ISOLATION AND STRUCTURE ELUCIDATION OF ANTIOXIDANTS IN THE FLOWER OF *RADERMACHERA IGNEA* (KURZ) STEENIS

Duangnapa Sompong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry

Suranaree University of Technology

Academic Year 2008

การแยกและการวิเคราะห์โครงสร้างของสารออกฤทธิ์ต่อต้านอนุมูลอิสระในดอกปีบทอง

นางดวงนภา สมพงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมี
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2551

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ดวงนภา สมพงษ์ : การแยกและการวิเคราะห์โครงสร้างของสารออกฤทธิ์ต่อต้านอนุมูลอิสระในดอกปีบทอง (ISOLATION AND STRUCTURE ELUCIDATION OF ANTIOXIDANTS IN THE FLOWER OF *RADERMACHERA IGNEA* (KURZ) STEENIS) อาจารย์ที่ปรึกษา : ผศ. ดร. พิชญา ตระการรุ่งโรจน์, 94 หน้า.

การวิจัยนี้มีวัตถุประสงค์เพื่อหาสารต้านอนุมูลอิสระในดอกปีบทอง (*Radermachera ignea* (Kurz) Steenis) ซึ่งเป็นดอกไม้ประจำมหาวิทยาลัยเทคโนโลยีสุรนารี จังหวัดนครราชสีมา จากการศึกษาในเบื้องต้นพบว่า สารสกัดเอทิลอะซิเตทจากดอกปีบทองมีฤทธิ์ต่อต้านอนุมูลอิสระ จึงได้นำสารสกัดดังกล่าวมาแยกสารองค์ประกอบ โดยใช้เทคนิคทางโครมาโทกราฟี และพิสูจน์โครงสร้างทางเคมีโดยอาศัยข้อมูลเทคนิคทางสเปกโทรสโกปี พบสารสำคัญซึ่งแสดงฤทธิ์ต่อต้านอนุมูลอิสระคือ สาร zeaxanthin ซึ่งเป็นสารประกอบในกลุ่มแคโรทีนอยด์ นอกจากนี้ยังพบสารประกอบที่ไม่มีฤทธิ์ซึ่งอยู่ในกลุ่มสเตียรอยด์อีก 2 ชนิดคือ β-sitosterol และ stigmasterol เมื่อเปรียบเทียบฤทธิ์ต่อต้านอนุมูลอิสระด้วยค่า IC_{50} ของสาร zeaxanthin กับสารมาตรฐาน ascorbic acid และ catechin พบว่า zeaxanthin มีฤทธิ์ต่อต้านอนุมูลอิสระสูง โดยมีค่า IC_{50} เท่ากับ 1.13 mol/mol DPPH ซึ่งอยู่ในระดับเดียวกันกับ catechin ซึ่งมีค่า IC_{50} เท่ากับ 1.10 mol/mol DPPH และมีฤทธิ์ที่แรงกว่า ascorbic acid ซึ่งมีค่า IC_{50} เท่ากับ 304.30 mol/mol DPPH มาก

สาขาวิชาเคมี ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2551 ลายมือชื่ออาจารย์ที่ปรึกษา_____

DUANGNAPA SOMPONG : ISOLATION AND STRUCTURE ELUCIDATION OF ANTIOXIDANTS IN THE FLOWER OF *RADERMACHERA IGNEA* (KURZ) STEENIS. THESIS ADVISOR: ASST. PROF. PICHAYA TRAKANRUNGROJ, PH.D., 95 PP.

RADERMACHERA IGNEA / ANTIOXIDANT ACTIVITY

This research was aimed to study the antioxidant activity of the flower of *Radermachera ignea* (Kurz) steenis, the emblem of Suranaree University of Technology, Nakhon Ratchasima. From the preliminary study, ethyl acetate extract was found to exhibit antioxidant activity; therefore, it was selected for further study. The chemical constituents in the ethyl acetate extract were isolated by chromatographic methods, and their structures were characterized by spectroscopic methods. The results showed that the main antioxidant component in the extract was the carotenoid zeaxanthin. In addition, two non-antioxidant steroids, β-sitosterol and stigmasterol, were isolated and identified.

The antioxidant activity of zeaxanthin was studied in comparison with two antioxidant standards; ascorbic acid and catechin. The results indicated that zeaxanthin demonstrated potent free radical scavenging activity on DPPH radical scavenging assay with the IC_{50} of 1.13 mol/mol DPPH, which was in the same level as that of catechin (IC_{50} 1.10 mol/mol DPPH), and was much stronger than that of ascorbic acid (IC_{50} 304.30 mol/mol DPPH).

School of Chemistry Student's Signature_____

Academic Year 2008 Advisor's Signature_____

ACKNOWLEDGMENTS

I would like to express my deep appreciation to my thesis advisor, Asst. Prof. Dr. Pichaya Trakanrungroj for her guidance and encouragement throughout my study and thesis. I would like to express my gratitude to all teachers of the School of Chemistry who taught and helped me during my study at SUT, especially, Assoc. Prof. Dr. Jatuporn Wittayakun and Asst. Prof. Dr. Thanaporn Manyum who also are my thesis committees. I wish to express my special thanks to Assoc. Prof. Dr. Malee Tangsathitkulchai for her encouragement and for giving me opportunity to study in the School of Chemistry. In addition, I would like to thank Suranaree University of Technology for providing a research grant to partially support my thesis research.

I would like to express sincere thanks to Prof. Dr. Robert Bates at the University of Arizona for his help with the NMR techniques, and to Assoc. Prof. Dr. James R. Ketudate-Cairns at the School of Biochemistry, SUT, for his help with the LC-MS technique. I also would like to thank to all of the staffs at Center for Scientific and Technological Equipment for their assistance, and to all of my good friends for their friendship and encouragement.

Finally, I would like to thank the principles of Ban Sumwhai School, Mr. Supoln Nakmun and Mr. Payup Anannin for their kindness of giving me an opportunity to study Master's Degree at SUT. I would also like to thank all of my brothers and sisters who work at Ban Sumwhai School for their cheerfulness and encouragement.

Duangnapa Sompong

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LIST OF ABBREVIATIONS

ACN	acetonitrile
brs	broad singlet
cm	centimeter
C_{18}	octadecane
^{o}C	degree celcius
$CDCl_3$	chloroform-d_1
CH_2Cl_2	dichloromethane
COSY	correlation spectroscopy
d	doublet
dd	doublet of doublets
DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EtOAc	ethyl acetate
Flash CC	flash column chromatography
FT-IR	fourier transform-infrared spectroscopy
g	gram
HMBC	heteronuclear multiple-bond correlation
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
Hz	hertz
IR	infrared spectroscopy

LIST OF ABBREVIATIONS (Continued)

J	coupling constant in hertz
LC-MS	liquid chromatography-mass spectrometry
m	multiplet
mL	milliliter
$M^{-1}sec^{-1}$	per molar per second
MeOH	methanol
MHz	megahertz
nm	nanometer
NMR	nuclear magnetic resonance spectroscopy
ppm	parts per million
PTLC	preparative thin-layer chromatography
q	quartet
R_f	flow rate
s	singlet
t	triplet
TLC	thin-layer chromatography
UV	ultraviolet radiation
UV-Vis	ultraviolet-visible radiation
δ	chemical shift in ppm
λ_{max}	maximum absorption wavelength
ν_{max}	maximum absorption wavenumber

CHAPTER I

INTRODUCTION

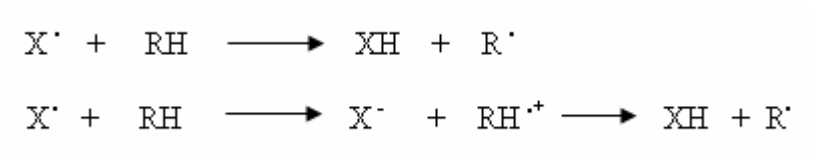
Presently, there has been increasing in awareness of healthy diet, especially the consumption of fruits, vegetables, and herbal supplements that contain antioxidants. Many degenerative human diseases such as cancer, diabetes, cardiovascular diseases and neurodegenerative diseases have been recognized as being a consequence of free radical damage. Several studies have been conducted to find the way to prevent or delay these diseases from the beginning stage. Although, practically, increasing of body antioxidant level can be achieved by consumption of fruits and vegetables, this may not be the most efficient method to fight against these diseases. Therefore, the search for potent antioxidant substances from a new source is still ongoing.

1.1 Free radicals and antioxidants

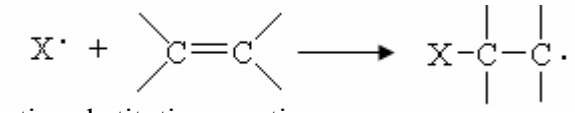
Free radicals are species that contain unpaired electrons. Molecules are composed of electrons, which are present generally in pairs. However, under certain conditions such as in lipid peroxidation process that occur in human body, molecules may contain unpaired electrons, which are called free radicals. Free radicals are generally reactive in seeking other electrons to become paired. They are highly reactive metabolites which oxidize the constituents of the cell, and in particular, the membrane, thus accelerating its aging and destruction (Saint-Crieq de Gaulejac, Glories, and Vivas, 1999). The reactive oxygen species, including superoxide radical ($O_2^{\cdot-}$),

hydroxyl radical ($^{\cdot}OH$), peroxyl radical ($ROO^{\cdot}$) and non-free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), are various forms of activated oxygen (Banerjee, Dasgupta, and De, 2005). This reactive oxygen species are essential for production of energy, synthesis of biologically essential compounds, and phagocytosis, a critical process of the immune system. However, they can induce some oxidative damages to biomolecules causing accelerative aging, cancer, diabetes, cardiovascular diseases, neurodegenerative diseases including Parkinson's diseases, Alzheimer's diseases, and inflammation (Ames, 1983, quoted in Banerjee et al., 2005; Stadtman, 1992; Sun, 1990, quoted in Banerjee et al., 2005). The important reactions of free radicals may be classified into 5 groups as follows:

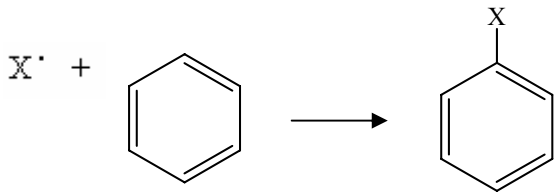
1. Hydrogen atom transfer reaction



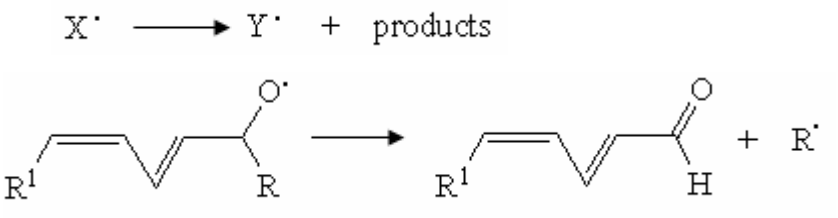
2. Addition reaction



3. Aromatic substitution reaction



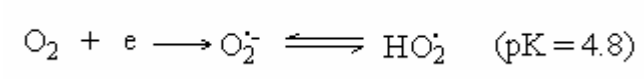
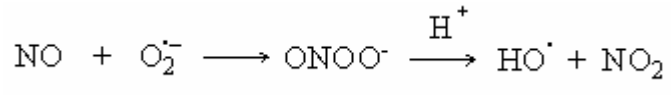
4. β-Scission reaction



5. Coupling reaction

Free radicals and reactive oxygen species are formed *in vivo* by various ways as summarized in Table 1.1.

Table 1.1 *In vivo* production of reactive oxygen species.

Reactive oxygen species	Formation
Superoxide ($O_2^{\cdot-}$), Hydoperoxyl radical, ($HO_2^{\cdot}$)	Enzymatic and non-enzymatic one electron reduction of oxygen 
hydroxyl radical, ($HO^{\cdot}$)	Radiolysis of water, metal-catalyzed decomposition of hydrogen peroxide, interaction of NO and superoxide 
Alkoxyl and peroxyl radicals, ($LO^{\cdot}$, $LO_2^{\cdot}$)	Metal-catalyzed decomposition of hydroperoxides
Hydrogen peroxide, (H_2O_2)	Dismutation of superoxide, oxidation of sugars
Singlet oxygen, ($^1O_2$)	Photosensitized oxidation, biomolecular interactions between peroxyl radicals
Nitrogen dioxide, ($NO_2^{\cdot}$)	Reaction of peroxyl radical and NO, polluted air and smoking
Protein radical, ($P^{\cdot}$)	Hydrogen atom transfer from protein

Reactive oxygen species can be trapped and destroyed by the body's antioxidant systems, including superoxide dismutase, catalase and glutathione peroxidase. Because of the change of environment, pollution, increasing stress, smoking, excessive exercise and/or dietary xenobiotics, the amount of free radicals are increased to the extent that are difficult to be controlled by these enzymes (Papas, 1999). The imbalance of free radicals versus antioxidant processes causes the subsequent cellular damage which leads to several diseases. The most effective approach to prevent degenerative disease is to improve body antioxidant status by higher consumption of vegetables and fruits which contain natural antioxidants. In addition, several synthetic antioxidants are commercially available, for example, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG) (Papas, 1999). Examples of antioxidants are shown below.

Endogenous components of the antioxidant system include:

- Glutathione (GHS, also present in foods) and Se-glutathione peroxidase
- Fe-catalase
- NADPH
- Coenzyme Q10
- Mn, Cu, Zn-superoxide dismutase (SOD)
- Uric acid
- Lipoic acid
- Melatonin
- Metal binding proteins including albumin, Fe and Cu-binding proteins (transferring, ceruloplasmin) and Fe-complex binding proteins (heptoglobin, hemopexin)

Dietary antioxidants include:

- α-Tocopherols (vitamin E)
- ascorbate or ascorbic acid (vitamin C)
- vitamin A and carotenoids (β-carotene, lycopene. Lutein, etc.)
- Dietary and other supplements (CoQ10, glutathione, lipoic acid, etc.)
- Food antioxidants (BHA, BHT, propyl gallate, rosemary extract)

In this thesis, L-ascorbic acid (vitamin C) and (+)-catechin were used as standard antioxidants.

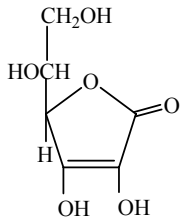


Figure 1.1 Structure of ascorbic acid

Ascorbic acid is a potent water-soluble antioxidant which is a required nutrient for humans. It is an excellent electron donor or reducing agent for chemical reactions that occur either inside or outside cells. It reduces superoxide, hydroxyl radical, and other reactive oxidant species. Ascorbic acid is reversibly oxidized with the loss of one electron to form the free radicals so called semidehydroascorbic acid (Figure 1.2). Semidehydroascorbic acid is relatively stable with a rate constant of decay of approximately 10^{-5} $M^{-1}sec^{-1}$ (Bielski, Richter, and Chan, 1975, quoted in Rumsey, Wang and Levine, n.d.). Further oxidation of semidehydroascorbic acids results in

dehydroascorbic acids which can be reduced back to ascorbic acids via the same pathway (Rumsey et al., n.d.).

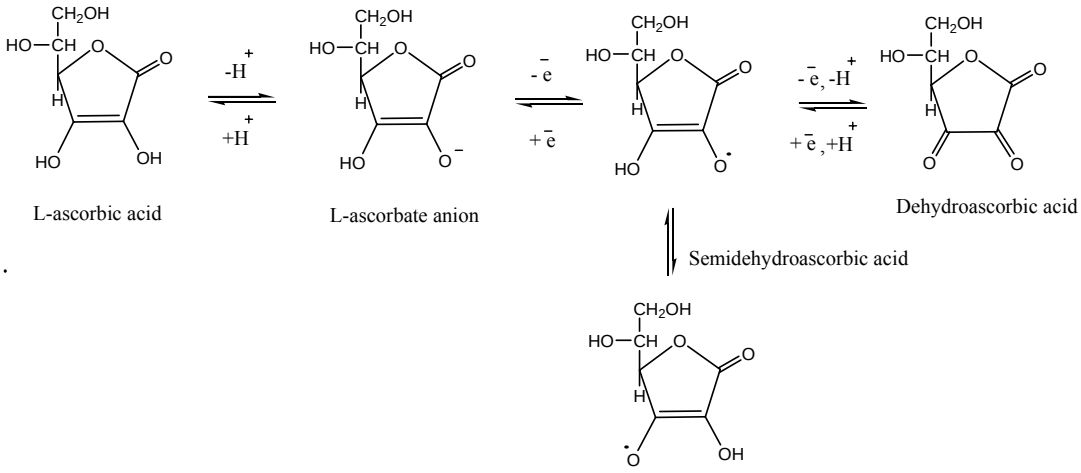


Figure 1.2 Ascorbic acid and its oxidation products

Catechin is a bioflavonoid with polyphenolic structure that is a powerful antioxidant. Several evidences have shown that catechin is related to inhibition of tumors and enhancement of immune system function due to its polyphenol antioxidant character.

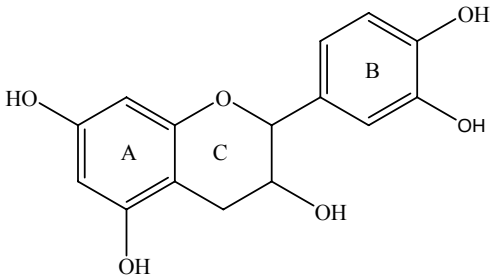


Figure 1.3 Structure of catechin

Sawai and Sakata (1998) have identified the antioxidant mechanism using a free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and found that the B ring of (+)-catechin has been changed to a quinone structure as shown in Figure 1.4.

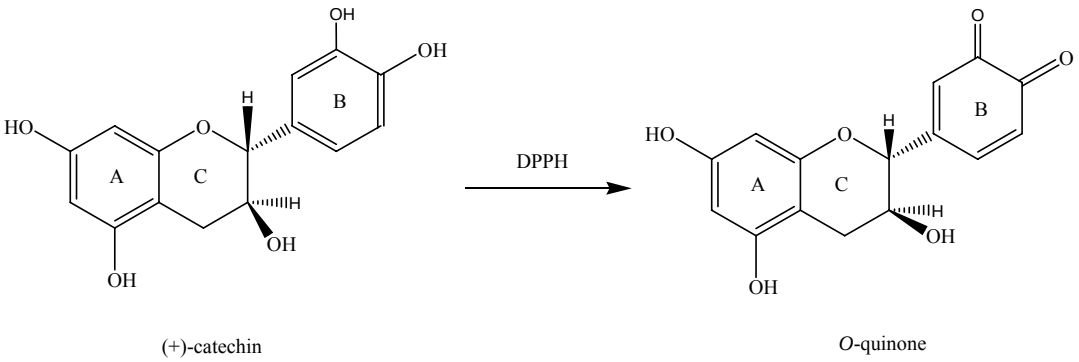


Figure 1.4 Reaction of (+)-catechin with DPPH

1.2 Evaluation of antioxidation activity

There are several methods for measurement of antioxidant activity. One common testing method is by using chemical assays, which are based on the ability to scavenge various kinds of free radicals (Kim, Lee, and Lee, 2002). The method of using DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging is among the most popular spectrophotometric methods for determining the antioxidant activity. DPPH radical is a stable radical due to the delocalization of the unpaired electron over the entire molecule. The delocalization also gives rise to the deep purple color that can be characterized by UV absorption spectroscopy. DPPH is scavenged by antioxidants either by donating a hydrogen atom or transferring of an electron to the odd electron of nitrogen to neutralize free radical character, leading to the loss of its purple color to yellow of a residual picryl group. The change in the color can be quantified by a decrease in absorbance at wavelength 517 nm. (Huang, Lin, Chen, and Lin, 2004).

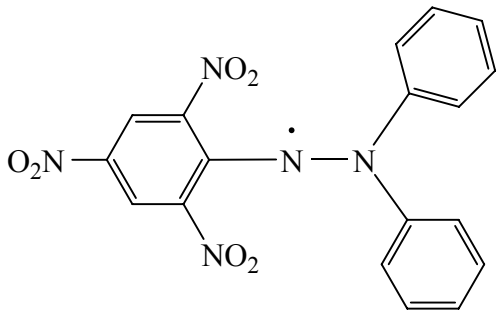


Figure 1.5 Structure of 1,1-diphenyl-2-picrylhydrazyl (DPPH)

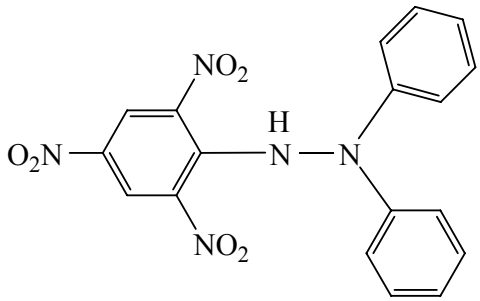


Figure 1.6 Structure of reduced form of 1,1-diphenyl-2-picrylhydrazyl (DPPH)

In a search for new antioxidants, numerous plants have been screened for their antioxidants and radical scavenging components. For this research, *Radermachera ignea* (Kurz) Steenis (Family-BIGNONIACEAE), whose common Thai name is "Peep Thong" (Suranaree University of Technology, 2003), was the target of the study. This plant is the emblem of Suranaree University of Technology. The color of *R. ignea* flowers is orange, normally indicating that flavonoids or carotenoids may be the major constituents. Several studies have suggested that these two natural product groups are potent antioxidants. As for *R. ignea*, there has been no previous study for its antioxidant activity reported in the literature. Therefore, this study was the first to investigate the antioxidant activity in this plant.

1.3 Specification of *Radermachera ignea* (Kurz) Steenis

The specification of this flower was the following;

Botanical Name: *Radermachera ignea* (Kurz) Steenis

Family: BIGNONIACEAE

Common Name: Tree Jasmine

Other Name: Peep Thong (Bangkok), Kasalong Kham (Chiang Rai), Khae Po, Kaki (Surat Thani), Samphao Lam Ton (Lampang), Chang Chuet (Chiang Mai), Saphao, Oichang (Northern) (เต็ม สมิตินันทน์, 2544).

Botanical Description: Evergreen tree, 6-15 m high, sometimes partly deciduous. *Leaves* 2-pinnate, 18-35 cm long, 3-4 jugate; leaflets elliptic-lanceolate, ovate-lanceolate, oblong-lanceorate, 5-12 by 2-4.6 cm usually unequal-sided, glabrous; apex acuminate to caudate; base acute; underpart with crowded minute glands at the base and few in the apical area. *Flowers* in a very short, pubescent raceme, almost reduced to a fascicle, 1-1.7 cm long, ramiflorous. *Calyx* tubular, 1.5-2.2 cm long, spathaceously slit halfway towards the base; apex of lobes acute with a few minute teeth. *Corolla* orange, tubular-funnel-shaped, 4.4-7 cm long, straight, the upper part barrel-shaped owing to a slight contraction, widest below the limb, with very short subequal lobes (Figure 1.7). *Stamens* hairy at insertion. *Capsule* 32-45 cm long; valves 4-6 mm broad. *Seeds* 13-15 by 2 mm. (Tem Smitinand and Lasen, 1987).

Distribution: South China (Yunnan), Burma, Laos, and Vietnam (Tonkin and Annam).

Ecology: Grow in the nature, frequent on the limestone hills and along the edges of evergreen forests; up to ca 1000 m elevation.

Propagation: Seed, cutting and budding.

Pharmacological study (กาสะลองคำ, www, 2548; สุทัศน์ จูงพงศ์, 2543)**:**

Barks: antidiarrhea.

Leaves: heal the sore of skin, anti-HIV-1 reverse transcriptase.

Stems: heal the infectious disease in early childhood.

Figure 1.7 Tree and flowers of *R. ignea*

1.4 Literature reviews

R. ignea belongs to the family of BIGNONIACEAE. Several plants of this family have been collected and studied for their biological activities as summarized in Table 1.2 (ลีนา ผู้พัฒนพงศ์, 2525; นันทวัน บุญประภัศร และอรนุช โชคชัยเจริญพร, 2541; Fujinori and Neil, 2003; Hase and Kawamoto,1995; Hase et al., 1996; Roy, 2001).

Table 1.2 Traditional use and chemical constituents of the plants in the family BIGNONIACEAE in Thailand.

Plant Species	Characteristics	Traditional Use	Chemical Constituents
Crescentia cujete Linn.(น้ำเต้าต้น)	Tree	Blended leaves: healing headache Boiled bark water: antidiarrhea Fruits: laxative	Sarcocarp: crescentic acid, tartaric acid (1), citric acid (2), chlorogenic acid (3), tannic acid (4)
Dolichandrone spathacea (Linn.f.) K. Schum. (แคทะเล)	Tree	Leaves: healing the wound and mouth wash Seeds: healing local pain Roots: expectorant, blood tonic	N/A
Millingtonia hort-ensis Linn. f. (ปีบ)	Tree	Roots: lung tonic,healing tuberculosis	apigenin-7-O-glucuronide , β-carotene (5), cirsimaritin (6),

Table 1.2 (Continued).

Plant Species	Charactistics	Traditional Use	Chemical Constituents
Millingtonia hort-ensis Linn. f. (ปีบ)	Tree		pectolinarigenin (7), dinatin (8), cornoside (9), dinatin-7-O-rutinoside, dinatin-7-rutinoside, *trans*-1-(2′-hydroxyl-ethyl)-cyclohexane-1- 4-diol, ellagic acid (10), lapachol (11), paulownin (12), p-coumaryl alcohol glucoside (13), isoeugenol-β-D-glucoside (14), β-sitosterol, scutellarein, hentriacontan-1-ol, n-hentriacontane, hispidulin-7-O-β-D-glucoside,

Table 1.2 (Continued).

Plant Species	Characteristics	Traditional Use	Chemical Constituents
Millingtonia hort-ensis Linn. f. (ปีบ)	Tree	-	halleridone hispidulin 7-O-methyl ester glu-curonide, salidroside (15), 2-phenylethyl ruti-noside (16), 2-(3,4-dihydroxy-phenyl)ethyl glucoside (17), rengyol (18), rengyoside A (19), rengyoside B (20), isorengyol (21), 8-O-β-D-glucopyranosyl isorengyol (22), millingtonine (23), millingtonia compound 14-21 (24)-(31), acetyl oleanolic acid

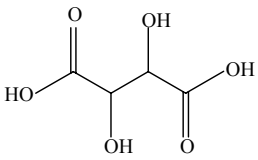
Table 1.2 (Continued).

Plant Species	Characteristics	Traditional Use	Chemical Constituents
Oroxylum indicum Vent. (เพกา)	Deciduous tree	Leaves: healing headache, enhancing appetite and healing joint pain Bark: diaphoresis and enhancing appetite Root: element tonic	Barks and Seeds: crystalline substance oroxylin Barks: alkaloid, glucoside, bitter substance, baicalein
Spathodea cam-panulata Beauv. (แคแสด)	Tree	Barks: healing skin disease and chronic ulcers	Barks, Leaves and Roots: hydrocyanic acid
Stereospermum fimbriatum A. DC. (แคยอดดำ)	Deciduous tree	Blended leaves with lemon juice: healing pruritus	N/A

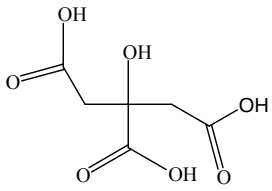
Table 1.2 (Continued).

Plant Species	Characteristics	Traditional Use	Chemical Constituents
Stereospermum personatum (Hassk.) Chatterjee (แคหิน)	Deciduous tree	Flowers: healing chronic ulcers Roots, leaves and flowers: healing fever	Barks: crystalline bitter substance

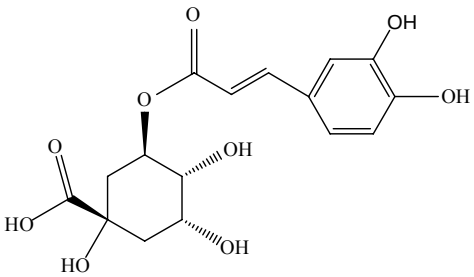
The following structures are the parts of chemical constituents of plants in family-BIGNONIACEAE:



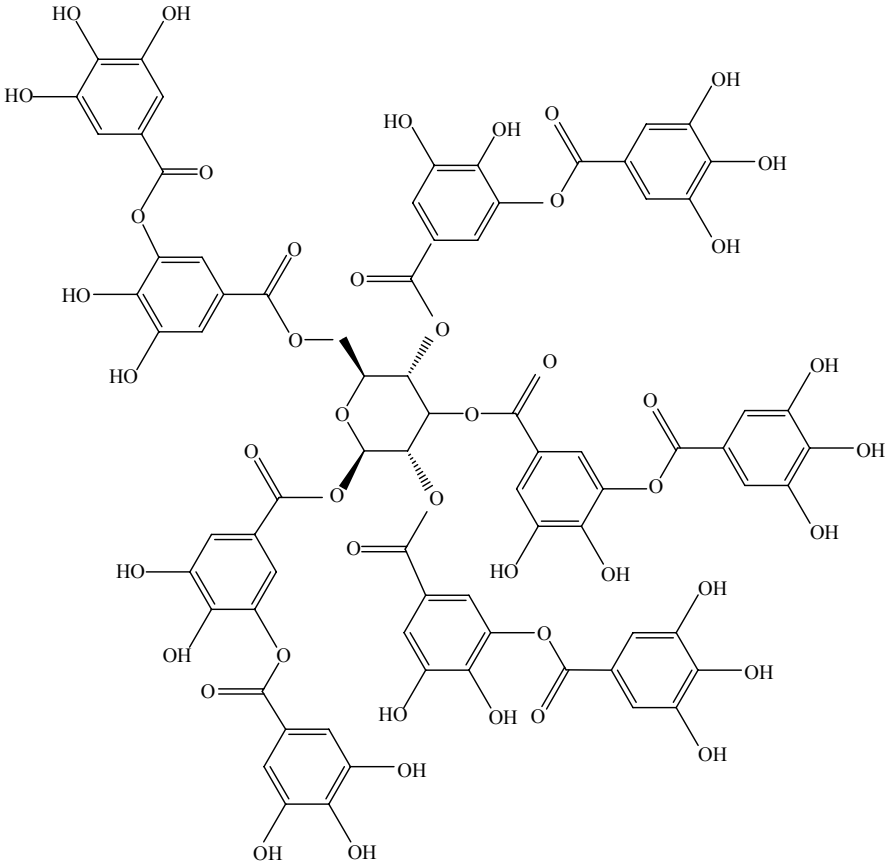
Tartaric acid (1)



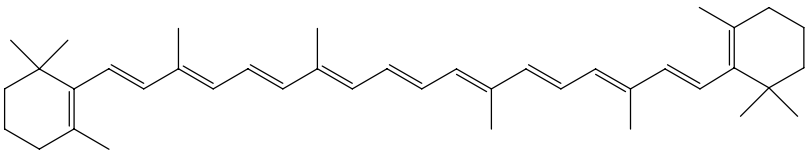
Citric acid (2)



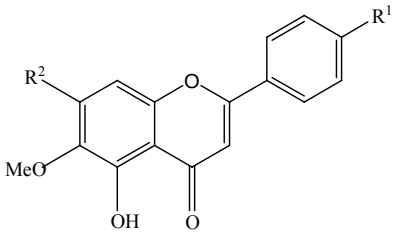
Chlorogenic acid (3)

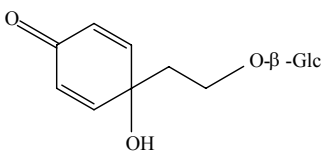


Tannic acid (4)



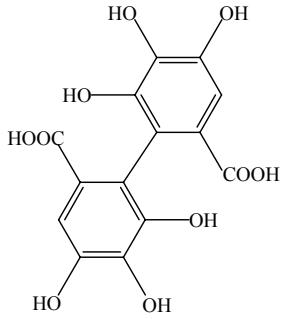
β -Carotene (5)



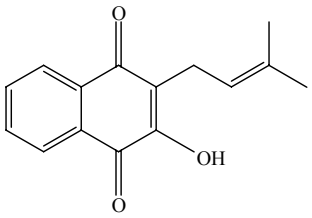


Cornoside (9)

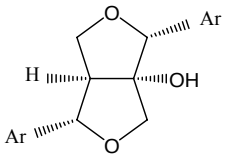
R^1= OH, R^2 = OMe: Cirsimaritin (6)
R^1= OMe, R^2= OH: Pectolinarigenin (7)
R^1= OH, R^2= OH: Dinatin (8)



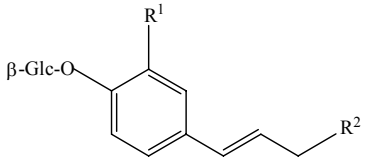
Ellagic acid (10)



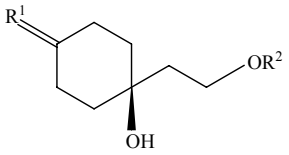
Lapachol (11)



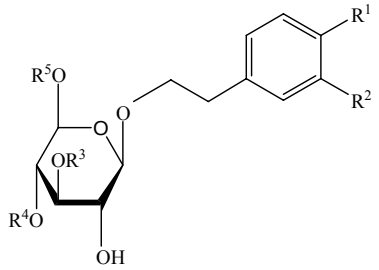
Ar = 3,4-Methylenedioxyphenyl:
Paulownin (12)



R^1	R^2	
H	OH	: p-Coumaryl alcohol glucoside (13)
OMe	H	: Isoeugenol glucoside (14)

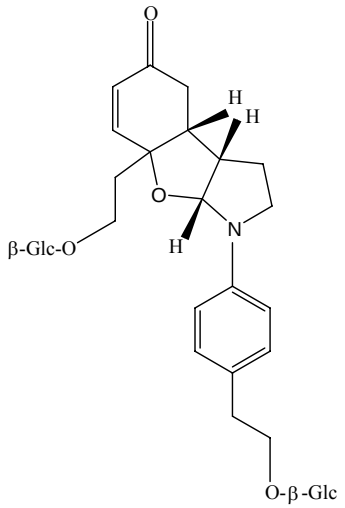


R^1	R^2	
O	β-Glc	: Rengyoside B (20)
	H	: Rengyol (18)
	β-Glc	: Rengyoside A (19)
	H	: Isorengyol (21)
	β-Glc	: 8-O-β-D-glucopyranosyl -isorengyol (22)

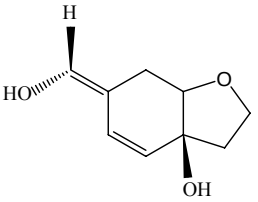


R^1	R^2	R^3	R^4	R^5	
OH	H	H	H	H	: Salidroside (15)
H	H	H	H	Rha	: 2-Phenylethyl rutinoside (16)
OH	OH	H	H	H	: 2-(3,4-Dihydroxyphenyl) -ethyl glucoside (17)

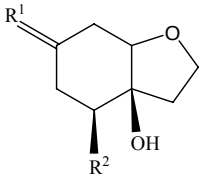
β-Glc = β-glucopyranosyl
Rha = α- rhamnopyranosyl



Millingtonine (23)



Millingtonia compound 14[a] (24)

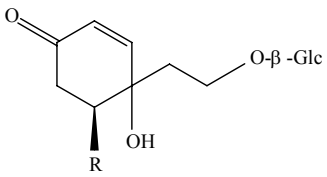


R^1 R^2

H
OH H : Millingtonia compound 15[b] (25)

H
OH H : Millingtonia compound 16[b] (26)

O H : Millingtonia compound 17[b] (27)



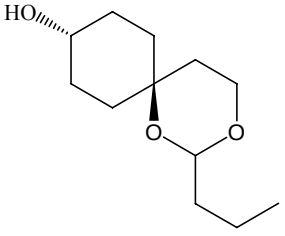
R

H : Millingtonia compound 18[c] (28)

OH : Millingtonia compound 19[c] (29)

OMe : Millingtonia compound 20[c] (30)

a) The structure is tentatively illustrated as one of enantiomers.
b) Compounds were racemic mixtures.
c) Aglycones of 18, 19 and 20 were racemic mixtures.



Millingtonia compound 21[b] (31)

1.5 Preliminary study

The preliminary screening for antioxidants in the flowers of *R. ignea* has been done by Asst. Prof. Dr. Pichaya Trakanrungroj's group at Suranaree University of Technology. Dried flowers of *R. ignea* were extracted with methanol to obtain methanol crude extract, which was then consecutively extracted with hexane and ethyl acetate. Each crude extract was tested for antioxidant activity by spectrophotometric assay using the protocols of Yen and Hsieh (Yen and Hsieh, 1997), which was based on scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical.

The preliminary testing results, as shown in Table 1.3, indicated that the ethyl acetate crude extract exhibited antioxidant activity with IC_{50} value of 70.81 μg/mL. Therefore, the ethyl acetate crude extract were selected for further study to identify the biologically active compound in this plant part.

Table 1.3 IC_{50} values of crude extracts and residue for antioxidant activity.

Extract	IC_{50} value (μg/mL)
Hexane extract	1.61×10^5
EtOAc extract	70.81
Residue of MeOH crude extract	76.71

1.6 Scope of this study

1.6.1 The fresh flowers of *R. ignea* were collected directly from the trees of *R. ignea* around the campus area of Suranaree University of Technology, Nakhon Ratchasima, Thailand.

1.6.2 Antioxidant was isolated from ethyl acetate extract of the flowers of *R. ignea.*

1.6.3 Antioxidant activity was measured by using the scavenging activity against DPPH radicals.

1.7 Objectives of this study

1.7.1 To isolate and identify main antioxidant component from ethyl acetate extract of the *R. ignea* flowers.

1.7.2 To evaluate the antioxidant activity of the main antioxidant component by using ascorbic acid and catechin as the standards.

CHAPTER II

AN OVERVIEW OF CHROMATOGRAPHIC AND SPECTROSCOPIC TECHNIQUES

2.1 Chromatographic techniques

Chemical constituents from the ethyl acetate extract of *R. ignea* flowers were analyzed and isolated by several chromatographic techniques as described below.

2.1.1 Thin layer chromatography (TLC)

Separation by TLC is based on the interaction of compounds with stationary phase and mobile phase. Compounds of a mixture will be separated according to their relative polarities. Normally, stationary phase or sorbent is alumina or silica gel coated on a glass or aluminum plate. There are two different types of TLC technique, which are analytical TLC and preparative TLC. Analytical TLC is mainly used for qualitative analysis whereas preparative TLC is used for isolation purpose. For this research, only analytical TLC was used for determination of suitable solvent systems for column chromatography and for evaluation of antioxidant activity.

Commercially available analytical TLC plate normally has a 0.2 mm thickness of silica sorbent coated on aluminum sheet. The plate (20 x 20 cm) is cut to a suitable size before spotting with a solution of sample. The plate is placed into a developing tank containing a solvent as the mobile phase. The solvent front then migrates up the plate through the sorbent by capillary action; a process is known as development.

The important factor in quantifying migration of a compound on a particular sorbent and solvent system is the R_f value, which can be defined as:

$$R_f = \frac{\text{Compound distance from origin (A)}}{\text{Solvent front distance from origin (B)}} \quad (2.1)$$

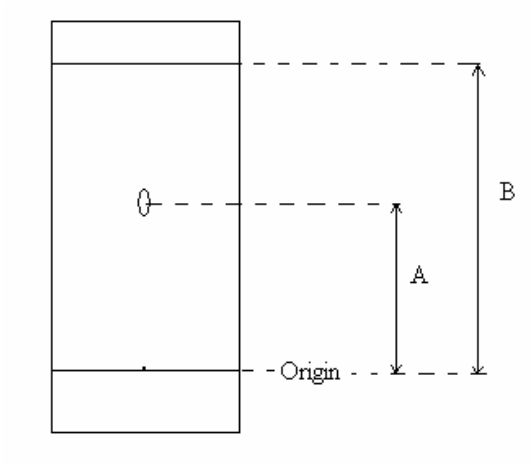


Figure 2.1 TLC plate

For a normal TLC plate, polar compounds has higher affinities for the sorbent (stationary phase), thereby moving slowly up the plate as the solvent (mobile phase) migrates. Conversely, nonpolar compounds with weaker affinities to the stationary phase will move comparatively quickly up the plate. As a consequence of development, compounds of a mixture can be separated from one another.

2.1.2 Column chromatography (CC) techniques

A. Conventional column chromatography

The conventional, gravity-driven, column chromatography method is still widely used for separation purpose. Like TLC, the concept of column chromatography techniques is based primarily on interaction of compounds with solid support or

stationary phase and mobile phase, and compounds of a mixture can be separated by their difference in polarity. The optimum ratio between the solid support and the sample can be ranging from 1:10 to 1:300 depending on how difficult components of the mixture can be separated from one another. The major drawbacks of this method are that it requires large amount of solvents in elution, and is a time-consuming process. Therefore, this method has been replaced by a more efficient flash chromatographic method as will be described in the next section.

B. Flash column chromatography (Flash CC)

Flash chromatography is also known as medium pressure chromatography. The main differences of this technique from the conventional column chromatography are that smaller-sized silica gel particles are used and the compressed air is applied to drive the solvent through the column of stationary phase. This method requires much less time in separation as compared to the conventional column chromatography method. Figure 2.2 shows the apparatus of flash column chromatography system that was used in this work.

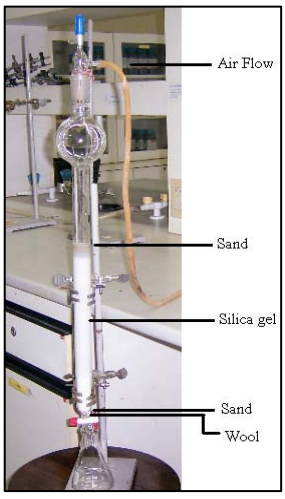


Figure 2.2 The apparatus of flash column chromatography

C. Gel filtration

Size exclusion chromatography or gel filtration is an important technique for isolation of biomolecules. The support or gel is a neutral, porous material that allows molecules of different sizes to pass through gel with different extents. Generally, molecules will be separated based on the difference of their molecular weight. The elution order from the column is reciprocal to the molecular weights of the compounds. However, for a small molecule, other factors such as polarity will also play a role in the separation process.

Sephadex LH-20 has become the most popular hydrophilic gels for isolation of various natural product compounds. It is particularly useful for removal of high molecular weight and polymeric material from a sample.

2.1.3. High performance liquid chromatography (HPLC)

HPLC is a vey useful technique for separation, identification, and quantification of compounds. The HPLC system, as represented in Figure 2.3, consists of a column packed with stationary phase, a pump that moves the mobile phase through the column, and a detector that gives out when molecules with certain functional groups have been detected. The chromatogram is a plot of peak intensity versus retention time as shown in Figure 2.4.

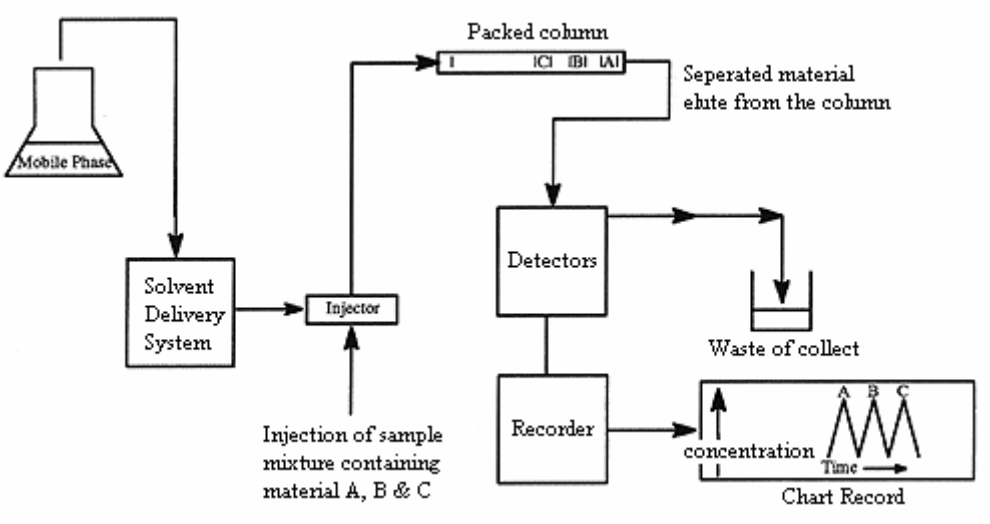


Figure 2.3 The components of HPLC instrument

HPLC techniques can be divided based on polarity of the stationary phase into two types, which are normal-phase HPLC, and reversed-phase HPLC. Normal phase HPLC (NP-HPLC) system consists of a polar stationary phase and a non-polar mobile phase. This method is suitable for analysis of relatively polar compounds. The separation of component in a mixture is based on polarity difference of compounds. Relatively polar components adsorb strongly on the stationary phase therefore they are retained in the column longer than less polar components. The retention time increases with the polarity of the component.

Another type of HPLC methods that has been routinely used for analysis and separation of organic compounds is the reversed phase HPLC (RP-HPLC or RPC). The system of RP-HPLC consists of a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is a silica gel which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}.

With these stationary phases, retention time is longer for molecules which are more non-polar, while polar molecules elute more readily. The retention time can be increased using a more polar solvent as the mobile phase. On the other hand, a decrease in retention time can be obtained with a more hydrophobic solvent.

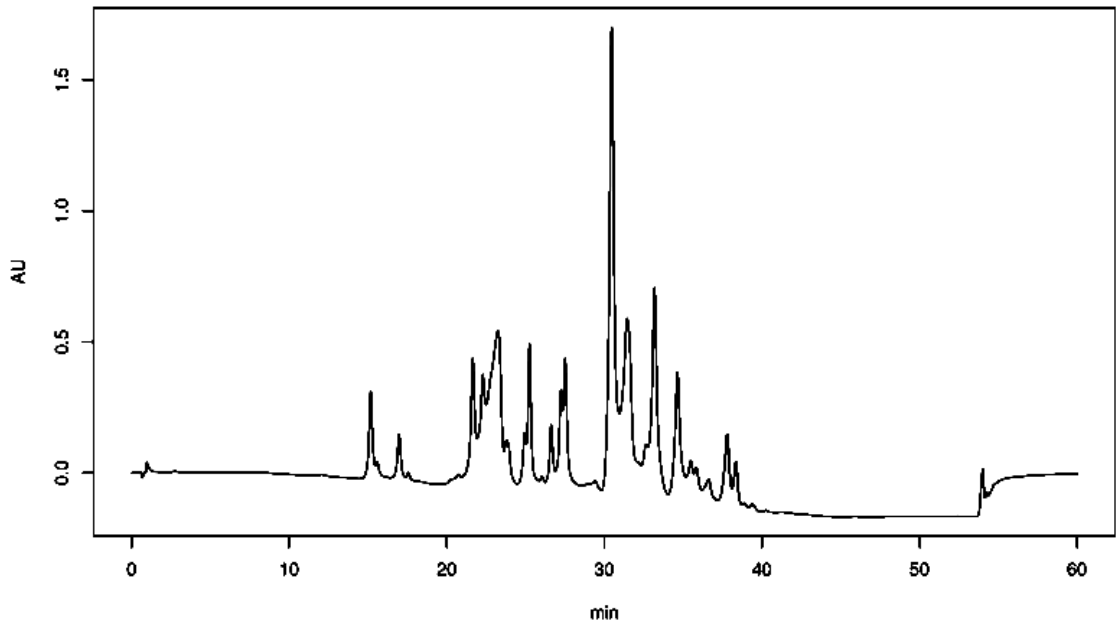


Figure 2.4 A chromatogram of complex mixture (perfume water) obtained by reversed phase HPLC

2.2 Spectroscopic techniques

Spectroscopic techniques were used to analyze and identify the compounds obtained from chromatographic separation. The related spectroscopy techniques that were utilized in this research are described as follows.

2.2.1 Ultraviolet-visible (UV-VIS) spectroscopy

UV-VIS spectroscopy is an analytical technique that provides useful information on absorption property of the molecules. The absorption occurs when the energy

required for an electronic transition matches with the energy of the incoming radiation. For most organic compounds, the observed wavelengths are in the 190-700 nm region. Compounds that can absorb UV or visible light must contain certain functional groups known as chromophores, which usually are unsaturated groups.

The diagram below shows a beam of monochromatic radiation of radiant power, P_0, directed at a sample solution. Absorption takes place and the beam of radiation leaving the sample has radiant power P.

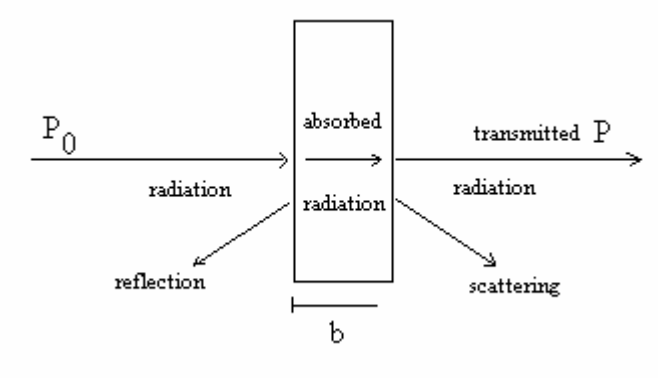


Figure 2.5 Interaction of compound with radiation

The amount of radiation absorbed may be measured as:

Transmittance, $T = P/P_0$

% Transmittance, $\%T = 100\ T$

$$A = \log_{10} P_0 / P \quad (2.2)$$

$$A = \log_{10} 1 / T \quad (2.3)$$

$$A = \log_{10} 100 / \%T \quad (2.4)$$

$$A = 2 - \log_{10} \%T \quad (2.5)$$

The relationship between absorbance and concentration can be determined by Beer-Lambert's law as:

$$A = \varepsilon bc \qquad (2.6)$$

where; A = absorbance (no units, since $A = \log_{10} P_0 / P$)

ε = the molar absorptivity with units of L mol^{-1} cm^{-1}

b = cell path length in cm

c = concentration in mol/L

This spectroscopic technique is one of the useful methods to determine the class of natural product compounds and to quantitatively study biological activity of the compounds. A UV-VIS spectrum can be measured by preparing a very dilute solution of sample in a suitable solvent, which does not give absorption band within the same area as the sample.

The most widely used solvent for UV-VIS spectroscopy is 95% ethanol. Other commonly used solvents are water, methanol, hexane, and petroleum ether. However, absolute alcohol, should be avoided because it contains residual benzene which absorbs in the short UV region. In addition, organic solvents such as chloroform and pyridine are avoided because they absorb strongly in the 200-260 nm region. A UV-VIS spectrum is a plot of absorbance versus the absorption wavelength. The absorption data are reported as λ_{max}, the absorption at peak maximum. An example of UV-VIS spectra is shown in Figure 2.6.

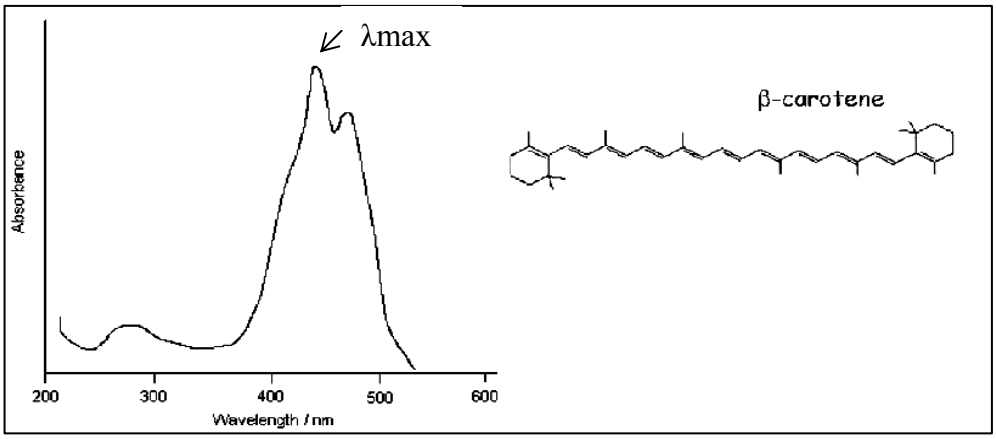


Figure 2.6 UV spectrum of β-carotene

The data from UV-VIS spectra can be very useful for identification of the chemical classes of plant pigments. Table 2.1 shows the absorption properties of some important classes of natural products.

Table 2.1 Spectral properties of the different classes of plant pigment[a].

Pigment class	Visible spectral range (nm)	Ultraviolet range (nm)
Chlorophylls (green)	640-660 and 430-470	intense short UV absorption due to protein attachment
Phycobilins (red and blue)	615-650 or 540-570	
Cytochromes (yellow)	545-605 (minor band sometimes at 415-440)	
Anthocyanins (mauve or red)	475-550	ca. 275

Table 2.1 (Continued).

Pigment class	Visible spectral range (nm)	Ultraviolet range (nm)
Betacyanins		
(mauve)	530-554	250-270
Carotenoids		
(yellow to orange)	400-500 (a major peak with two minor peaks or inflections)	-
Anthraquinones		
(yellow)	420-460	3-4 intense peak between 220-290
Chalcones and Aurones		
(yellow)	365-430	240-260
Yellow flavonols		
(yellow)	365-390	250-270

[a] Mean data from Phytochemical Methods (20), Harborne, J. B., (1998), London: Chapman & Hall.

2.2.2 Fourier transform-infrared (FT-IR) spectroscopy

The infrared region of the electromagnetic spectrum extends from 14,000 cm^{-1} to 10 cm^{-1}. The absorption region of most organic compounds is in the mid-infrared region (4,000 cm^{-1} to 400 cm^{-1}). The concept of IR spectroscopy is based on the vibrational transition energies of chemical bonds within the molecules. The infrared spectrum of a sample is measured by passing a beam of infrared light through the sample. The transmitted light shows how much energy was absorbed at each wavelength. From this process, a transmittance or absorbance spectrum can then be produced to show the positions of the absorbed wavelengths.

There are several techniques to prepare the sample of different physical forms for IR measurements. For solid samples, the two common methods of preparation are Nujol mull, and KBr disk. In addition, the sample can be prepared as a solution by dissolving in a suitable solvent that does not absorb in the region of interest. For this study, all solid samples were prepared as KBr disks. General procedure to prepare a KBr disk is by mixing and grinding 1-2 mg of a solid sample with KBr until very fine particles are obtained. The mixture is then placed in a small die and put under pressure mechanically for several minutes before removing the die and the KBr disk. The disk is then placed in a sample holder for IR measurement.

In an IR spectrum; the functional group information of the molecule can be obtained from the characteristic absorption peaks in the "functional group region", which is ranging from 4000-1300 cm^{-1}. The infrared absorption frequencies of some classes of natural products are summarized in Table 2.2. In addition, an IR spectrum can provide specific characteristic for each molecule in the fingerprint region at the frequency below1300 cm^{-1}. The Intensities of the IR bands are reported as strong (S), medium (M) and weak (W).

Table 2.2 Characteristic infrared frequencies of some classes of natural product[a].

Class of compound	**Approximate positions of characteristic bands above 1300 cm^{-1}**
Alkanes	2940 (S), 2860 (M), 1455 (S), 1360 (M)
Alkenes	3050 (W-M), 1850 (W), 1650 (W-M), 1410 (W)
Aromatics	3050 (W-M), 2100-1700 (W), 1600, 1580, 1500 (W-M)
Acetylenes	3310 (M), 2225 (W), 2150 (W-M), 1300 (W)

Table 2.2 (Continued).

Class of compound	Approximate positions of characteristic bands above 1300 cm^{-1}
Alcohols and Phenols	3610 (W-M), 3600-2400 (broad), 1410 (M)
Aldehydes and Ketones	2750 (W), 2680 (W), 1820-1650 (S), 1420 (W-M)
Esters and Lactones	1820-1680 (S)
Carboxylic acids	3520 (W), 3400-2500 (broad, M), 1760 (S), 1710 (S)

[a] Mean data from Phytochemical Methods (22), Harborne, J. B., (1998), London: Chapman & Hall.

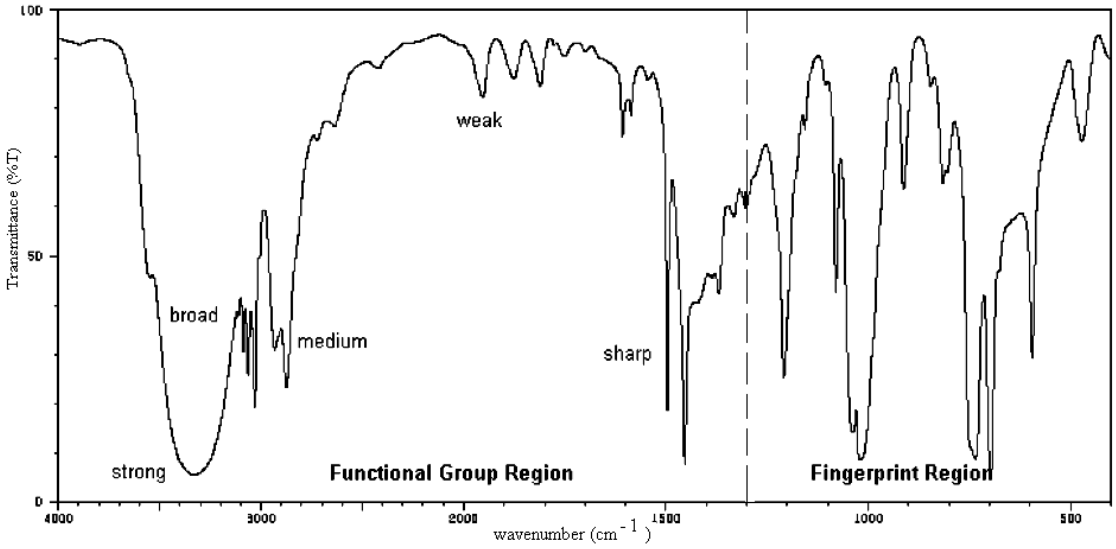


Figure 2.7 IR spectrum (benzyl alcohol)

Although, IR spectroscopy alone can not be used for structure elucidation of an unknown compound, it can provide useful structural information when used in combination with other spectroscopic techniques such as NMR.

2.2.3 Liquid chromatography-mass spectrometry (LC-MS)

Liquid chromatography-mass spectrometry (LC-MS) is an analytical technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. Mass spectrometry is an analytical technique that identifies the chemical composition of a compound or sample based on the mass-to-charge ratio of charged particles. A sample undergoes chemical fragmentation to form charged particles (ions). The ratio of mass to charge of the particles is calculated by passing them through electric and magnetic fields in a mass spectrometer. The diagram of the components of a mass spectrometer is shown in Figure 2.8.

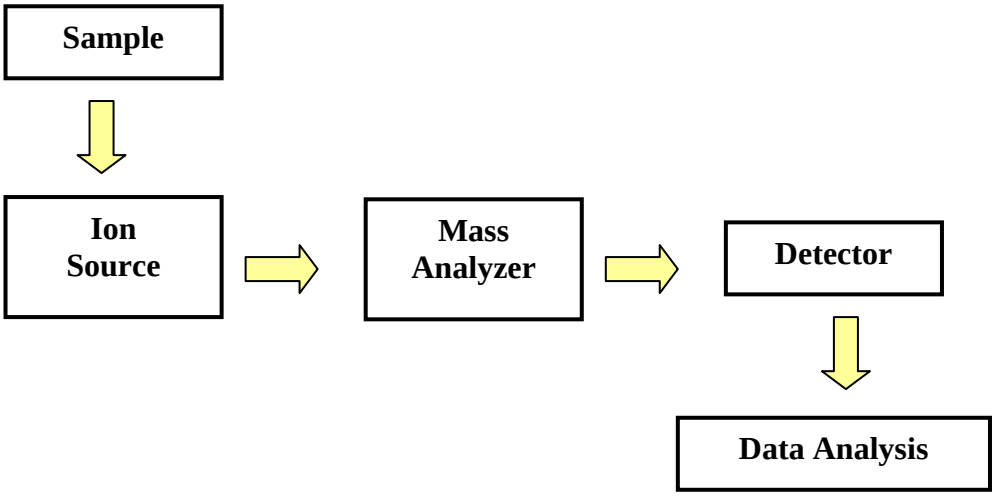


Figure 2.8 The diagram of components of mass spectrometer

Mass spectrometer has three necessary parts: an ion source, which transforms the molecules in a sample into ionized fragments; a mass analyzer, which sorts the ions by their masses by applying electric and magnetic fields; and a detector, which

measures the value of some indicator quantity and thus provides data for calculating the abundances of each ion fragment present.

A. Ionization techniques

Different types of ionization techniques and their applications are concluded in Table 2.3.

Table 2.3 Application of ionization methods[a].

Ionization method	Typical Analytes	Samples Introduction	Method Highlights
Electron Impact (EI)	Relatively small volatile	GC or liquid/ solid probe	Hard method
Chemical Ionization (CI)	Relatively small volatile	GC or liquid/ solid probe	Soft method
Electrospray(ESI)	Non-volatile	Liquid chromatography or syringe	Soft method
Fast Atom Bombardment(FAB)	Non-volatile	Sample mixed in viscous matrix	Soft method but harder than ESI or MALDI
Matrix Assisted Laser Desorption MALDI	Peptide Proteins Nucleotides	Sample mixed in solid matrix	Soft method

[a] Mean data from Bioactivities and Chemical Constituents of a Vietnamese Medicinal Plant Jasminum Subtriplinerve Blume (38), Ngan, D. H., (2005). Denmark: Roskilde.

I. Chemical Ionization (CI)

Chemical ionization uses reagent gas to react with electron beam to form reagent gas ions, which will react further with neutral reagent gas before reacting with the sample to form protonated molecular ion. Some common reagent gases include: methane, ammonia, and isobutane.

$$CH_4 + \bar{e} \longrightarrow CH_4^{+\cdot} + 2\bar{e}$$

$$CH_4 + CH_4^{+\cdot} \longrightarrow CH_5^{+\cdot} + CH_3$$

$$M + CH_5^{+\cdot} \longrightarrow CH_4 + [M+H]^{+\cdot}$$

Chemical ionization for gas phase analysis is either positive or negative: Positive reagent gives $[M+H]^+$ or $[M+NH_4]^+$, and negative reagent gives $[M-H]^-$ or $[M+X]^-$.

II. Atmospheric pressure chemical ionization (APCI)

Atmospheric pressure chemical ionization is a form of chemical ionization which takes place at atmospheric pressure.

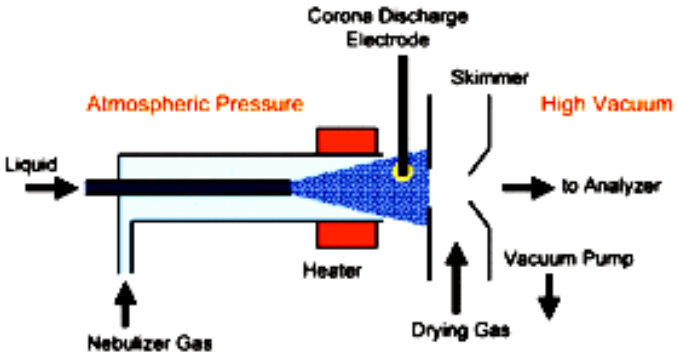


Figure 2.9 The diagram of atmospheric pressure chemical ionization

Typically, the mobile phase containing eluting analyte is heated to relatively high temperatures (above 400 ^{o}C), and sprayed with high-flow rate beam of nitrogen to generate the aerosol cloud, which is subjected to a Corona discharge electrode to create ions.

B. Mass analyzer

There are several mass analyzers that are commonly used in the mass spectrometer, some of which are shown in Table 2.4.

Table 2.4 Some kinds of mass analyzers[a].

Analyzer	System Highlights
Quadrupole Mass Filter and Quadrupole ion storage (ion trap)	Unit mass resolution, fast scan
Sector (Magnetic and/or Electrostatic); Double focusing	High resolution, exact mass
Time-of-Flight (TOF)	No limitation for m/z maximum, high throughput
Ion Cyclotron Resonance (ICR)	Very high resolution, exact mass

[a] Mean data from Bioactivities and Chemical Constituents of a Vietnamese Medicinal Plant Jasminum Subtriplinerve Blume (38), Ngan, D. H., (2005). Denmark: Roskilde.

C. Detector

Different types of detectors can be classified in either of two categories. The photographic plate and the Faraday cage allow a direct measurement of the charges

that reach the detector, whereas electron or photonmultiplier detectors and array detectors increase the intensity of the signal.

I. Photographic Plates and Faraday Cylinders

Photographic plates located behind the analyzer as detectors: ion sharing the same m/z ratio all reach the plate at the same place. A calibration scale allows the determination of m/z and m values.

A Faraday cylinder can also be used. Ions reach the inside of the cylinder where they give up their charge. The discharge current is then amplified and measured.

II. Electron multipliers

A positive or negative ion reaching the plate (conversion dynode) causes the emission of several secondary particles. These secondary particles can include positive ions, negative ions, electron and neutrals. When positive ions strike the negative high-voltage conversion dynode, the secondary particles of interest are negative ions and electrons. When negative ions strike the positive high-voltage conversion dynode, the secondary particles of interest are positive ions. These secondary particles are accelerated into the continuous-dynode electron multiplier. They strike the cathode with sufficient energy to dislodge electrons as they collide with its curving inner walls. These electrons pass further into the electron multiplier, again striking the walls, causing the emission of more and more electrons as they travel toward the ground potential. A cascade of electrons is created that finally results in a measurable current at the end of the electron multiplier. The amplification may reach 10^7.

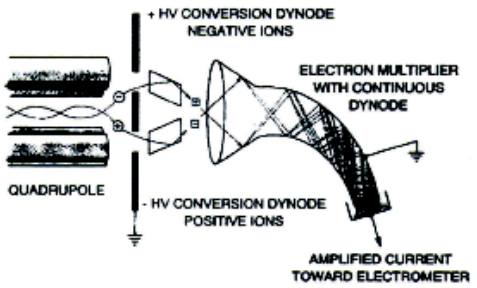


Figure 2.10 Electron multiplier. 0 = incident ions; □ = secondary particles

In this research, the detector in the LC/MS is a high-energy-dynode (HED) electron multiplier. The detector receives the ions exiting the quadrupole mass filter which separates ions according to their mass-to-charge ratio (m/z). It generates a signal current proportional to the number of ions it receives.

III. Array detectors

An array detector is a plate where parallel cylindrical channels have been drilled. The plate input side is kept at a negative potential of about 1 kV compared with the output side. Electron multiplication is insured by a semiconductor substance covering each channel and giving off secondary electrons. The amplification can reach 10^8.

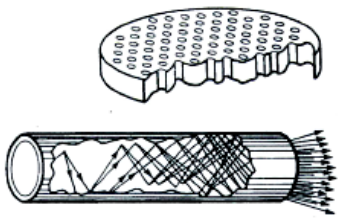


Figure 2.11 Cross-section of an array plate and electron multiplication within a Channel

IV. Photon multipliers

Photon multiplier is made up of two conversion dynodes, a phosphorescent screen and a photon multiplier. This device allows the detection of both positive and negative ions. The amplification ranges from 10^4 to 10^5.

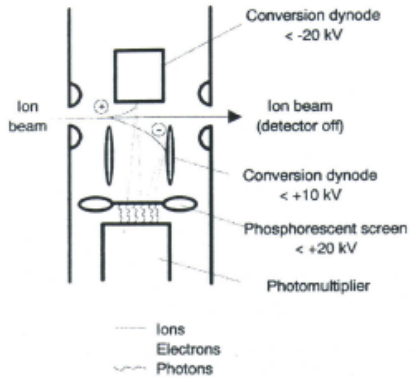


Figure 2.12 Photon multiplier

2.2.4 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is one of the versatile analytical tools for elucidation of molecular structures. This technique defines the number and types of nuclei present in a molecule according to their chemical environments. The resonance frequency is reported as chemical shift in δ scale with a unit in parts per million (ppm). Chemical shift can be defined as the frequency that shifts from the chemical shift of reference compound. The most widely used reference compound is tetramethylsilane (TMS). Chemical shifts of nuclei are influenced by the surrounding electrons, called shielding effect. The factors that determine the shielding around nuclei are the electron density around each nucleus as well as the orientation of molecules in the magnetic field. In modern NMR spectroscopy, pulsed fourier transform (FT) method has been used to obtain the NMR signals. The NMR signals are collected as FID (free induction de-

cay) as a function of time before they can be processed with the NMR software to the peaks as a function of frequency.

A. One dimensional NMR

I. Proton NMR (^{1}H NMR)

^{1}H NMR is a plot between proton resonance frequencies versus the intensities of the signal. ^{1}H NMR is one of the routine techniques for structure determination of organic compounds. It provides information about the number of protons of difference environment within the molecules. The number of neighboring protons can be determined from the splitting pattern of NMR signals. The peak area of each signal, termed as integration, provides information about number of protons present in the molecule. The normal chemical shift range in a ^{1}H NMR spectrum is between 0-12 ppm depending on the functional groups around protons.

II. ^{13}C NMR

^{13}C NMR is another one dimensional NMR technique that gives useful structural information of the molecules. Due to low natural abundance ^{13}C isotope, only 1.1 % in nature, this technique has a lower sensitivity than ^{1}H NMR. In normal ^{13}C NMR experiment, the attached protons are decoupled from the ^{13}C nuclei; therefore, only a singlet peak representing each carbon can be observed. The range of ^{13}C NMR chemical shift values is normally from 0-240 ppm.

B. Two-dimensional (2D) NMR

2D-NMR experiments are normally used for compounds of complex molecular structures. The major advantage of 2D-NMR techniques is that they show connec-

tivity between nuclei of the same on different types. For this study, the 2D-NMR experiments that were used to characterize the chemical structures of the isolated compounds include homonuclear correlated spectroscopy (^{1}H-^{1}H COSY), heteronuclear single quantum coherence spectroscopy (HSQC) and heteronuclear multiple bond correlation spectroscopy (HMBC).

- **^{1}H-^{1}H COSY**

The COSY experiment is a powerful method to observe spin-spin couplings between nearby protons within a molecule. A COSY spectrum has a projection of ^{1}H NMR along diagonal, conventionally from top right to bottom left. The connectivity between protons can be seen from cross peaks of diagonal. To determine which proton is coupling to which, a line from a diagonal peak can be drawn to cross peaks of each axis and that should show the connectivity to another proton peak along diagonal.

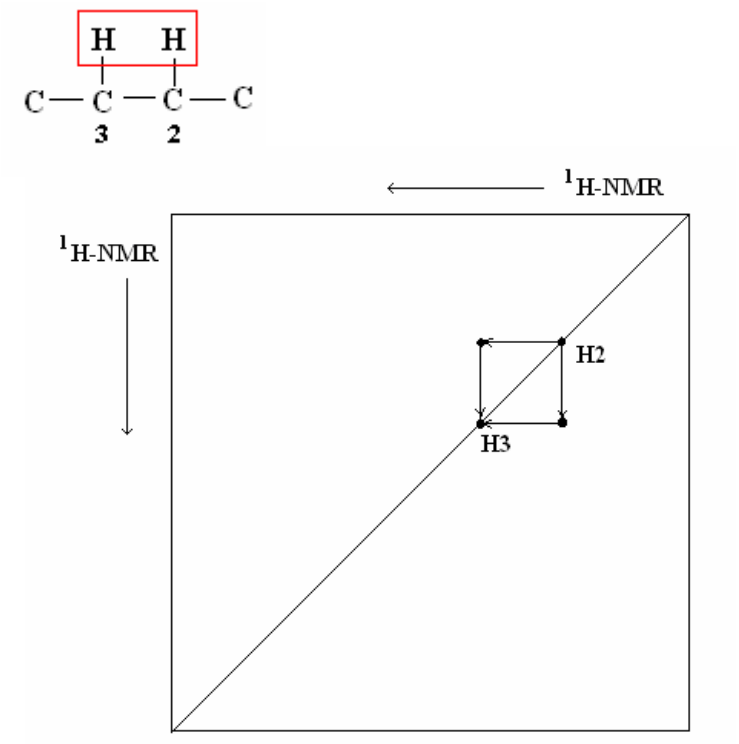


Figure 2.13 ^{1}H-^{1}H COSY spectrum

- **Heteronuclear single quantum correlation spectroscopy (HSQC)**

This 2D experiment correlates the chemical shift of proton with the chemical shift of the directly bonded carbon. This experiment provides one-bond ^{13}C-^{1}H correlation information. On the horizontal axis is the ^{1}H spectrum and on the vertical axis is the ^{13}C spectrum of the molecule. Cross peaks show which proton is attached to which carbon.

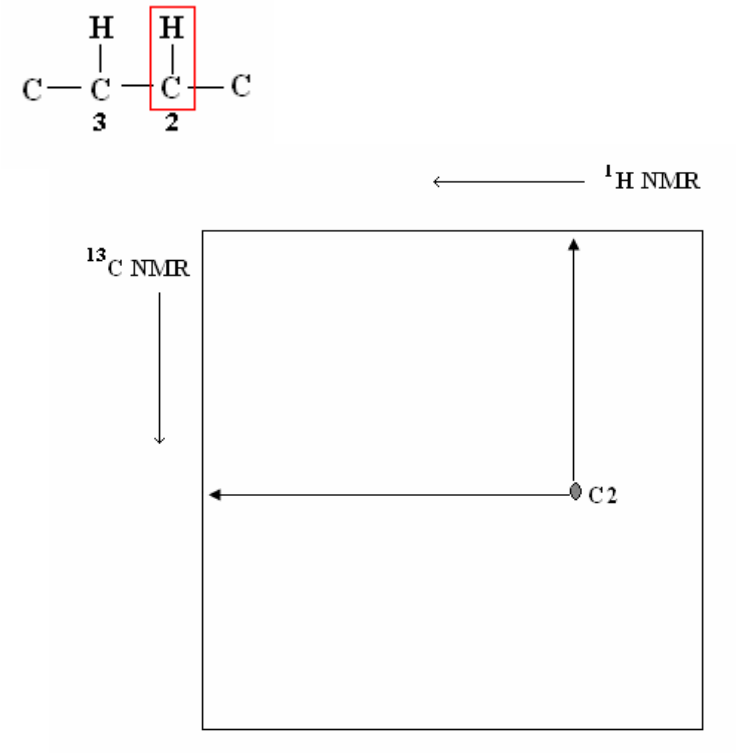


Figure 2.14 HSQC spectrum

- **Heteronuclear multiple bond correlation (HMBC)**

The HMBC experiment provides long-range coupling between proton and carbon of 2-3 bonds apart. The experiment can be adjusted to detect relatively large coupling constants (4-10 Hz) or smaller. This experiment in conjunction with ^{1}H-^{1}H COSY enables the elucidation of the skeleton of the compound.

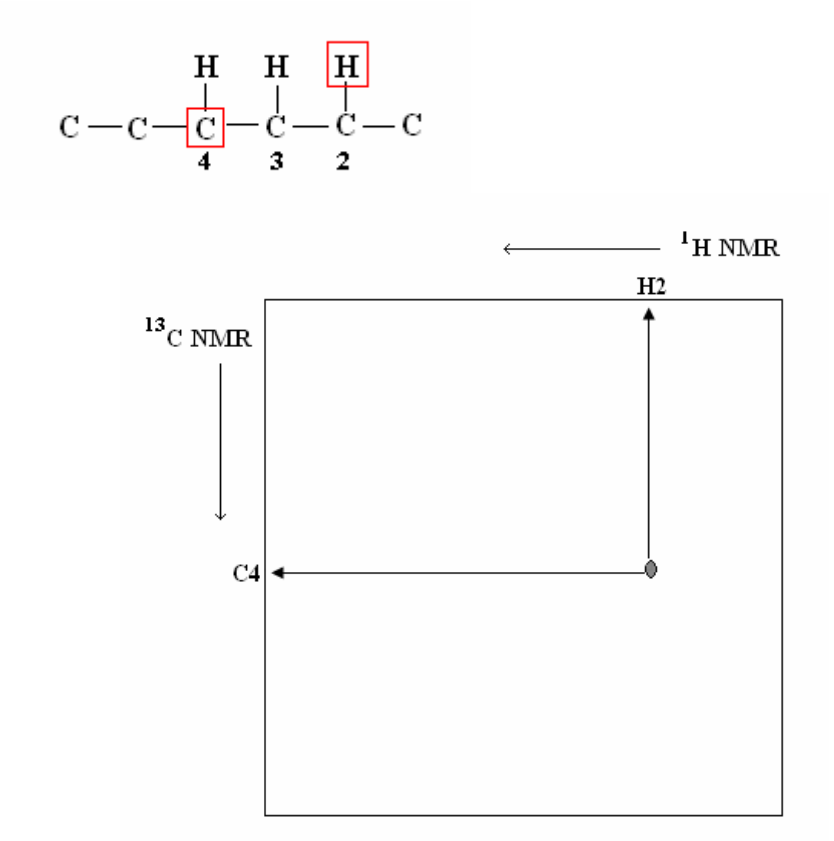


Figure 2.15 HMBC spectrum

CHAPTER III

EXPERIMENTAL

3.1 Chemicals and instruments

3.1.1 Chemicals

All commercial grade solvents were distilled before used. Other chemicals as listed below were analytical grade reagents.

Absolute Ethanol	CARLO ERBA
DPPH (1,1-Diphenyl-2-picryl-hydrazyl)	Fluka
Ethyl acetate	CARLO ERBA
Hexane	CARLO ERBA
Methanol	CARLO ERBA
Acetonitrile (HPLC grade)	CARLO ERBA
Isopropanol	CARLO ERBA
Dichloromethane	CARLO ERBA
Perchloric acid ($HClO_4$)	Fluka
L-ascorbic acid (Vitamin C)	Fluka
(+)-Catechin	Sigma
Deuterated Chloroform	Aldrich
Deuterated methanol	Aldrich
Dimethyl sulfoxide	CARLO ERBA
Silica gel 60 (0.043-0.063 mm)	Merck

Silica gel 60 (0.063-0.200 mm)	Merck
Silica gel 60 F_{254} pre-coated 20 x 20 cm^2	Merck
Sephadex LH-20	Amersham Biosciences

3.1.2 Instruments

UV/VIS Spectrophometer series CARY 1E	Varian
UV/VIS Spectrophotometer series 6405	Jenway
UV-Cabinet II	Camag
HPLC (Semi-Prep) Model 600	Waters
Pump: Waters 600 Controller	
Injector: Waters Delta 600	
Detector: Waters 2487 (Dual λ Absorbance Detector)	
Program: Empower Software	
Column: Waters PrepLC™ 25mm Module	Waters
(25 mm x 100 mm)	
: Bio-sil C18 HL 90-5 S	BIO-RAD
(4.6 mm x 250 mm)	
Guard: Waters Guards. Pak™	
Nylon membrane filter : 0.45 μm	Whatman
FT-IR Spectrophotometer Model Spectrum GX	Perkin-Elmer.
NMR Spectrometer:	
INOVA 300 (at Suranaree University of Technology)	Varian
DRX-500 (at University of Arizona)	Bruker
DRX-600 (at University of Arizona)	Bruker

INOVA 600 (at University of Arizona)	Varian
LC-MS series 1100	Agilent
Rotary evaporator	BüCHI
Heating bath: BüCHI Heating bath B-490	
Rotavapor: BüCHI Rotavapor R-200	
Controller: BüCHI Vacuum Controller V-800	
Vac® V-500	
Thermostat: Heto HMT200	
Ultrasonic bath Model 6900	CREST
Vortex mixer Model 232	Fisher Scientific
Centrifuge: Labofuge 200	-
Labopette	HIRSCHMANN
Electronic Analytical Balance	Sartorius
Filter paper #1	Whatman
Hot air oven	Memmert
Blender: BE-112	OTTO
Soxhlet apparatus	-
Distilled apparatus	-

3.2 Chromatography techniques

3.2.1 Thin layer chromatography (TLC)

Adsorbent: Silica gel 60 F_{254} pre-coated on aluminum plates (Merck) with layer thickness of 0.2 mm were used for analytical TLC and preparative TLC.

Detection on chromatography plate:

1. Ultraviolet detection: Spots on TLC plates were detected under ultraviolet light at wavelengths 254 nm and 366 nm.

2. Spray detection: A 20% (w/v) aqueous solution perchloric acid reagent was sprayed to TLC plate, and the plate was heated at 100 °C until coloration appeared. This spray reagent is useful for detection of steroids and triterpenes.

3.2.2 Flash column chromatography (Flash CC)

Column: Glass columns with dimensions of 10 x 160 mm, 15 x 160 mm, and 30 x 575 mm were used for separation and purification purposes.

Adsorbent: Silica gels (particle sizes 0.040-0.063 mm, and 0.063-0.200 mm).

Packing method: Dry packing; Dry powder of silica gels was added into the column before filling the column with hexane. Hexane was passed the column until silica gels saturated with solvent. Then the sample was added.

Sample loading: The sample was dissolved in a small amount of elution solvent and added gently onto the top of the column.

Elution:

1. Gradient system: The column was eluted with gradient solvents between hexane and EtOAc at the total volume of 8 mL (hexane portion was decreased 5% per loading).

2. Isocratic system: The column was eluted with isocratic solvent. In both systems, air pressure was applied through the inlet (flow rate ~ 100 drops/min).

Fraction collection: Each fraction was collected and combined on the basis of TLC analysis.

3.2.3 Gel filtration

Column: Glass column with dimensions of 10 x 160 mm was used.

Adsorbent: Sephadex LH-20

Packing method: Slurry packing; Dry powder of sephadex LH-20 was swollen before used by suspending in MeOH for 24 hours before loading the slurry into the column in one continuous motion.

Sample loading: Sample was dissolved in a small amount of MeOH and applied onto the top of column.

Elution: The column was eluted with MeOH.

3.2.4 HPLC

Injection volume: 50 µL (Analytical-HPLC), and 5000 µL (SemiPrep-HPLC)

Column: C_{18} at 25 °C

Detection wavelength: 450 nm

Mobile Phase:

Solvent A: CH_2Cl_2 / MeOH / ACN / water in 5:85:5.5:4.5 v/v ratio

Solvent B: CH_2Cl_2 / MeOH / ACN / water in 22:28:45.5:4.5 v/v ratio

Table 3.1 HPLC condition.

Time			**Flow rate (mL/min)**	
(minutes)	**%A**	**%B**	**Anal-HPLC**	**Prep-HPLC**
0	100	0	0.7	10.0
14	100	0	0.7	10.0
20	0	100	0.7	10.0
50	0	100	0.7	10.0

3.2.5 LC-MS

Instrument: Agilent

Column: C_{18}

Flow rate: 0.4 mL/min Time: 5 min

Mobile phase: 100% ACN Mode: Positive mode

Injection Volume: 100 µL Detection Wavelength: 450 nm

Ionization: Atmospheric pressure chemical ionization (APCI)

Mass Analyzer: Quadrupole mass analyzer Scan Range: 350-700 m/z

Detector: High energy dynode (HED) electron multiplier

3.3 Plant identification

The flowers, leaves and capsules of *R. ignea* were collected from the campus area of Suranaree University of Technology, Nakhon Ratchasima, Thailand. The plant materials were dried at 40 °C for 48 hours before submitting for plant authentication at the Forest Herbarium, Bangkok, Thailand.

3.4 Preparation of plant materials

The whole flowers without calyces of *R. ignea* were collected from the trees around the campus area of Suranaree University of Technology, Nakhon Ratchasima, Thailand, during the month of December 2006 to January 2007. The flowers were washed and dried in the oven at 40 °C for 48 hours. The dried plant materials were stored at room temperature before using.

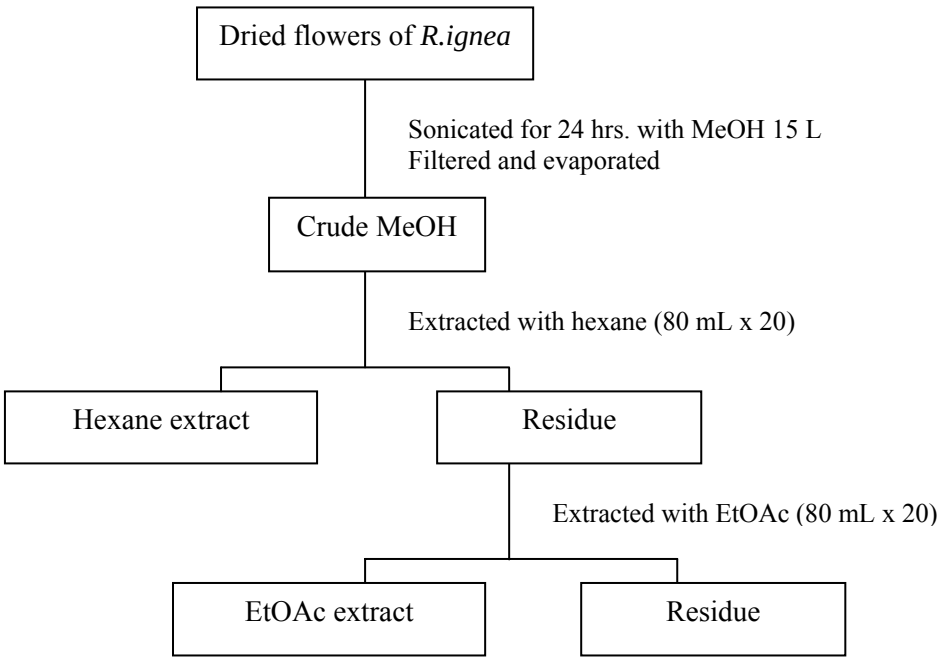
3.5 Extraction

In this research, two extraction methods; sonication and soxhlet extraction were used for isolation of the antioxidant component from the plant materials. The two methods were compared and evaluated by the amount of ethyl acetate extract obtained, the amount of solvents used for extractions, and TLC analysis of the components.

A. Extraction procedures

I. <u>Sonication</u>

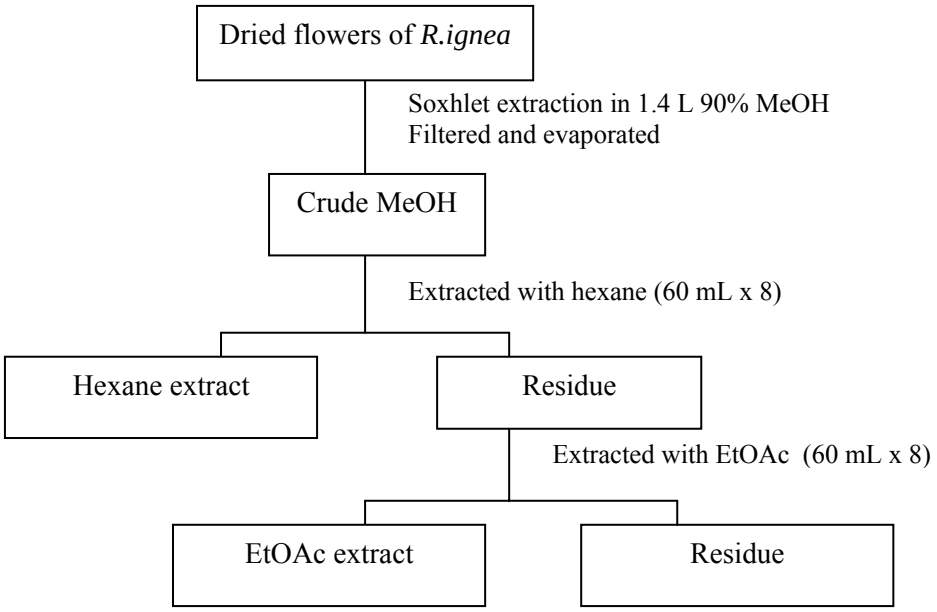
Dried flowers 300 g were cut to small pieces and extracted with methanol 15 L by sonicating at 40 °C for 24 hours. The obtained crude extract was filtered and evaporated by rotary evaporator. The dried crude extract was extracted consecutively with *n*-hexane and ethyl acetate to give ethyl acetate extract as shown in Scheme 3.1.



Scheme 3.1 Extraction of the flowers of *R. ignea* by sonication

II. Soxhlet extraction

The dried flowers 50 g were milled to powder and extracted with 90% methanol (1.4 L) by soxhlet extractor at 100 °C for 7 hours. The solution was filtered and evaporated by rotary evaporator to give methanol crude extract. The dried crude extract was extracted consecutively with *n*-hexane and ethyl acetate to give hexane extract and ethyl acetate extract as shown in Scheme 3.2.



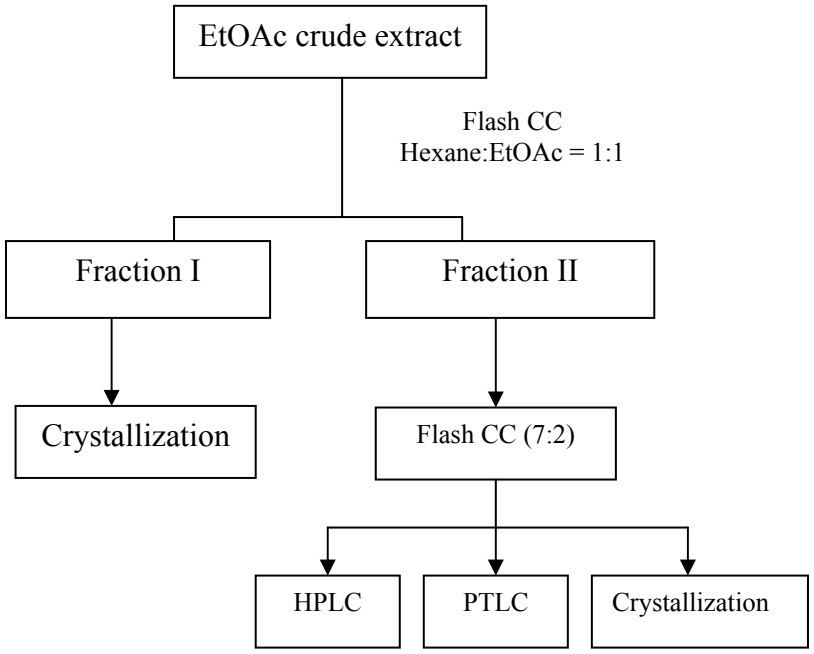
Scheme 3.2 Extraction of the flower *R. ignea* by soxhlet extraction

B. TLC analysis of ethyl acetate extract

The components of ethyl acetate extract from sonication and soxhlet extraction methods were compared by TLC analysis using the mixture of hexane and ethyl acetate (1:1) as the mobile phase. The developed TLC plates were sprayed with 0.2 mM DPPH to indicate the position of antioxidant component.

3.6 Isolation and purification

Ethyl acetate extract was dissolved using minimum volume of the elution solvent (hexane:ethyl acetate = 1:1). The solution was then added onto the top of column and the sample was allowed to soak into silica gel (~0.5 g of sample per 200 mL of silica gel). Next, the elution solvent was added to the column and pressure was applied to force the solvent through the column. Each fraction was analyzed by using TLC technique. The fractions of the same components were combined and concentrated by rotary evaporator. The isolation and purification diagram was presented in Scheme 3.3.



Scheme 3.3 Diagram of isolation and purification

3.7 Structure elucidation

The structures of the isolated compounds were elucidated by using spectrometry techniques UV, FT-IR, LC-MS and NMR. The results were discussed in the Chapter IV.

UV: All samples were dissolved in absolute ethanol (abs. EtOH).

FT-IR: The spectra of all samples were measured as KBr pallets.

LC-MS: All samples were dissolved in acetonitrile and filtered with 0.45 μm nylon membrane filter before they were injected to the LC-MS system.

NMR: All samples were dissolved in deuterated chloroform ($CDCl_3$) with TMS as the internal reference.

3.8 Antioxidant activity study

3.8.1 Preparation of DPPH solution

In this study, the antioxidant activity was measured based on DPPH radical scavenging activity.

For TLC screening test

The 0.2 mM DPPH stock solution was prepared daily by dissolving 0.0039 g of DPPH radical with MeOH and the volume was adjusted to 50 mL in volumetric flask.

For spectrophotometric assay

The 0.0631 mM DPPH stock solution was prepared daily by dissolving 0.0025 g of DPPH radical with DMSO and the volume was adjusted to 100 mL in volumetric flask. To retain free radical activity of DPPH, the solutions were stored at 4 °C in the dark until they were ready to be used.

3.8.2 TLC screening test for antioxidants

This method was used to screen for the antioxidant activity of components in crude extracts and isolated compounds. This TLC screening method was prepared according to the protocol reported by Hostettmann, Terreauv, Marton, and Potteral (1997). A solution of the sample was spot on a TLC plate then the plate was put into a

developing chamber, which was saturated with vapor of developing solvents. The developed TLC plate was sprayed with 0.2 mM methanolic solution of DPPH.

At the position of compounds with antioxidant activities, the color of spots turned into yellow or retains their original colors on the purple background of the TLC plate after spraying with DPPH indicating the interaction of antioxidants with DPPH.

3.8.3 Spectrophotometric assay

To avoid the interference of compounds with a chromophoric system that have similar light absorbance to the reagent (DPPH) at the maximum absorbance at 515 nm, the protocols of Escrig *et al*. (2000) was used and modified by using DMSO as the solvent. Added to 3.9 mL of 6.31 x 10^{-5} M methanolic DPPH radical were 0.1 mL of samples at different concentrations (0.1 x 10^{-4}, 0.3 x 10^{-4}, 0.4 x 10^{-4}, 0.9 x 10^{-4} and 1.2 x 10^{-4} M in DMSO). The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark. The absorbance of the solutions was measured at 580 nm, by using MeOH to set zero absorbance. The percentage of radical scavenging was calculated according to the following equation:

$$\% \text{ radical scavenging} = [1-A_{sample}/A_{control}] \times 100 \qquad (3.1)$$

where A_{sample} = absorbance with extract

$A_{control}$ = absorbance without extract

Determination of IC_{50} Values

The term IC_{50} is defined as the concentrations of samples required to scavenge 50% of free radicals. A lower IC_{50} value indicates greater antioxidant activity. IC_{50} values were calculated from the regression equations prepared by plotting a graph

between concentrations of samples or moles of samples per moles of DPPH. The percentage of radical scavenging was calculated by using the program Microsoft Excel to create trendline that showed equation for IC_{50} value.

CHAPTER IV

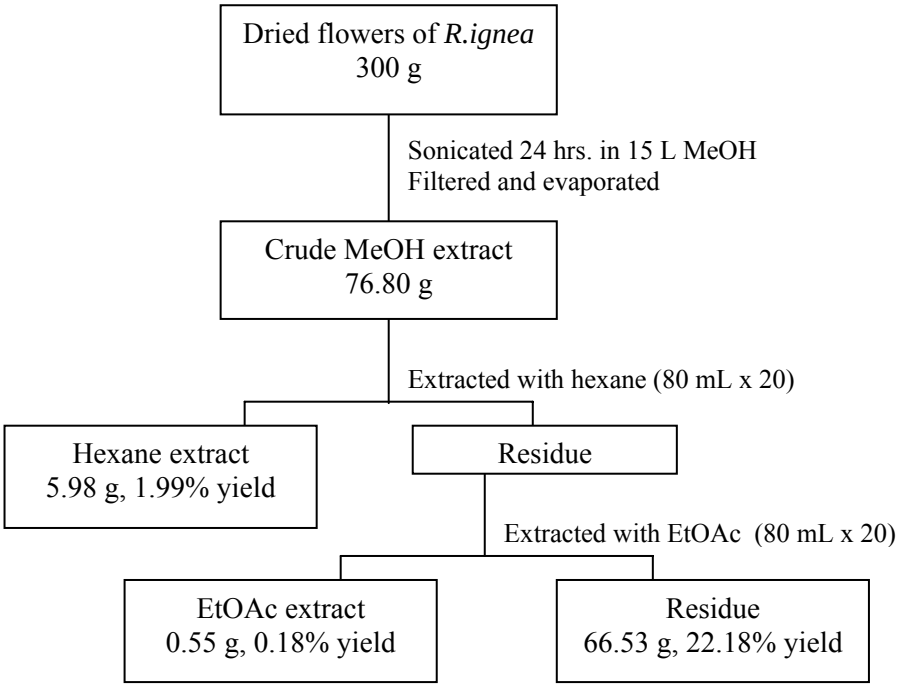
RESULTS AND DISCUSSION

4.1 Extraction

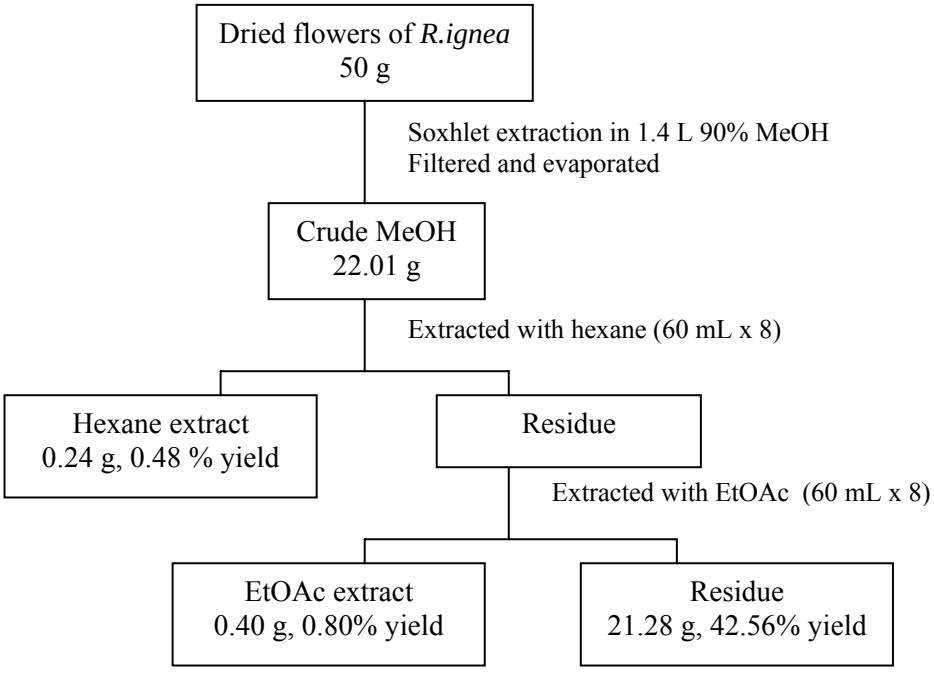
The investigation of chemical constituents that show antioxidant activities from the ethyl acetate extract of the flowers of *Radermachera ignea* (Kurz) Steenis was done by two extraction methods, which were sonication, and soxhlet extraction. These two methods were studied in comparable to each other. The results were described as follows:

A. <u>Comparison of extraction methods</u>

The result from sonication was shown in scheme 4.1. By using this extraction method, dried flowers 300 g were extracted by 15 L of methanol yielding 76.80 g of methanol crude extract. After this methanol crude extract was subsequently extracted with hexane followed by ethyl acetate, the extractions yielded 0.55 g of ethyl acetate extract (0.18% based on weight of dried flowers). While, the result from soxhlet extraction was shown in scheme 4.2. By using this extraction method, dried flowers 50 g were extracted with 1.4 L of 90% methanol to obtained 0.40 g of ethyl acetate extract (0.80% based on weight of dried flowers). We found that sonication showed lower percent yield of ethyl acetate extract than soxhlet extraction and sonication used a lot of solvent and much more time than soxhlet extraction for solvent evaporation to obtain crude extract.



Scheme 4.1 The result of extraction by sonication



Scheme 4.2 The result of extraction by soxhlet extraction

Table 4.1 Comparison of extraction methods.

Method	Solvent (mL)/g sample	% Yield (ethyl acetate extract)
sonication (300g sample)	50	0.18
soxhlet extraction (50g sample)	28	0.80

B. TLC Comparison of the crude extracts

By comparing the TLC analysis results of the ethyl acetate extract obtained from sonication and soxhlet extraction, both extraction methods gave similar results before and after spraying with a solution of 0.2 mM DPPH as demonstrated in Figure 4.1.

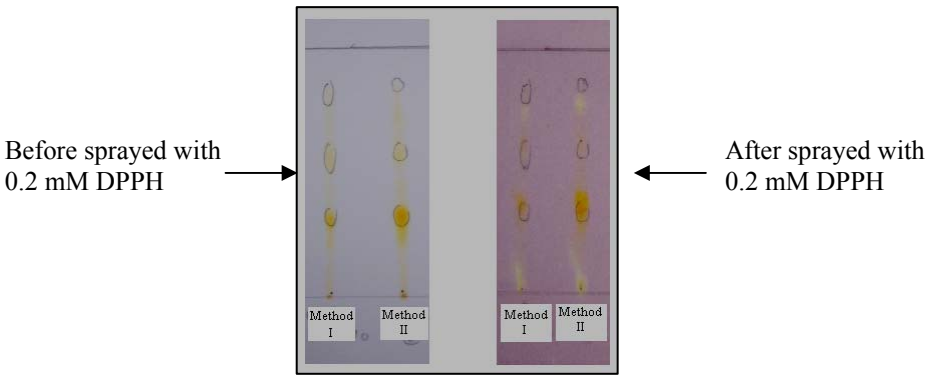
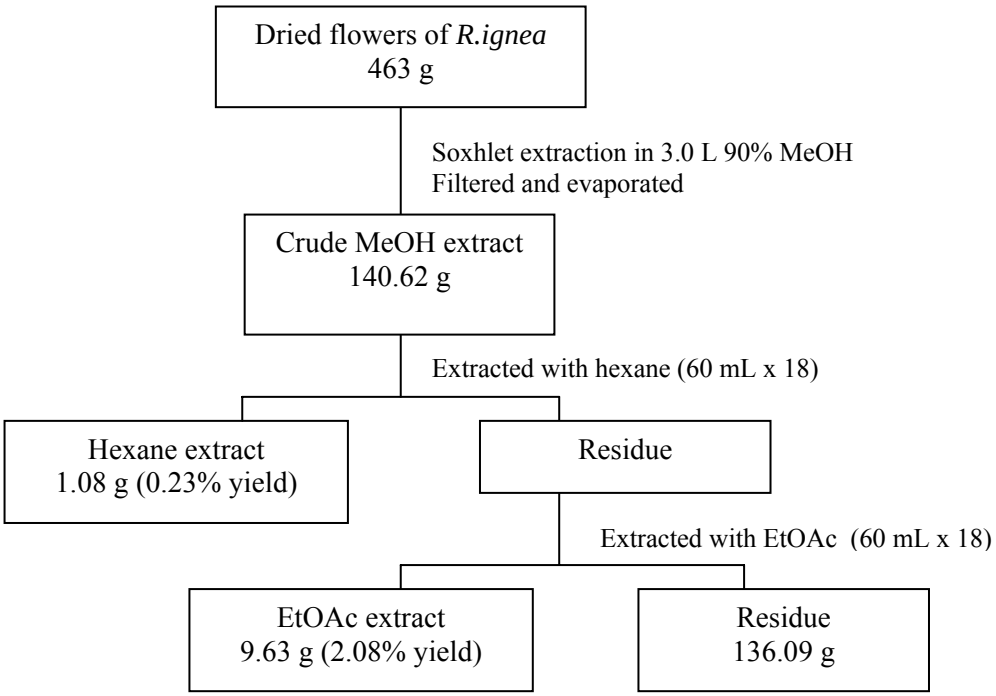


Figure 4.1 Comparison of the components of ethyl acetate extract between sonication (method I) and soxhlet extraction (method II) with the mixture of hexane:ethyl acetate (1:1) as mobile phase

By considering the higher yield percentage of ethyl acetate extract obtained, the amount of solvent used, and the TLC analysis results, soxhlet extraction was selected

as the methods for further isolation process. Scheme 4.3 showed the result of the extraction by soxhlet extraction.

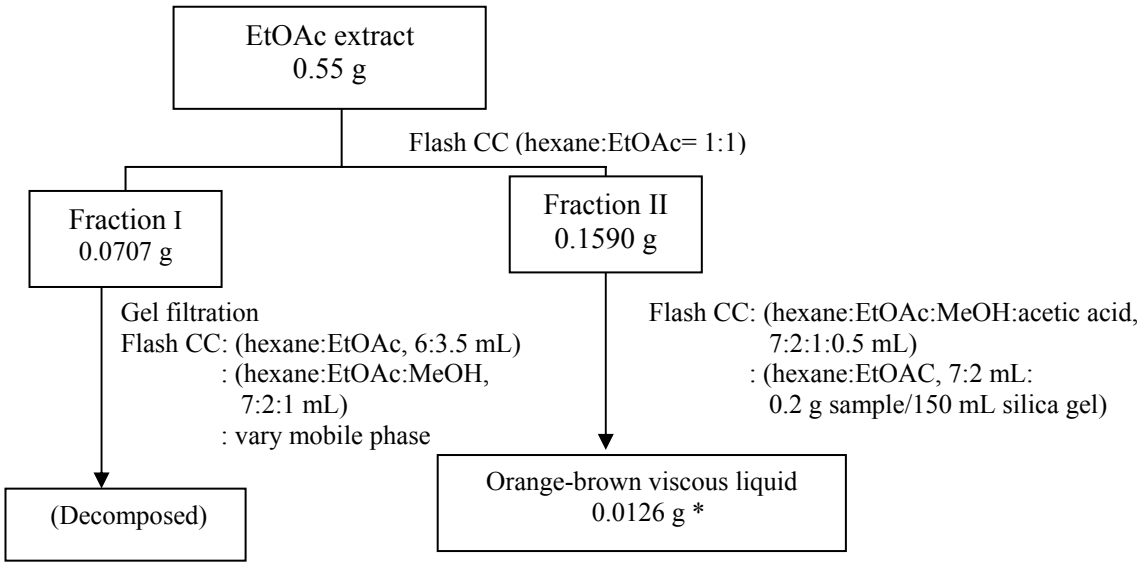


Scheme 4.3 The result of extraction by soxhlet extraction

4.2 Isolation and purification

Flash column chromatography technique was used to isolate and purify chemical constituents from ethyl acetate extract. Both methods gave the same fractions.

From sonication, fraction I (0.0707 g) and fraction II (0.1590 g) were used to study the purifying method. The result of isolation and purification was shown in Scheme 4.4.



* Fraction was collected and mixed with the fraction from soxhlet extraction

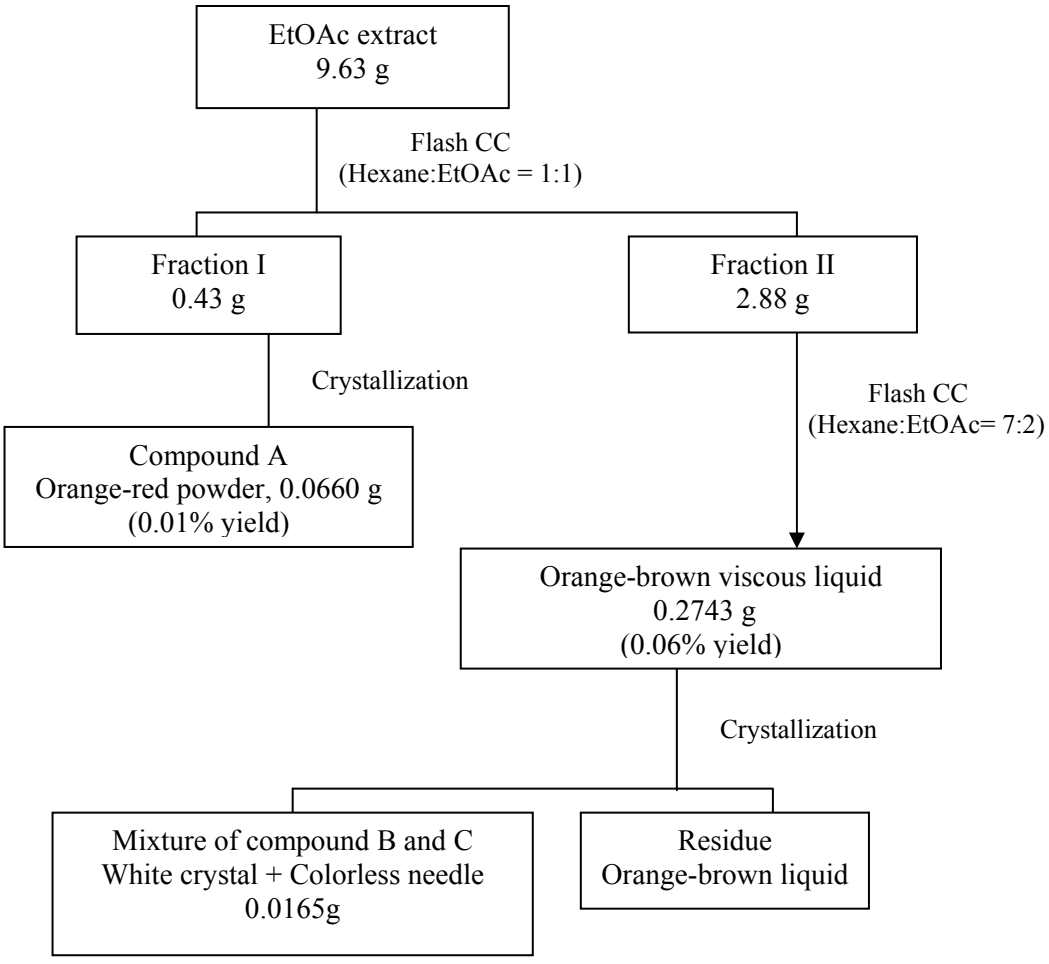
Scheme 4.4 The result of isolation and purification by sonication

Fraction I (0.0707 g) was purified by gel filtration but it was not successful. Therefore, the sample was collected and was purified again by flash column chromatography with the mixture of hexane:ethyl acetate at 6:3.5 mL and hexane:ethyl acetate:methanol at 7:2:1 mL as isocratic systems, respectively. However, purification under conditions was still not successful. Finally, sample was purified by flash column chromatography with gradient system. The ratio of solvent mixture between hexane and ethyl acetate was varied to drive the sample into the column. The purification was still not successful and this fraction decomposed.

Fraction II (0.1590 g) was separated by flash column chromatography with the mixture of hexane, ethyl acetate, methanol and acetic acid at the ratio of 7:2:1:0.5 mL as mobile phase. 0.2 g sample/80 mL silica gel was used. The separation was unsuccessful. Therefore, the sample was collected again, then separated by flash column

chromatography with the mixture of hexane and ethyl acetate (7:2) as isocratic system and 0.2 g sample/150 mL silica gel was used. This procedure obtained 0.0126 g of orange-brown viscous liquid which was collected and mixed with the same fraction from soxhlet extraction.

Ethyl acetate extract from soxhlet extraction was separated and purified to obtain the interested compounds. The result of this procedure is shown in Scheme 4.5.



Scheme 4.5 The result of isolation and purification by soxhlet extraction

From this experiment, 2 fractions, fraction I (0.43 g) and fraction II (2.88 g) were obtained from ethyl acetate extract from soxhlet extraction.

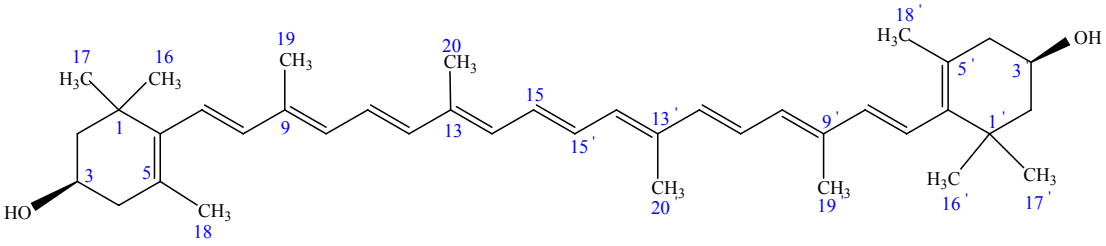
Fraction I was purified by crystallization to obtain orange-red powder (compound A, R_f = 0.33). The crystal was washed with cold ethyl acetate and cold methanol, respectively.

For fraction II, orange-brown viscous liquid (0.2743 g) was obtained after purified by flash column chromatography using a mixture of hexane and ethyl acetate as the mobile phase. The TLC analysis indicated that this fraction contains two main components (compound B and C). The methods of purification to obtain pure compounds were reverse-phase HPLC and Prep-TLC. For reverse-phase HPLC, column Bio-sil C18 HL 90-5 S (4.6 mm x 250 mm) was used for analytical-HPLC and Waters PrepLC^{TM} 25mm Module (25 mm x 100 mm) was used for preparative-HPLC. The mobile phase consisted of mixture of dichloromethane, methanol, acetonitrile and water in 5:85:5.5:4.5 v/v ratio (A) and mixture of dichloromethane, methanol, acetonitrile and water in 22:28:45.5:4.5 v/v ratio (B). The gradient system was shown in Table 3.1 (see Chapter III). For Prep-TLC, silica gel 60 F_{254} pre-coated on aluminum plates size 10 x 10 cm was used as the stationary phase with a mixture of hexane and isopropanol at 24:3 ratio (v/v) as the mobile phase. However, the purification by these two methods still not successful to obtain compound B and C. The mixture of compound B and C was obtained as a mixture of white crystal and colorless needle from crystallization by dissolving this orange-brown viscous liquid with ethyl acetate extract and a few drops of acetonitrile. The mixture of compound B and compound C was washed with cold acetonitrile and dried by storage in desicator.

4.3 Structural elucidation

From series of chromatographic isolation, ethyl acetate extract yield an antioxidant component (compound A), and two non-antioxidant components. The isolated compounds were characterized by spectroscopic techniques, which the obtained spectral data were compared with those previous reported in the literature.

4.3.1 Structural elucidation of compound A (Zeaxanthin)



Compound A

Compound A was obtained as orange-red crystalline powder (0.07 g, 0.02% yield based on the weight of dried flowers). The compound was insoluble in water and methanol and slightly soluble in ethanol and chloroform. From spectral data, compound A was identified as zeaxanthin. The observed spectroscopic data were described as follows:

UV (abs.EtOH); The observed λ_{max} were at 450 and 477 nm (*see* Figure A-1 in the Appendix section). The data suggested that compound A is a carotenoid, which normally showed the absorption peaks in 400-500 nm range. (Soares, 2006 and Cannell, 1998)

FT-IR (KBr); The absorption peaks were observed at ν 3435 cm^{-1}(O–H stretch ing), 2921 cm^{-1} (C–H stretching), 1628 cm^{-1} (C=C stretching), 1384 cm^{-1} (CH_3 bending), 1049 cm^{-1} (C–O stretching), 958 cm^{-1} and 688 cm^{-1} (C=C–H out of plane bending) (*see* Figure A-2 in the Appendix section) .

LC/APCI-MS; The spectrum showed the molecular ion $[M+H]^+$ peak at m/z 569.4 Therefore, this compound has the molecular weight at 568 (*see* Figure A-3 in the Appendix section).

NMR spectra of compound A were measured in $CDCl_3$ with TMS as the internal reference. The chemical structure of compound A was characterized by using ^1H–, ^{13}C– NMR and two-dimensional NMR techniques (^1H-^1H-COSY, HSQC and HMBC). The NMR spectra were presented in the Appendix section.

^{13}C NMR ($CDCl_3$ at 150 MHz); The chemical shifts (δ in ppm unit) were presented in Table 4.2. The obtained data were corresponded with those of zeaxanthin as reported by Eisenreich *et al*. (2002). The spectrum was presented in Figure A-4 (*see* the Appendix section).

Table 4.2 ^{13}C NMR data of compound A (in $CDCl_3$) comparing with those of zexanthin.

Carbon Position	**δ_C (ppm)**	
	Zeaxanthin[a]	**Compound A**
1, 1’	37.1	37.1
2, 2’	48.2	48.4
3, 3’	65.1	65.1
4, 4’	42.6	42.6

Table 4.2 (Continued).

Carbon Position	δ_C (ppm)	
	Zeaxanthin[a]	Compound A
5, 5’	126.2	126.2
6, 6’	137.8	137.7
7, 7’	125.6	125.6
8, 8’	138.5	138.5
9, 9’	135.7	135.7
10, 10’	131.3	131.3
11, 11’	124.9	124.9
12, 12’	137.6	137.6
13, 13’	136.5	136.5
14, 14’	132.6	132.6
15, 15’	130.1	130.1
16, 16’	30.3	30.3
17, 17’	28.7	28.7
18, 18’	21.6	21.6
19, 19’	12.8	12.8
20, 20’	12.8	12.8

[a] Mean data from “Biosynthesis of zeaxanthin via mevalonate in **Paracoccus** species strain PTA-3335”, Eisenreich *et al*., 2002**,** Journal of Organic Chemistry, 871-5.

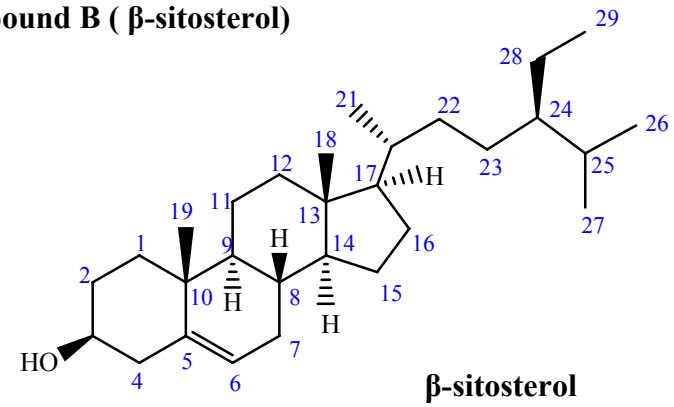
^{1}H NMR ($CDCl_3$ at 600 MHz); The chemical shifts (δ in ppm unit) were presented in Table 4.3. The chemical shifts of protons were assigned from HSQC and HMBC spectra. The spectra were presented in Figure A-5–A-8. (*see* the Appendix section).

Table 4.3 ^{1}H NMR data compound A (in $CDCl_3$).

Proton Position		δ_H (ppm) Compound A
1, 1’	C	- (q)
2, 2’	CH_2	1.77(dd, J=12, 2.4 Hz),
3, 3’	CH	1.48(t, J=12 Hz)
4, 4’	CH_2	2.39(dd, J=16.8, 5.4 Hz)
4, 4’	CH_2	2.05(dd, J=16.8,10.2 Hz)
5, 5’	C	- (q)
6, 6’	C	- (q)
8, 8’	CH	6.13 (d, J=15.6 Hz)
9, 9’	C	- (q)
10, 10’	CH	6.16 (d, J=10.8 Hz)
11, 11’	CH	6.65 (dd, J=13.2, 10.8Hz)
12, 12’	CH	6.63 (d, J=15 Hz)
13, 13’	C	- (q)
14, 14’	CH	6.25 (brd, 9 Hz)
15, 15’	CH	6.64 (d, J=15 Hz)
16, 16’	CH_3	1.07 (s)
17, 17’	CH_3	1.07 (s)
18, 18’	CH_3	1.74 (s)
19, 19’	CH_3	1.97 (s)
20, 20’	CH_3	1.97 (s)

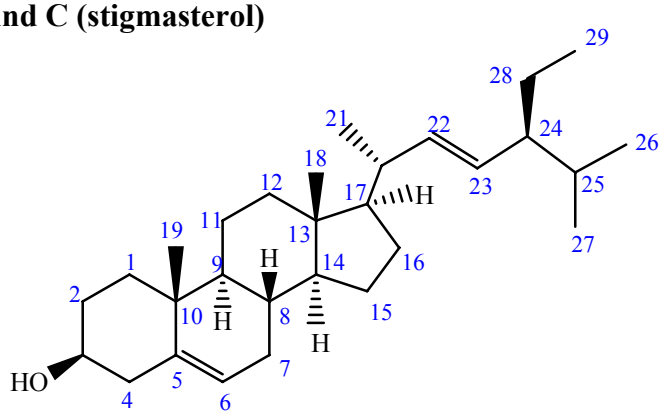
4.3.2 Structural elucidation of compound B and C

Compound B (β-sitosterol)



β-sitosterol

Compound C (stigmasterol)



stigmasterol

Compound B and C were obtained as a mixture of white crystal and colorless needle. The spots on TLC showed positive color when spraying with a solution of 20% $HClO_4$ indicating steroid-type compounds. From spectral data, compound B and C were identified as β-sitosterol and stigmasterol, respectively. The observed spectroscopic data were described as follows:

LC/APCI-MS; The spectrum showed $[M+H]^+$ peaks at m/z 415.4 and 413.3, which corresponded to the molecular weight of β-sitosterol and stigmasterol, respectively. (*see* Figure B-1 in the Appendix section).

NMR spectra of the mixture of compound B and C were measured in $CDCl_3$ with TMS as the internal reference. The chemical structure of compound A was characterized by using ^1H–, ^{13}C– NMR data to compare with the reported data in the literature. The NMR spectra were presented in the Appendix section.

^{13}C NMR ($CDCl_3$ at 125.6 MHz); The chemical shifts (δ in ppm unit) were presented in Table 4.4. The obtained data suggested that the mixture contained mostly β-sitosterol.

Table 4.4 ^{13}C NMR (125.6 MHz) data of the mixture of compound B and C comparing with those of β-sitosterol.

Carbon Position	δ_C (ppm)	
	β-Sitosterol [a]	Compound B & C
1	37.7	37.3
2	32.3	31.9
3	72.2	71.8
4	42.8	42.4
5	141.2	140.8
6	122.1	121.7
7	32.1	31.7
8	32.3	31.9
9	50.6	50.2
10	36.9	36.5
11	21.5	21.1
12	40.2	39.8
13	42.8	42.4
14	57.2	56.8
15	24.7	24.3
16	28.7	28.3
17	56.5	56.1
18	12.4	12.0
19	19.8	19.4
20	36.6	36.2
21	19.2	18.8
22	34.4	34.0
23	26.5	26.1
24	46.2	45.8
25	29.6	29.2
26	20.2	19.8
27	19.5	19.1
28	23.5	23.1
29	12.3	11.9

[a] Mean data from "Chemical constituents of dichloromethane extract of cultivated satureja khuzis tanica", Moghaddam *et al*., 2007, Evidence-based Complementary and Alternative Medicine, 4(1), 95-98.

^{1}H NMR ($CDCl_3$ at 600 MHz); The chemical shifts (δ in ppm unit) of compound B and C were presented in Table 4.5, and 4.6 by comparing with the data reported by Moghaddam *et al*. (2007) for β-sitosterol (500 MHz, $CDCl_3$) and by Fokou (2006) for stigmasterol (500 MHz, $CDCl_3$).

From ^{1}H NMR spectra as presented in Figure B-3 (*see* Appendix section), the chemical shifts of protons of the same position in β-sitosterol and stigmasterol were virtually the same except for the olefinic protons at the positions 22 (δ 5.01) and 23 (δ 5.15), which were clearly marked as those only contained in stigmasterol. By using integral estimation, the ratio of β-sitosterol to stigmasterol was approximately 80:20.

Several attempts were done to separate compound B and C from each other by crystallization, prep-TLC, and HPLC. However, these two compounds could not be isolated as pure compounds, which this observation was corresponding with the report of Xu, Huang, Qian, Sha and Wang (2005).

Table 4.5 Comparison of ^{1}H NMR data of compound B with β-sitosterol.

Proton Position	**δ_H (ppm)**	
	β-Sitosterol [a]	**Compound B**
3	3.56 (m)	3.52 (m)
6	5.39 (s)	5.35 (brs)
18	0.72 (s)	0.67
19	1.05 (s)	0.99
21	0.96 (d, *J*= 6.5Hz)	0.92 (*J*=6.5 Hz)
22	-	-
23	-	-
26	0.87 (d, *J* = 6.7 Hz)	0.84 (d, *J*=6.8 Hz)
27	0.85 (d, *J* = 6.7 Hz)	0.81 (d, *J*=6.8 Hz)
29	0.89 (t, *J* = 7.4 Hz)	0.85 (t, *J*=7.4 Hz)

[a] Mean data from "Chemical constituents of dichloromethane extract of cultivated satureja khuzis tanica", Moghaddam *et al*., 2007, Evidence-based Complementary and Alternative Medicine, 4(1), 95-98.

Table 4.6 Comparison of ^{1}H NMR data of compound C with stigmasterol.

Proton Position	δ_H (ppm)	
	Stigmasterol [b]	Compound C
3	3.55 (m, *J* = 5.23 Hz)	3.52 (m)
6	5.33 (s)	5.35 (brs)
18	0.53 (s)	0.70 (s)
19	0.8 (s)	0.80
21	1.02 (d, *J* = 6.67 Hz)	1.02 (d, *J*=6.8 Hz)
22	5.00 (dd, *J*=15.07, 8.79 Hz)	5.01 (dd, J=15.1, 8.7 Hz)
23	5.12 (dd, *J*=15.07, 8.79 Hz)	5.15 (dd, J=15.1, 8.7 Hz)
26	0.79 (d, *J* = 6.45 Hz)	0.79 (d, *J*=6.4 Hz)
27	0.93 (d, *J* = 6.45 Hz)	0.94 (d, *J*=6.8 Hz)
29	0.83 (t, *J* = 6.5 Hz)	0.84 (t, *J*=6.3 Hz)

[b] Mean data from "Chemical investigation of three plants used in cameroonian traditional medicine: *Maesopsis eminii* (Rhamnaceae), *Autranella congolensis* (Sapotaceae) and *Pentadesma grandifolia* (Guttiferae)", Fokou, P. A.,2006, . Ph.D. Dissertation, Bielefeld University, Germany.

4.4 Antioxidant activity

One of the main purpose of this research was to isolate and elucidate the structure of the compounds that show antioxidant activity and to compare the activity with standard antioxidants. The measurement of antioxidant activity were done on basis of DPPH scavenging activity. The isolation was directed by the results of TLC screening test, and the activity of antioxidant component was measured as IC_{50} value by spectrophotometric assay.

From Figure 4.1, the TLC screening tests showed that fraction I and II contained antioxidant components. Fraction I was isolated to yield compound A, which was assigned as zeaxanthin (*see* Section 4.3.1). The antioxidant component in Fraction II, however, appeared to be decomposed during chromatographic separations, and yield

only two non-antioxidant compound B and C, which were assigned as β-sitosterol and stigmasterol, respectively (*see* Section 4.3.2).

The antioxidant activity of compound A was further studied by spectrophotometric assay to measure the IC_{50} values. Due to solubility problem and structural effect of carotenoid-type compound that interferes with the UV absorption properties, the method specifically for antioxidant activity study of carotenoids proposed by Escrig *et al*. (2000) was adapted to be used in this study (*see* Section 3.8.3 for experimental details). Figure 4.2 showed the plot of percentage of radical scavenging versus concentration of a solution of compound A in DMSO. The activity was studied in comparison with standard antioxidants, which were catechin and ascorbic acid. The result of scavenging effect of catechin and ascorbic acid were shown in Figure 4.3 and 4.4. The IC_{50} value of compound A (zeaxanthin) was presented in Table 4.7.

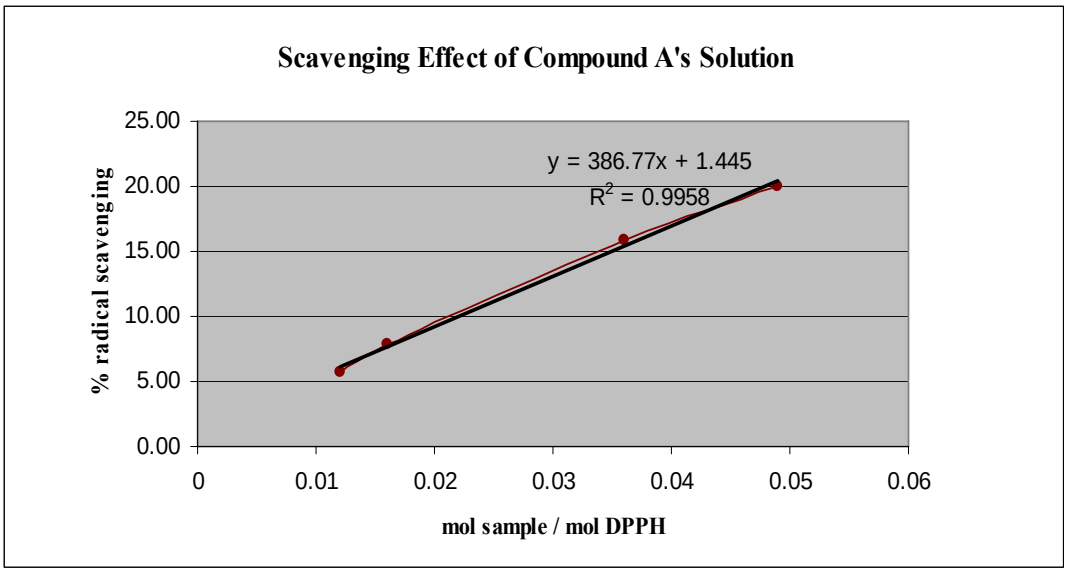


Figure 4.2 Scavenging effect of compound A's solution in DMSO

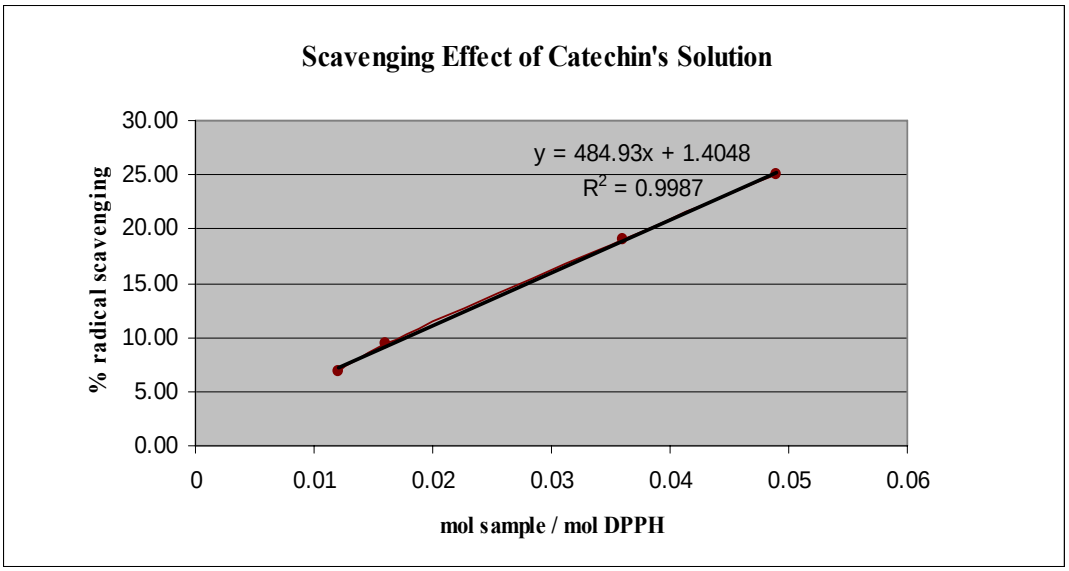


Figure 4.3 Scavenging effect of catechin's solution

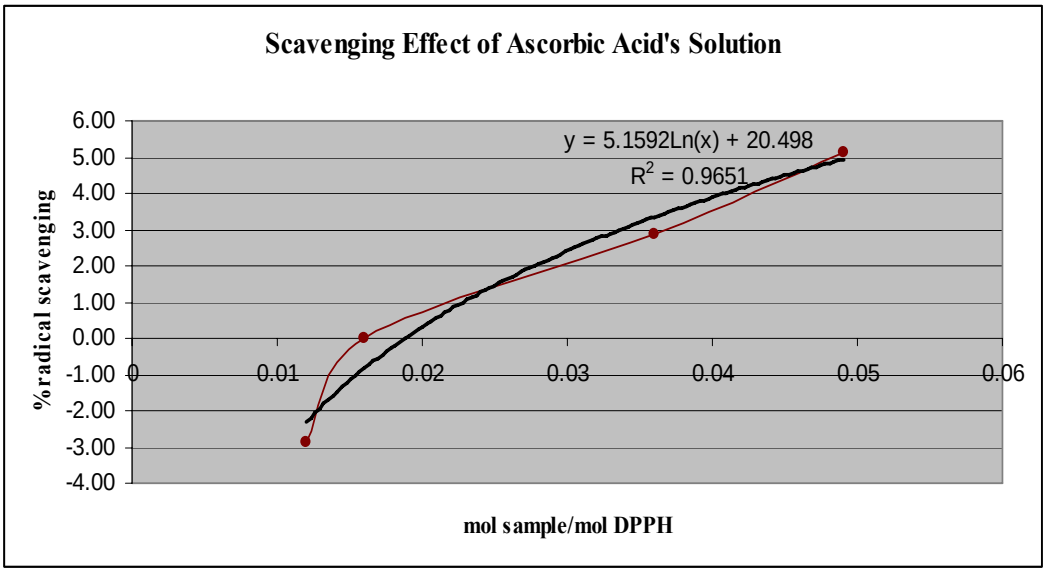


Figure 4.4 Scavenging effect of ascorbic acid's solution

Table 4.7 Antioxidant activity study of compound A.

Compound	IC_{50} (mol antioxidant/mol DPPH)
Compound A (zeaxanthin)	1.13
Zeaxanthin [a]	2.15
Lycopene[a]	0.16
Lutein[a]	3.29
(+)-Catechin	1.10
Ascorbic acid	304.30

[a] Mean data from, "Evaluation of free radical scavenging of dietary carotenoids by the stable radical 2,2- diphenyl-1-picrylhydrazyl", Escrig *et al*., 2000, Journal of the Science of Food and Agriculture: 1686-1690.

The results suggested that compound A has a potent free radical scavenging activity on DPPH radical scavenging assay with IC_{50} 1.13 mol/mol DPPH, which was in the same level as that of catechin (IC_{50} 1.10 mol /mol DPPH), and was much stronger than that of ascorbic acid (IC_{50} 304.30 mol /mol DPPH). The obtained IC_{50} value of compound A showed a small deviation from that zeaxanthin as reported by Escrig, presumably due to the influent of the solvent that was replacing THF by DMSO. Among the carotenoids, compound A or zeaxanthin showed less antioxidant activity than lycopene but stronger than lutein.

CHAPTER V

CONCLUSIONS

The ethyl acetate extract of the flower of *Radermachera ignea* (Kurz) Steenis, the emblem of Suranaree University of Technology, was used in this study to investigate antioxidant activity. The antioxidant activity was measured on the basis of DPPH scavenging activity.

The ethyl acetate extract was isolated and purified by flash column chromatography, and the chemical structures of the isolated compounds were characterized by spectroscopic techniques including UV, FT-IR, LC-MS, and NMR. The results indicated that the antioxidant component from the extract was zeaxanthin. Non-antioxidant compounds, β-sitosterol and stigmasterol, were also obtained as a mixture.

The antioxidant activity of zeaxanthin was studied in comparison with two antioxidant standards; ascorbic acid and catechin. The results showed that zeaxanthin exhibited a potent free radical scavenging activity on DPPH radical scavenging assay with the IC_{50} of 1.13 mol /mol DPPH, which was in the same level as that of catechin (IC_{50} 1.10 mol /mol DPPH), and was much stronger than that of ascorbic acid (IC_{50} 304.30 mol /mol DPPH).

For the flower of *R. ignea*, this is the first study to identify its chemical constituents and to report its antioxidant activity, which the results as described above suggested it to be a new source of potent antioxidant.

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APPENDIX

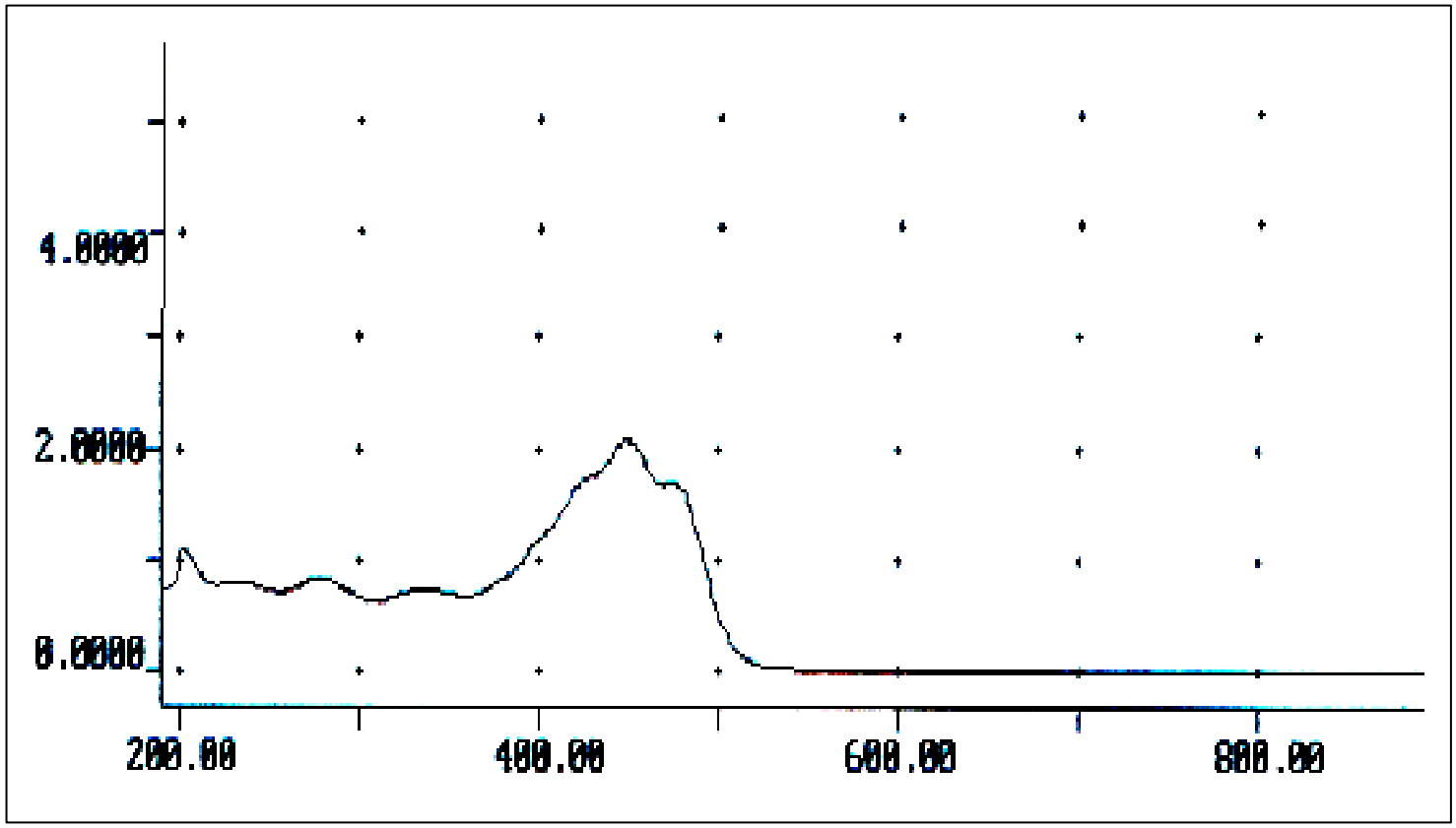

Figure A-1 The maximum absorption chromatogram of compound A (in abs.EtOH**)**

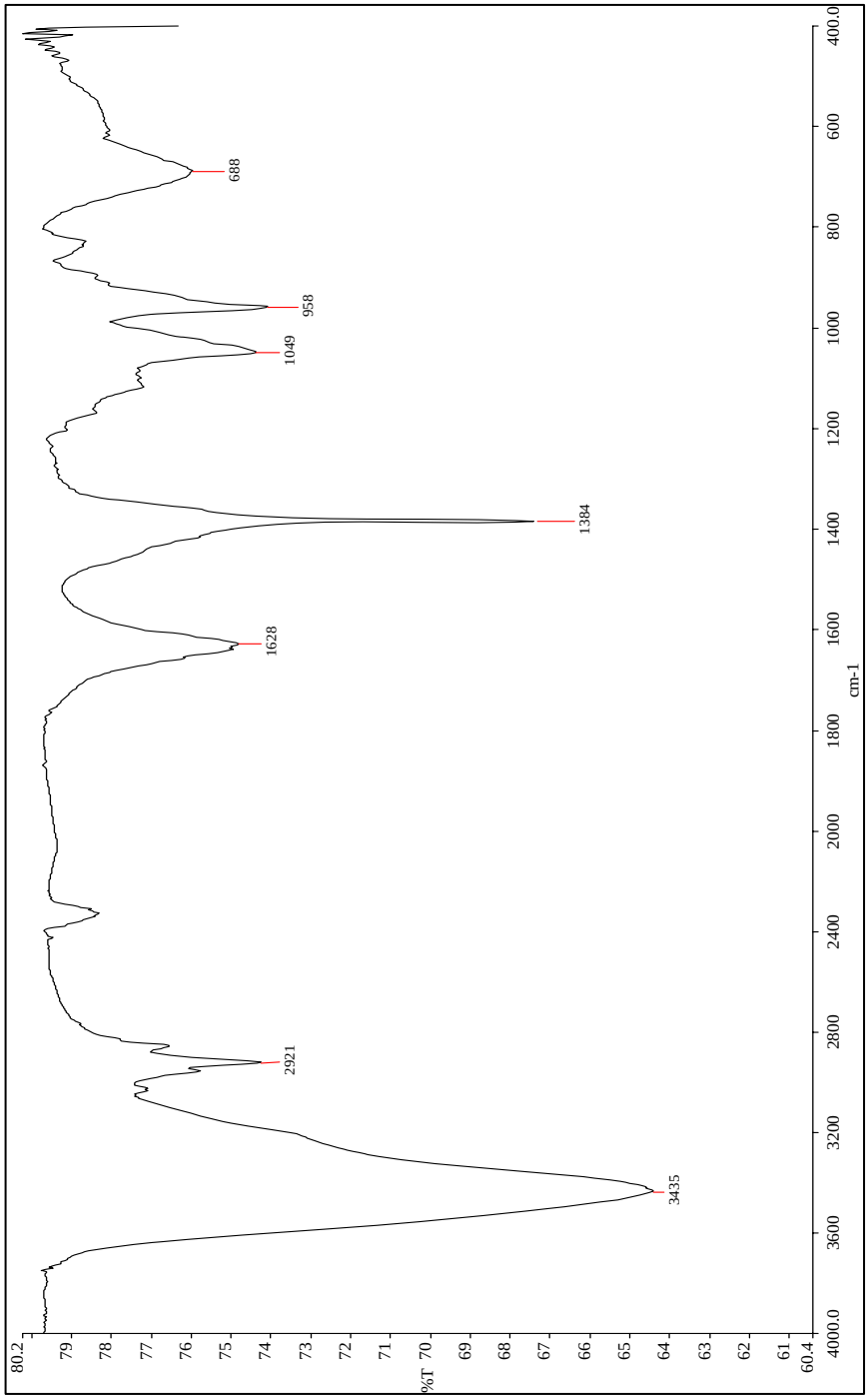

Figure A-2 FT-IR Spectrum of compound A (KBr pellet)

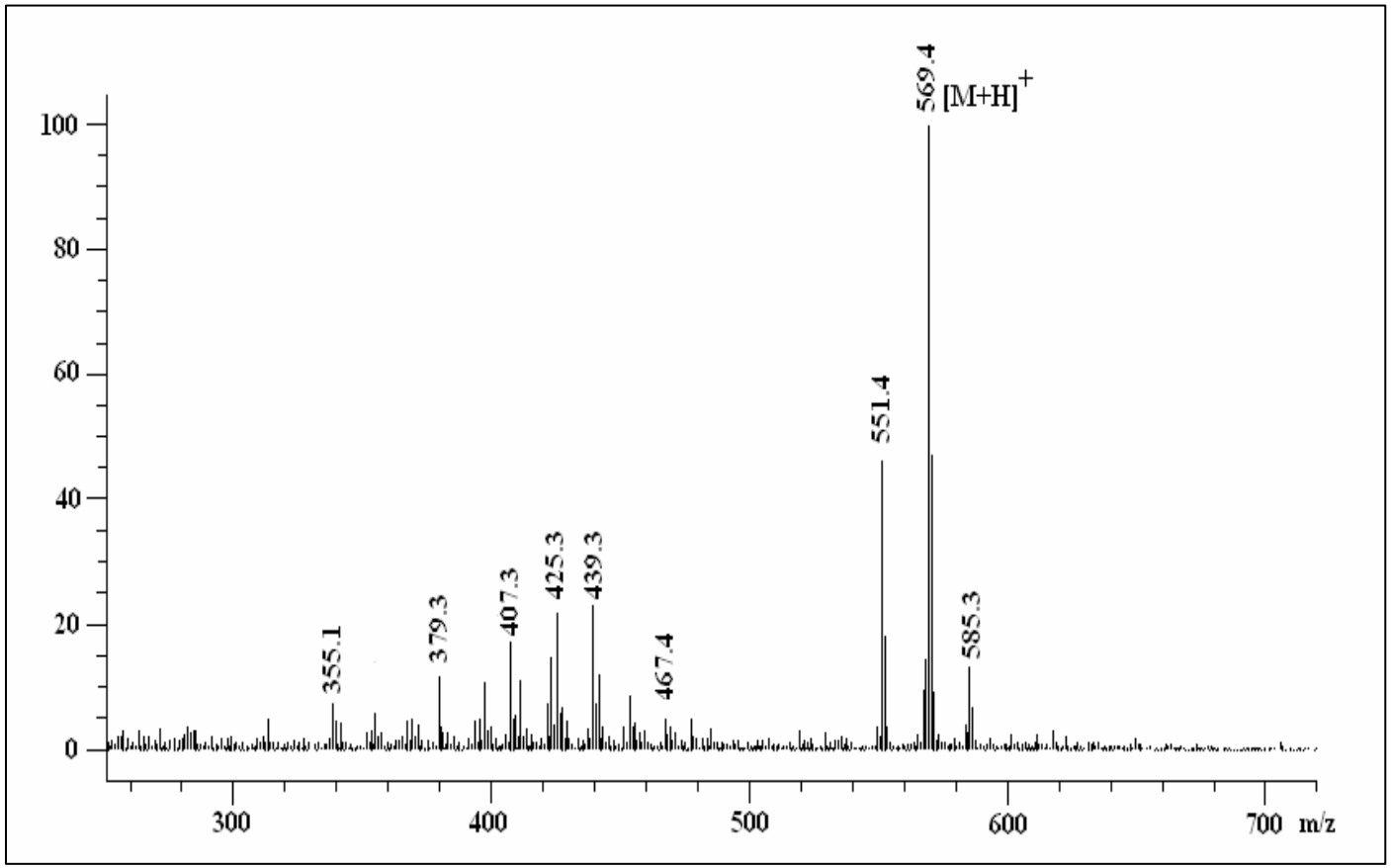

Figure A-3 LC-MS spectrum of compound **A**

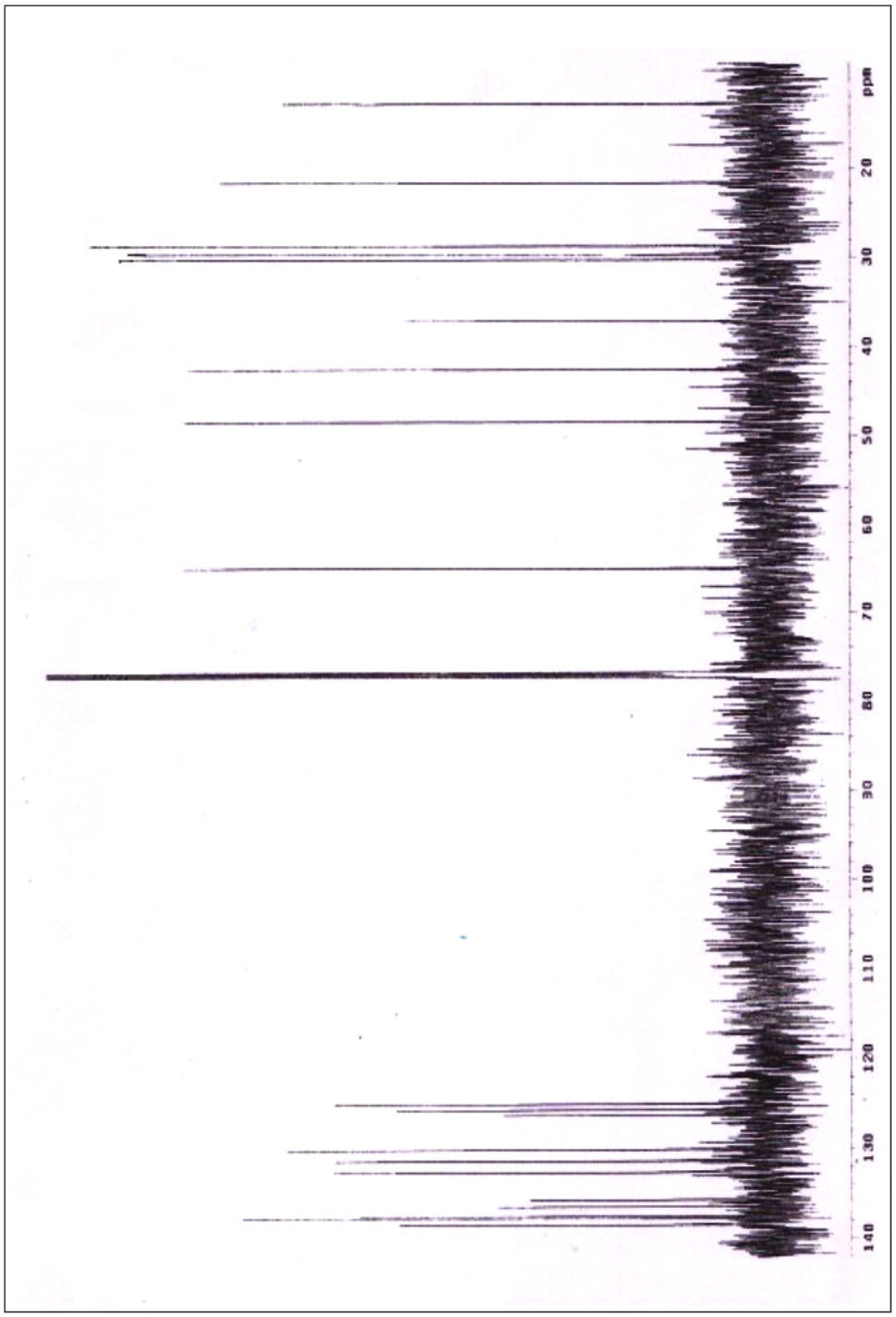

Figure A-4 ^{13}C NMR spectrum of compound A in $CDCl_3$ at 600 MHz

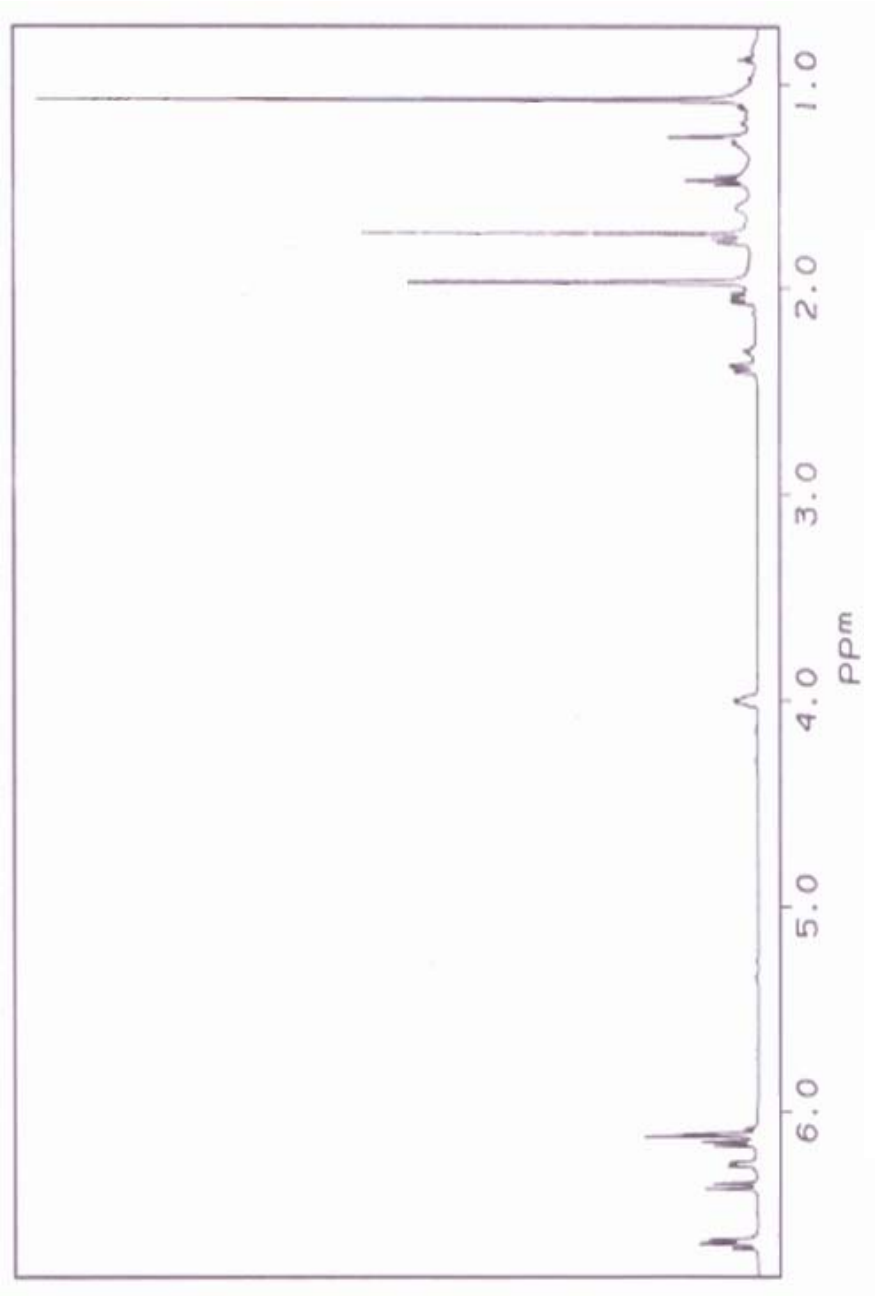

Figure A-5 ^{1}H NMR spectrum of compound A in $CDCl_3$ at 600 MHz

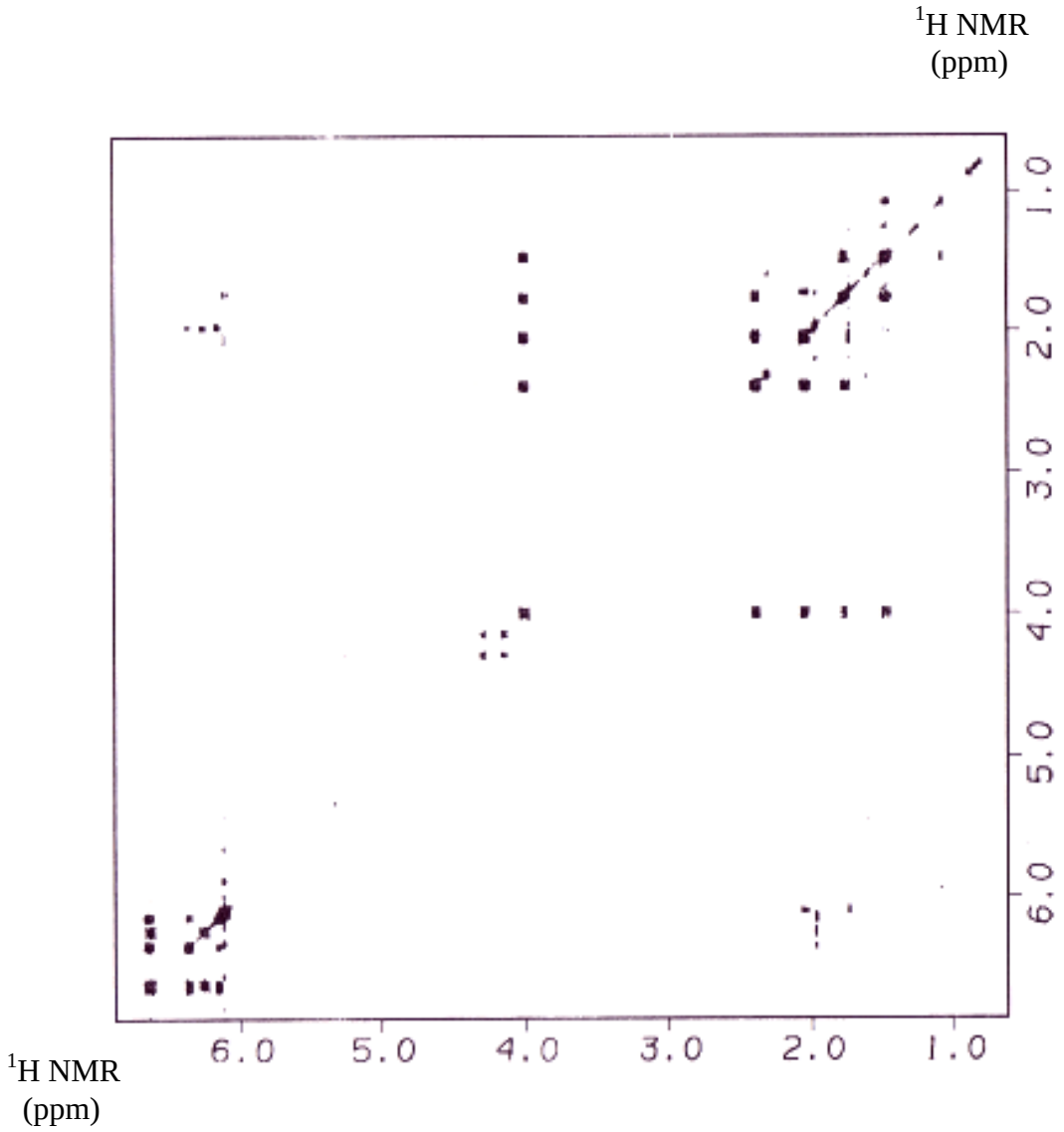

Figure A-6 ^{1}H-^{1}H COSY spectrum of compound A in $CDCl_3$ at 600 MHz

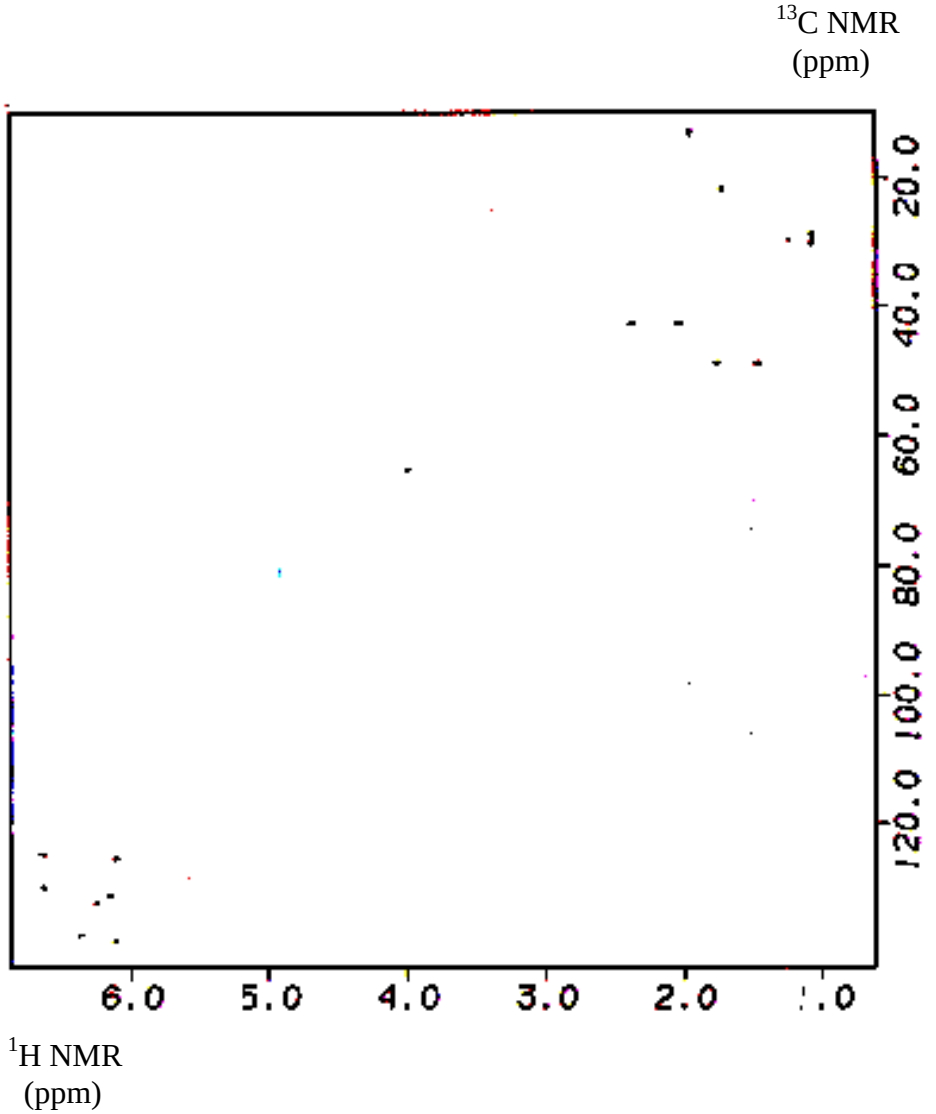

Figure A-7 HSQC spectrum of compound A in $CDCl_3$ at 600 MHz

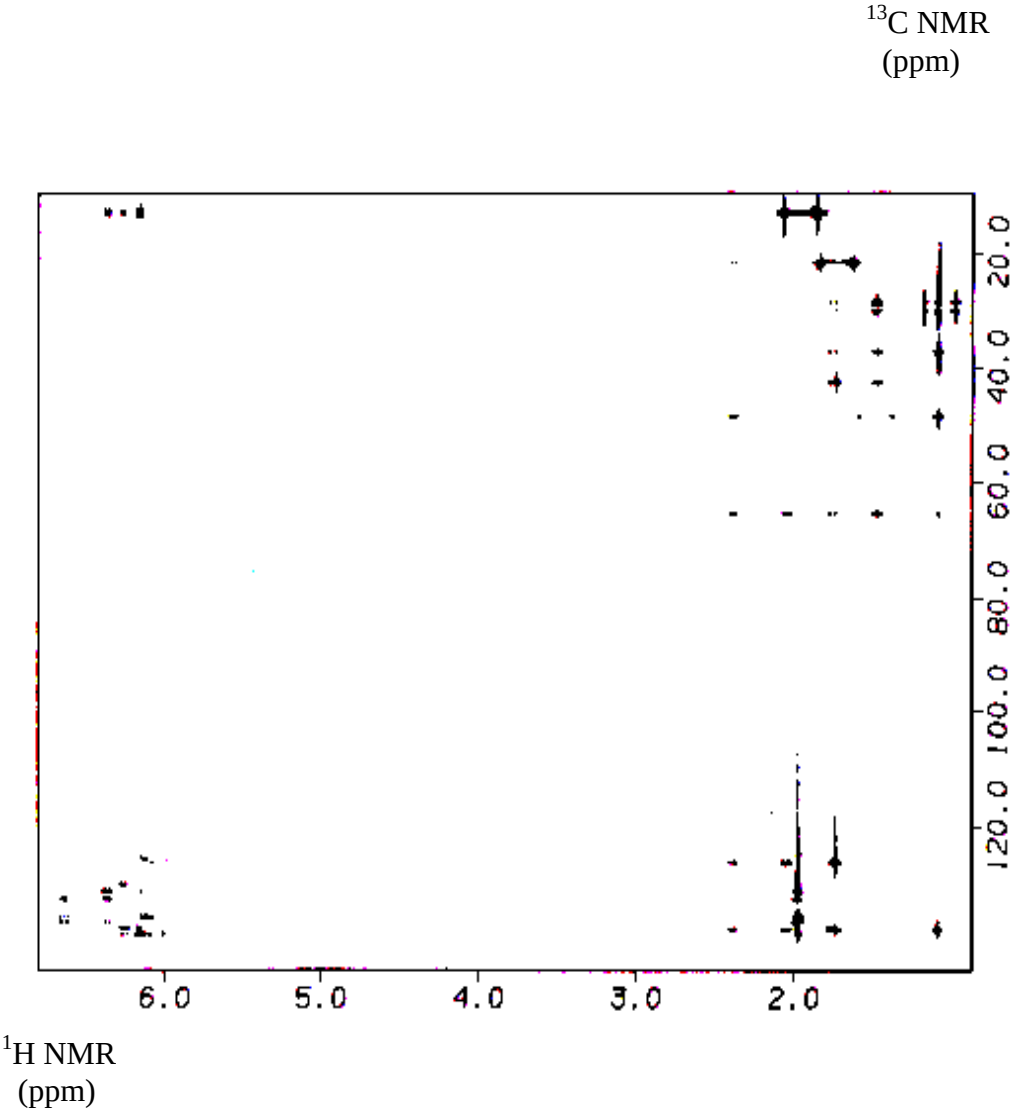

Figure A-8 HMBC spectrum of compound A in $CDCl_3$ at 600 MHz

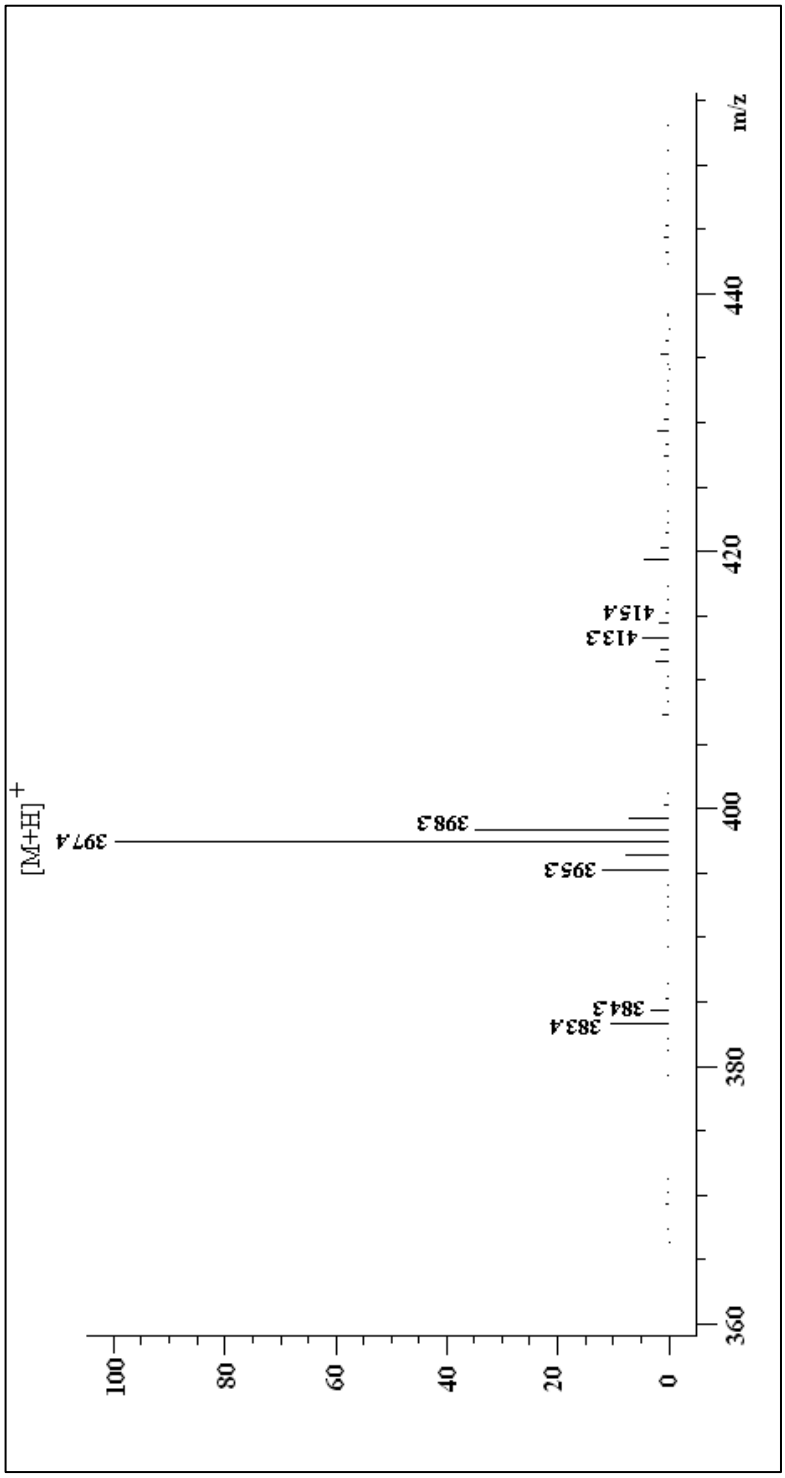

Figure B-1 LC-MS spectrum of compound B

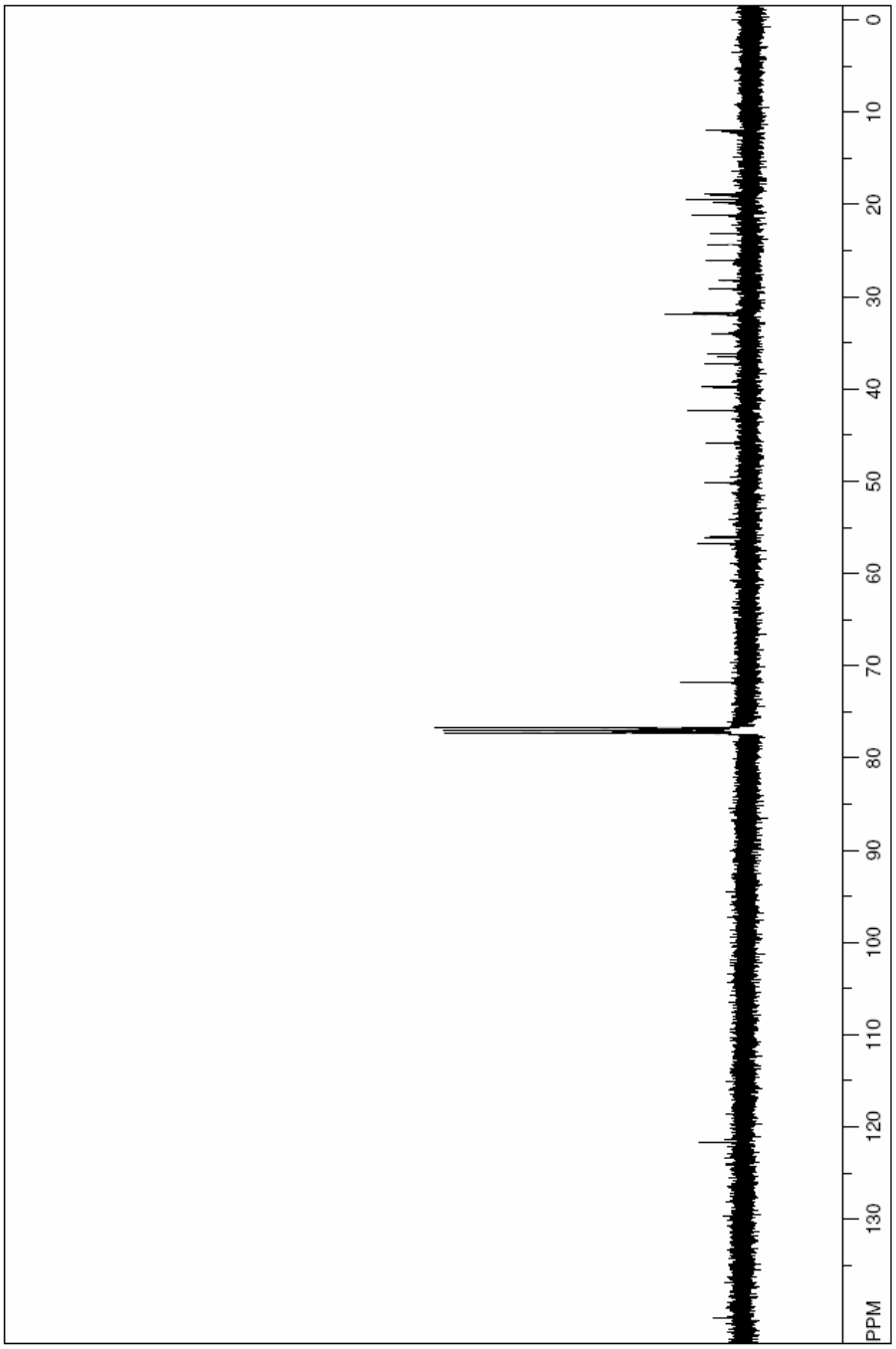

Figure B-2 ^{13}C NMR spectrum of Compound B in $CDCl_3$ at 500 MHz

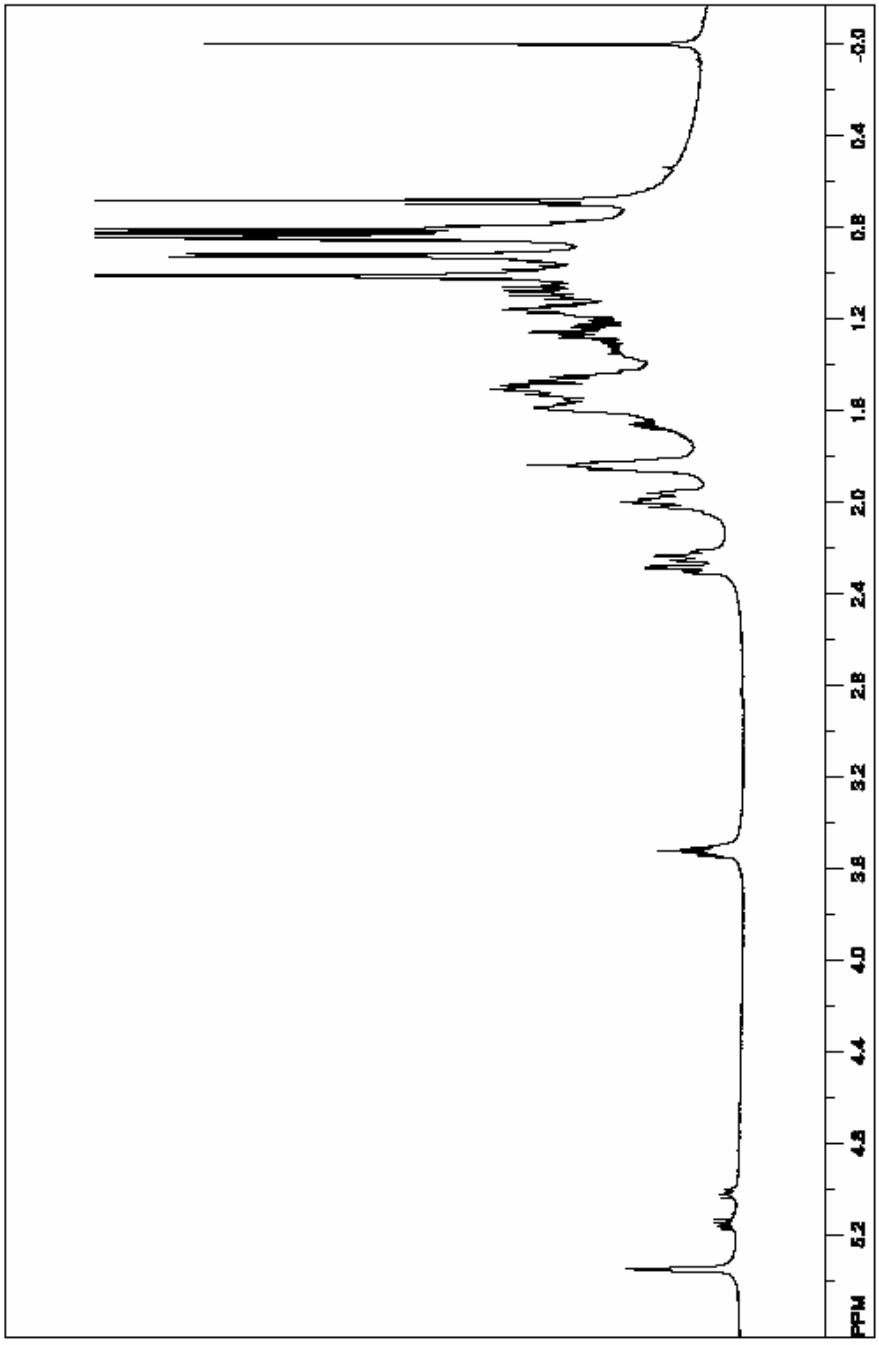

Figure B-3 ^{1}H NMR spectrum of Compound B in $CDCl_3$ at 600 MHz

ที่ ทส 0943/๑๑๗

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ตามหนังสือที่อ้างถึง สาขาวิชาเคมี สำนักวิชาวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี ขอความอนุเคราะห์ตรวจสอบชนิดพันธุ์พืช ตามที่ได้ส่งตัวอย่างพรรณไม้แห้งและภาพถ่ายประกอบ นั้น

สำนักงานหอพรรณไม้ ได้ตรวจสอบแล้ว พบว่าเป็นพืชในวงศ์ Bignoniaceae คือ ปีบทอง หรือ กาสะลองคำ ชื่อวิทยาศาสตร์ *Radermachera ignea* (Kurz) Steenis

จึงเรียนมาเพื่อทราบ

ขอแสดงความนับถือ

(นางก่องกานดา ชยามฤต)
หัวหน้าสำนักงานหอพรรณไม้

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