_7 = OH, R_2, = prenyl, R_4 = geranyl, R_5, R_8 = H
11 R_1, R_3 = prenyl, R_2, R_4, R_6, R_7 = OH, R_5, R_8 = H
12 R_1, R_3, R_6, R_7 = OH, R_2, R_4 = prenyl, R_5, R_8 = H





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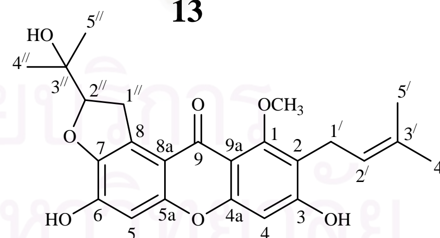

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Figure 2.1 Isolated xanthones from *C. cochinchinense* stems

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2.2 Structure Elucidation of New Compounds

2.2.1 Cratoxylumxanthone A (1)

Cratoxylumxanthone A was isolated as a pale yellow powder (mp 231-233 °C). Its pseudomolecular ion of *m/z* $[M+H]^+$ 409.1644 (calcd 409.1651) was in agreement with the molecular formula $C_{24}H_{24}O_6$. The UV spectrum showed absorption bands at 254, 266, 331, and 380 nm which were characteristics of a hydroxylated xanthone (Nguyen and Harrision, 1998). The ^{1}H NMR spectrum (**Table 2.1**) exhibited signals of a hydrogen-bonded hydroxy proton at δ 13.26 (s, 1-OH), two isolated aromatic protons at δ 6.28 (s, H-4) and 6.75 (s, H-5) and one methoxy group at δ 3.84 (s, 3-OCH_3). The methoxy group was accommodated at C-3 of ring A based on HMBC cross peak. The characteristic signals of protons in a prenyl unit were displayed at δ 3.29 (d, H-1′), 5.16 (m, H-2′), 1.61 (s, H-4′) and 1.72 (s, H-5′). In addition, the presence of dimethylchromene ring was indicated from two *cis*-olefinic protons (δ 7.97 and 5.75). The correlation of H-1′ to C-1, C-2 and C-3 in the HMBC spectrum (**Table 2.1**) established that the location of the prenyl unit was at C-2, whereas the correlation of H-1″ to C-7 and H-2″ to C-8 indicated the connectivity of the dimethylchromene moiety of ring B with ether linkage at C-7. From the molecular formula ($C_{24}H_{24}O_6$), a remaining hydroxyl group (δ 6.15) was placed at C-6 of ring A which confirmed by HMBC data (**Table 2.1**).

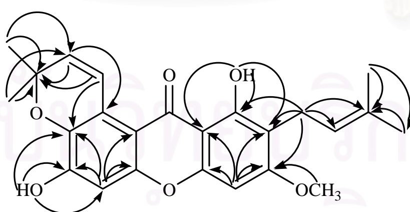
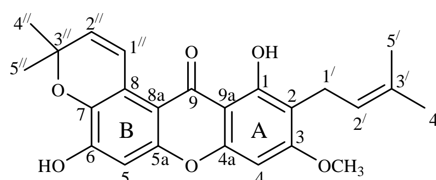


Figure 2.2 HMBC correlations of cratoxylumxanthone A

Thus, the complete structure of cratoxylumxanthone A was deduced as shown.



Cratoxylumxanthone A (**1**)

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Table 2.1 ^{1}H, ^{13}C and HMBC NMR data of cratoxylumxanthone A (**1**) in $CDCl_3$

Position	δ_H (J in Hz)	δ_C	HMBC correlations
1	-	159.2	-
2	-	110.5	-
3	-	163.0	-
4	6.28, s	88.7	C-2, C-3, C-4a, C-9a
4a	-	155.1	-
5	6.75, s	101.2	C-5a, C-6 ,C-7, C-8a
5a	-	153.4	-
6	-	149.7	-
7	-	137.3	-
8	-	119.2	-
8a	-	108.3	-
9	-	182.0	-
9a	-	103.6	-
1′	3.29, d (7.2)	20.3	C-1, C-2, C-3, C-2′, C-3′
2′	5.16, m	120.0	-
3′	-	130.7	-
4′	1.61, s	24.7	C-3′, C-2′, C-5′
5′	1.72, s	16.8	C-3′, C-4′, C-2′
1″	7.97, d (10.0)	21.3	C-7, C-3″
2″	5.75, d (10.4)	131.8	C-8, C-3″
3″	-	77.1	-
4″	1.43, s	26.3	C-2″, C-3″
5″	1.43, s	26.3	C-2″, C-3″
1-OH	13.26, s	-	C-1, C-2, C-9a
3-OCH_3	3.84, s	54.8	C-3
6-OH	6.15, s	-	C-5, C-6, C-7

2.2.2 Cratoxylumxanthone B (9)

Cratoxylumxanthone B was obtained as a yellow powder (mp 147-149 °C) and showed a molecular formula of $C_{28}H_{32}O_6$ [HRESIMS] which showed a quasi-molecular ion peak at *m/z* 487.2077 $[M+Na]^+$ (calcd 487.2091). The UV spectrum exhibited absorption bands at 229, 265, 321, and 377 nm. The ^{1}H NMR spectrum (**Table 2.2**) showed signals of hydrogen-bonded hydroxy proton at δ 13.09 (s, 1-OH) and three aromatic protons which coupled as an ABX system at δ 7.56 (d, J = 2.8 Hz, H-8), 7.48 (d, J = 8.8 Hz, H-5) and 7.34 (d, J = 7.6 Hz, H-6). The characteristic signals of protons in a geranyl side chain were displayed at δ 3.46 (m, H-1″), 5.32 (m, H-2″), 1.98 (m, H-4″), 2.05 (m, H-5″), 5.02 (m H-6″), 1.52 (s, H-8″), 1.88 (s, H-9″) and 1.50 (s, H-10″). In addition, the presence of 1-hydroxy-

1-methylethyldihydrofuran group was indicated from the resonances at δ 3.20 (m. H-1′), 4.86 (m, H-2′), 1.29 (s, H-4′ and H-5′) and 3.84 (s, 3′-OH). The correlation of H-1″ to C-3, C-4 and C-4a in the HMBC (**Table 2.2**) established that the location of geranyl unit was at C-4, whereas the correlation of H-1′ to C-1, C-2 and C-3 indicated that the 1-hydroxy-1-methylethyldihydrofuran group was fused to C-2 and C-3 of ring A of xanthone nucleus with ether linkage at C-3. The remaining hydroxyl group (δ 8.97) was placed at C-7 of ring B which confirmed by HMBC data (**Table 2.2**).

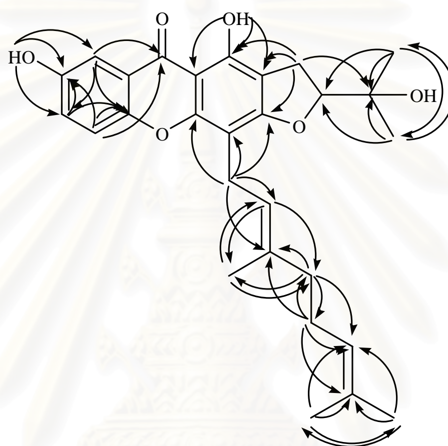
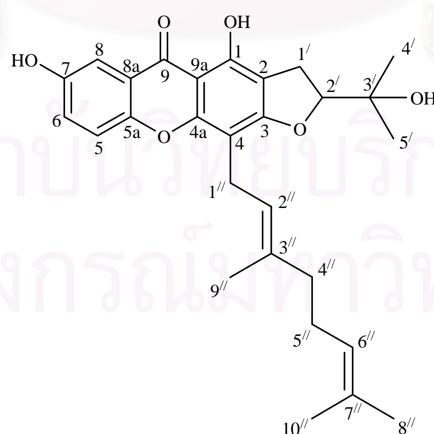


Figure 2.3 HMBC correlations of cratoxylumxanthone B

Therefore, the complete structure of cratoxylumxanthone B was assigned as shown.



Cratoxylumxanthone B (**9**)

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Table 2.2 ^{1}H, ^{13}C and HMBC NMR data of cratoxylumxanthone B (**9**) in acetone-d_6

Position	δ_H (J in Hz)	δ_C	HMBC correlations
1	-	155.7	-
2	-	108.3	-
3	-	165.9	-
4	-	101.7	-
4a	-	154.9	-
5	7.48, d (8.8)	118.3	C-5a, C-7, C-8a, C-9
5a	-	149.9	-
6	7.34, d (7.6)	124.7	C-5a, C-7
7	-	153.9	-
8	7.56, d (2.8)	108.3	C-5a, C-6, C-7, C-9
8a	-	120.5	-
9	-	180.6	-
9a	-	103.3	-
1′	3.20, m	26.4	C-1, C-2, C-3, C-3′,C-2′
2′	4.86, m	91.6	C-4′, C-5′
3′	-	70.3	-
4′	1.29, s	24.2	C-2′, C-3′, C-5′
5′	1.29, s	25.3	C-2′, C-3′, C-4′
1″	3.46, m	21.7	C-3, C-4, C-4a, C-2″, C-3″
2″	5.32, m	121.9	C-1″, C-4″, C-9″
3″	-	134.9	-
4″	1.98, m	39.5	C-2″, C-3″, C-5″, C-6″, C-9″
5″	2.05, m	26.4	C-3″, C-4″, C-6″, C-7″
6″	5.02, m	123.7	C-8″, C-10″
7″	-	130.7	-
8″	1.52, s	24.8	C-6″, C-7″, C-10″
9″	1.88, s	15.5	C-2″, C-4″, C-3″
10″	1.50, s	16.8	C-6″, C-7″, C-8″
1-OH	13.09, s	-	C-1, C-2, C-9a
7-OH	8.97, s	-	C-6, C-7, C-8
3′-OH	3.84, s	-	C-2′, C-3′, C-5′

2.2.3 Cratoxylumxanthone C (10)

Cratoxylumxanthone C was isolated as a yellow powder (mp 176-178 °C). It showed a pseudomolecular ion peak at *m/z* 487.2100 $[M+Na]^+$ (calcd 487.2091), which was accounted for a molecular formula of $C_{28}H_{32}O_6$. The UV spectrum of cratoxylumxanthone C exhibited absorption bands at 229, 262, 321, and 375 nm. The ^{1}H NMR spectrum (**Table 2.3**) showed signals of hydrogen-bonded hydroxy proton at δ 13.34 (s, 1-OH) and two isolated aromatic protons at δ 6.85 (s, H-5) and 7.40 (s, H-8). The presence of prenyl group was suggested by

signals at δ 3.30 (d, J = 6.8 Hz, H-1′), 5.12 (m, H-2′), 1.52 (s, H-4′) and 1.64 (s, H-5′). Moreover, the presence of geranyl group was suggested by following spectral data: δ 3.44 (d, J = 6.8 Hz, H-1″), 5.12 (m, H-2″), 1.85 (m, H-4″), 1.94 (m, H-5″), 4.88 (m, H-6″), 1.36 (s, H-8″), 1.77 (s, H-9″) and 1.38 (s, H-10″). The HMBC correlations of H-1′ to C-1, C-2 and C-3 and H-1″ to C-3, C-4 and C-4a indicated that the location of prenyl and geranyl groups were at C-2 and C-4 of ring A, respectively. From the basis of molecular formula ($C_{28}H_{32}O_5$), the three remaining hydroxyl groups were placed at C-3 of ring A and C-6, C-7 of ring B.

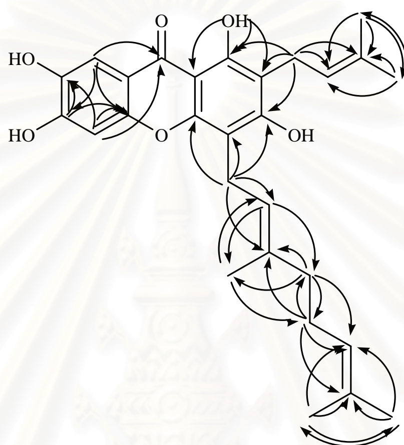
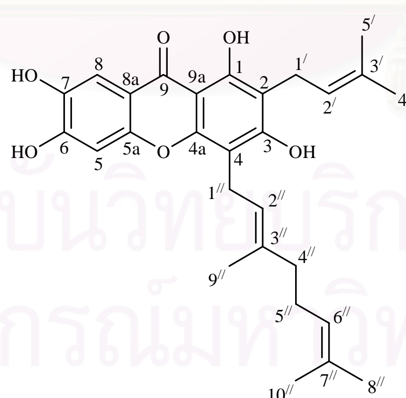


Figure 2.4 HMBC correlations of cratoxylumxanthone C

Thereby, the overall structure of cratoxylumxanthone C was elucidated as shown.



Cratoxylumxanthone C (**10**)

Table 2.3 ^{1}H, ^{13}C and HMBC NMR data of cratoxylumxanthone C (**10**) in acetone-d_6

Position	δ_H (*J* in Hz)	δ_C	HMBC correlations
1	-	158.0	-
2	-	110.0	-
3	-	159.3	-
4	-	105.9	-
4a	-	152.8	-
5	6.85, s	103.0	C-5a, C-6, C-7, C-8a, C-9
5a	-	153.6	-
6	-	151.9	-
7	-	143.6	-
8	7.40, s	107.9	C-5a, C-6, C-7, C-9
8a	-	113.0	-
9	-	180.9	-
9a	-	103.0	-
1′	3.30, d (6.8)	22.2	C-1, C-2, C-3, C-3′
2′	5.12, m	122.2	C-4′, C-5′
3′	-	131.4	-
4′	1.52, s	25.1	C-2′, C-3′, C-5′
5′	1.64, s	17.1	C-2′, C-3′, C-4′
1″	3.44, d (6.8)	23.1	C-3, C-4, C-4a, C-4″, C-9″
2″	5.12, m	122.2	C-1″, C-4″, C-9″
3″	-	135.0	-
4″	1.85, m	39.5	C-3″, C-5″, C-9″
5″	1.94, m	26.4	C-4″
6″	4.88, m	124.0	C-4″, C-5″
7″	-	130.8	-
8″	1.36, s	24.5	C-6″, C-7″, C-10″
9″	1.77, s	15.5	C-2″, C-5″
10″	1.38, s	16.8	C-6″, C-7″, C-8″
1-OH	13.34, s	-	C-1, C-2, C-9a

2.2.4 Cratoxylumxanthone D (11)

Cratoxylumxanthone D was obtained as a yellow powder (mp 133-135 °C). The molecular formula of compound **11**, $C_{23}H_{24}O_6$, was deduced from its HRESIMS quasi-molecular ion peak at *m/z* = 395.1491 $[M-H]^-$ (calcd 395.1500). The UV spectrum showed absorption bands at 217, 256, 320, and 370 nm. The absence of the signal of hydrogen-bonded hydroxy proton in low field suggested that the structure disappeared of hydroxyl group at C-1 or C-8. This spectrum also showed two isolated aromatic protons at δ 6.84 (s, H-5) and 7.43 (s, H-8). The characteristic signals of protons in two prenyl groups were displayed at 3.45 (m,

H-1′), 5.11 (m, H-2′), 1.54 (s, H-4′), 1.77 (s, H-5′) and 3.31 (d, J = 6.8 Hz, H-1″), 5.13 (m, H-2″), 1.54 (m, H-4″), 1.67 (s, H-5″) (**Table 2.4**). The correlations of H-1′ to C-2, C-4a and C-9a and H-1″ to C-2, C-3 and C-4 in HMBC spectrum (**Table 2.4**) established that two prenyl groups were at C-1 and C-3 of ring A, respectively. The four remaining hydroxyl groups were located at C-2, C-4 of ring A and C-6, C-7 of ring B according to its molecular formula ($C_{23}H_{24}O_6$).

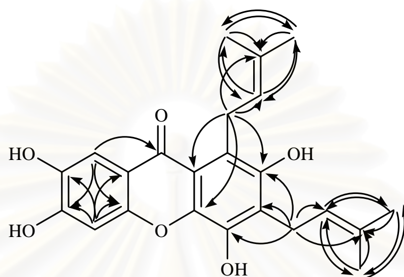
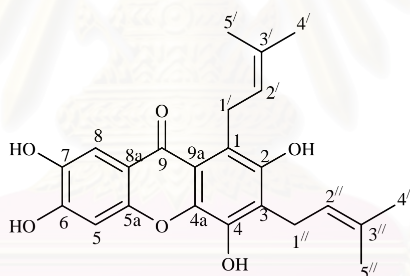


Figure 2.5 HMBC correlations of cratoxylumxanthone D

Therefore, the complete structure of cratoxylumxanthone D was deduced as shown.



Cratoxylumxanthone D (**11**)

Table 2.4 ^{1}H, ^{13}C and HMBC NMR data of cratoxylumxanthone D (**11**) in acetone-d_6

Position	δ_H (J in Hz)	δ_C	HMBC correlations
1	-	135.0	-
2	-	159.0	-
3	-	109.7	-
4	-	157.5	-
4a	-	152.9	-
5	6.84, s	102.5	C-5a, C-6, C-7, C-8a
5a	-	142.5	-
6	-	152.9	-

Table 2.4 (Cont.)

Position	δ_H (*J* in Hz)	δ_C	HMBC correlations
7	-	151.5	-
8	7.43, s	108.2	C-5a, C-6, C-7,C-9
8a	-	112.5	-
9	-	179.6	-
9a	-	105.5	-
1′	3.45, m	21.5	C-2, C-4a, C-9a, C-2′, C-3′
2′	5.11, m	121.9	C-4′, C-5′
3′	-	130.8	-
4′	1.54, s	24.8	C-2′, C-3′, C-5′
5′	1.77, s	16.8	C-2′, C-3′, C-4′
1″	3.31, d (6.8)	21.2	C-3, C-4, C-5, C-2″, C-3″
2″	5.13, m	121.9	C-4″, C-5″
3″	-	131.3	-
4″	1.54, s	24.8	C-2″, C-3″, C-5″
5″	1.67, s	16.8	C-2″, C-3″, C-4″

2.2.5 Cratoxylumxanthone E (13)

Cratoxylumxanthone E was isolated as a yellow powder (mp 190-192 °C). The molecular formula was determined as $C_{24}H_{26}O_7$ by HRESIMS, which showed a pseudomolecular ion at *m/z* 427.1773 $[M-H]^-$ (calcd 427.1762). The UV spectrum displayed absorption bands at 218, 247, 256, 315, and 360 nm. The ^1H NMR spectrum (**Table 2.5**) demonstrated signals of hydrogen-bonded hydroxy proton at δ 13.64 (s, 1-OH), two isolated aromatic protons at δ 6.34 (s, H-4) and 6.69 (s, H-5) and one methoxy group at δ 3.81 (s, 3-OCH_3). On the basis of HMBC cross peaks, this methoxy group was placed at C-3 of ring A. The characteristic signals of prenyl group were showed at δ 3.19 (d, *J* = 6.0 Hz, H-1′), 5.08 (m, H-2′), 1.51 (s, H-4′) and 1.64 (s, H-5′). Furthermore, the presence of 3-hydroxy-3-methylbutyl group was determined from the resonances of δ 3.36 (dd, *J* = 7.2 Hz, H-1″), 1.72 (dd, *J* = 7.2 Hz, H-2″) and 1.17 (s, H-4″ and H-5″). The correlations of H-1′ to C-1, C-2 and C-3 (**Table 2.5**) revealed that the prenyl unit was located at C-2 of ring A, while those of H-1″ to C-7, C-8 and C-8a indicated that the 3-hydroxy-3-methylbutyl group was attached at C-8 of ring B. The two remaining hydroxyl groups were placed at C-6 and C-7 of ring B which suggested was by its molecular formula ($C_{24}H_{26}O_7$).

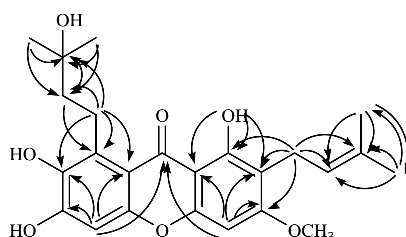
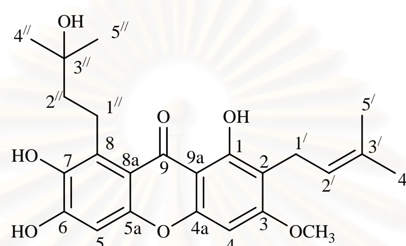


Figure 2.6 HMBC correlations of cratoxylumxanthone E

Thus, the complete assignment of cratoxylumxanthone E was determined as structure below.



Cratoxylumxanthone E (**13**)

Table 2.5 ^{1}H, ^{13}C and HMBC NMR data of cratoxylumxanthone E (**13**) in acetone-d_6

Position	δ_H (J in Hz)	δ_C	HMBC correlations
1	-	159.5	-
2	-	110.7	-
3	-	163.5	-
4	6.34, s	89.8	C-2, C-3, C-4a, C-9, C-9a
4a	-	154.6	-
5	6.69, s	100.2	C-6, C-7, C-8a, C-9
5a	-	148.0	-
6	-	153.0	-
7	-	140.3	-
8	-	130.0	-
8a	-	111.0	-
9	-	182.0	-
9a	-	103.5	-
1′	3.19, d (6.0)	21.0	C-1, C-2, C-3, C-2′, C-3′
2′	5.08, m	123.5	C-1′, C-5′
3′	-	130.4	-
4′	1.51, s	25.0	C-2′, C-3′, C-5′
5′	1.64, s	17.2	C-2′, C-3′, C-4′
1″	3.36, dd (7.2)	21.6	C-7, C-8, C-8a, C-2″, C-3″
2″	1.72, dd (7.2)	43.1	C-8, C-3″
3″	-	69.8	-

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Table 2.5 (Cont.)

Position	δ_H (*J* in Hz)	δ_C	HMBC correlations
4″	1.17, s	24.6	C-2″, C-3″
5″	1.17, s	24.4	C-2″, C-3″
1-OH	13.64, s	-	C-1, C-2, C-9a
3-OCH_3	3.81, s	55.5	C-3

2.2.6 Cratoxylumxanthone F (14)

Cratoxylumxanthone F was obtained as a yellow powder (mp 186-188 °C) which showed a quasi-molecular ion peak at *m/z* 425.1553 $[M-H]^-$ (calcd 425.1606). The molecular formula established as $C_{24}H_{26}O_7$ by HRESIMS. The UV spectrum displayed absorption bands at 217, 247, 255, 314, and 366 nm. The absence of the signal of hydrogen-bonded hydroxy proton in low field suggested that this structure disappeared of hydroxyl group at C-1 or C-8. The ^{1}H NMR spectrum showed two isolated aromatic protons at δ 6.34 (s, H-4) and 6.65 (s, H-5) and one methoxy group at δ 3.84 (s, 1-OCH_3). According to the HMBC correlation, the location of methoxy group was at C-1 of ring A. The characteristic signals of prenyl group were exhibited at δ 3.20 (d, *J* = 6.8 Hz, H-1′), 5.10 (m, H-2′), 1.52 (s, H-4′) and 1.67 (s, H-5′). In addition, the presence of 1-hydroxy-1-methylethyldihydrofuran group was confirmed from the resonance at δ 3.62 (m, H-1″), 4.69 (m, H-2″), 1.15 (s, H-4″) and 1.20 (s, H-5″) (**Table 2.6**). The HMBC correlations of H-1′ to C-1, C-2 and C-3 indicated that the prenyl group was located at C-2 of ring A, whereas the correlation H-1″ to C-7 and C-8 indicating that the 1-hydroxy-1-methylethyldihydrofuran ring was fused to C-7 and C-8 of xanthone nucleus with an ether linkage at C-7 of ring B. The two remaining hydroxyl groups were placed at C-3 of ring A and C-6 of ring B based on its molecular formula ($C_{24}H_{26}O_7$).

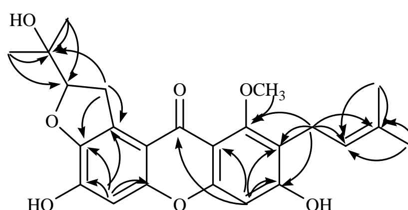
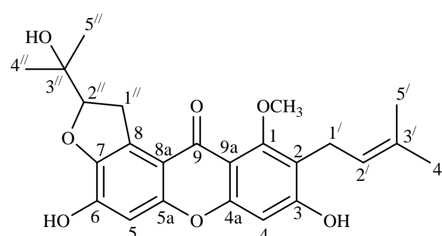


Figure 2.7 HMBC correlations of cratoxylumxanthone F

Thereby, the complete structure of cratoxylumxanthone F was concluded as follow.



Cratoxylumxanthone F (**14**)

Table 2.6 ^{1}H, ^{13}C and HMBC NMR data of cratoxylumxanthone F (**14**) in acetone-d_6

Position	δ_H (J in Hz)	δ_C	HMBC correlations
1	-	163.7	-
2	-	110.9	-
3	-	157.0	-
4	6.34, s	89.9	C-2, C-3, C-9, C-9a
4a	-	156.0	-
5	6.65, s	102.4	C-5a, C-6, C-7, C-8
5a	-	148.7	-
6	-	152.6	-
7	-	145.2	-
8	-	111.0	-
8a	-	126.1	-
9	-	180.2	-
9a	-	103.7	-
1′	3.20, d (6.8)	20.9	C-1, C-2, C-3, C-2′, C-3′
2′	5.10, m	122.3	-
3′	-	130.7	-
4′	1.52, s	25.2	C-2′, C-3′
5′	1.67, s	17.7	C-2′, C-3′
1″	3.62, m	32.2	C-7, C-8a, C-3″
2″	4.69, m	91.0	-
3″	-	70.0	-
4″	1.15, s	24.5	C-2″, C-3″
5″	1.20, s	24.5	C-2″, C-3″
1-OCH_3	3.84, s	55.7	C-1

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2.3 Experimental Section

2.3.1 General Experimental Procedure

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

2.3.2 Plant material

The stems of *Cratoxylum cochinchinense* (Lour.) Blume. (Tiu Kliang) were collected in April 2004 at Nakornpanom province, Thailand. The plant material was identified by Dr. Chumpol Khunwasi, and the voucher specimen (BCU 011803) has been deposited in the herbarium of Department of Botany, Faculty of Science, Chulalongkorn University.

2.3.3 Extraction and Purification

The air-dried powdered stems of *Cratoxylum cochinchinense* (1.8 kg) were extracted twice with hexane and CH_2Cl_2, respectively. All of these crude extracts of *C. cochinchinense* were further purified by chromatographic techniques. The maceration and isolation procedure of *C. cochinchinense* were summarized in **Scheme 2.1**

The hexane extract (12.6 g) was chromatographed on normal phase (vacuum liquid chromatography). Elution with hexane, CH_2Cl_2-hexane, CH_2Cl_2 and MeOH-CH_2Cl_2, afforded 8 fractions. Fraction 7 was subjected to silica gel CC using CH_2Cl_2-hexane and MeOH-CH_2Cl_2, yielding cratoxylumxanthone A (**1**, 15 mg) and dulcisxanthone B (**2**, 65 mg) (Deachatthai *et al.*, 2005). Fraction 8 was also chromatographed on Sephadex LH-20 and radial chromatography on silica gel (Chromatotron®) to give 2-geranyl-1,3,7-trihydroxy-4-(3-methyl-but-2-enyl)xanthone (**3**, 1.2 g) (Bennett *et al.*, 1993).

The CH_2Cl_2 extract (11.1 g) was fractionated on normal phase (VLC), eluting with CH_2Cl_2-hexane, CH_2Cl_2, and EtOAc-CH_2Cl_2 to afford 10 fractions. Fraction 3 was further purified on silica gel CC eluting with EtOAc-CH_2Cl_2 to afford β-mangostin (**5**, 35 mg) (Yates and Bhat, 1968). Fraction 5 was purified on Chromatotron® to yield α-mangostin (**4**, 19 mg) (Yates and Stout, 1958).

The all remaining fractions from the isolation of hexane extract were recombined to get fraction 1. Fraction 1 was concentrated and fractionated on silica gel CC, eluting with CH_2Cl_2-MeOH to give 5 fractions. Fraction 2 was further purified on Chromatotron® to afford cochinchinone A (**6**, 12 mg) (Mahabusarakam *et al.*, 2006) and garcinone A (**7**, 17 mg) (Ashis *et al.*, 1982).

The stems of *C. cochinchinense* (0.8 kg) were also extracted with hexane by soxhlet apparatus. This extract was further chromatographed on normal phase (VLC). Elution with hexane, CH_2Cl_2 and EtOAc, gave 10 fractions. Fraction 4 was purified by Chromatotron® to yield cratoxylumxanthone A (**1**, 7 mg) and β-mangostin (**5**, 5 mg). Fraction 5 was recrystallized to give dulcisxanthone B (**2**, 10 mg). Fraction 6 was further isolated by Chromatotron® to get 2 subfractions, 61 and 62. Subfraction 61 was purified by Chromatotron® to afford 2-geranyl-1,3,7-trihydroxy-4-(3-methyl-but-2-enyl)xanthone (**3**, 6.2 g) and α-mangostin (**4**, 17 mg). Subfraction 62 was separated by column chromatography to give 2 fractions, 621 and 622. The fraction 622 was purified by HPLC, eluting with 100% acetonitrile to obtain cratoxylumxanthone B (**9**, 11 mg). The fraction 8 was chromatograped by Chromatotron® to furnish cochinchinone B (**8**, 23 mg) (Mahabusarakam *et al.*, 2006), cratoxylumxanthone C (**10**, 13 mg), cratoxylumxanthone D (**11**, 15 mg), cudratricusxanthone E (**12**, 6 mg) (Zou *et al.*, 2004a), cratoxylumxanthone E (**13**, 5 mg) and fraction 30-7. This fraction was

also rechromatographed by Sephadex LH-20 to give 5 fractions. The fraction 1 was purified by Chromatotron® to yield cratoxylumxanthone F (**14**, 6 mg). The soxhlet extraction and isolation procedure soxhlet extractor were summarized in **Scheme 2.2**

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Cratoxylumxanthone A (**1**): pale yellow crystals; ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.26 (1H, s, 1-OH), 7.97 (1H, d, J = 10.0 Hz, H-1″), 6.75 (1H, s, H-5), 6.28 (1H, s, H-4), 6.15 (1H, s, 6-OH), 5.75 (1H, m, H-2″), 5.16 (1H, m, H-2′), 3.84 (3H, s, 3-OCH_3), 3.29 (2H, d, J = 7.2 Hz, H-1′), 1.72 (3H, s, H-5′), 1.61 (3H, s, H-4′) and 1.43 (6H, s, H-4″ and H-5″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.0 (C-9), 163.0 (C-3), 159.2 (C-1), 155.1 (C-4a), 153.4 (C-5a), 149.7 (C-6), 137.3 (C-7), 131.8 (C-2″), 130.7 (C-3′), 120.0 (C-2′), 119.0 (C-8), 110.5 (C-2), 108.3 (C-8a), 103.6 (C-9a), 101.2 (C-5), 88.7 (C-4), 77.1 (C-3″), 54.8 (3-OCH_3), 26.3 (C-4″ and C-5″), 24.7 (C-4′), 21.3 (C-1″), 20.3 (C-1′) and 16.8 (C-5′).

Dulcisxanthone B (**2**): yellow powder; ^{1}H NMR (400 MHz, acetone-d_6) δ 13.80 (1H, s, 1-OH), 6.86 (1H, s, H-5), 6.45 (1H, s, H-4), 5.35 (1H, m, H-2″), 5.23 (1H, m, H-2′), 4.20 (2H, d, J = 6.8 Hz, H-1″), 3.96 (3H, s , 3-OCH_3), 3.33 (2H, d, J = 7.2 Hz, H-1′), 1.87 (3H, s, H-5″), 1.80 (3H, s, H-5′) and 1.67 (6H, s, H-4′ and H-4″); ^{13}C NMR (100 MHz, actone-d_6) δ 182.3 (C-9), 163.4 (C-3), 156.6 (C-1), 155.2 (C-4a), 151.6 (C-6), 151.5 (C-5a), 140.8 (C-7), 130.5 (C-3′), 130.4 (C-3″), 127.9 (C-8), 123.5 (C-2″), 122.5 (C-2′), 111.2 (C-8a), 110.5 (C-2), 103.3 (C-9a), 100.2 (C-5), 88.7 (C-4), 55.6 (3-OCH_3), 25.0 (C-4′, C-1″ and C-4″), 21.0 (C-1′), 17.4 (C-5″) and 17.0 (C-5′).

2-Geranyl-1,3,7-trihydroxy-4-(3-methylbut-2-enyl)xanthone (**3**): pale yellow crystals; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.95 (1H, s, 1-OH), 7.61 (1H, s, H-8), 7.22 (2H, s, H-5 and H-6), 6.56 (1H, s, 3-OH), 5.29 (2H, m, H-2′ and H-2″), 5.09 (1H, m, H-6′) 3.55 (2H, d, J = 7.2 Hz, H-1″), 3.47 (2H, d, J = 6.8 Hz, H-1′), 2.13 (2H, m, H-5′), 2.01 (2H, m, H-4′), 1.90 (3H, s, H-9′), 1.88 (3H, s, H-5″), 1.80 (3H, s, H-4″), 1.67 (3H, s, H-10′) and 1.61 (3H, s, H-8′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.9 (C-9), 161.2 (C-3), 158.2 (C-1), 153.0 (C-7), 152.9 (C-4a), 150.2 (C-5a), 138.0 (C-3′), 134.9 (C-3″), 131.8 (C-7′), 124.2 (C-6), 123.8 (C-6′), 121.5 (C-2′ and C-2″), 120.5 (C-8a), 118.8 (C-5), 109.2 (C-2), 108.8 (C-8), 105.0 (C-4), 103.1 (C-9a), 39.7 (C-4′), 26.4 (C-5′), 25.9 (C-4″), 25.6 (C-10′), 21.7 (C-1″), 21.6 (C-1′), 17.9 (C-5″), 17.2 (C-8′) and 16.7 (C-9′).

***α*-Mangostin** (**4**): yellow powder; ^{1}H NMR (400 MHz, acetone-*d₆*) δ 13.80 (1H, s, 1-OH), 6.79 (1H, s, H-5), 6.38 (1H, s, H-4), 5.26 (4H, m, H-2′ and H-2″), 4.10 (2H, d, *J* = 6.4 Hz, H-1″), 3.77 (3H, s, 7-OCH_3), 3.31 (2H, d, *J* = 6.8 Hz, H-1′), 1.81 (3H, s, H-5″), 1.77 (3H, s, H-5′) and 1.64 (6H, s, H-4′ and H-4″); ^{13}C NMR (100 MHz, acetone-d_6) δ 181.8 (C-9), 162.2 (C-3), 160.7 (C-1), 156.6 (C-6), 155.2 (C-5a), 154.0 (C-4a), 143.5 (C-7), 137.1 (C-8), 130.4 (C-3′ and C-3″), 123.9 (C-2″), 122.6 (C-2′), 110.9 (C-8a), 110.1 (C-2), 102.5 (C-9a), 101.7 (C-5), 92.2 (C-4), 60.3 (7-OCH_3), 26.0 (C-1″), 25.1 (C-4′), 25.0 (C-4″), 21.1 (C-1′), 17.4 (C-5″) and 17.0 (C-5′).

***β*-Mangostin** (**5**): yellow powder; ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.40 (1H, s, 1-OH), 6.82 (1H, s, H-5), 6.33 (1H, s, H-4), 5.27 (1H, m, H-2″), 5.23 (1H, m, H-2′), 4.09 (2H, d, *J* = 6.4 Hz, H-1″), 3.90 (3H, s, 7-OCH_3), 3.80 (3H, s, 3-OCH_3), 3.35 (2H, d, *J* = 7.6 Hz, H-1′), 1.83 (3H, s, H-5″), 1.80 (3H, s, H-5′) and 1.68 (6H, s, H-4′ and H-4″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.9 (C-9), 163.5 (C-3), 159.7 (C-1), 155.7 (C-6), 155.4 (C-5a), 155.2 (C-4a), 142.5 (C-7), 137.0 (C-8), 132.1 (C-3″), 131.7 (C-3′), 123.2 (C-2″), 112.3 (C-8a), 111.5 (C-2), 103.8 (C-9a), 101.5 (C-5), 88.8 (C-4), 26.5 (C-1″), 25.8 (C-4′ and C-4″), 21.3 (C-1′), 18.2 (C-5″) and 17.8 (C-5′).

Cochinchinone A (**6**): yellow powder; ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.00 (1H, s, 1-OH), 7.54 (1H, d, *J* = 2.8 Hz, H-8), 7.29 (1H, d, *J* = 9.2 Hz, H-5), 7.17 (1H, d, *J* = 3.2 Hz, H-6), 6.40 (1H, s, 3-OH), 5.22 (1H, m, H-2′), 5.19 (1H, m, H-2″), 4.98 (1H, m, H-6″), 3.49 (2H, d, *J* = 7.2 Hz, H-1″), 3.39 (2H, d, *J* = 7.2 Hz, H-1′), 2.03 (2H, m, H-5″), 1.99 (2H, m, H-4″), 1.81 (3H, s, H-9″), 1.78 (3H, s, H-5′), 1.69 (3H, s, H-4′), 1.56 (3H, s, H-8′) and 1.49 (3H, s, H-10′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.9 (C-9), 161.0 (C-3), 158.2 (C-1), 152.9 (C-4a), 152.0 (C-7), 150.5 (C-5a), 137.8 (C-3″), 135.1 (C-3′), 131.8 (C-7″), 123.8 (C-6 and C-6″), 121.5 (C-2′), 121.4 (C-2″), 120.7 (C-8a), 119.0 (C-5), 109.1 (C-2 and C-8), 105.5 (C-5a), 103.2 (C-9a), 39.7 (C-4″), 26.4 (C-5″), 25.9 (C-4′), 25.6 (C-8″), 21.8 (C-1″), 21.5 (C-1′), 17.9 (C-5′), 17.6 (C-10″) and 16.3 (C-9″).

Garcinone A (**7**): yellow powder; ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.03 (1H, s, 1-OH), 7.57 (1H, d, *J* = 2.8 Hz, H-8), 7.27 (1H, d, *J* = 3.2 Hz, H-6), 7.16 (1H, d, *J* = 2.8 Hz, H-5), 6.41 (1H, s, 3-OH), 5.20 (2H, m, H-2′ and H-2″), 1.81 (3H, s, H-5″), 1.78 (3H, s, H-5′), 1.70 (3H, s, H-4′) and 1.67 (3H, s, H-4″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.9 (C-9), 160.9 (C-3), 158.2 (C-1), 153.0 (C-4a), 152.1 (C-6), 150.4 (C-5a), 135.4 (C-3′), 133.8 (C-3″), 123.9 (C-7), 121.6 (C-2″), 121.4 (C-2), 120.6 (C-8a), 118.9 (C-5), 109.0 (C-8), 108.7 (C-2), 105.2 (C-4), 103.2 (C-9a), 25.9 (C-4′), 25.8 (C-4″), 21.6 (C-1″), 21.0 (C-1′) and 17.9 (C-5′ and C-5″).

Cochinchinone B (**8**): yellow powder; ^{1}H NMR (400 MHz, acetone-d_6) δ 13.34 (1H, s, 1-OH), 7.34 (1H, s, H-8), 6.40 (1H, s, H-4), 5.17 (1H, m, H-2″), 5.14 (1H, m, H-2′), 4.80 (2H, m, H-6″), 3.48 (1H, d, *J* = 7.2 Hz, H-1″), 3.21 (2H, d, *J* = 6.8 Hz, H-1′), 1.91 (2H, m, H-5″), 1.84 (2H, m, H-4″), 1.76 (3H, s, H-9″), 1.64 (3H, s, H-4′), 1.50 (3H, s, H-5′), 1.36 (3H, s, H-10″) and 1.34 (3H, s, H-8″); ^{13}C NMR (100 MHz, acetone-d_6) δ 179.8 (C-9), 162.0 (C-3), 159.3 (C-1), 155.7 (C-4a), 150.7 (C-6), 149.8 (C-5a), 142.0 (C-7), 135.1 (C-3″), 130.8 (C-3′), 130.5 (C-7″), 124.0 (C-6″), 122.6 (C-2′), 121.6 (C-2″), 115.7 (C-5), 112.6 (C-8a), 109.9 (C-2), 105.5 (C-8), 102.1 (C-9a), 93.0 (C-4), 39.5 (C-4″), 26.4 (C-5″), 25.0 (C-5′), 24.8 (C-10″), 22.1 (C-1″), 21.1 (C-1′), 17.0 (C-4′), 16.8 (C-8″) and 15.6 (C-9″).

Cratoxylumxanthone B (**9**): yellow powder; ^{1}H NMR (400 MHz, acetone-d_6) δ 13.09 (1H, s, 1-OH), 8.97 (1H, s, 7-OH), 7.56 (1H, s, H-8), 7.48 (1H, d, *J* = 8.8 Hz, H-5), 7.34 (1H, d, *J* = 7.6 Hz, H-6), 5.32 (1H, m, H-2″), 5.02 (1H, m, H-6″), 4.86 (1H, m, H-2′), 3.84 (1H, s, 3′-OH), 3.46 (2H, m, H-1″), 3.20 (2H, m, H-1′), 2.05 (2H, m, H-5″), 1.98 (2H, m, H-4″), 1.88 (3H, s, H-9″), 1.52 (3H, s, H-8″), 1.50 (3H, s, H-10″) and 1.29 (6H, s, H-4′ and H-5′); ^{13}C NMR (100 MHz, acetone-d_6) δ 180.6 (C-9), 165.9 (C-3), 155.7 (C-1), 154.9 (C-4a), 153.9 (C-7), 134.9 (C-3″), 130.7 (C-7″), 124.7 (C-6), 123.7 (C-6″), 121.9 (C-2″), 120.5 (C-8a), 118.3 (C-5), 108.3 (C-2 and C-8), 103.3 (C-9a), 101.7 (C-4), 91.6 (C-2′), 70.3 (C-3′), 39.5 (C-4″), 26.4 (C-5″ and C-1′), 25.3 (C-5′), 24.8 (C-8″), 24.2 (C-4′), 21.7 (C-1″), 16.8 (C-10″) and 15.5 (C-9″).

Cratoxylumxanthone C (**10**): yellow powder; ^{1}H NMR (400 MHz, acetone-d_6) δ 13.34 (1H, s, 1-OH), 7.40 (1H, s, H-8), 6.85 (1H, s, H-5), 5.12 (2H, m, H-2′ and H-2″), 4.88 (1H, m, H-6″), 3.44 (2H, d, J = 6.8 Hz, H-1″), 3.30 (2H, d, J = 6.8 Hz, H-1′), 1.94 (2H, m, H-5″), 1.85 (2H, m, H-4″), 1.77 (3H, s, H-9″), 1.64 (3H, s, H-5′), 1.52 (3H, s, H-4′), 1.38 (3H, s, H-10″) and 1.36 (3H, s, H-8″); ^{13}C NMR (100 MHz, acetone-d_6) δ 180.9 (C-9), 159.3 (C-3), 158.0 (C-1), 153.6 (C-5a), 152.8 (C-4a), 151.9 (C-6), 143.6 (C-7), 135.0 (C-3″), 131.4 (C-3′), 130.8 (C-7″), 124.0 (C-6″), 122.2 (C-2′ and C-2″), 113.0 (C-8a), 110.0 (C-2), 107.9 (C-8), 105.9 (C-4), 103.0 (C-5 and C-9a), 39.5 (C-4″), 26.4 (C-5″), 25.1 (C-5′), 24.5 (C-10″), 23.1 (C-1″), 22.2 (C-1′), 17.1 (C-4′), 16.8 (C-8″), 15.5 (C-9″).

Cratoxylumxanthone D (**11**): yellow powder; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.43 (1H, s, H-8), 6.84 (1H, s, H-5), 5.13 (1H, m, H-2″), 5.11 (1H, m, H-2′), 3.45 (2H, m, H-1′), 3.31 (2H, d, J = 6.8 Hz, H-1″), 1.77 (3H, s, H-5′), 1.67 (3H, s, H-5″) and 1.54 (6H, s, H-4′ and H-4″); ^{13}C NMR (100 MHz, acetone-d_6) δ 179.6 (C-9), 159.0 (C-2), 157.5 (C-4), 152.9 (C-4a and C-6), 151.5 (C-7), 142.5 (C-5a), 135.0 (C-1), 131.3 (C-3″), 130.8 (C-3′), 121.9 (C-2′ and C-2″), 112.5 (C-8a), 109.7 (C-3), 108.2 (C-8), 105.5 (C-9a), 102.5 (C-5), 24.8 (C-4′ and C-4″), 21.5 (C-1′), 21.2 (C-1″) and 16.8 (C-5′ and C-5″).

Cudratricusxanthone E (**12**): yellow powder ^{1}H NMR (400 MHz, acetone-d_6) δ 13.36 (1H, s, 1-OH), 7.43 (1H, s, H-8), 6.86 (1H, s, H-5), 5.10 (2H, m, H-2′ and H-2″), 3.44 (2H, d, J = 6.8 Hz, H-1″), 3.31 (2H, d, J = 7.2 Hz, H-1′), 1.76 (3H, s, H-5″), 1.68 (3H, s, H-5′), 1.53 (6H, s, H-4′ and H-4″).

Cratoxylumxanthone E (**13**): yellow powder; ^{1}H NMR (400 MHz, acetone-d_6) δ 13.64 (1H, s, 1-OH), 6.69 (1H, s, H-5), 6.34 (1H, s, H-4), 5.08 (1H, m, H-2′), 3.81 (3H, s, 3-OCH_3), 3.36 (2H, m, H-1″), 3.19 (1H, d, J = 6.0 Hz, H-1′), 1.72 (2H, m, H-2″) 1.64 (3H, s, H-5′), 1.51 (3H, s, H-4′) and 1.17 (6H, s, H-4″ and H-5″); ^{13}C NMR (100 MHz, acetone-d_6) δ 182.0 (C-9), 163.5 (C-3), 159.5 (C-1), 154.6 (C-4a), 153.0 (C-6), 148.0 (C-5a), 140.3 (C-7), 130.4 (C-3′), 130.0 (C-8), 123.5 (C-2′), 111.0 (C-8a), 110.7 (C-2), 103.5 (C-9a), 100.2 (C-5), 89.8 (C-4), 69.8 (C-3″), 55.5 (3-OCH_3), 43.1 (C-2″), 24.6 (C-4″), 24.4 (C-4′ and C-5″), 21.6 (C-1″), 21.0 (C-1′) and 17.2 (C-5′).

Cratoxylumxanthone F (**14**): yellow powder; ^{1}H NMR (400 MHz, acetone-d_6) δ 6.65 (1H, s, H-5), 6.34 (1H, s, H-4), 5.10 (1H, m, H-2′), 4.69 (1H, m, H-2″), 3.84 (3H, s, 1-OCH_3), 3.62 (2H, m, H-1″), 3.20 (2H, d, J = 6.8 Hz, H-1′), 1.67 (3H, s, H-5′), 1.52 (3H, s, H-4′), 1.20 (3H, s, H-5″) and 1.15 (3H, s, H-4″); ^{13}C NMR (100 MHz, acetone-d_6) δ 180.2 (C-9), 163.7 (C-1), 157.0 (C-3), 156.0 (C-4a), 152.6 (C-6), 148.7 (C-5a), 145.2 (C-7), 130.7 (C-3′), 126.1 (C-8a), 122.3 (C-2′), 111.0 (C-8), 110.9 (C-2), 103.7 (C-9a), 102.4 (C-5), 91.0 (C-2″), 89.9 (C-4), 70.0 (C-3″), 55.7 (1-OCH_3), 32.2 (C-1″), 25.2 (C-5′), 24.5 (C-4″ and C-5″), 20.9 (C-1′) and 17.7 (C-4′).

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CHAPTER III

ANTIOXIDANT ACTIVITY OF ISOLATED COMPOUNDS FROM *Cratoxylum cochinchinense* STEMS

3.1 Antioxidant Activity of Crude extracts

The hexane and dichloromethane crude extracts of *C. cochinchinense* stems were preliminary evaluated using TLC autographic method for screening of antioxidants with 2,2-Diphenyl-1-picryhydrazyl (DPPH) which both crude extracts showed to have promising activity.

3.2 Antioxidant Activity of Isolated Compounds

The isolation and purification of crude extracts from *C. cochinchinense* stems led to the isolation of 14 xanthones including six new xanthones (**1**, **9-11**, and **13-14**). The antioxidant activity of all isolated xanthones determined by DPPH radical scavenging activity, superoxide radicals generated by xanthine/xanthine system and Lipid peroxidation inhibition, were all expressed as IC_{50} (mM) by using spectroscopic method. The biological activities results of all isolated xanthones are shown in **Table 3.1**.

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Table 3.1 Antioxidant activity of all isolated xanthones

Isolated compounds	**IC_{50} (mM)**	
	DPPH scavenging	**Lipid peroxidation**
Curcumin*	-	0.082
Ascorbic acid*	0.168	-
Cratoxylumxanthone A (**1**)	> 1	> 1
Dulcisxanthone B (**2**)	0.135	0.039
2-geranyl-1,3,7-trihydroxy-2,4-di(3-methoxyl-but-2-enyl)xanthone (**3**)	> 1	> 1
α-mangostin (**4**)	> 1	> 1
β-mangostin (**5**)	> 1	> 1
Cochinchinone A (**6**)	> 1	> 1
Garcinone A (**7**)	>1	> 1
Cochinchinone B (**8**)	0.117	0.130
Cratoxylumxanthone B (**9**)	> 1	> 1
Cratoxylumxanthone C (**10**)	0.030	0.013
Cratoxylumxanthone D (**11**)	0.100	0.180
Cudratricusxanthone E (**12**)	0.190	0.029
Cratoxylumxanthone E (**13**)	0.120	0.190
Cratoxylumxanthone F (**14**)	> 1	> 1

* Standard antioxidant

- Not determined

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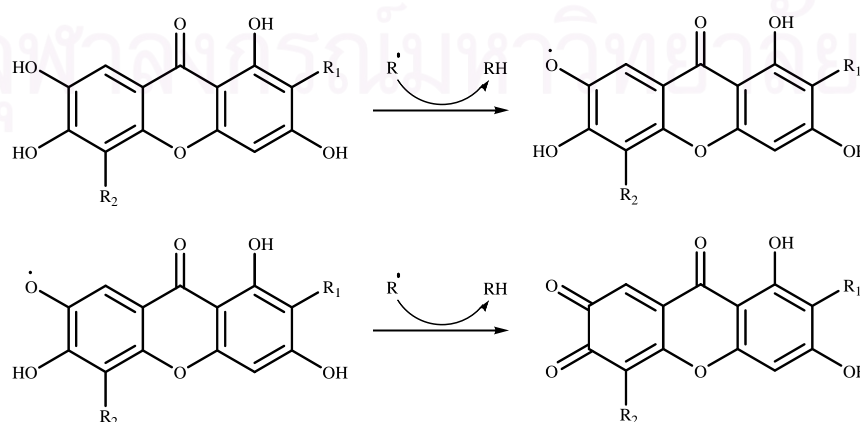
3.3 Discussion

3.3.1 DPPH radicals scavenging activity

Antioxidative activity of isolated xanthones were tested against various radical sources by UV-Vis spectroscopy. Anti-radical property of xanthones was examined with DPPH, which is widely used for assessing the ability of polyphenol to transfer labile H-atom to radicals. From ring B, all isolated xanthones from *C. cochinchinense* can be classified into catecholic (**2**, **8**, **10**, **11**-**13**), *O*-protected catecholic (**1**, **4**, **5**, **14**) and non-catecholic or 7-hydroxy xanthones (**3**, **6,** **7**, **9**).

The scavenging activity on DPPH results in **Table 3.1** indicated that compound **10** was the most potent antioxidant (IC_{50} = 0.030 mM). Compounds **11** (IC_{50} = 0.100 mM), **8** (IC_{50} = 0.117 mM), **13** (IC_{50} = 0.120 mM) and **2** (IC_{50} = 0.135 mM) also showed higher activity in DPPH scavenging than that of ascorbic acid (IC_{50} = 0.168 mM), which was used as reference antioxidant. Thus, compound **12** (IC_{50} = 0.190 mM) showed slightly weaker than ascorbic acid. In addition, compounds **1**, **3**, **4**, **5**, **6**, **7**, **9** and **14** showed no activity (IC_{50} > 1 mM).

Xanthone, which has 6,7-dihydroxyl groups in ring B, exhibited strong free radical scavenging activity. The greater effectiveness of compounds was possible due to the presence *ortho*-dihydroxy groups which upon donating hydrogen radicals will give higher stability to their radical forms (Mahabusarakum *et al.*, 2006). It can be rationalized that *O*-protected catechol could not transfer to quinone, while vicinal dihydroxyl groups could transfer to quinone easily by releasing two electrons in **Scheme 3.1** (Lee *et al.*, 2005; Pietta *et al.*, 2000; Cai *et al.*, 2002).



Scheme 3.1 Scavenging of ROS ($R^{\bullet}$) by xanthones.

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Experimental data revealed that most catecholic xanthones (**10**, **11**, **8**, **13**, **2** and **12**) exhibited strong scavenging activity, while *O*-protected and non-catecholic xanthones (**1**, **3**, **4**, **5**, **6**, **7**, **9** and **14**) showed no activity. Catecholic xanthones polyphenols have been extensively exploited both because of their wide ranging pharmacological properties and also because they serve as important units for donating electrons. The catecholic group could also be oxidized in an enzymatic or a non-enzymatic manner to yield a quinone-methide type prooxidant which is responsible for cancer prevention and apoptosis (Lee *et al*., 2005).

3.3.2 Lipid peroxidation inhibition

Lipid peroxidation, the nonenzymatic autocatalytic interaction of polyunsaturated fatty acids (PUFA) with molecular oxygen, is a process typical of all biological systems. Products generated as a consequence of lipid peroxidation are involved in pathophysiological diseases such as cancer, atherosclerosis and aging (Phuwapraisirisan *et al*., 2006).

In order to clarify the active component of *C. cochinchinense*, the fourteen isolated xanthones were examined *in vitro*. From the Fe^{2+} ascorbic acid stimulated lipid peroxidation in rat brain homogenate (Chang *et al*., 1994).

The lipid peroxidation inhibition results in **Table 3.1**, showed that compound **10** (IC_{50} = 0.013 mM) was remarkable inhibitory of malondialdehyde (MDA) formation of lipid peroxidation. Compound **10** showed highest activity and the activity decreased following order: **12**, **2**, **8**, **11** and **13** which revealed IC_{50} 0.029, 0.039, 0.130, 0.180 and 0.190 mM, respectively, whereas compounds **1**, **3**, **4**, **5**, **6**, **7**, **9** and **14** had no effect (IC_{50} > 1 mM). However, compounds **10, 12**, **2** and **8** showed higher active than that of curcumin (IC_{50} = 82 μM), which was used as reference antioxidant.

The antilipid peroxidation (ALP) of the xanthones was evaluated using rat brain homogenate as the test model. The test xanthones inhibited the Fe^{2+} ascorbic acid mediated lipid peroxidation of rat brain homogenate in a concentration dependent manner. The antioxidant activities of test xanthones were related to the number of free phenolic hydroxyls present in them. In addition, the results indicated that, the presence of catechol moiety enhances the antioxidant activity of polyphenolic which has the better electron-donating properties (Patro *et al*., 2005).

From the view of structure and activity relationship, it is note worthy that the inhibitory effects of catecholic (*ortho*-dihydroxy) xanthone is the most effective compounds and more active than reference antioxidant at the same concentration (Chang *et al*., 1994).

3.3.3 Superoxide radical scavenging activity

The gallic acid showed an activity to scavage superoxide radicals at IC_{50} = 0.24 mM. All isolated xanthones from *C. cochinchinense* exhibited no activity because all of these compounds can be formed with NBT and then precipitated in aqueous test media and its interference with the spectroscopic measurement. From these reasons, all of isolated xanthones could not be determined by this method.

From previous literature review of xanthone for superoxide radical scavenging activity, it was suggested to use *N*-(1-naphthyl)-ethylenediamine dihydrochloride for color reagent instead of NBT. It could also be explained that activity of superoxide radical is effected not only by converted H-atom abstraction (electron donation) but also by catalyzing the dismutation of $O_2^{\bullet -}$ (protonation effect) (Patro *et al*., 2005).

3.3.4 Inhibition of xanthine oxidase

The xanthine oxidase (XO) is the enzyme that catalyses the metabolism of hypoxanthine and xanthine into uric acid. It is responsible for the medical condition known as gout, which is caused by the deposition of uric acid in the joints leading to painful inflammation. Inhibition of XO leads to remission in gout (Chiang *et al*., 1994). XO also serves as an important biological source of oxygen-derived free radicals that contribute to oxidative damage to living tissues involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging. *In vitro* bioassays are used may be potentially useful for the treatment of gout or other XO induced diseases (Sweeney *et al*., 2001; Filha *et al*., 2006).

All isolated xanthones showed no activity of xanthine oxidase (IC_{50} > 1 mM) compared with allopurinol (IC_{50} = 0.089 mM), which powerful inhibitor of the xanthine oxidase and used as medication, was used as a positive control.

Xanthine oxidase further oxidase xanthine to produce uric acid, superoxide and hydrogen peroxide (Parks and Granger, 1986). Thus, xanthine oxidase inhibitors have been subjected to extensive by scrutiny with respect to antioxidant

potential. Allopurinol is oxidized by xanthine oxidase to oxypurinol, which binds to the active site of xanthine oxidase causing xanthine inhibition (Warner *et al*., 2004).

The superoxide radicals are normally the first ROS produced during cellular oxidation and their effects are usually magnified as they may generate other toxic ROSs including the hydroxyl radicals. Apart from oxygen metabolism, xanthine oxidase is one of their main biological sources *in vivo*. It is established that superoxide anion, directly or indirectly damages biomacromolecules. In addition, the uric acid produced during its generation from hypoxanthine oxidation promotes human gout. Scavenging of the radical and/or inhibition of xanthine oxidase would be a promising remedy for these diseases (Patro *et al*., 2005).

From previous reports, the structure-activity relationship of flavonoids as inhibitors of xanthine oxidase and as scavengers of the superoxide radicals, produced by the action of the enzyme xanthine oxidase, has been investigated. The hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 were essential for a high inhibitory activity on xanthine oxidase. For a high superoxide scavenging activity on the other hand, a hydroxyl group at C-3′ in ring B and at C-3 were essential (**Figure 3.1**). Flavones showed slightly higher inhibitory activity than flavonols (Cos *et al*., 1998).

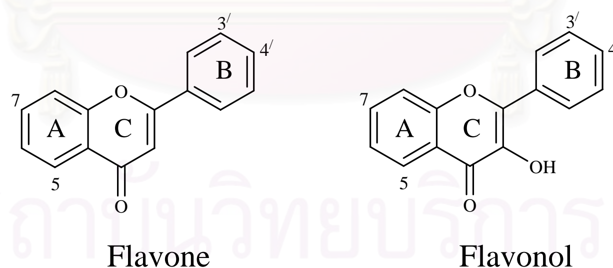


Figure 3.1 Structures of Flavone and Flavonol

3.4 Experimental Section

3.4.1 General Experimental Procedure

The pH values were measured by MP220 pH meter Mettler Toledo. UV-visible adsorption spectra were recorded on UV-2552PC UV-Vis spectrometer (Shimadzu, Kyoto, Japan), UV-spectrometer, microtiter plate reader, model sunrise (Tecan, Austria GmbH) and centrifuge model Chermle Z 230A.

3.4.2 DPPH radical scavenging activity

3.4.2.1 TLC autographic assay

Using 2,2-Diphenyl-1-picryhydrazyl (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 0.2 % DPPH solution in methanol. The plates are considered 30 minutes after spraying. Active compounds occur as yellow spots on a purple background (Hostettmann *et al.*, 1997).

3.4.2.2 Spectrophotometric assay

After isolation and purification, activities of pure compounds were quantified in this assay. Various concentrations of samples dissolved in methanolic solution (50 μL) were added to DPPH radical methanolic solution (0.3 mM, 200 μL). After 30 minutes incubation at room temperature in the dark, the absorbance was measured at 517 nm with a spectrophotometer. All tests were run in triplicate and averaged. The scavenging activity was evaluated from the decrease value of 517 nm absorption, which was calculated by the following equation. The activity was shown as IC_{50} values that donate the concentration of sample required scavenging 50 % DPPH free radicals (Yen and Hsieh, 1997).

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

3.4.3 Lipid peroxidation inhibition

The assay was conducted by analysis of rat brain lipid peroxidation using the previous methods (Hung and Yen, 2002) with modification. Rat brain homogenated (0.5 mg protein/mL) was indicated by protein content assay (Lowry's method). Test sample solution (100 μL) was incubated at 37 °C for 2 h

with rat brain homogenate (500 μL) and a mixture containing 10 mM KCl (100 μL), 0.05 mM ascorbic acid (100 μL), phosphate buffer pH 7.4 (100 μL) and 5 mM $FeSO_4$ (100 μL). After incubation, the reaction mixture was terminated by heat at 100 °C for 20 min. After the reaction mixture cool down, then add 3M HCl (500 μL) and 1% thiobarbituric acid (500 μL), respectively. The ratio of reaction mixture and saturated butanol was 1:1 and then centrifuged them at 3000 rpm for 5 min. The butanolic extract was detected at 532 nm for TBA-MDA formation. The percent inhibition of lipid peroxidation was calculated by from regression line.

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

3.4.4 Xanthine oxidase –related activity

3.4.4.1 Assay for scavenging of $O_2^{\bullet -}$ by xanthine oxidase

Superoxide anion radical was generated from xanthine-xanthine oxidase method (Okamura, 1994) with a slight modification. The reaction mixture consisted samples at various concentrations in DMSO (150 μL), 0.4 mM xanthine (400 μL), 0.24 mM nitroblue tetrazolium (NBT) (480 μL), xanthine oxidase (0.1 unit/mL, 100 μL) and 0.1 M phosphate buffer pH 8.0 (120 μL). After being incubated at 37 ºC for 30 minutes, the reaction was terminated by adding of 69 mM sodium dodecyl sulfate (SDS) (50 μL). 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.

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

3.4.4.2 Assay for inhibition activity against xanthine oxidase

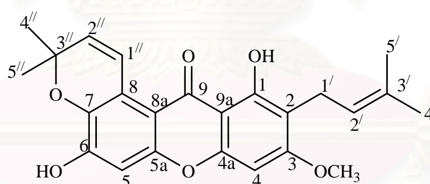
The method described by Schuldt (2004), for studying of xanthine oxidase inhibitory activity, the arise in the absorbance at 290 nm due to uric acid production was measured in the absence of nitroblue tetrazolium. Allopurinol, which is a drug for gout treatment, was used as a standard for this assay. The inhibitory activity was shown as percent inhibition, which was estimated from the following equation. The IC_{50} values were determined from regression line.

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

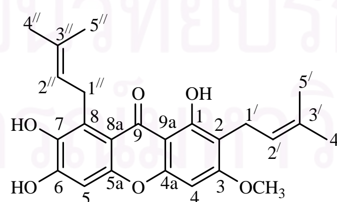
CHAPTER IV

CONCLUSION

The purifications crude hexane and dichloromethane extracts of *Cratoxylum cochinchinense* afforded fourteen xanthones which were six new compounds (**1**, **9**-**11**, **13**-**14**). The structures of all isolated xanthones were characterized by MS and NMR experiment as well as comparison with the previous reports. They were cratoxylumxanthone A (**1**), dulcisxanthone (**2**), 2-geranyl-1,3,7,-trihydroxy-4-(3-methylbut-2-enyl)xanthone (**3**), α-mangostin (**4**), β-mangostin (**5**), cochinchinone A (**6**), garcinone A (**7**), cochinchinone B (**8**), cratoxylumxanthone B (**9**), cratoxylumxanthone C (**10**), cratoxylumxanthone D (**11**), cudratricusxanthone E (**12**), cratoxylumxanthone E (**13**) and cratoxylumxanthone F (**14**). In addition, compound **3** was a major component of *C. cochinchinense* stems (0.28 % of both crude extracts). The structures of these isolated xanthones are shown as followed.



Cratoxylumxanthone A (**1**)



Dulcisxanthone B (**2**)

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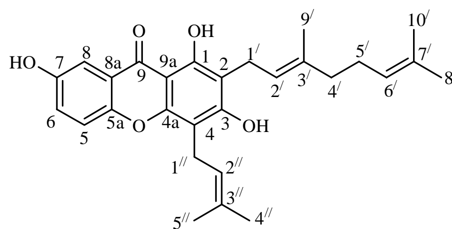
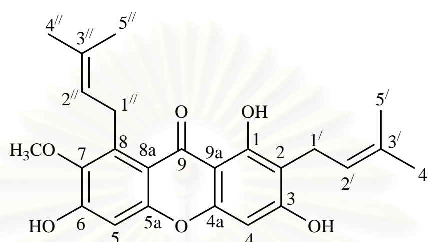
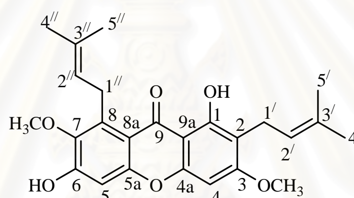


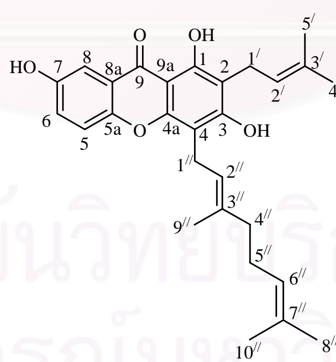
2-geranyl-1,3,7-trihydroxy-4-(3-methylbut-2-enyl)xanthone (**3**)



α-Mangostin (**4**)



β-Mangostin (**5**)



Cochinchinone A (**6**)

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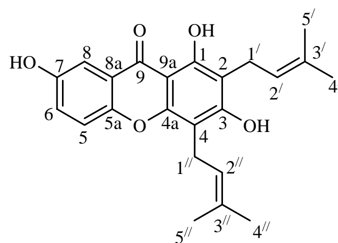
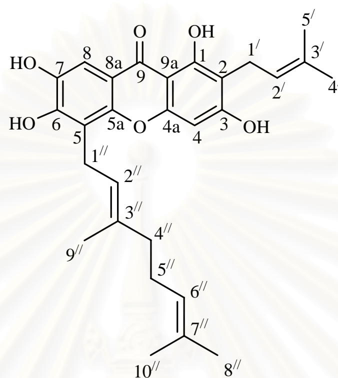
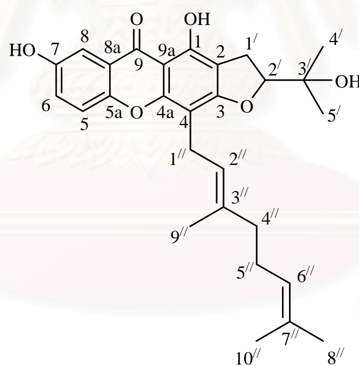


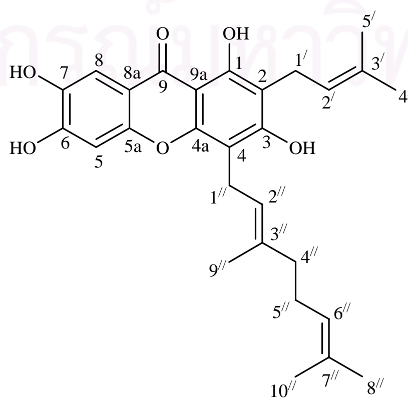
Garcinone A (**7**)



Cochinchinone B (**8**)



Cratoxylumxanthone B (**9**)



Cratoxylumxanthone C (**10**)

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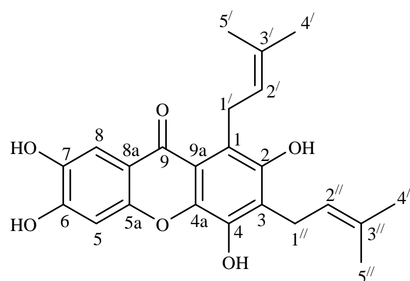
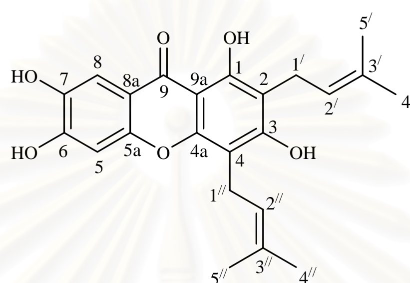
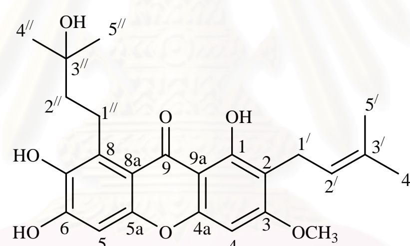


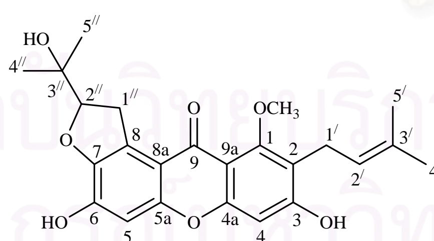
Cratoxylumxanthone D (**11**)



Cudratricusxanthone E (**12**)



Cratoxylumxanthone E (**13**)



Cratoxylumxanthone F (**14**)

The DPPH radical scavenging activity indicated that compound **10** (IC_{50} = 0.03 mM) showed the most potent activity followed by **11** (IC_{50} = 0.10 mM), **8** (IC_{50} = 0.117 mM), **13** (IC_{50} = 0.12 mM), **2** (IC_{50} = 0.135 mM) and **12** (IC_{50} = 0.19 mM) respectively, whereas compound **1**, **3**, **4**, **5**, **6**, **7**, **9** and **14** showed no

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activity (IC_{50} > 1 mM). Moreover, compound **10** (IC_{50} = 13 μM) also showed the most effect on lipid peroxidation inhibition activity followed by **12** (IC_{50} = 29 μM), **2** (IC_{50} = 39 μM), **8** (IC_{50} = 130 μM), **11** (IC_{50} = 180 μM) and **13** (IC_{50} = 190 μM) whereas, compounds **1**, **3, 4**, **5**, **6**, **7**, **9** and **14** showed weak activity (IC_{50} > 1 mM). While, all of isolated xanthones showed inactive of superoxide scavenging activity and inhibition of xanthine oxidase activity.

Compounds **10**, **11** and **13** are new xanthones which obtained promising DPPH radical scavenging activity and lipid peroxidation inhibition activity. Compounds **4** and **5** showed antibacterial activity (Boonnak *et al*., 2006). Moreover, compound **5** also showed antimalarial activity (Laphookhieo *et al*., 2006). Compound **12** showed significant inhibitory effects on human tumor cell lines (HCT-116, SMMC-7721, SCG 7901 and BGC-823) (Zou *et al*., 2004a).

In summary, Cratoxylumxanthone C (**10**) revealed the most potent and higher than reference antioxidant (ascorbic acid and curcumin) in both DPPH radical scavenging activity and lipid peroxidation inhibition. In addition, dulcisxanthone B (**2**), cochinchinone B (**8**), cratoxylumxanthone D (**11**), cudratricusxanthone E (**12**) and cratoxylumxanthone E (**13**) were also highly active. On the other hand, the other xanthones showed no activity. These results suggested that the presence of *ortho*-dihydroxy moiety of C-6 and C-7 (catecholic group) on ring B of xanthone nucleus enhanced scavenging activity more than other xanthones which, without *ortho*-dihydroxy moiety. For the role of a superoxide scavenging activity and xanthine oxidase inhibitor, all isolated xanthones showed inactive.

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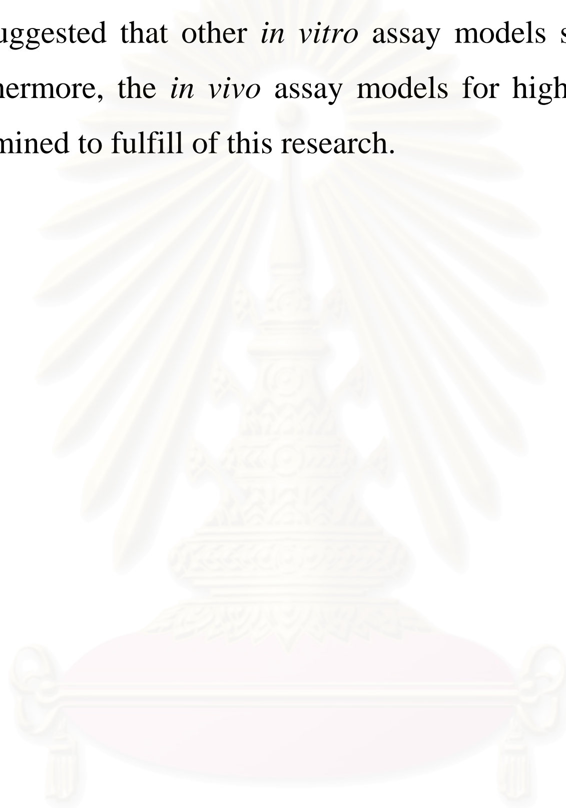
Proposal for Further Work

According to xanthones can be reacted with NBT, all isolated xanthones from *C. cochinchinense* failed to exhibit superoxide radicals. In order to solve this problem, using other appropriate color reagent instead of NBT such as *N*-(1-naphthyl)-ethylenediamine dihydrochloride, which was not effect to xanthones might be successfully evaluated for superoxide radicals scavenging activity.

Concerning the antioxidant activity, this research determined *in vitro* assay. It was suggested that other *in vitro* assay models should be additionally examines. Furthermore, the *in vivo* assay models for high effective compounds should be determined to fulfill of this research.



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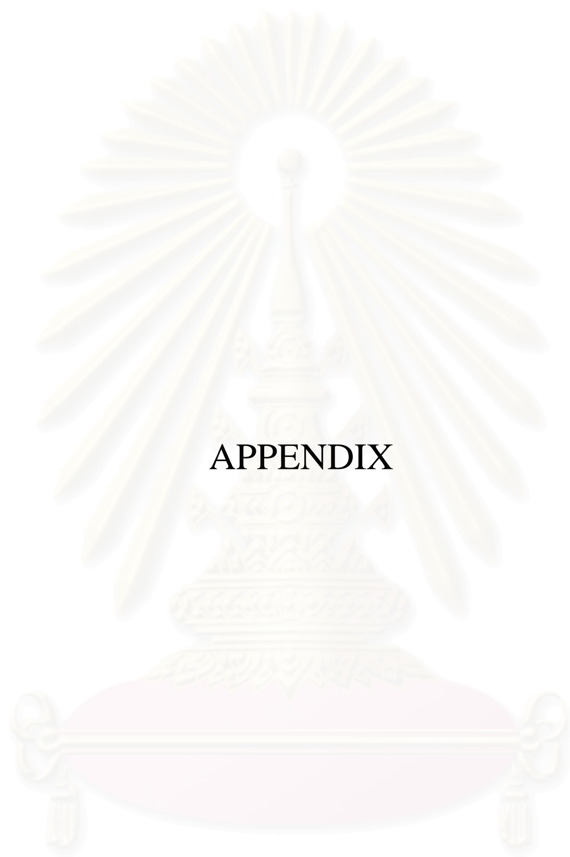

APPENDIX

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Appendix

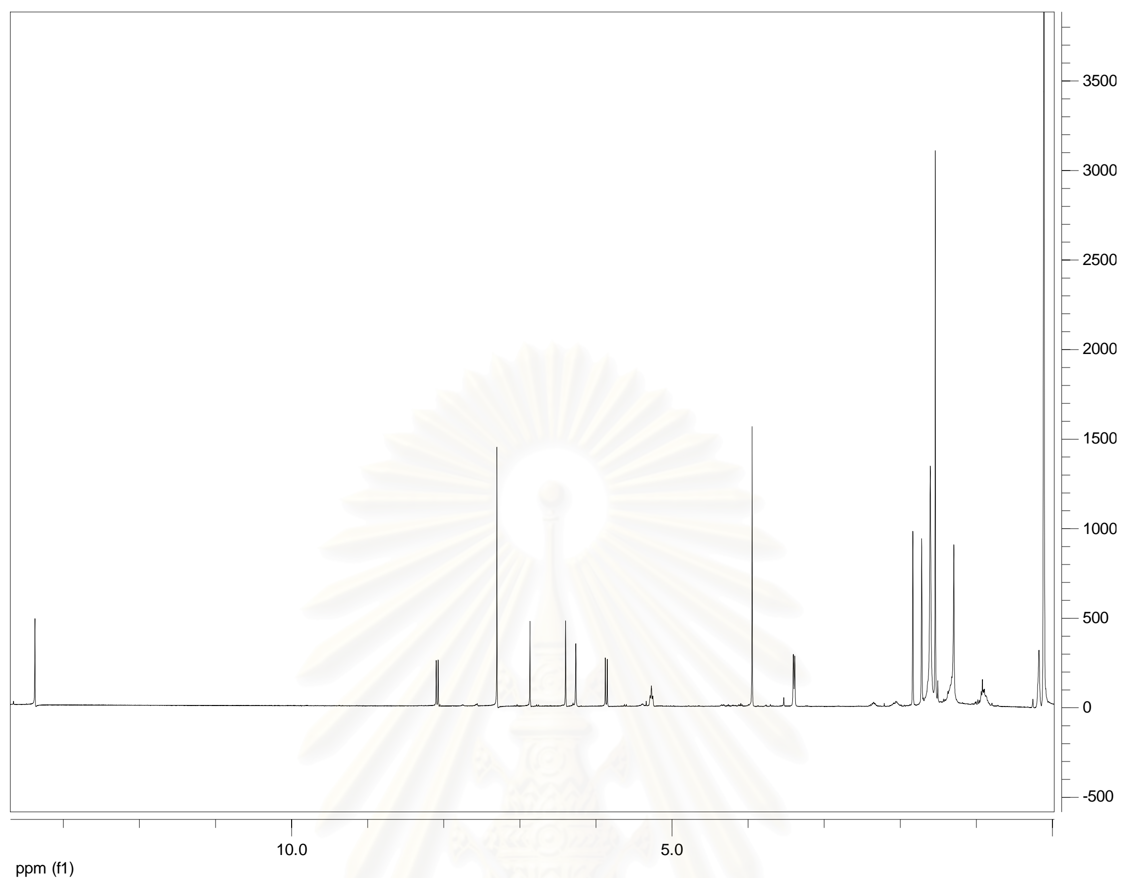

Figure 1 ^{1}H NMR spectrum ($CDCl_3$) of cratoxylumxanthone A (**1**)

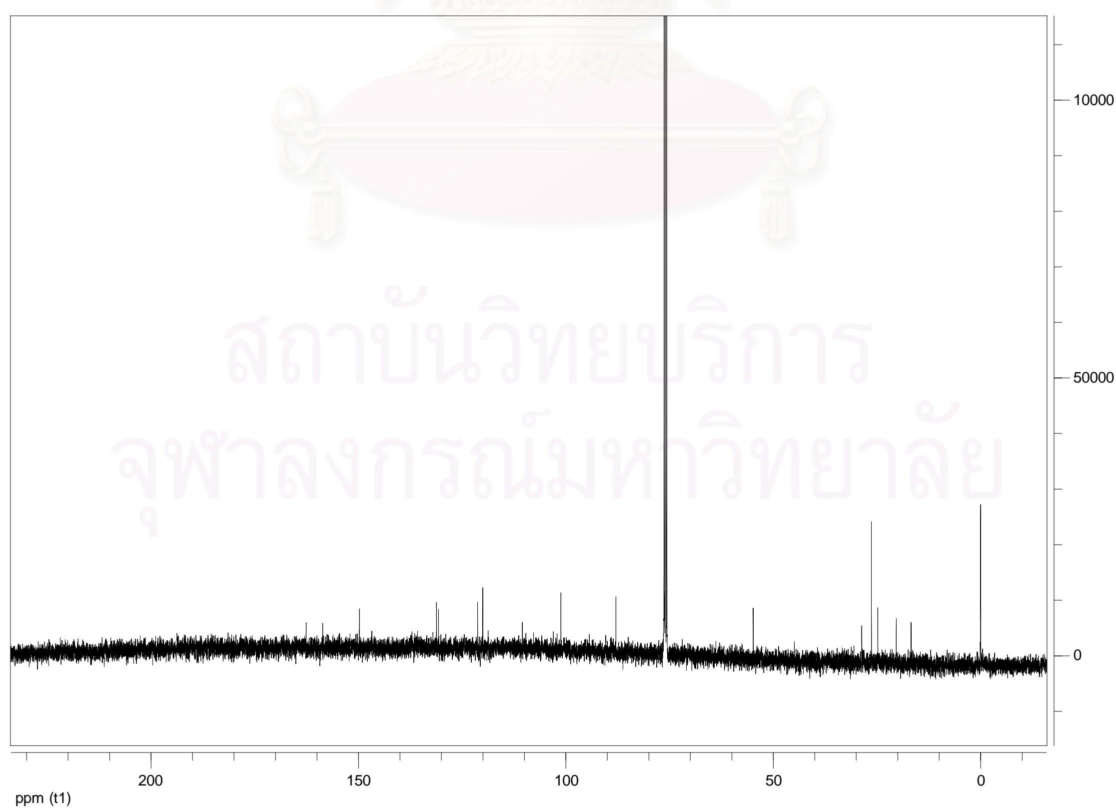

Figure 2 The ^{13}C NMR ($CDCl_3$) of cratoxylumxanthone A (**1**)

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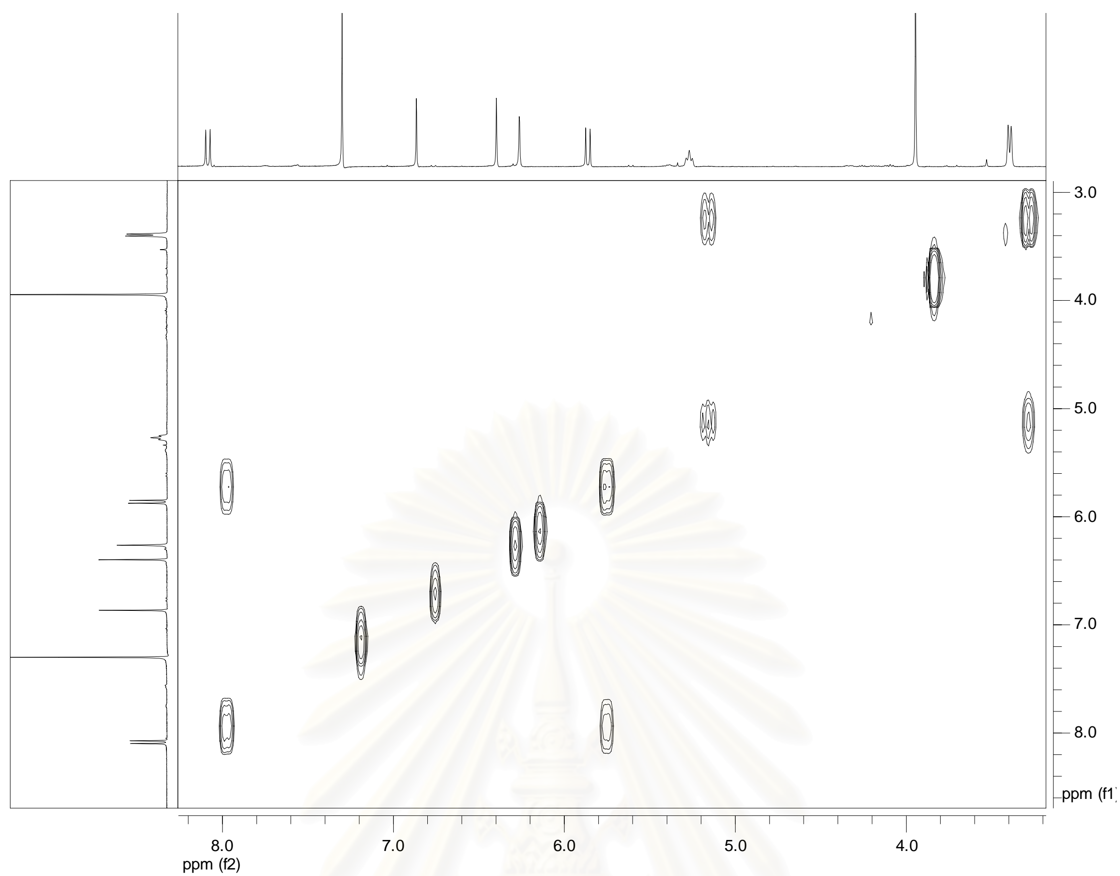

Figure 3 The COSY spectrum ($CDCl_3$) of cratoxylumxanthone A (**1**)

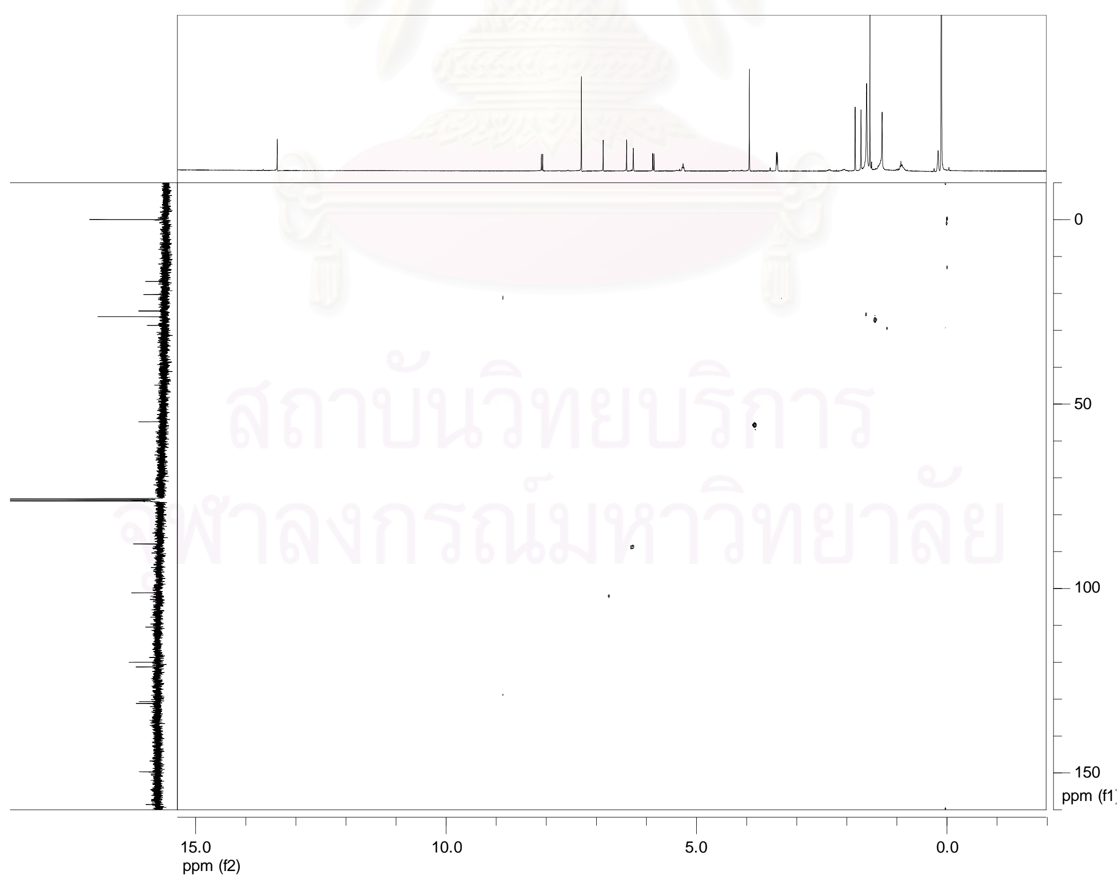

Figure 4 The HSQC spectrum ($CDCl_3$) of cratoxylumxanthone A (**1**)

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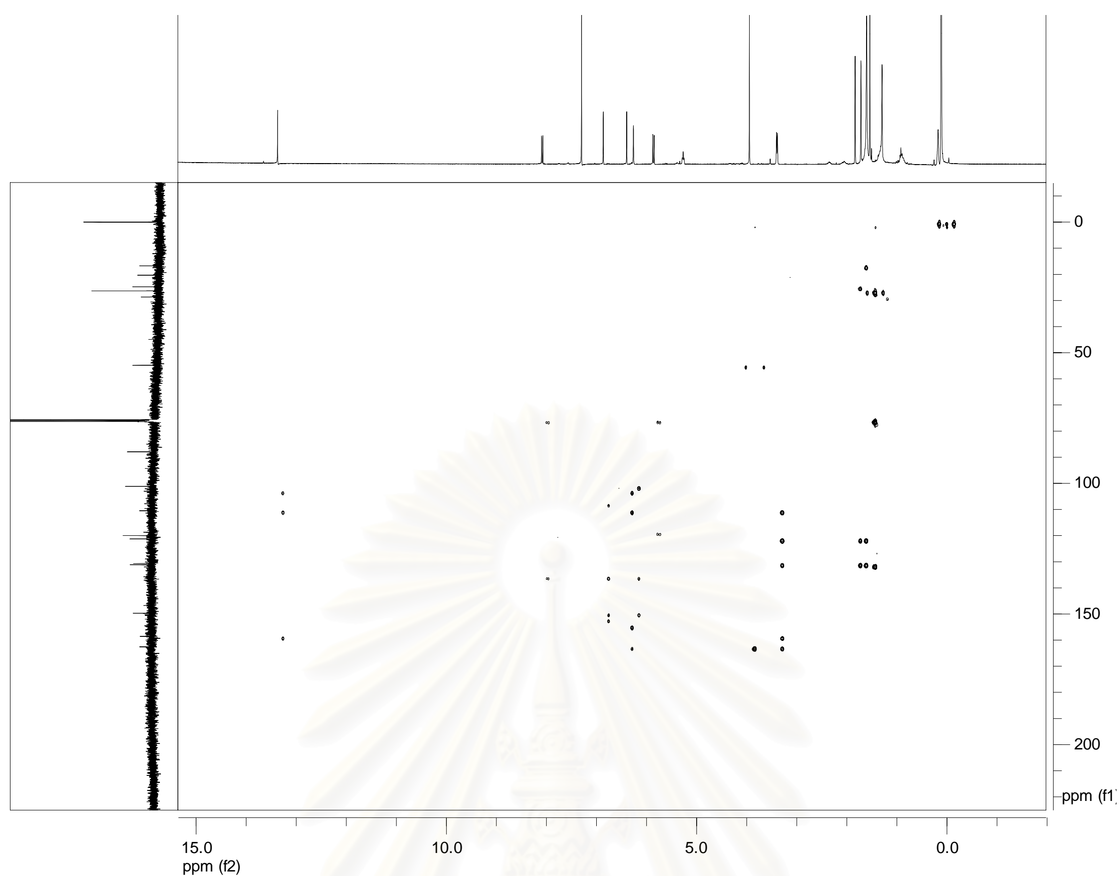

Figure 5 The HMBC spectrum ($CDCl_3$) of cratoxylumxanthone A (**1**)

Figure 6 The High resolution mass spectrum of cratoxylumxanthone A (**1**)

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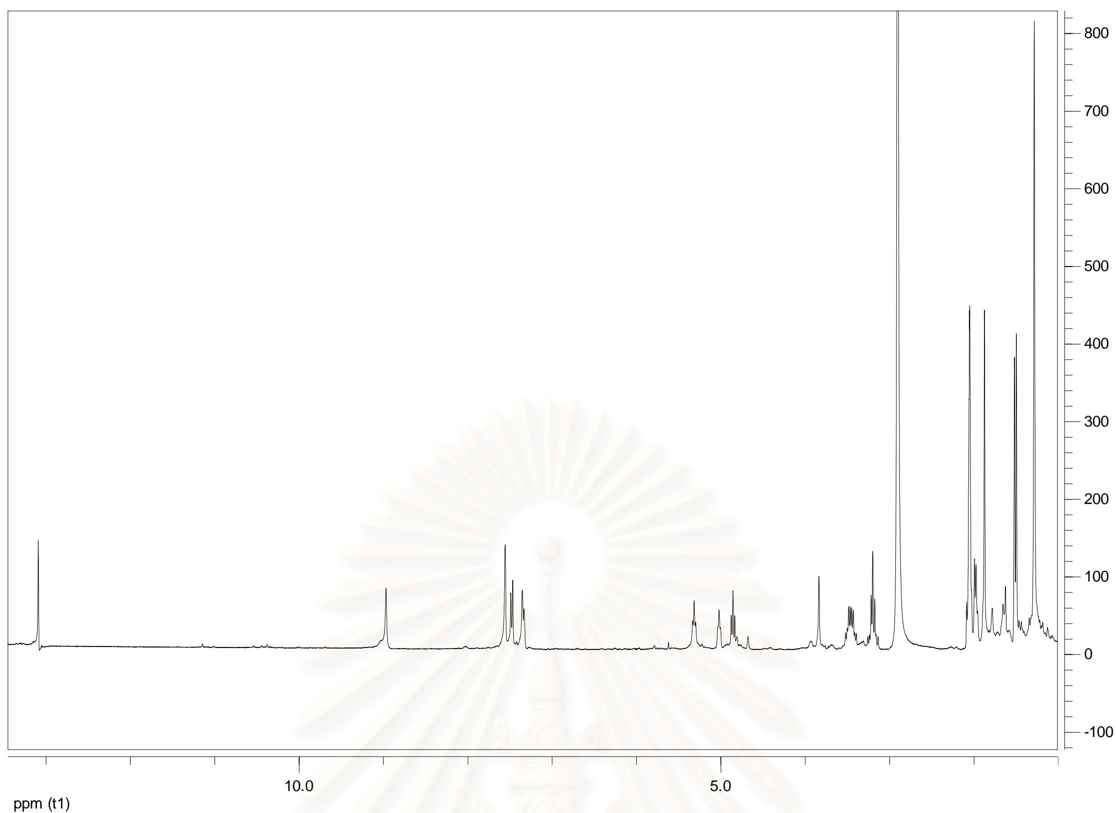

Figure 7 The ^{1}H NMR spectrum (acetone-d_6) of cratoxylumxanthone B (**9**)

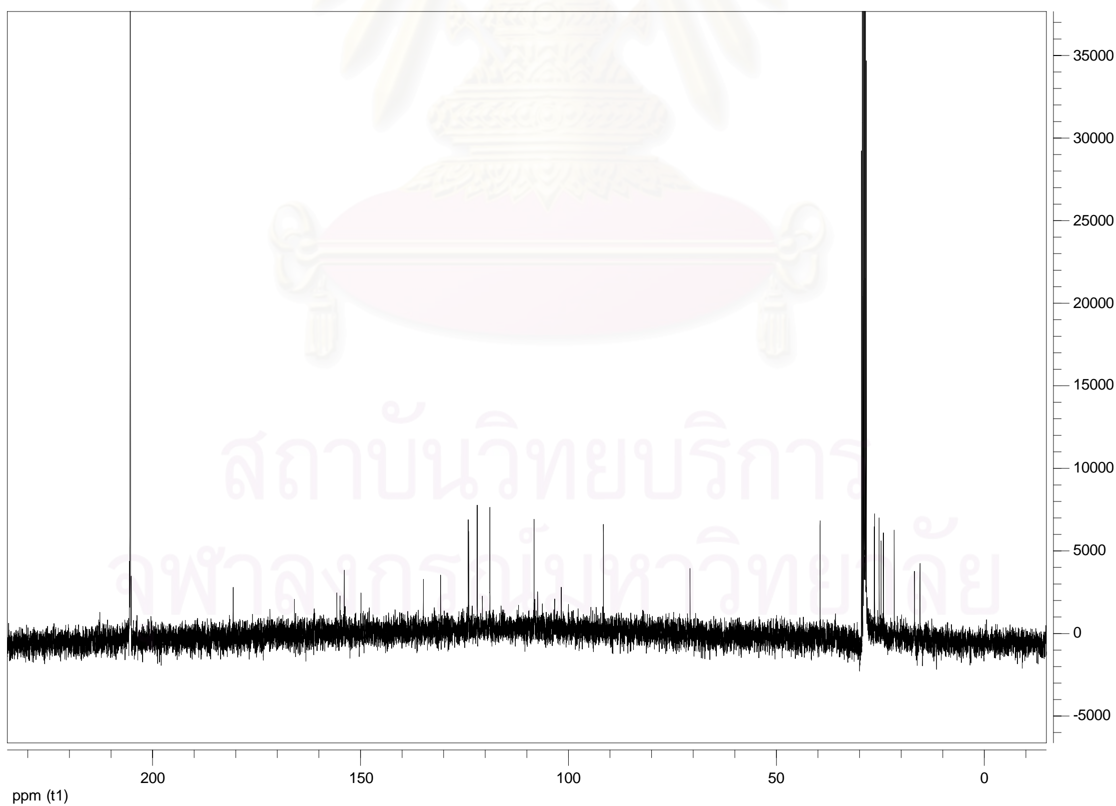

Figure 8 The ^{13}C NMR spectrum (acetone-d_6) of cratoxylumxanthone B (**9**)

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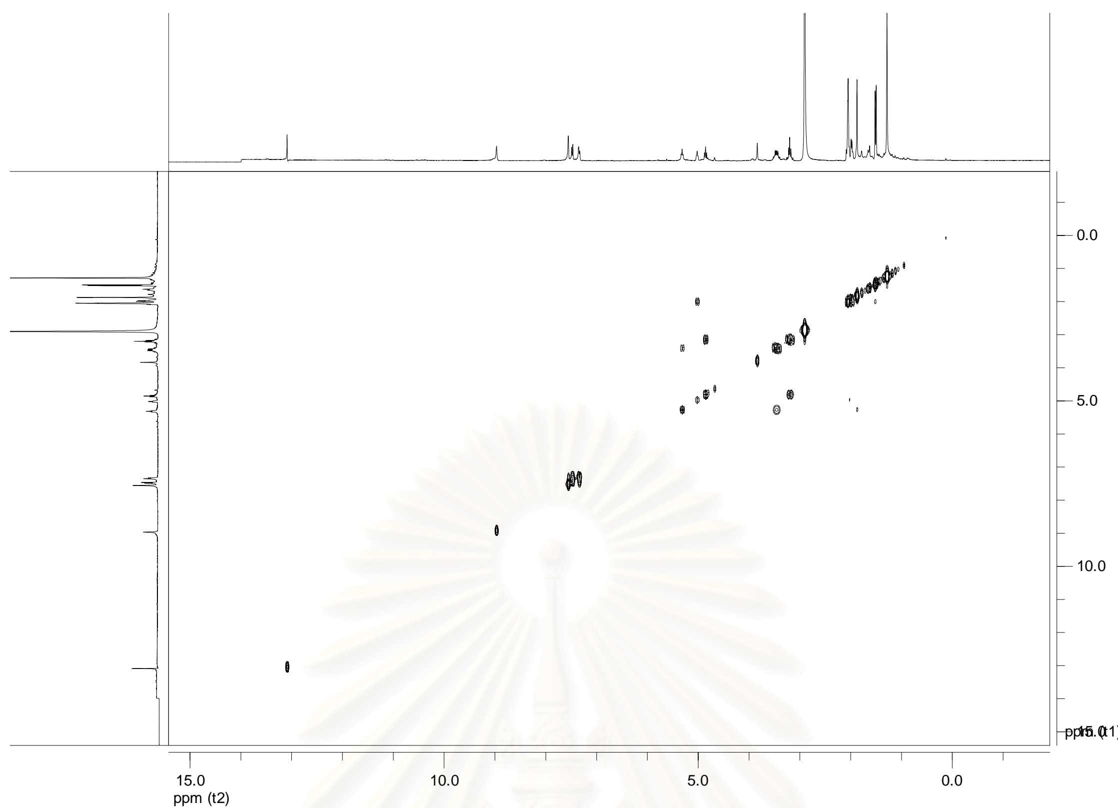

Figure 9 The COSY spectrum (acetone-d_6) of cratoxylumxanthone B (**9**)

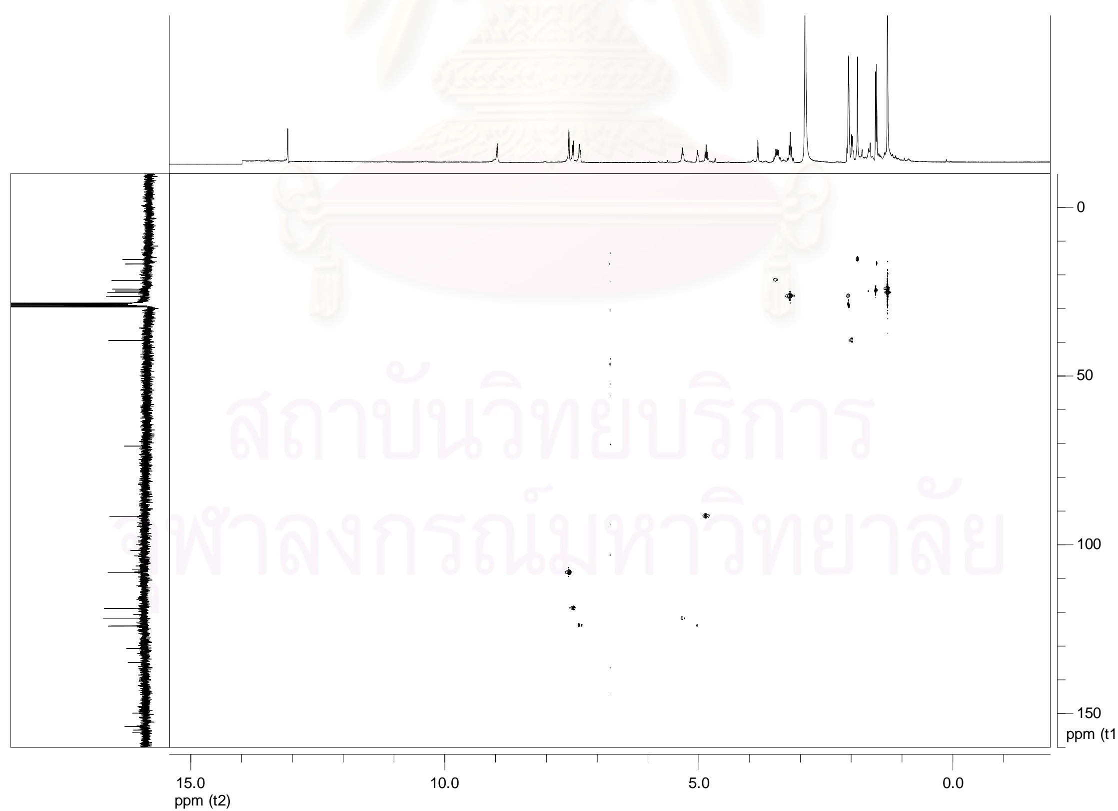

Figure 10 The HSQC spectrum (acetone-d_6) of cratoxylumxanthone B (**9**)

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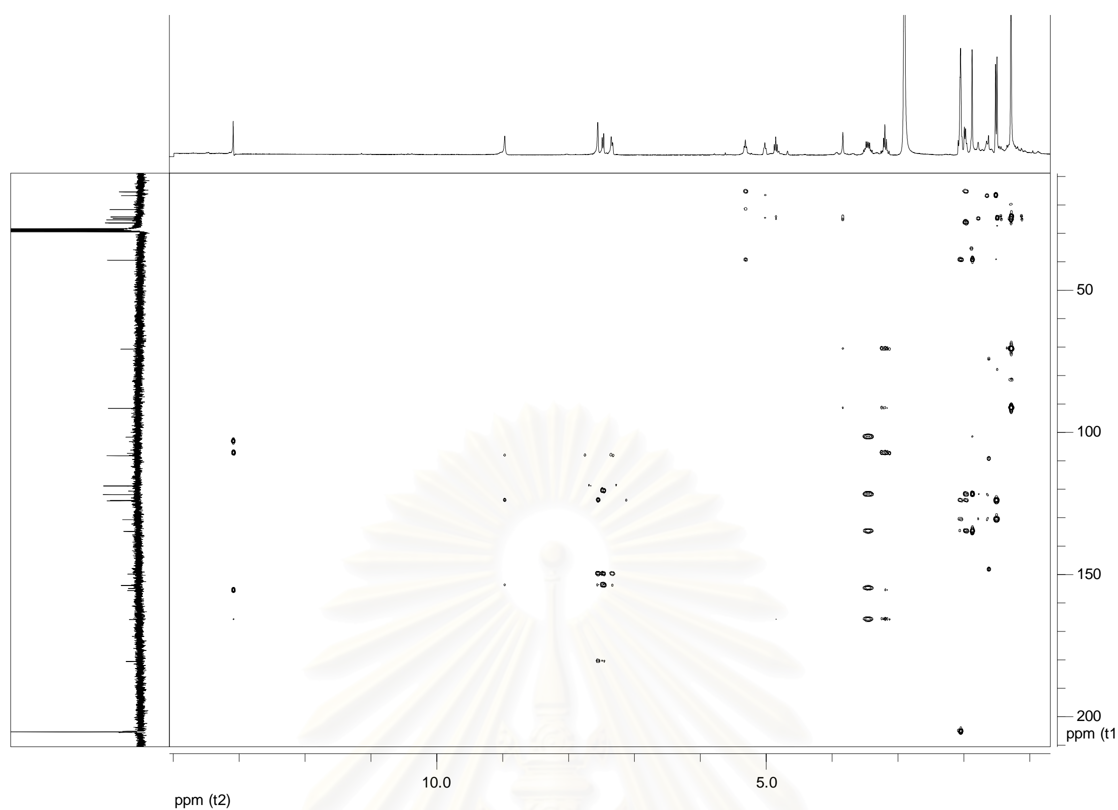

Figure 11 The HMBC spectrum (acetone-d_6) of cratoxylumxanthone B (**9**)

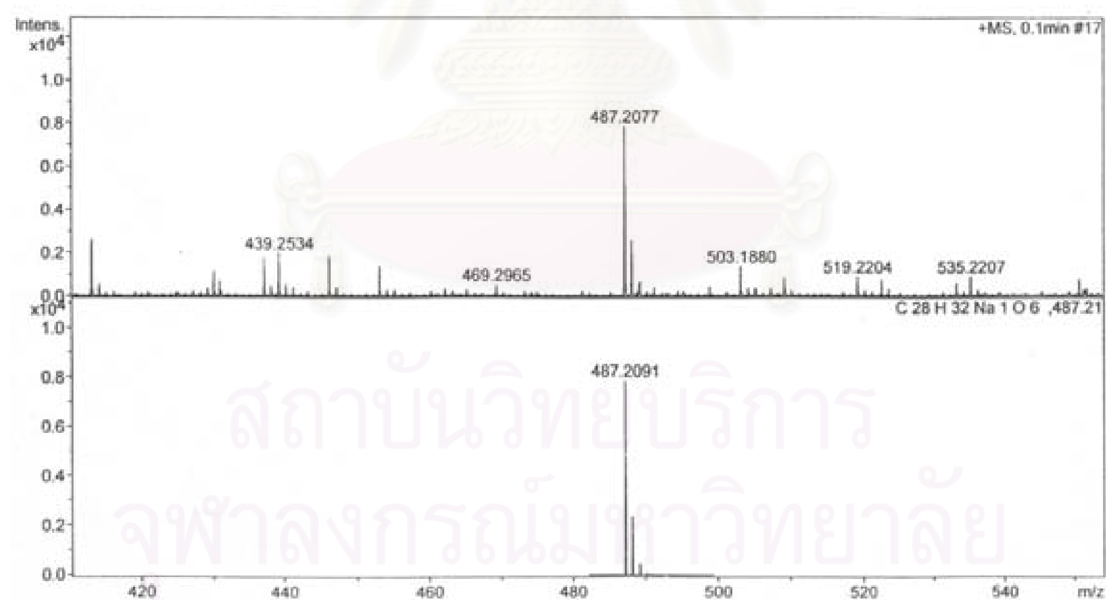

Figure 12 The High resolution mass spectrum of cratoxylumxanthone B (**9**)

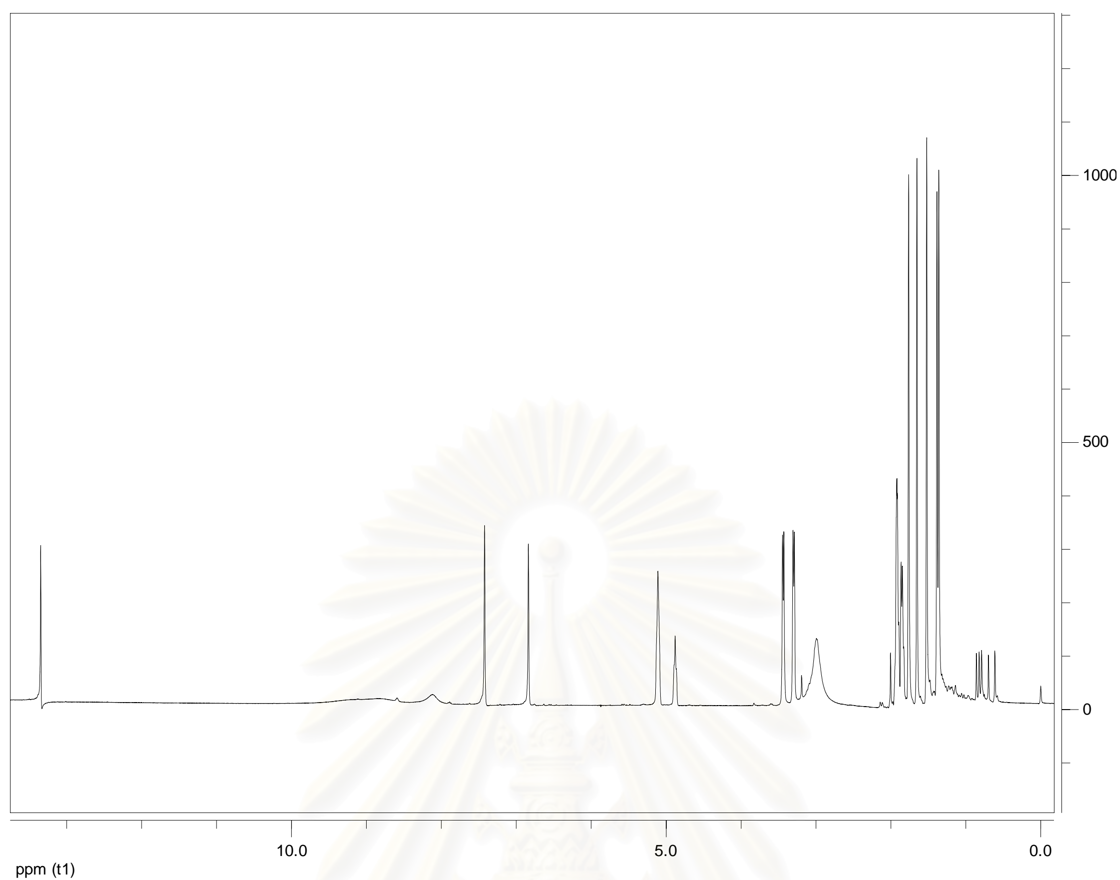

Figure 13 The ^{1}H NMR spectrum (acetone-d_6) of cratoxylumxanthone C (**10**)

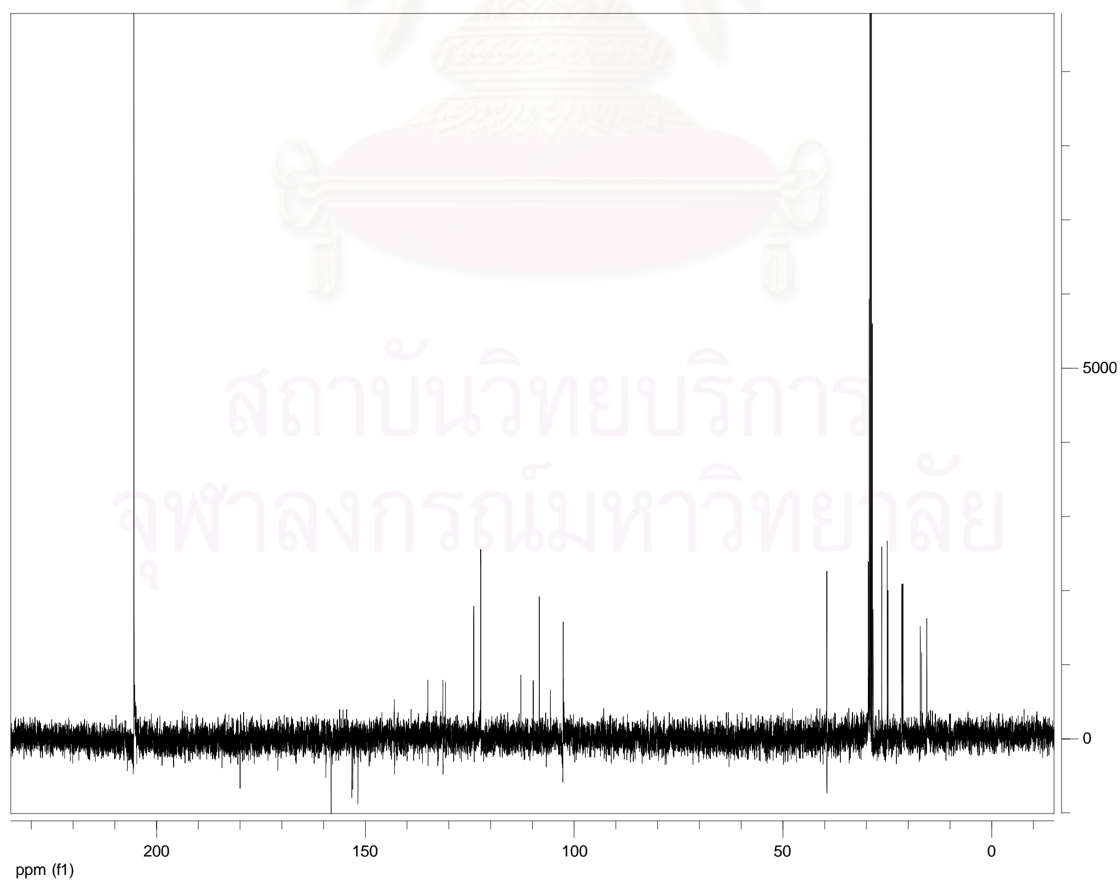

Figure 14 The ^{13}C NMR spectrum (acetone-d_6) of cratoxylumxanthone C (**10**)

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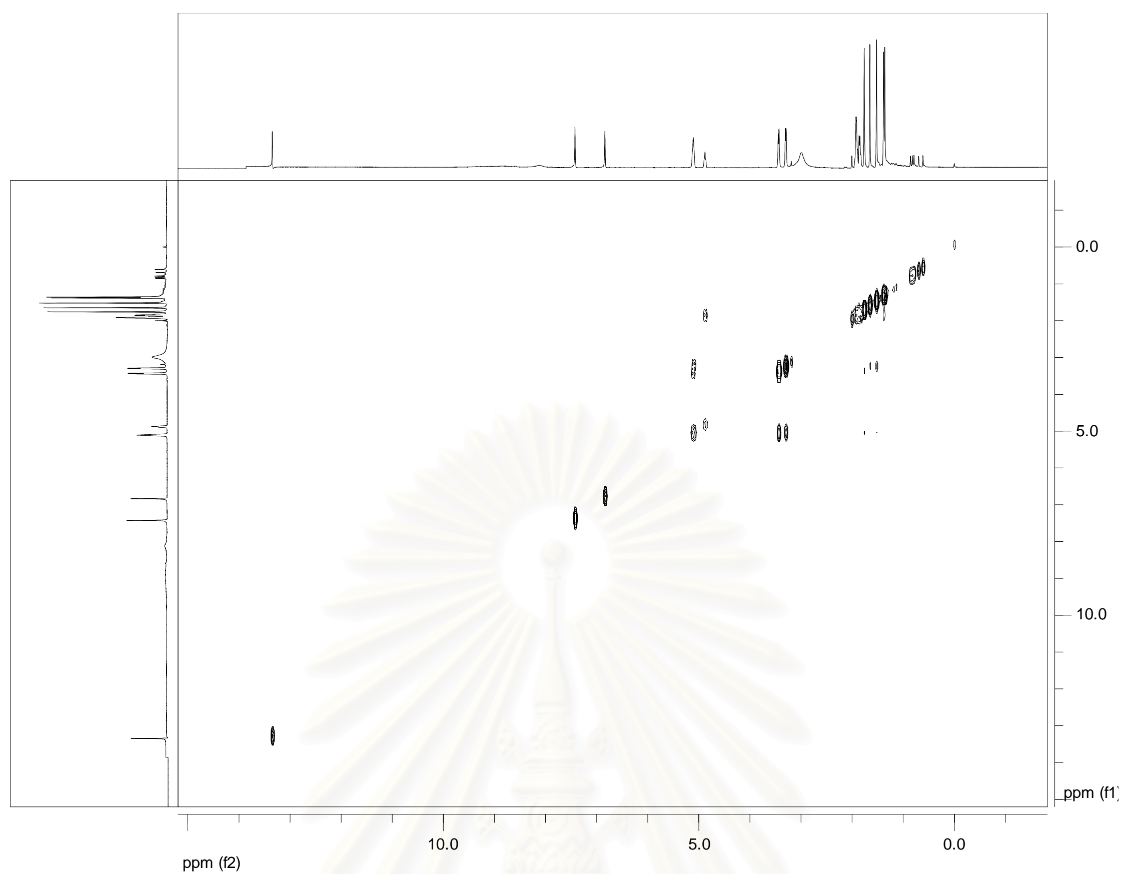

Figure 15 The COSY spectrum (acetone-d_6) of cratoxylumxanthone C (**10**)

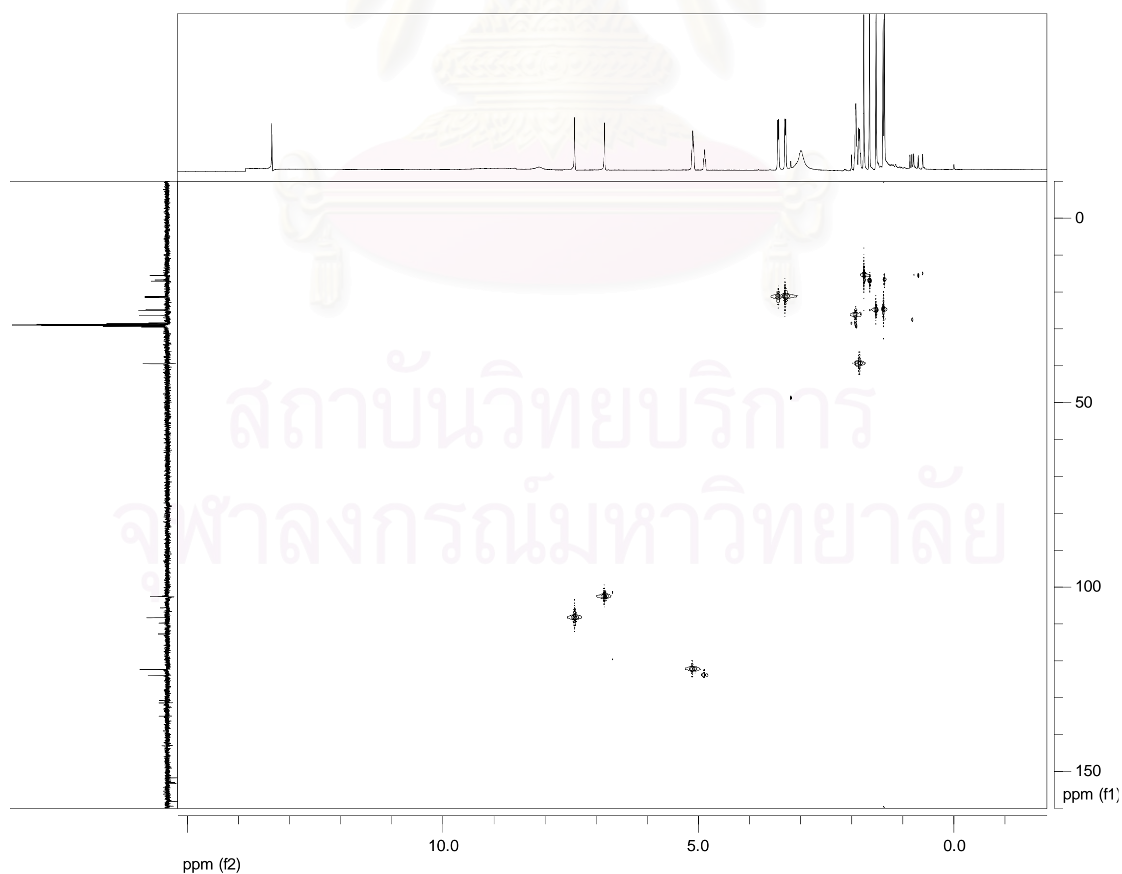

Figure 16 The HSQC spectrum (acetone-d_6) of cratoxylumxanthone C (**10**)

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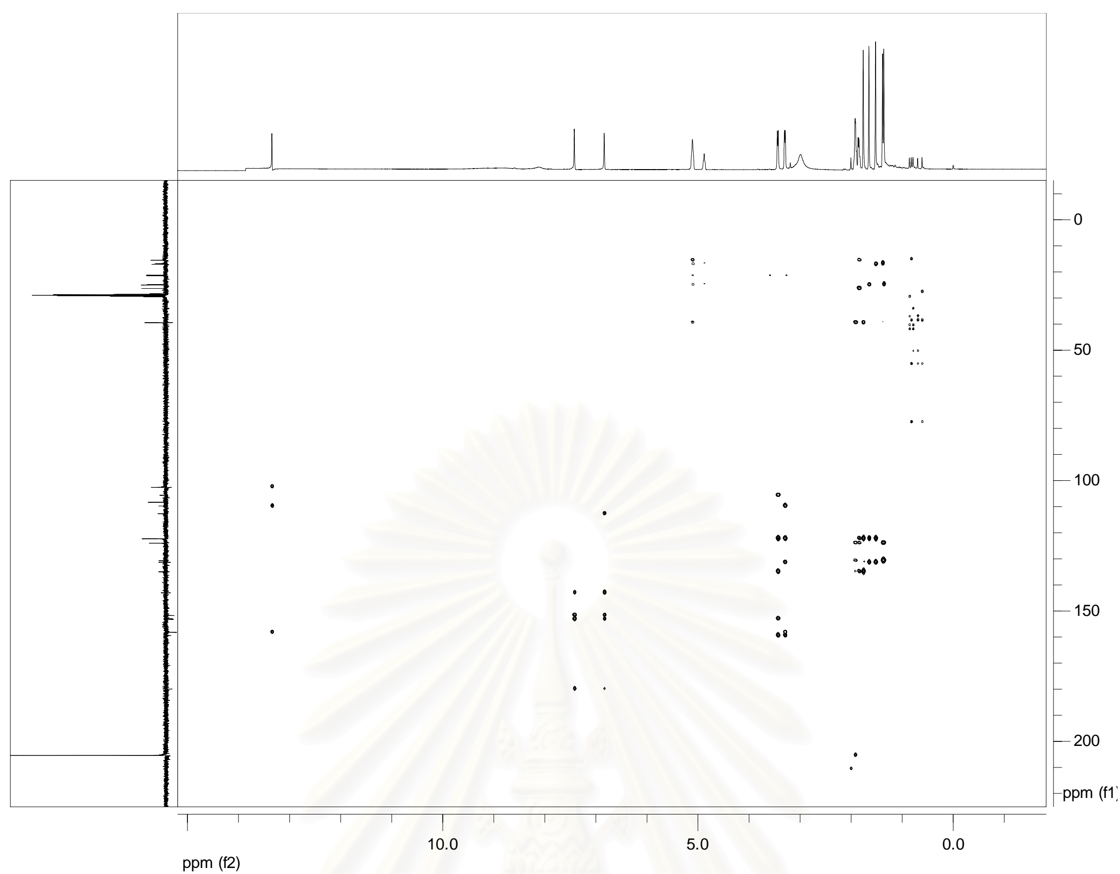

Figure 17 The HMBC spectrum (acetone-d_6) of cratoxylumxanthone C (**10**)

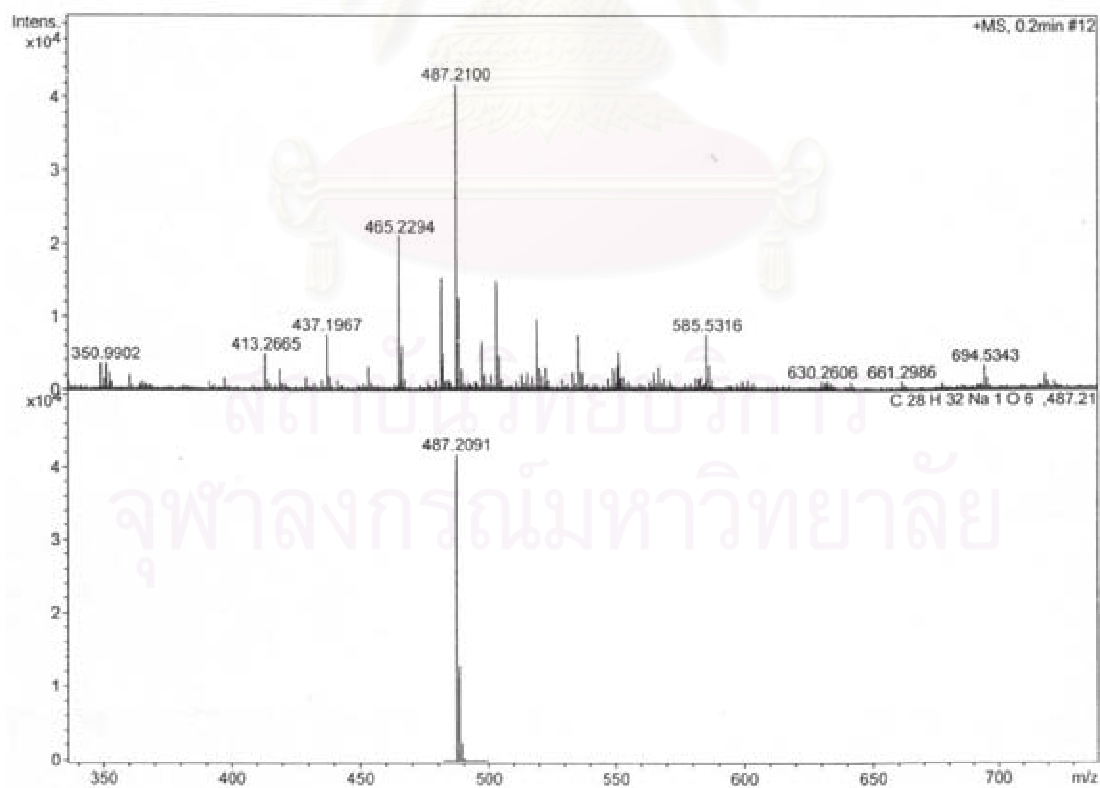

Figure 18 The High resolution mass spectrum of cratoxylumxanthone C (**10**)

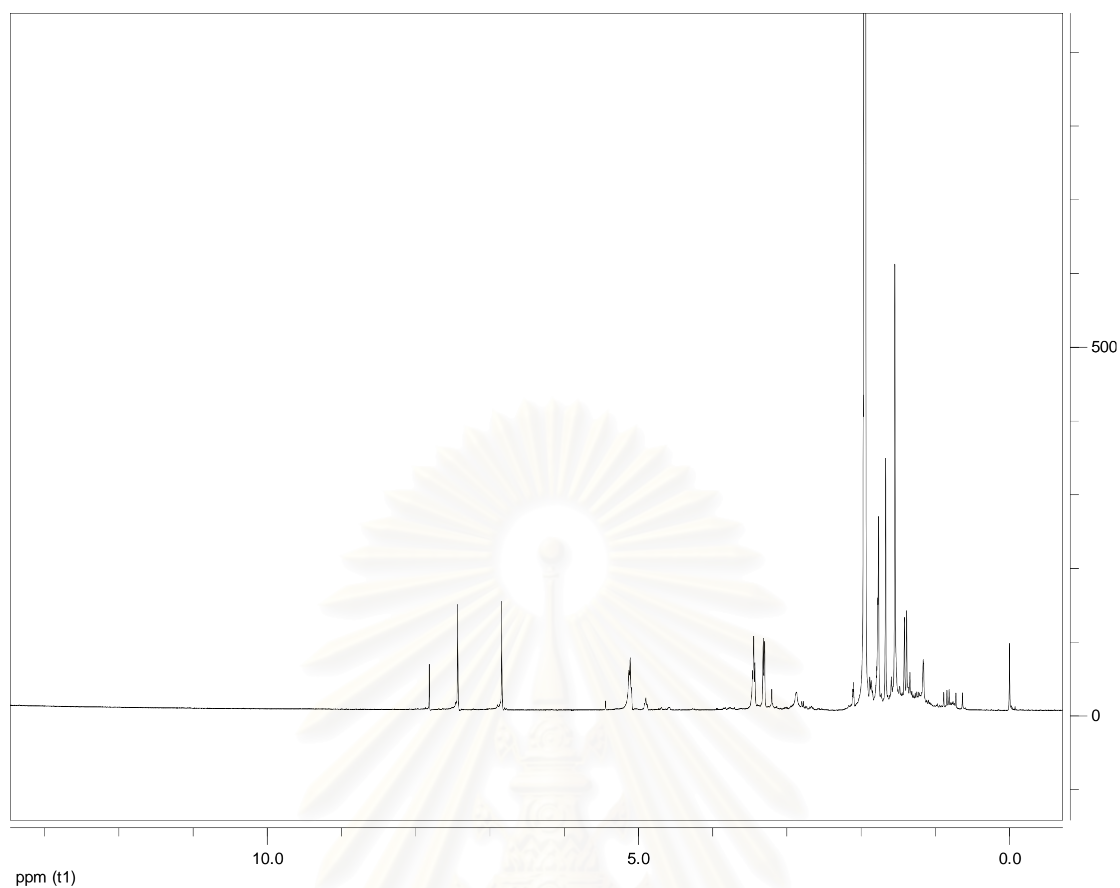

Figure 19 The ^{1}H NMR spectrum (acetone-d_6) of cratoxylumxanthone D (**11**)

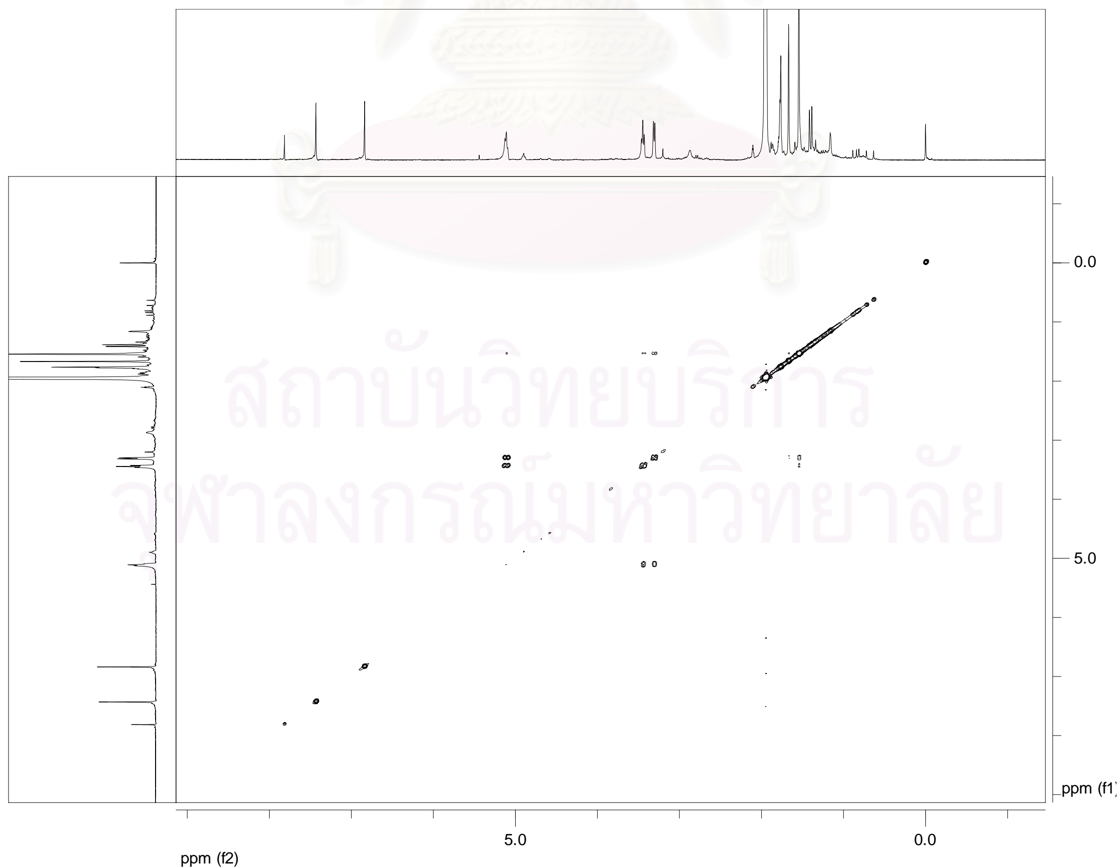

Figure 20 The COSY spectrum (acetone-d_6) of cratoxylumxanthone D (**11**)

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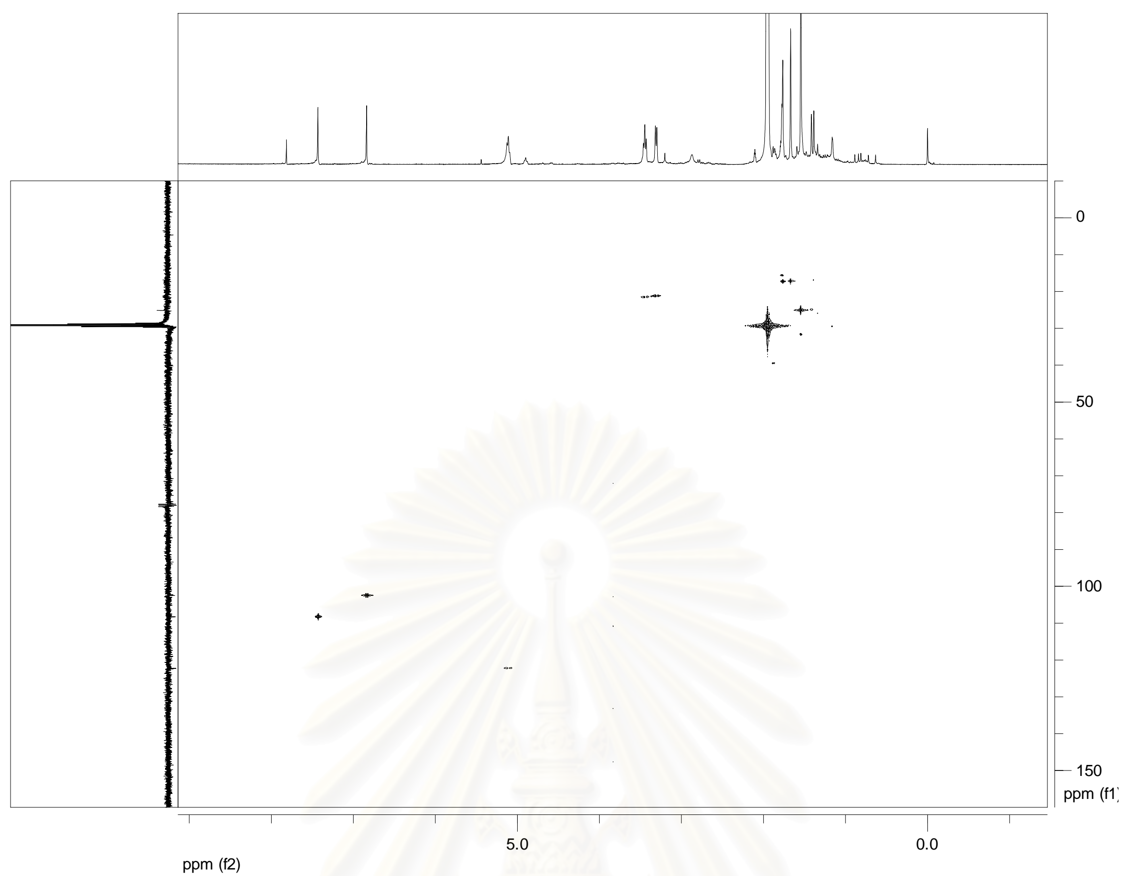

Figure 21 The HSQC spectrum (acetone-d_6) of cratoxylumxanthone D (**11**)

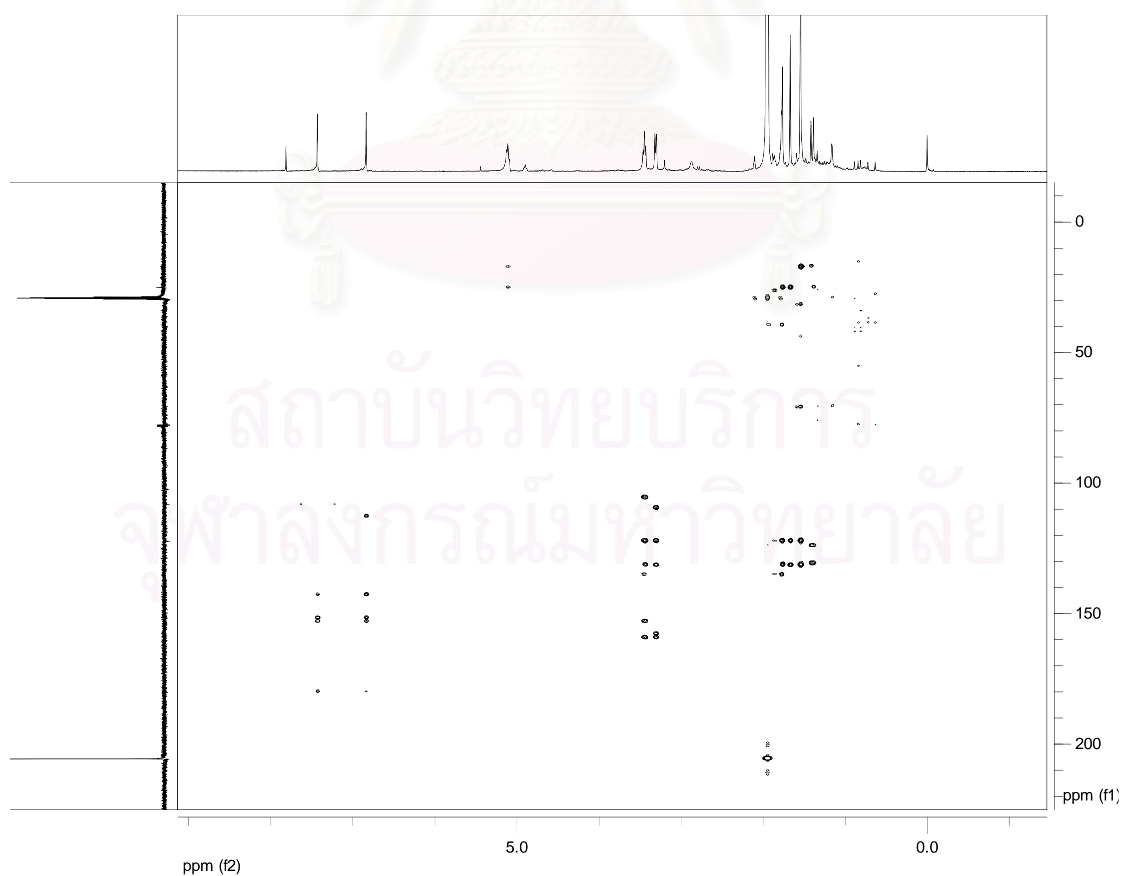

Figure 22 The HMBC spectrum (acetone-d_6) of cratoxylumxanthone D (**11**)

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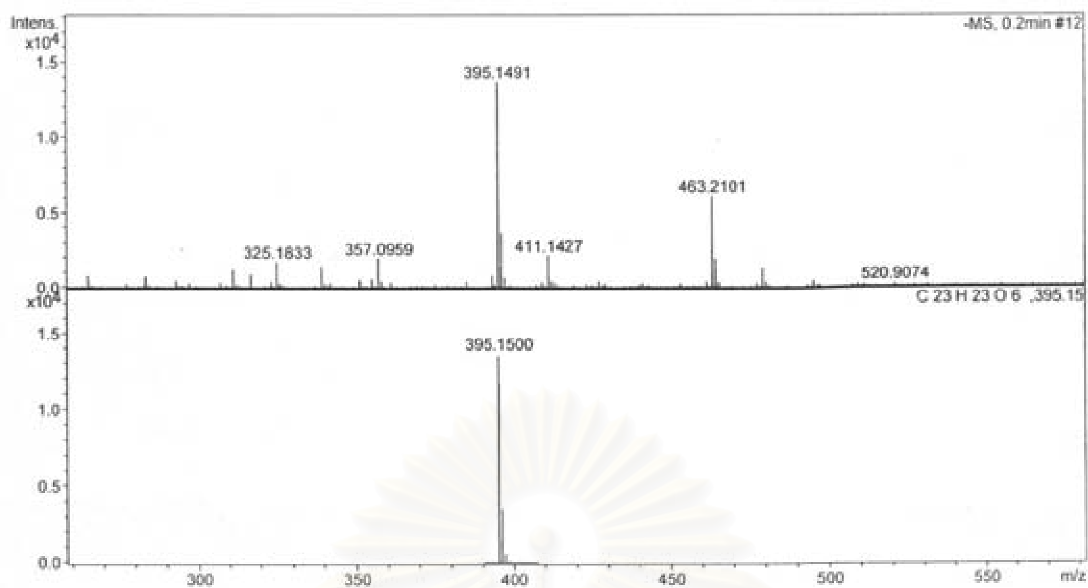

Figure 23 The High resolution mass spectrum of cratoxylumxanthone D (**11**)

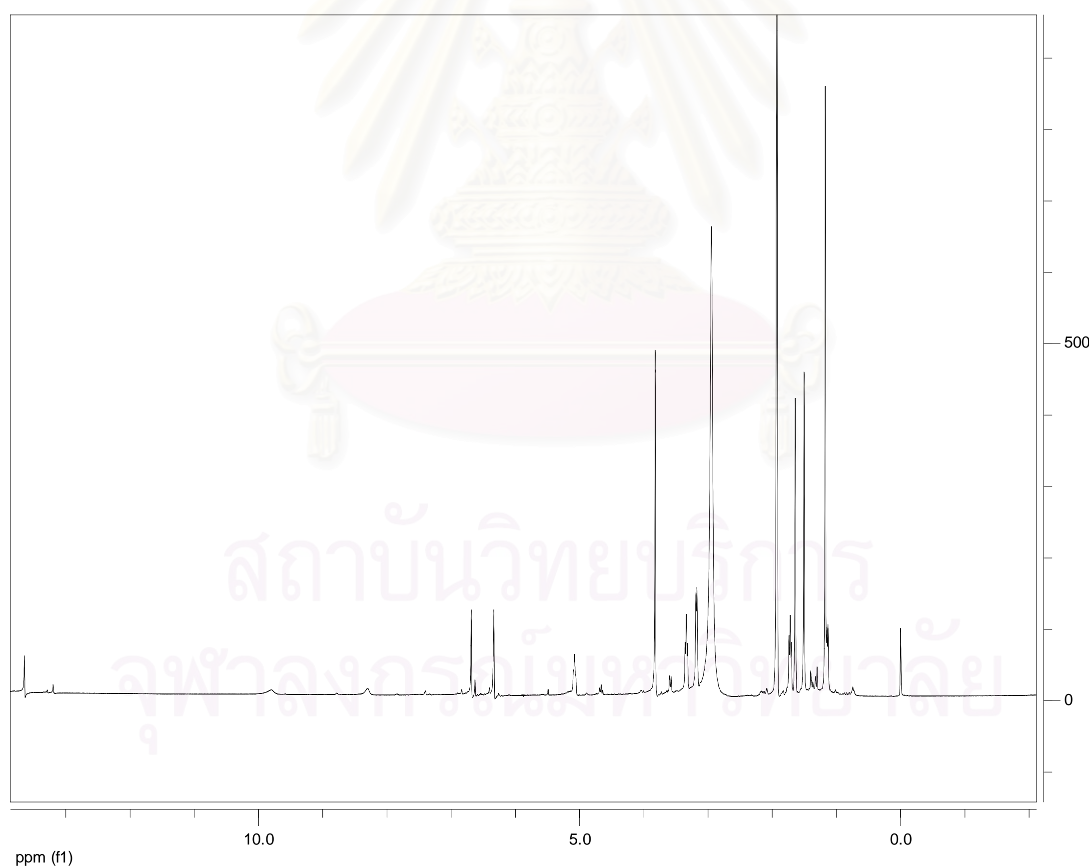

Figure 24 The ^{1}H NMR spectrum (acetone-d_6) cratoxylumxanthone E (**13**)

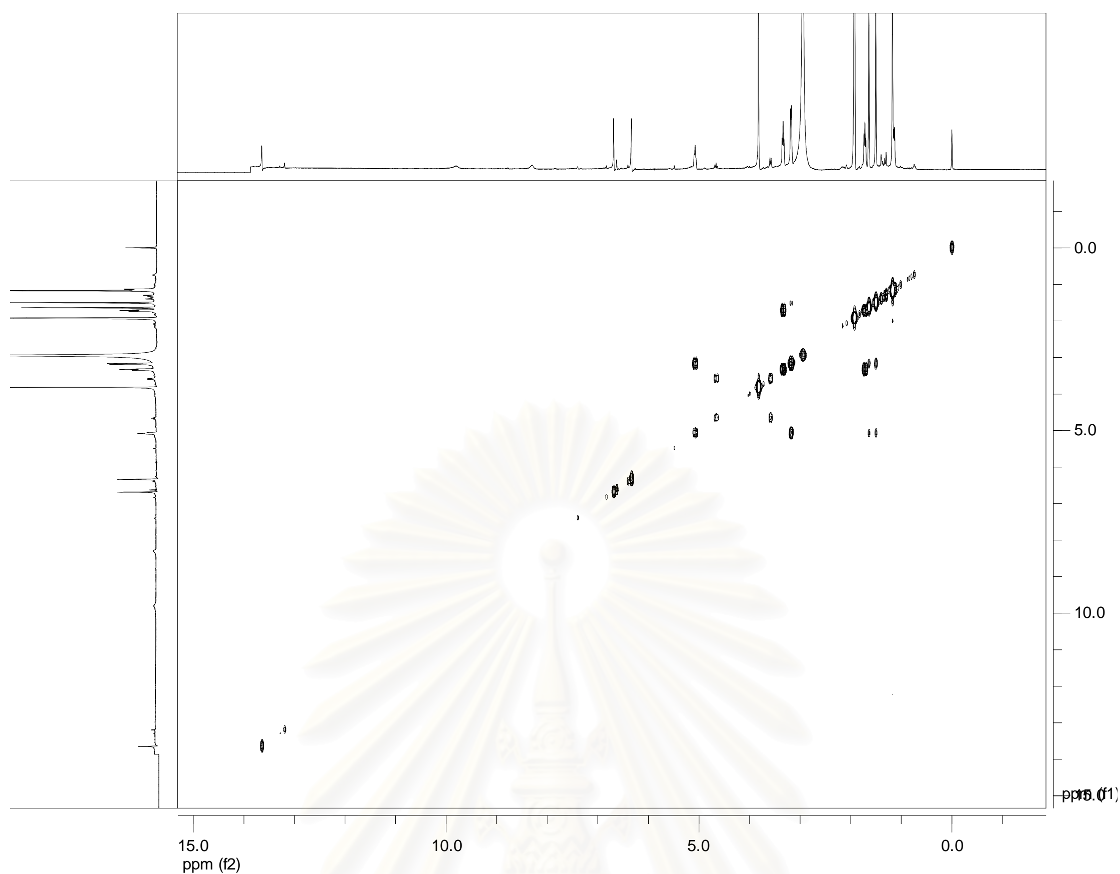

Figure 25 The COSY spectrum (acetone-d_6) of cratoxylumxanthone E (**13**)

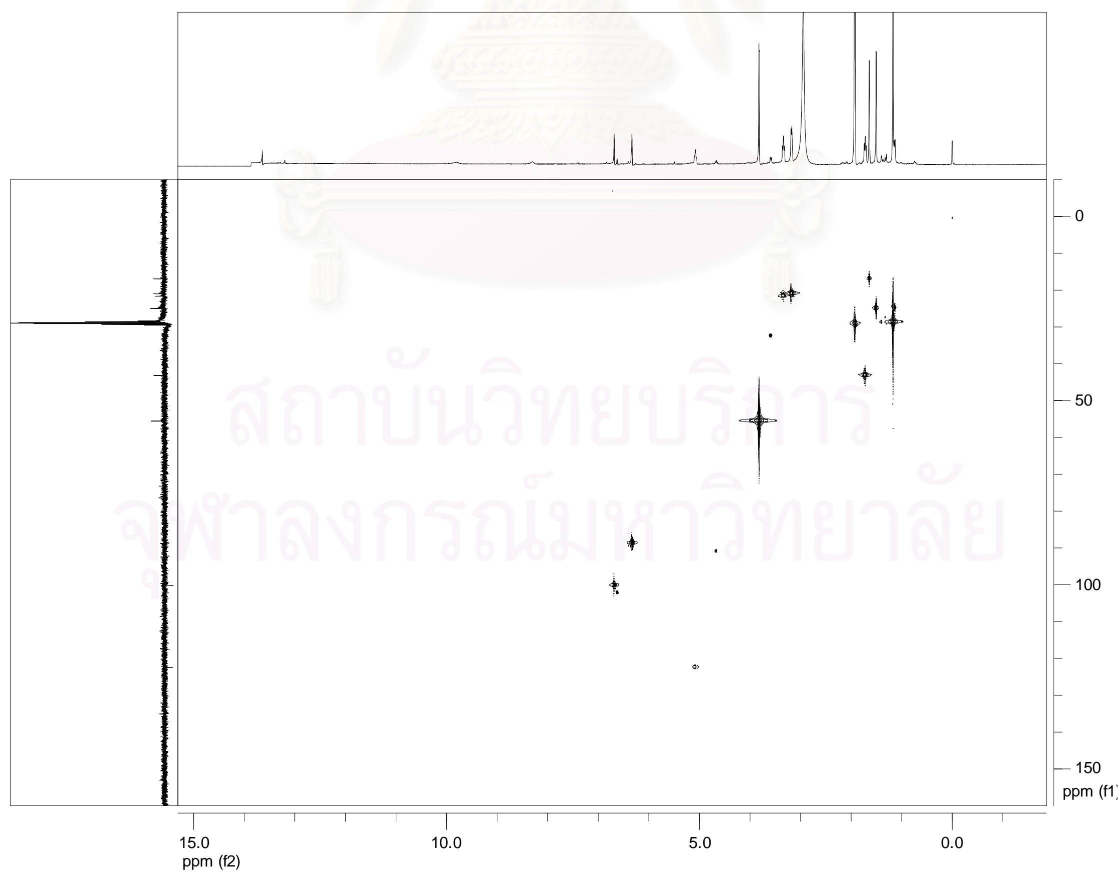

Figure 26 The HSQC spectrum (acetone-d_6) of cratoxylumxanthone E (**13**)

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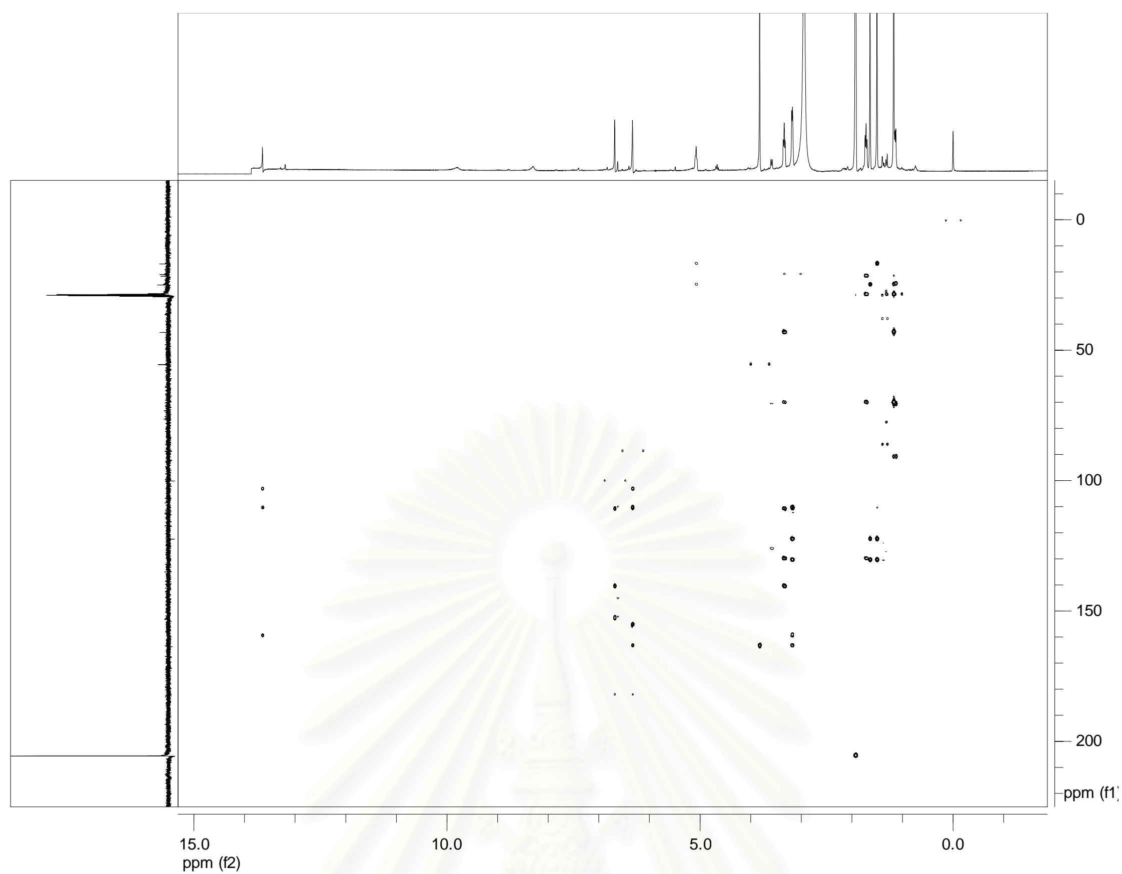

Figure 27 The HMBC spectrum (acetone-d_6) of cratoxylumxanthone E (**13**)

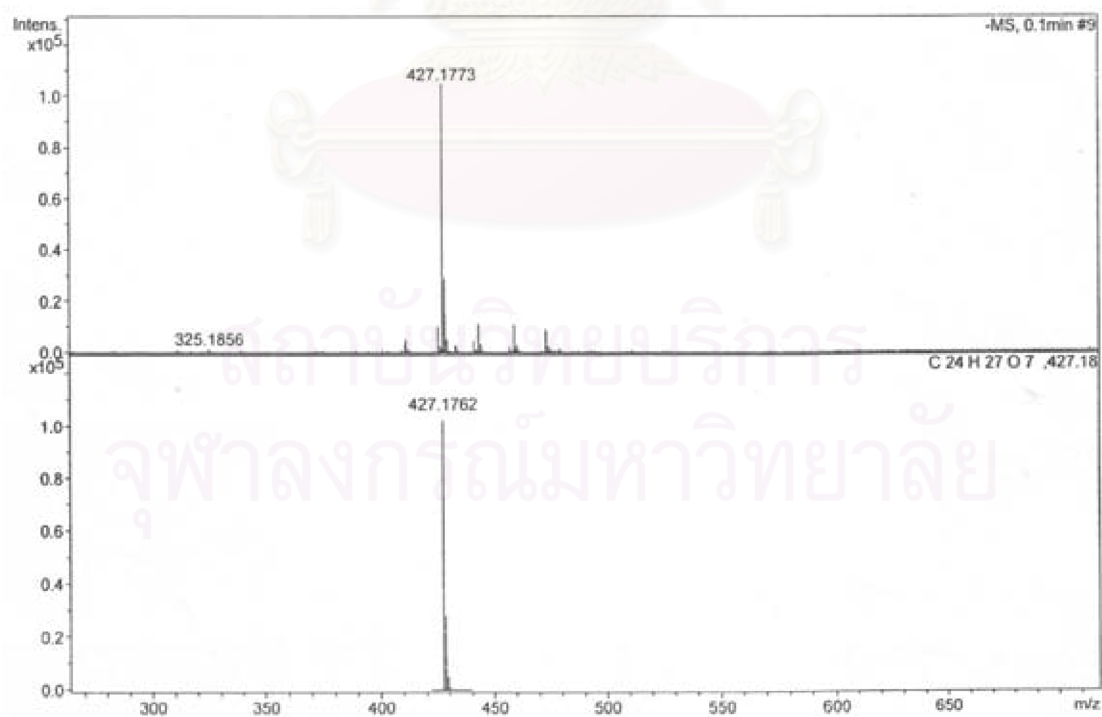

Figure 28 The High resolution mass spectrum of cratoxylumxanthone E (**13**)

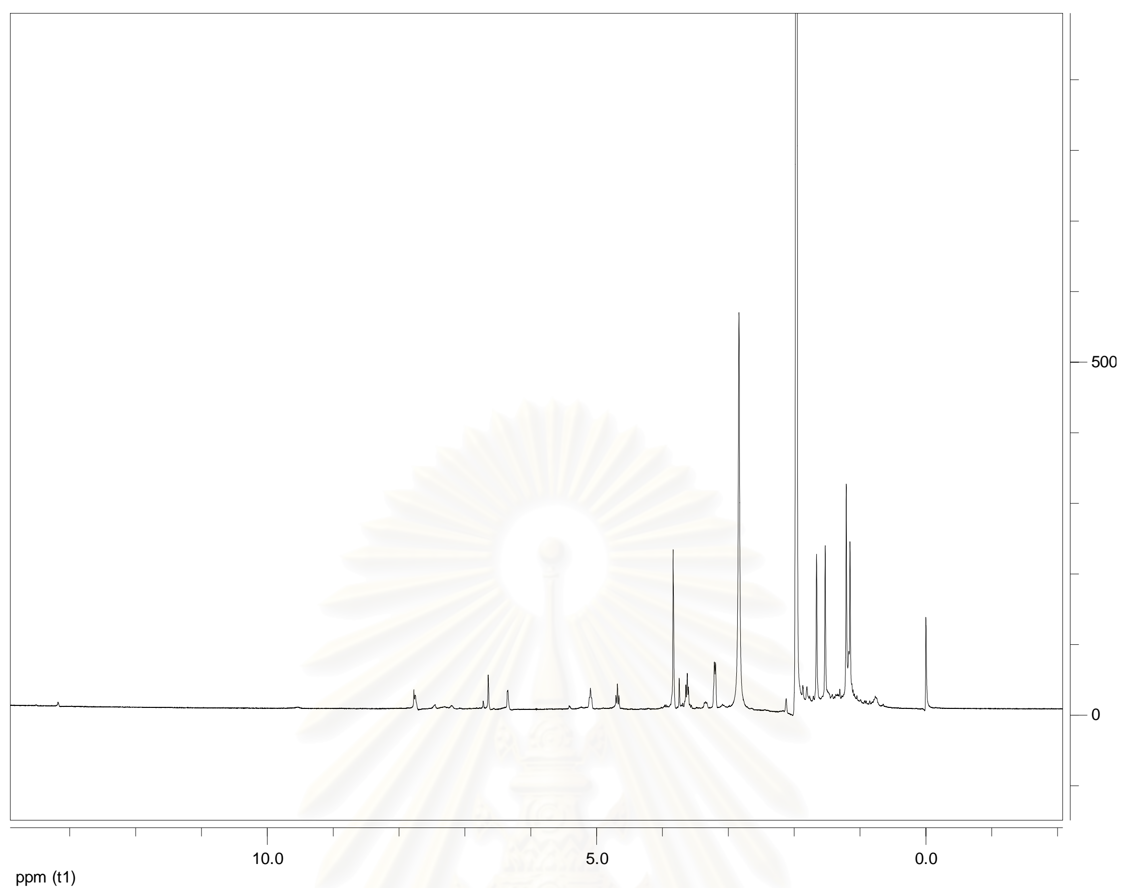

Figure 29 The ^{1}H spectrum (acetone-d_6) of cratoxylumxanthone F (**14**)

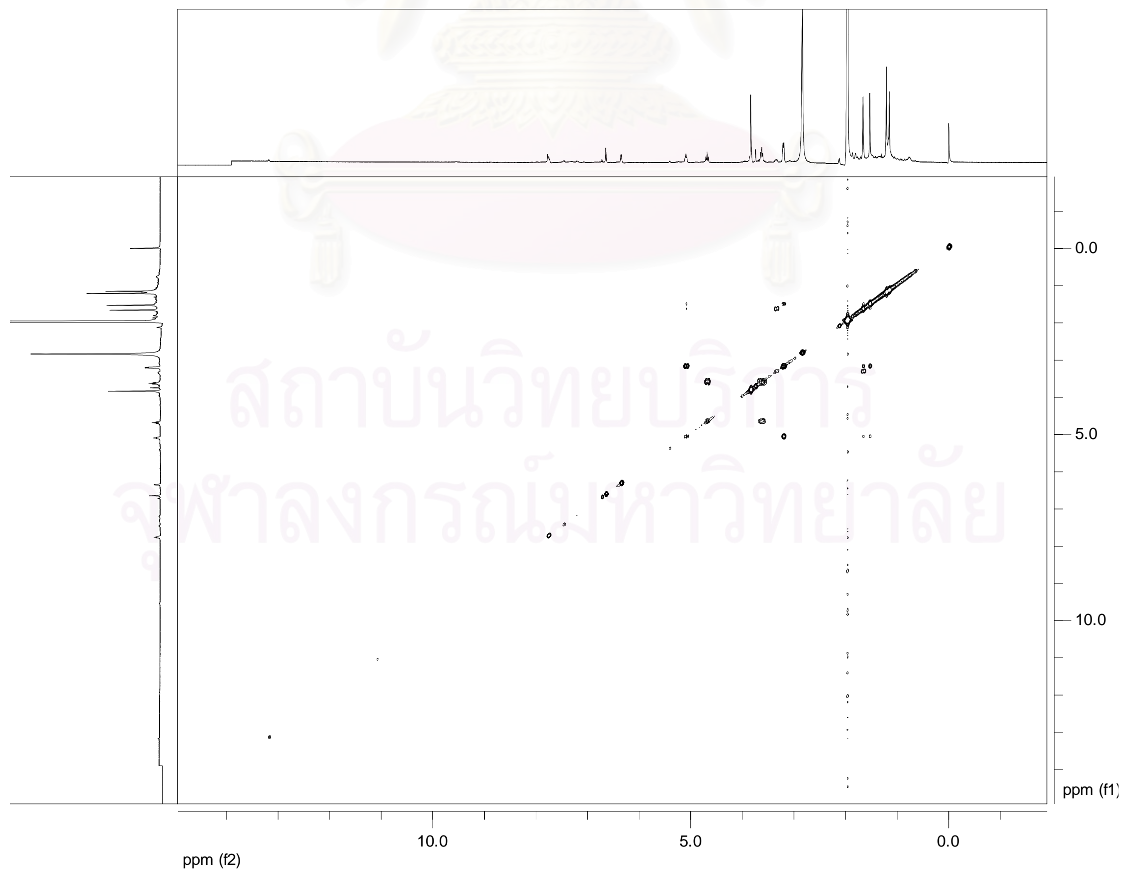

Figure 30 The COSY spectrum (acetone-d_6) of cratoxylumxanthone F (**14**)

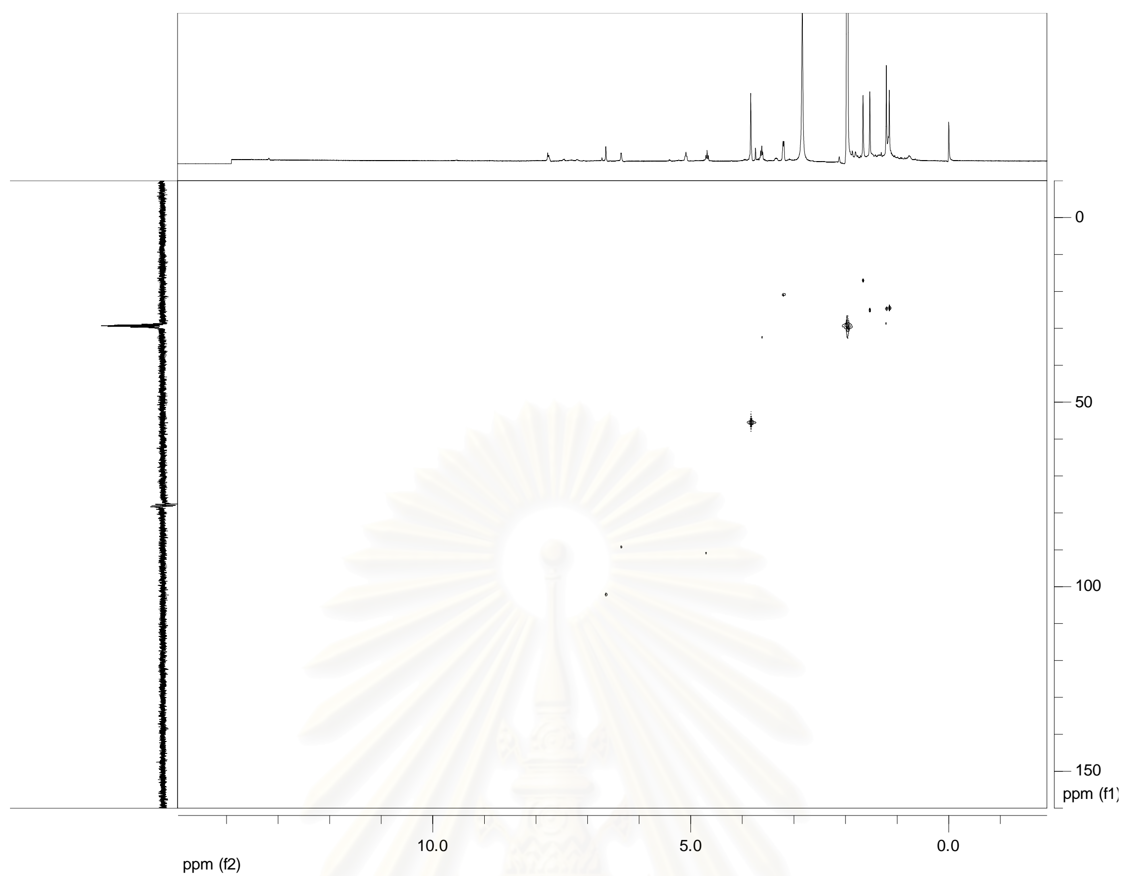

Figure 31 The HSQC spectrum (acetone-d_6) of cratoxylumxanthone F (**14**)

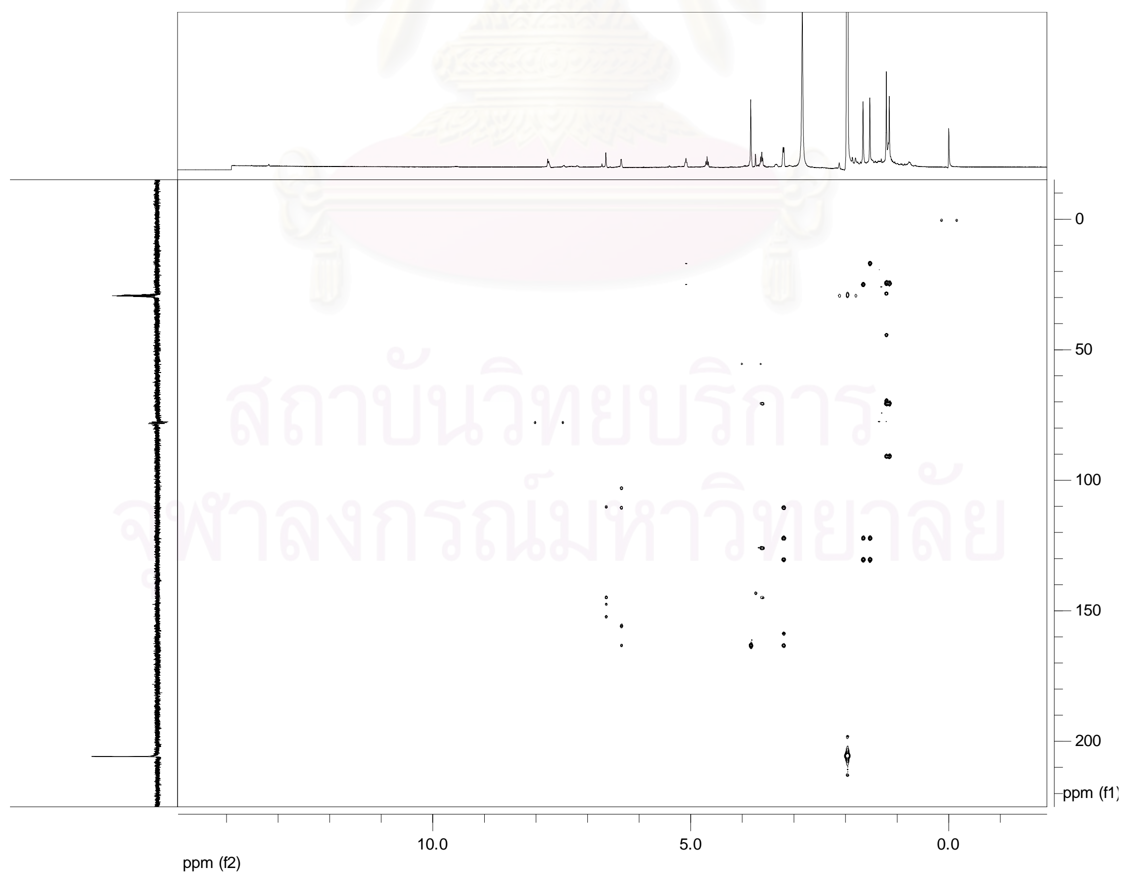

Figure 32 The HMBC spectrum (acetone-d_6) of cratoxylumxanthone F (**14**)

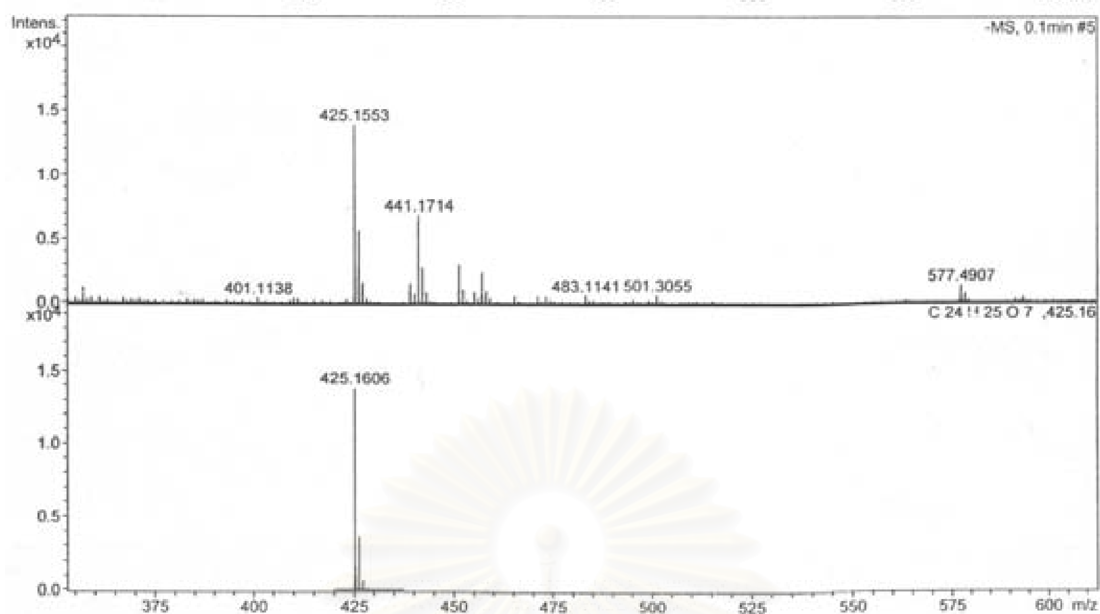

Figure 33 The High resolution mass spectrum of cratoxylumxanthone F (**14**)

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VITA

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