สารออกฤทธิ์ทางชีวภาพจากเนื้อไม้ของต้นกำลังวัวเถลิง *Anaxagorea luzonensis* A. Gray.

นางสาวชลิศา ทรัพย์พร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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BIOACTIVE COMPOUNDS FROM THE HEARTWOOD OF DALAIRO *Anaxagorea luzonensis* A. Gray.

Miss Chalisa Sabphon

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	BIOACTIVE COMPOUNDS FROM THE HEARTWOOD OF DALAIRO *Anaxagorea luzonensis* A. GRAY.
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ชลิศา ทรัพย์พร : สารออกฤทธิ์ทางชีวภาพจากเนื้อไม้ของต้นกำลังวัวเถลิง *Anaxagorea luzonensis* A. Gray (BIOACTIVE COMPOUNDS FROM THE HEARTWOOD OF DALAIRO *Aaxagorea lusonensis* A. Gray.) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ดร.พัฒทรา สวัสดี, 102 หน้า

การศึกษาองค์ประกอบทางเคมีของเนื้อไม้ของต้นกำลังวัวเถลิง สามารถแยกสารได้ทั้งหมด 17 ชนิด โดยอาศัยสมบัติทางกายภาพและข้อมูลทางสเปกโทรสโกปี สามารถหาโครงสร้างของสารที่แยกได้ คือ 1,3,5-trihydroxy-4-(3-hydroxy-3- metylbutyl)xanthone (1), orobol (2), naringenin (3), aromadendrin (4), keampferol (5), quercetin (6), taxifolin (7), 1,3,6-trihydroxyxanthone (8), 1,3,5-trihydroxy-4-prenylxanthone (9), 1,3,5,6-tetrahydroxyxanthone (10), daidzein (11), biochanin A (12), genistein (13), 3,6-dihydroxy-1,5-dimethoxyxanthone (14), 8-prenylnaringenin (15), stigmasterol (16) และ 6-deoxy-isojacareubin (17) โดยสารที่แยกได้ทั้งหมดนี้ เป็นครั้งแรกที่พบว่า สาร (3), (4), (11), (13), (16) และ (17) เป็นองค์ประกอบทางเคมีของต้นกำลังวัวเถลิง จากการทดสอบฤทธิ์ทางชีวภาพต่างๆ พบว่า สาร (1) และ (2) มีฤทธิ์ที่ดี ด้วยค่า IC_{50} เท่ากับ 2.36 และ 2.57 ไมโครโมลาร์ ต่อเอนไซม์แอซีทิลโคลีนเอสเทอเรสและ ค่า IC_{50} เท่ากับ 2.25 และ 3.66 ไมโครโมลาร์ ต่อเอนไซม์บิวทีริวโคลีนเอสเทอเรส ตามลำดับ นอกจากนี้ สาร (6) และ (9) แสดงฤทธิ์ที่ดี ด้วยค่า IC_{50} เท่ากับ 1.27 และ 4.90 ไมโครโมลาร์ ต่อเอนไซม์บิวทีริวโคลีนเอสเทอเรส ตามลำดับ สาร (6) ยังแสดงฤทธิ์ต้านอนุมูลอิสระต่อ DPPH แรดดิเคิลที่สูง มีค่า IC_{50} เท่ากับ 0.09 มิลลิโมลาร์ และฟอกจากสี ABTS ด้วยค่า TEAC เท่ากับ 2.88 สารแซนโทน (1), (8), (9) และ (10) แสดงฤทธิ์ต้านเอนไซม์ฟอสโฟไดเอสเทอเรสได้ดีกว่าสารในกลุ่มฟลาโวนอยด์อื่นๆ

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

ปีการศึกษา.....2551.....ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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CHALISA SABPHON: BIOACTIVE COMPOUNDS FROM THE HEARTWOOD OF DALAIRO *Aaxagorea lusonensis* A. Gray. THESIS ADVISOR: PATTARA SAWASDEE, Ph.D., 102 pp.

The chemical constituent investigation from the heartwood of *Anaxagorea luzonensis* A. Gray disclosed seventeen compounds. By means of physical properties and spectroscopic evidences, the structures of seventeen compounds could be elucidated as 1,3,5-trihydroxy-4-(3-hydroxy-3-metylbutyl)xanthone (1), orobol (2), naringenin (3), aromadendrin (4), keampferol (5), quercetin (6), taxifolin (7), 1,3,6-trihydroxy-5-methoxyxanthone (8), 1,3,5-trihydroxy-4-prenylxanthone (9), 1,3,5,6-tetrahydroxyxanthone (10), daidzein (11), biochanin A (12), genistein (13), 3,6-dihydroxy-1,5-dimethoxyxanthone (14), 8-prenylnaringenin (15), stigmasterol (16) and 6-deoxy-isojacareubin (17). Among of them, compounds (3), (4), (11), (13), (16) and (17) have been first reported as chemical constituents in *A. luzonensis*. The results of bioactivity test revealed that compounds (1) and (2) had strong activity with IC_{50} 2.36, and 2.57 μM against acetylcholinesterase and 2.25, 3.66 μM against butyrylcholinesterase, respectively. Additionally, compounds (6) and (9) revealed strong activity with IC_{50} 1.27 and 4.90 μM against butyrylcholinesterase, respectively. Compound (6) exhibited the highest scavenging activity toward DPPH radical with IC_{50} 0.09 mM and decolorizing ABTS with TEAC value 2.88. Xanthones (1), (8), (9) and (10) exhibited high inhibitory activity against phosphodiesterase comparing with other flavonoids.

Field of Study:.....Biotechnology…….. Student's Signature:……..
Academic Year:……2008…….. Advisor's Signature:.....

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List of Schemes

List of Abbreviations

ABTS	=	2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Diammonium salt
AChE	=	acethylcholinesterase
ACh	=	acetylcholine
AChEI	=	acetylcholinesterase inhibitor
AD	=	Alzheimer's disease
BChE	=	butyrylcholinesterase
br	=	broad
BuOH	=	butanol
ºC	=	Degree Celsius
$CDCl_3$	=	Deuterated chloroform
*c*GMP	=	cyclic nucleotides guanosine monophosphate
*c*AMP	=	cyclic nucleotides adenine monophosphate
$CHCl_2$	=	dichloromethane
$CHCl_3$	=	Chlorofrom
ChEI	=	cholinesterase inhibitor
^{13}C NMR	=	Carbon-13 Nuclear Magnetic Resonance
d	=	doublet (NMR)
dd	=	Doublet of doublet (NMR)
DPPH	=	2,2-Diphenyl-1-picryhydrazyl
DMSO-d_6	=	Deuterated dimethylsulfoxide
EtOAc	=	Ethyl acetate
g	=	Gram (s)
^1H NMR	=	Proton-1 nuclear magnetic resonance
H_2O	=	Water
His	=	Histidine
Hz	=	Hertz (NMR)
J	=	Coupling constan
Kg	=	Kilogram (s)
K_m	=	Michaelis constant
LC MS MS	=	Liquid Chromatography mass spectrometry

m	=	multiplet (NMR)
MeOH	=	Methanol
mg	=	Milligram (s)
min	=	Minute
mL	=	Milliliter (s)
mm	=	Millimeter (s)
m.p.	=	Melting point
MS	=	Mass Spectrometry
MW	=	Molecular weight
PDE	=	Phosphodiesterase
ppm	=	Part per million
R_f	=	Retarding factor in chromatography
s	=	Singlet (NMR)
t	=	Triplet (NMR)
TLC	=	Thin Layer Chromatography
THA	=	Tracrine
V_{max}	=	maximal reaction velocity
δ	=	Unit of chemical shift

CHAPTER I

INTRODUCTION

Natural products have served as an important source of drugs since ancient times and half of the useful drugs today are derived from natural sources. Chemo-diversity in nature, e.g. in plants, microorganisms and marine organisms, still offers a valuable source for novel lead discovery [1]. Although the use of bioactive nature products as herbal drug preparations dates back hundreds, of years ago, their application as isolated and characterized compounds to modern drug discovery and development only started in the 19th century, the dawn of the chemotherapy era. It has been well documented that nature products played critical roles in modern drug development, especially for antibacterial and antitumor agents. More importantly, natural products presented scientists with unique chemical structures, which are beyond human imagination most of the time, and inspired scientist to pursue new chemical entities with completely different structures from known drugs [2].

Fortunately, Thailand is located in the tropical region of the world and has abundant kinds of plants, especially herbs which are used as medicinal plants. Therefore natural products research in Thailand is targeted toward the goal of studying the biological activity and chemical constituents of medicinal plants for both to obtain new drug leads and to contribute to the economic development of Thailand by developing them into new pharmaceuticals that provide new industry.

Anaxagorea luzonensis A. Gray is a tree indigenous of Thailand. It was used as a traditional medicinal plant that belongs to the Annonaceae family. As herbal medicine, the heartwood of this plant has been used as a blood tonic, stomachic, antipyretic agents and for treatment of muscular pain. Previous phytochemical investigations of the plant led to the isolation of xanthones and flavonoids [3]. A prenylflavonoid, 8-isopentenylnaringenin, obtained from this plant was found to possess estrogen uterotropic effectiveness [4]. Estrogen has been shown to exert a variety of beneficial effect on men and women. It is recognized to provide protection

against osteoporosis, heart attack, and other cardiovascular problems, and possibly Alzheimer's disease [4]. As part of our search for compounds that are active against cholinesterase, it was found that a methanol extract of the heartwood of *A. luzonensis* exhibited significant anticholinesterase inhibitory activity using TLC-bioautographic method. This report deals with the isolation and structural determination of the active anticholinesterase components of this plant including antioxidant and phosphodiesterase inhibitory activites.

1.1 Botanical aspects and distribution of *Anaxagorea luzonensis A. Gray* [5]

Family Name: Annonaceae

Common Name: Dalairo

Local Name: Kam-lang-wua-thaloeng (กำลังวัวเถลิง)

Origin: Philipines and Thailand

In Thailand *A. luzonensisi* has been known as Kam-lang-wua-thalueng. Other names are Poonta, Chamab and other. The plant was traditional medicinal plant as blood tonic, stomachic, antipyretic and for treatment of muscular pain. The botanical characteristics can be described as follows:

Tree: deciduous tree to 20 (rarely 30) m with delicate foliage and open spreading crown when mature.

Flowers: solitary, leaf-opposed, and about 2 centimeters in diameter

Leaves: oblong, 7 to 15 centimeters long, 3 to 5 centimeters wide and smooth

Heartwood: reddish-brown, hard, a little of the whitish bark still adheres and easily split.

(a) (b) (c) (d)

Figure 1.1 (a) Tree, (b) flowers, (c) leaves and (d) heartwood of *A. luzonensis*

1.2 Chemical constituents and literature reviews of *A. luzonensis.*

From the literature reviews, only two reports studied on the chemical constituents of *A. luzonensis* which can be summarized as follows:

In 1998, Kitaoka *et al*. [6] could be obtained one new flavonoid named 8-isopentenylnaringenin from the methanol extract of the heartwoods of *A. luzonensis*. This compound had estrogenic activity greater than genistein, a standard compound.

In 2000, Gonda *et al*. [4] isolated 20 natural products, five new xanthones, 1,3,6-trihydroxy-5-methoxy-4-prenylxanthone, 1,3,5-trihydroxy-6-methoxy-2-prenylxanthone, 1,3,5-trihydroxy-4-(3-hydroxy-3-methylbutyl)xanthone, 1,3,6-trihydroxy-4-prenylxanthone, 3,6-dihydroxy-1,5-dimethoxyxanthone and one new flavonoid, 3,5,7,4′-tetrahydroxy-2′-methoxyflavone were isolated from the heartwood of *A.luzonensis* together with seven known xanthones, 1,3,5-trihydroxy-4-prenylxanthone, 1,3,5-trihydroxy-2-prenylxanthone, 1,3,6-trihydroxy-5-methoxyxanthone, genistein (1,3,7-trihydroxyxanthone), 1,3,5-trihydroxyxanthone, 3,5-dihydroxy-1-methoxyxanthone, and 1,3,5,6-tetrahydroxy xanthone and seven known flavonoids, biochanin A, chrysin, 3′-methylorobol, orobol, taxifolin, kaempferol and quercetin. Almost all flavonoids and 1,3,5,6-tetrahydroxy xanthone showed antioxidant activity toward DPPH radical.

The structures of isolated compounds from the heartwood of *A.luzonensis* were shown in Figure 1.2.

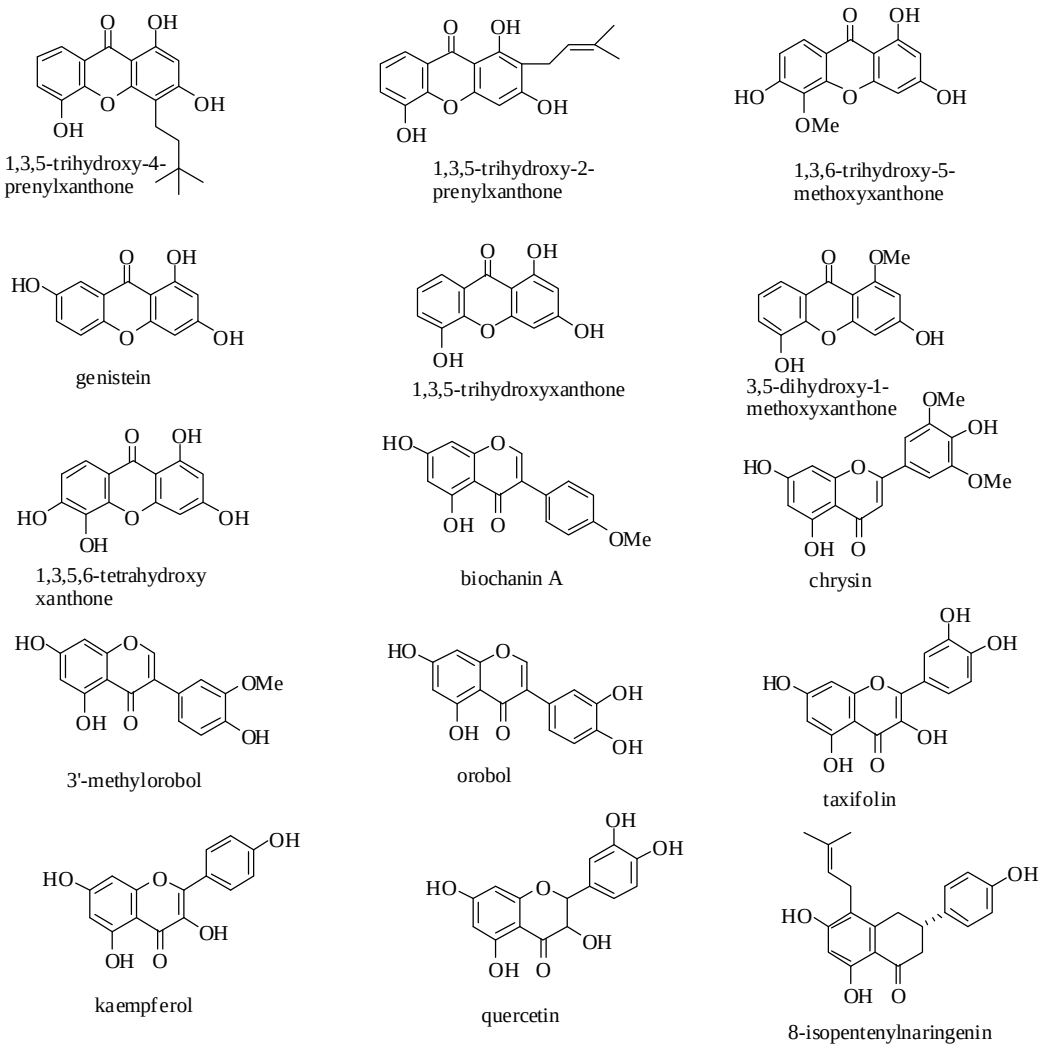


Figure 1.2 The structures of isolated compounds from *A.luzonensis.*

1.3 Anticholinesterase inhibitory activity

Alzheimer's disease (AD) is a progressive degenerative neurological disorder resulting in impaired memory and cognitive function. In AD patients, there are decreased levels of acethylcholine in the brain areas releated to memory and learning. The primary approach in treating AD has aimed at augmenting the cholinergic system.[7] The process begins when acetylcholine is released to travel across the synaptic cleft (Figure 1.3) where it binds to a receptor on the other side of the synapse (post-synaptic terminal). Once the signal is triggered acetylcholine is rapidly broken down by an enzyme, acetylcholinesterase (AChE), and liberating choline. This is

followed by a rapid hydrolysis of the acylated enzyme yielding acetic acid (Scheme 1.1), and the restoration of the esteratic cells.

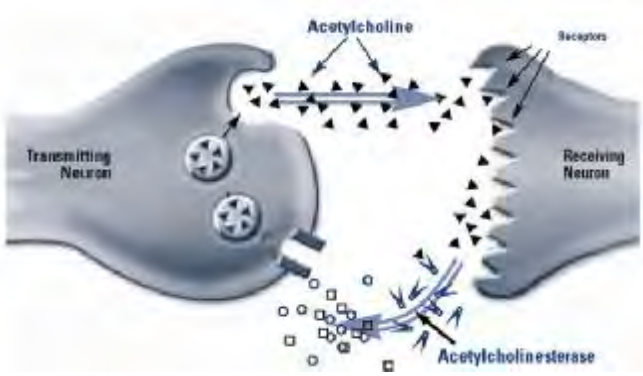
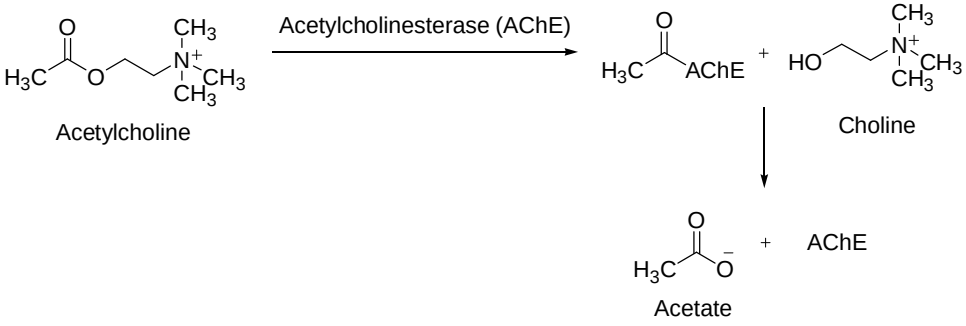


Figure1.3 The action mechanism of acetylcholinesterase

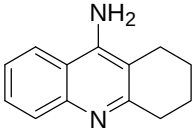
In spite of the multi-factorial nature of AD, most current agents follow one therapeutic approach, based on the so-called cholinergic hypothesis of cognitive dysfunction. This hypothesis postulates that at least some of the cognitive decline experienced by patients of AD results from a deficiency in neurotransmitter acetylcholine and thus in cholinergic neurotransmission, which seems to play a fundamental role in memory. In late stages of AD, levels of AChE decline by up to 85% and BChE represents the predominant cholinesterase in the brain. Such studies have targeted BChE as a new approach to intercede in the progression of AD

However, currently cholinesterase inhibition is the major treatment for the symptoms of AD and inhibition of AChE and BChE are therapeutic targets for improving the cholinergic deficit. [8]

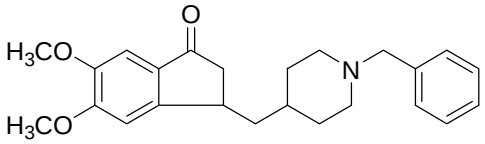


Scheme 1.1 Overall reaction during hydrolysis of acetylcholine

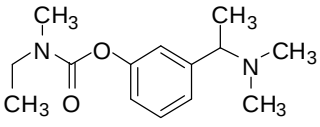
To date, the use of acetylcholinesterase inhibitors (tacrine, donepezil, rivastigmine, and galantamine) is the only therapy to have shown consistent positive results in the treatment of Alzheimer's disease [9]. Tacrine (THA, Cognex®) was the first synthetic drug approved by the USA Food and Drug Administration for AD treatment [10]. This substance is a centrally-acting cholinesterase inhibitor (ChEI) that shows moderate effect in relieving AD symptoms of mild and medium intensity. However, its application became limited due to serious side effects, like hepatotoxicity that forced patients to discontinue the treatment. Besides THA, currently there are three other ChEI drugs available in USA and Europe for AD treatment: donepezil (Aricept®), rivastigmine (Exelon®), and galanthamine (Reminyl®). Of these, tacrine, donepezil and galanthamine are reversible inhibitors, while rivastigmine acts pseudo-irreversibly. These compounds also differ with regard to selectivity for acetyl-(AChE) versus butyrylcholinesterase (BChE) and central versus peripheral activity. Galantamine is a natural product that has the additional effect of allosterically potentiating nicotinic acetylcholine receptors, and it has been used as a prototype in anti-cholinesterase drug development. However, the present drugs (galantamine, tacrine, rivastigmine and donepezil) with AChE inhibitory activity possess some side effects and are effective only against the mild type of AD and there has been no drug available with BChE inhibitory activity to present, yet. Consequently, it is compulsory to develop new drugs in order to combat AD.



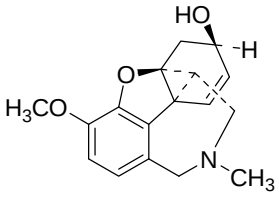
Tacrine



Donepezil



Rivastigmine



Galantamine

Figure1.4 The chemical structure of drugs for AD treatment

1.4 Antioxidant compounds: DPPH and ABTS assay

Oxidative stress is involved in the pathology of cancer, arteriosclerosis, malaria and rheumatoid arthritis, and could play a role in neurodegenerative diseases and ageing processes. The protection that fruits and vegetables provide against several diseases has been attributed to the various antioxidants, flavonoids, polyphenols, carotenoids, fiber, vitamins A, B, C and E. tocopherols and selenium [11]. The example structures of antioxidants are shown in Figure 1.5.

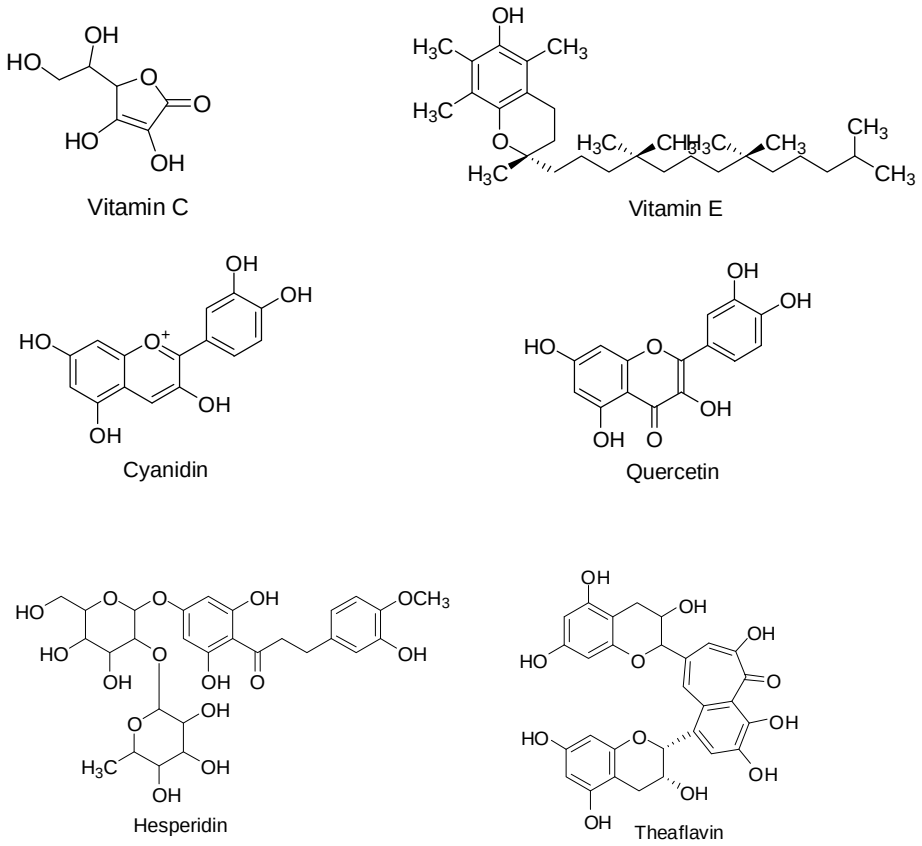


Figure 1.5 Structures of some antioxidant compounds

Several methods have been developed to evaluate the antioxidant potential of natural antioxidant form plant; the trolox equivalent antioxidant capacity (TEAC) using ABTS (2,2-azino-bis-3ethylbenzothiazoline-6-sulfonic acid) as an oxidant, the ferric reducing antioxidant power (FRAP), the DPPH (2.2′-diphenyl-1-picrylhydrazyl) free radical scavenging potential, the oxygen radical absorption capacity (ORAC) and the total radical absorption potentials (TRAP) assays are some of the most commonly

used [12]. The mechanism of ABTS, FRAP and DPPH assays are measuring the reduce power of single electron transfer whereas ORAC and TRAP assays measured that of hydrogen atom transfer. ABTS and DPPH are widely and commonly used to assess antioxidant activity although both of these are foreign to biological system. Both methods required simple equipment and costless than other method. However, both methods could be useful for assessing antioxidant activities of natural extracts or compounds only if color interference is not significant.

1.5 Cyclic GMP phosphodiesterase

Cyclic nucleotide phosphodiesterase (PDEs, E. C. 3.1.4.17), by specifically hydrolyzing cyclic nucleotides (Fig. 1.6) which degrade the second messenger cyclic 3′, 5′-adenosine monophosphate (cyclic AMP) and cyclic 3′, 5′- guanosine monophosphate (cyclic GMP) to 5′-AMP and 5′-GMP, nucleotides which are unable to activate the protein kinase cascade. As second messengers, cyclic AMP and cyclic GMP play key roles in the functional responses of cells to many hormones and neurotransmitter. PDE nomenclature (PDE1 to PDE11) was established according to the gene of which they are products, their biochemical properties, regulation, and sensitivity to pharmacological agents [13].

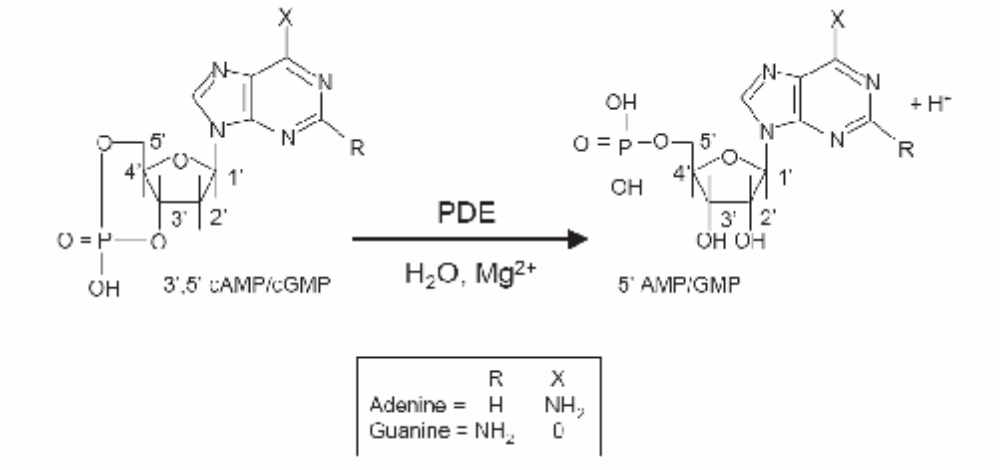


Figure 1.6 Cyclic nucleotide hydrolysis by cyclic nucleotide phosphodiesterase.

Phosphodiesterases occur widely in biological systems. They are present in nearly all mammalian tissues with the possible exceptions of red blood cells and isolated rat adrenal cells. These two cellular systems provide excellent models for

studying cyclic nucleotide synthesis uncomplicated by the interference of phosphodiesterase. These enzymes have been found also in bacteria, yeast, insect, higher plants and several marine organisms. In general, they are found wherever the cyclic nucleotides exist. Phosphodiesterase activity is detected early in embryonic development, increases up to maturity, seems to decrease with age, and varies in activity with the cell cycle [14].

PDE-5, previously named cGMP-PDE, PDE-5, was firstly characterized as a cGMP-binding protein different from protein kinase co-purifying with a cGMP phosphodiesterase in rat platelets and in rat lung [15]. PDE5 was firstly implicated in vasorelaxation, since the specific inhibition of PDE5 by zaprinast was shown to induce an increase in cGMP associated with a vasorelaxing effect [16]. The potentiation of a PDE5 inhibitor relaxing effect obtained on the aorta containing functional endothelium or treated with NO donors suggested that PDE5 mediates the NO/cGMP relaxing effect. In that way, new PDE5 inhibitors derived from zaprinast were designed as antihypertensive compounds or coronary vasodilators; unexpectedly, during clinical studies, sildenafil ameliorated erectile dysfunction, pointing out PDE5 as a new target for treatment of erectile dysfunction and increasing the development of PDE5 inhibitors. The high level of PDE5 encountered in the lung, as well the observation that PDE5 was activated in pulmonary hypertension, has contributed to propose also PDE5 as a new target for the treatment of pulmonary hypertension and respiratory distress. Furthermore, in agreement with PDE5 characterization in the brain, it was recently shown that PDE5 inhibition improves early memory consolidation of object information [17].

1.6 The goals of this research

According to the literature reviews, the isolated substances from *A. luzonensis* previously studied displayed interesting biological activities such as phytoestrogen, antioxidant activity *etc.*, but none has been reported on cholinesterase inhibitory activity. Thus, the goals of this research could be summarized as follows:

1.6.1 To extract and isolate the chemical constituents from the heartwood of *A. luzonensis.*

1.6.2 To elucidate the structures of isolated compounds from the heartwood of *A. luzonensis.*

1.6.3 To investigate the bioactivities; anticholinesterase, antioxidation and antiphosphodiesterase activities, of the isolated compounds from *A. luzonensis*.

CHAPTER II

Experimental

2.1 Plant materials

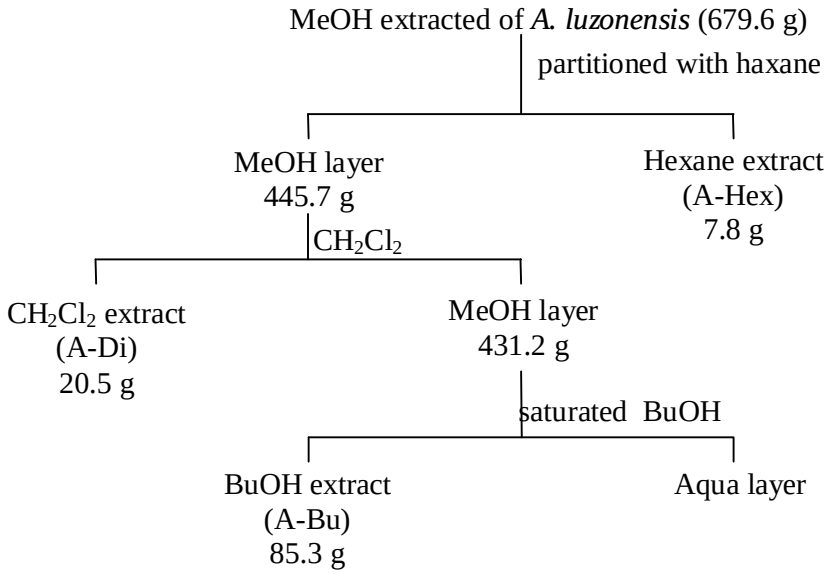
The dreid heartwood of *Anaxagorea luzonensis A.Gray* was purchased in Bangkok from a commercial supplier in 2006 and voucher specimen was No. 117345 deposited at the herbarium of the Royal Forestry Department, Thailand.

2.2 Instruments and equipment

The ^{1}H and ^{13}C-NMR spectra (in $CDCl_3$, methanol-*d4* and acetone-d6) were determined with a nuclear magnetic resonance spectrometer of Varian model Mercury+ 400. Melting points were recorded with Fisher-John melting point apparatus and are uncorrected. Adsorbents used for separation were silica gel 60 Merck, No. 7734 and 7731 for column chromatography and quick column chromatography, respectively. Thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 PF254). The spots on plate were detected under UV light or visualized by spraying with 10% H_2SO_4 in ethanol. Preperative Thin layer chromatography (PTLC) was coated on glass sheets precoated with silica gel (Merck, No 7730). Gel filtration chromatography was performed on sephadex LH-20.

2.3 Extraction procedure of the heartwood from *A. luzonensis*

The dried heartwood (5 kg) of *A. luzonensis* was extracted with methanol by Soxhlet's extractor. The extract was further partitioned with hexane, dichloromethane and butanol, yielding after evaporation hexane (7.8 g), dichloromethane (20.5 g), and butanol (85.3 g) extracts, respectively. The extraction procedure was summarized as shown in Scheme 2.1.



Scheme 2.1 Extraction procedure of the methanol extract from *A. luzonensis*

2.4 Bioassay procedures

2.4.1 Cholinesterase inhibitory assay

This assay was based on Ellman's method with modification [18]. The test relies on the cleavage by cholinesterase of acetylthiocholine to form thiocholine which in turn reacts with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to give a yellow colored 2-nitrobenzoate-5-mercaptothiocholine (Scheme 2.2). Inhibitors are naturally occurring or synthetic molecules that decrease enzyme activity or prevents any substrate molecules from reacting with the enzyme

2.4.1.1 Chemical reagents

All commercial solvents used in this research were distilled prior to use except for those which were reagent grades.

Acetylcholinesterase (AChE) from electric eels (Type-VI-S lypophilized powder, EC 3.1.1.7), butyrylcholinesterase (BChE) from horse serum (lypophilized powder EC 3.1.1.8), acetylthiocholine iodide (ATCl), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), galanthamine and 2,2-Diphenyl-1-picryhydrazyl (DPPH) radical were purchased from Sigma (St. Louis, MO, USA).

Tris-(hydroxymethyl)-aminomethane (*Tris*-HCl) was purchased from Merck (Darmstadt, Germany).

2.4.1.2 Chemical preparation

1. Buffers:

The following buffers were used;

Buffer A:50 mM *Tris*–HCl, pH 8;

Buffer B: 50 mM *Tris*–HCl, pH 8 containing 0.1% bovine serum albumin (BSA);

Buffer C: 50 mM *Tris*–HCl, pH 8 containing 1 M NaCl and 0.2 mM $MgCl_2.6H_2O$.

2. Enzymes:

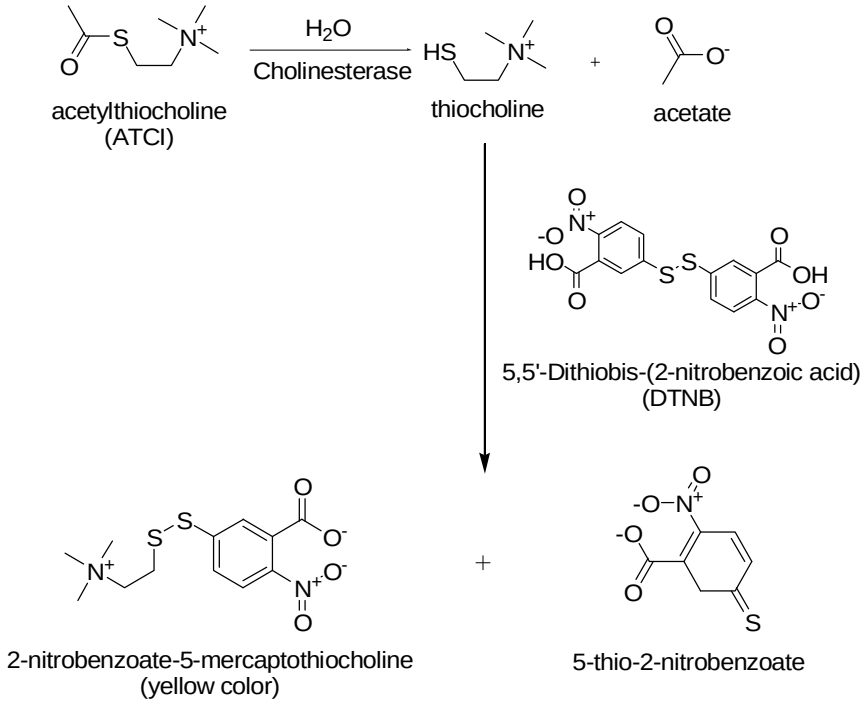
Cholinesterase enzymes (AChE and BChE) were dissolved in buffer A to make 1130 U/ml stock solution, and further diluted with buffer B to get 0.28 U/ml enzymes for the microplate assay, or diluted with buffer A to get 3 U/ml enzyme for the TLC assay.

3. Substrate:

5 mM ATCI in buffer A was used for the TLC assay and 1.5 mM in MilliQ water was used for the microplate assay.

4. Ellman reagent:

5 mM DTNB in buffer A was used for the TLC assay and 3 mM DTNB in buffer C was used for the microplate assay.



Scheme 2.2 Cholinesterase catalyzed hydrolysis of acetylthiocholine.

2.4.1.3 Cholinesterase inhibitory method

a) TLC bioautography method [19]

The samples were dissolved in appropriated solvent to a concentration of 10 mg/mL for extract, 1 mg/mL for isolated compounds and reference compounds (galantamine and eserine). Then 10 µL of each sample was spotted on silica gel TLC plate and developed in the appropriate solvent, dried 1-2 hr. After developing the TLC plate, the plate was sprayed with the mixture of DTNB and ATCI (1:1) solution until the silica was saturated with the solvent, but not so much that the spots ran off. It was allowed to dry for 3–5 min. and then 3 U/ml of enzyme solution was sprayed. A yellow background appeared, with white spots for inhibiting compounds becoming visible after 5 min. These were observed and recorded within 15 min because they disappeared in 20–30 min.

b) Microplate method

AChE and BChE inhibitory activities were measured by slightly modifying the spectrophotometric based on Ellman's method [20] In the 96-well plates, 25 µl of 1.5 m*M* ATCI in water, 125 µl of 3 m*M* DTNB in buffer C, 50 µl of buffer B, 25 µl of sample (1 mg/ml in MeOH diluted ten times with buffer A, to give a concentration of

0.1 mg/ml), 25 µl of 0.28 U/ml of enzyme were added and the absorbance was measured at 415 nm over 2 min with a 5-s interval. The resulting velocity was calculated and used for the determination of the enzyme and inhibitory activities. The IC_{50} was calculated using software package Prism (Graph Pad Inc., San Diego, CA). Figure 2.1 as an instance shows the curve of cholinesterase inhibition of inhibitors.

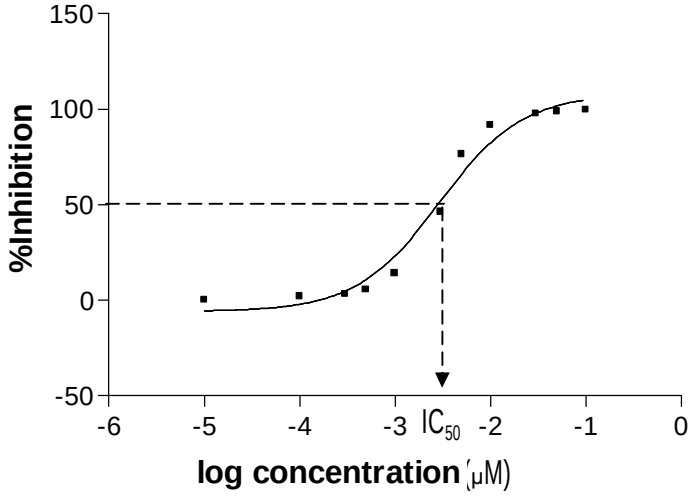
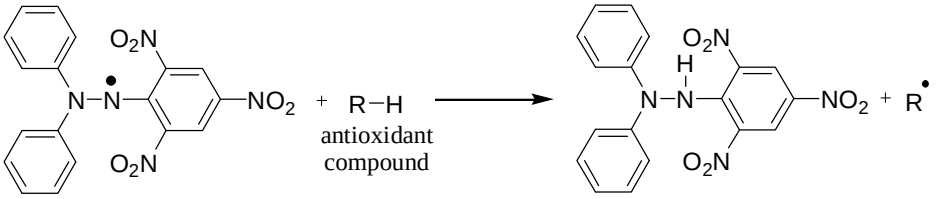


Figure 2.1 Cholinesterase inhibitory activity of inhibitor

2.4.2 Radical scavenging of DPPH assay

DPPH radical (purple color, λ_{max} 517 nm) is considered to be very stable and to be destroyed only by antioxidants or radical scavengers which can donate hydrogen atom (Scheme 2.3.). The different color between radical form (purple) and non-radical form (colorless) of DPPH was used for measuring their activity. Scavenging effect toward DPPH radical consisted of two assays for qualitative and quantitative analysis. The assay for qualitative analysis employed TLC bioautographic method while spectrophotometric (microplate) assay was used for quantitative analysis.



2,2-Diphenyl-1-picryhydrazyl (DPPH) radical (Purple color)

2,2-Diphenyl-1-picryhydrazine (DPPH) non radical (Colorless)

Scheme 2.3 The reaction of DPPH radical

2.4.2.1 Chemical reagents

2,2-Diphenyl-1-picryhydrazyl (DPPH) radical was purchased from Sigma (St. Louis, MO, USA).

2.4.2.2 Radical scavenging of DPPH method

a) TLC autographic method [21]

The samples were dissolved in approximately solvent to a concentration of 10 mg/mL for extract, 1 mg/mL for isolated compounds and standard (Vitamin C). Then 10 µL of each sample were spotted on silica gel TLC plate and developed in the appropriated solvent, dried and sprayed with 0.3 mM DPPH in methanolic solution. The plate was examined for 5 minutes after spraying. Active compounds appeared as yellow spots against purple background.

b) Microplate method

Radical scavenging activity of compound was measured by slightly modified method of Brand-Williams and Gisely [22]. In the 96-well plates, 200 µl of 0.03 m*M* DPPH in MeOH, 50 µl of each sample (1 mg/ml in MeOH, various concentrations by diluted two times with MeOH) were added. The samples were kept in the dark for 30 min at room temperature and then the decrease in absorption was measured. The absorbance was measured at 518 nm against a corresponding blank. Inhibition percentage of free radical DPPH (% I) was calculated in the following way:

$$\%\,I = \frac{[1 - A_{sample}]}{A_{blank}} \times 100\%.$$

Where A_{blank} is the absorbance of the control reaction (a reaction with all the reagents except sample), and A_{sample} is the absorbance of the test compound. Tests were carried out in triplicate and the compound concentration providing 50% inhibition (IC_{50}) was obtained by plotting extract solution concentration *versus* inhibition percentage. Figure 2.2 as an instance showed the curve of radical scavenging effect of antioxidants.

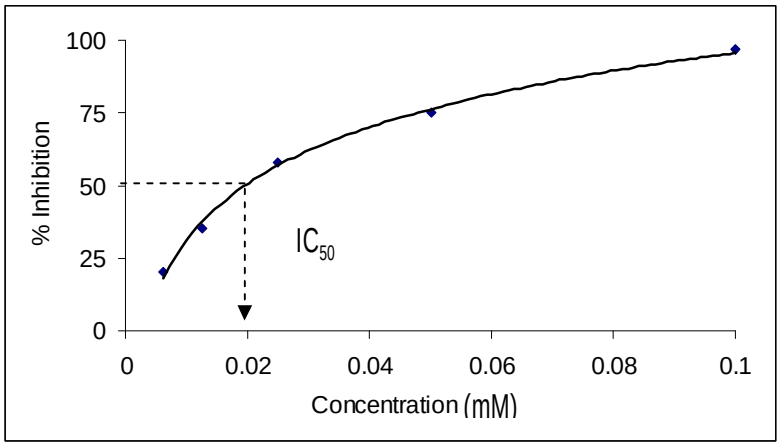
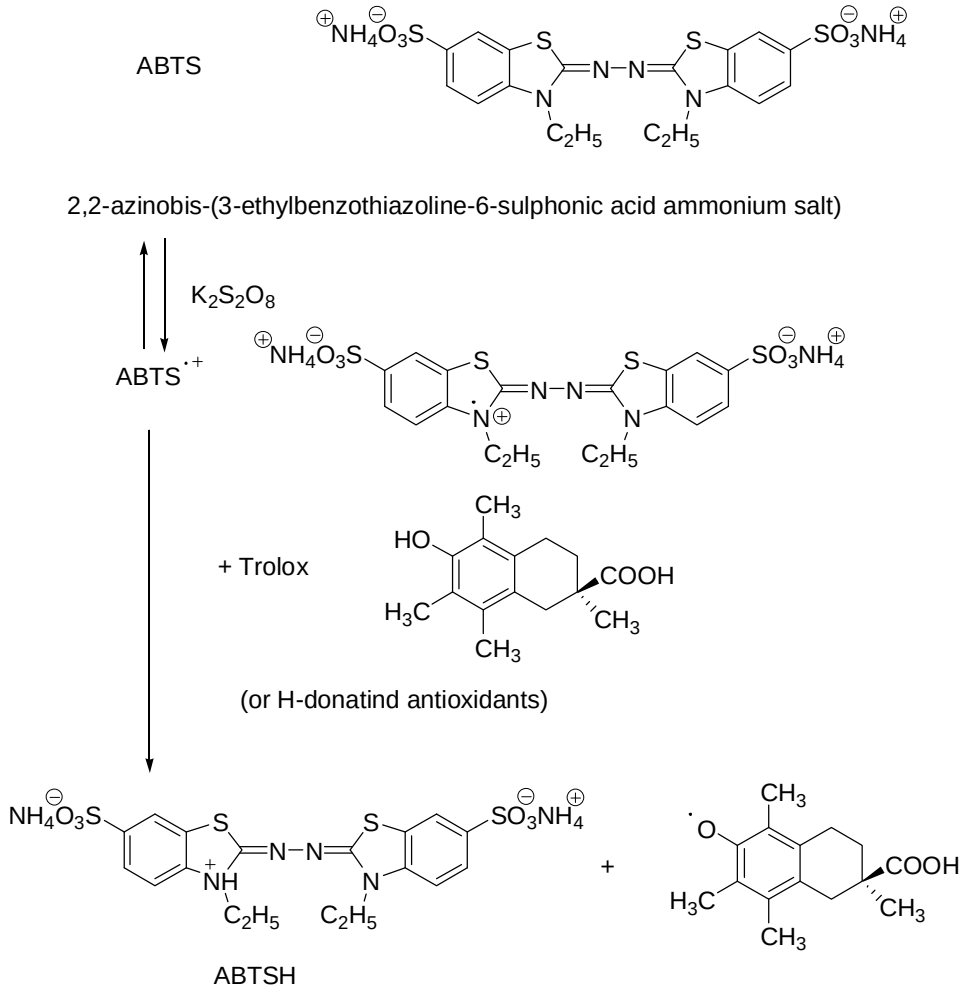


Figure 2.2 DPPH radical scavenging effect of antioxidants

2.4.3 ABTS decolorization assay

The assay was carried out by interacting the antioxidants with the ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation prepared as described in Re *et al* [23]. An antioxidant activity is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids and plasma antioxidants. The ABTS assay measures the relative ability of antioxidant to scavenge the $ABTS^{\bullet+}$ generated in aqueous phase, as compared with a Trolox (water soluble vitamin E analogue) standard. The $ABTS^{\bullet+}$ is generated by reaction a strong oxidizing agent (e.g., potassium permanganate or potassium persulfate) with the ABTS salt. The reduction of the blue-green $ABTS^{\bullet+}$ radical by hydrogen-donating antioxidant is measured by the suppression of its characteristic long wave absorption spectrum [23]. The method is usually expressed as Trolox equivalent antioxidant capacity (TEAC). It also has good repeatability and is simple to perform; hence, it is widely reported [24]. The TEAC values are obtained from the capacity of an individual.



Scheme 2.4 The assay for measuring TEAC (Trolox equivalent antioxidant capacity)

2.4.3.1 Chemical reagents

1. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Diammonium salt (ABTS) was purchased from Fluka Chemical Company.

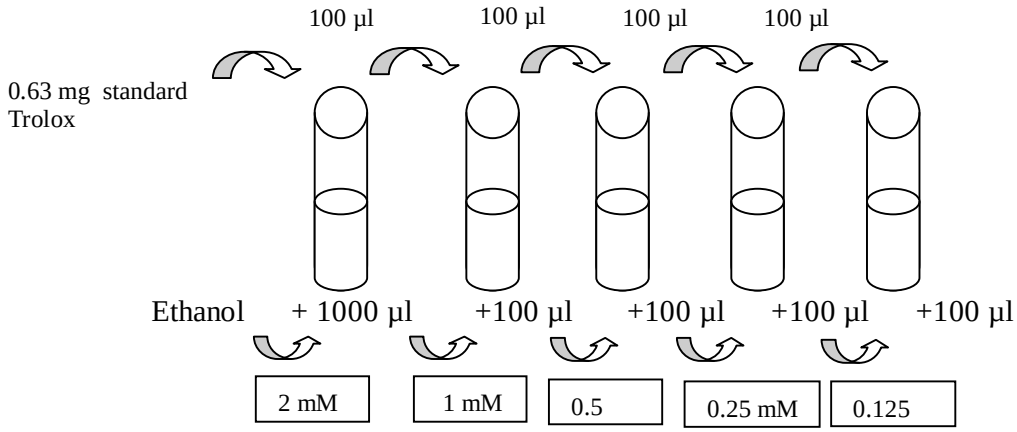
2. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chemical Company.

3. Potassium persulfate was purchased from Sigma-Aldrich Company.

2.4.3.2 Preparation of standard Trolox and sample

A standard curve was obtained by using Trolox standard solution at all doses standard test (2, 1, 0.5, 0.25 and 0.125 mM) with ethanol. This profile was prepared with stock Trolox standard (2 mM). Pipette ethanol 100 µl into 5 tubes. Using the

newly diluted stock Trolox solution, prepare a dilution series as depicted below. The 2 mM stock dilution serves as the highest standard. The overall procedure of dilution test for standard Trolox can be shown in Scheme 2.5.



Scheme 2.5 The profile of dilute test

- Sample preparation

Antioxidants solution were prepared by dissolution in ethanol and subsequently diluted in ethanol for introduction into the assay system at concentrations within the activity range of the assay (0.75 µM to 50 µM final concentration). The antioxidants are mixed in the cuvette with the $ABTS^{\cdot+}$solution, to give absorbance value at 734 nm. Results are calculated at 1 min and 6 min. The extent of radical cation present at 1 min and 6 min is calculated from the absorbance recorded.

2.4.3.3 ABTS decolorization method

A stock solution of 7 mM ABTS was prepared in DI. To this solution potassium persulphate (2.45 mM – final concentration) was added and the solutions allowed to react for a duration of 12-16 h at room temperature in the dark. ABTS and potassium persulphate react stoichiometrically at 1:0.5 leading to an incomplete oxidation to generate $ABTS^{\cdot+}$. The ABTS radical cation solution was diluted in ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Measurement of absorbance change is triggered as soon as the cuvette is filled which triggers a signal to the spectrophotometer. The measuring was done in a Cary 50 prove UV-Visible spectrophotometer connected to a Julabo F 33 thermostatted bath at 25 °C (Julabo, Seelbach/ Germany). Absorbance changes are monitored every 1 min for a duration of

6 min at 734 nm. Prior to the testing with the antioxidants, a baseline is obtained by monitoring the change in absorbance between $ABTS^{\cdot+}$ and ethanol. This reading is use absorbance as the basal value for calculating the antioxidant activity of the compounds.

2.4.3.4 TEAC value

The concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation at 734 nm as 1 mM Trolox was calculated in terms of the Trolox equivalent antioxidant activity (TEAC) at each specific time-point. To calculate the TEAC, the gradient of the plot of the percentage inhibition of absorbance vs. concentration plot for the antioxidant in question is divided by the gradient of the plot for Trolox.

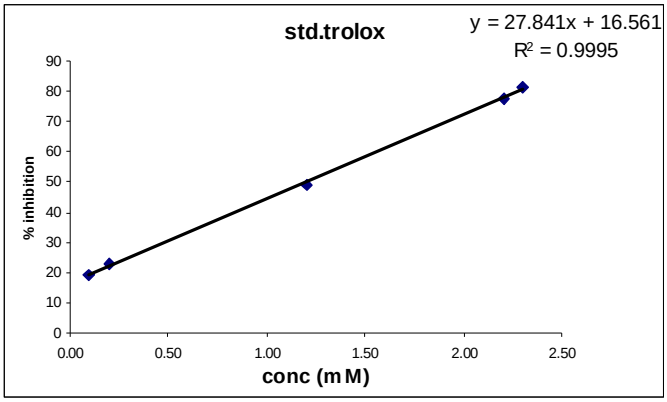
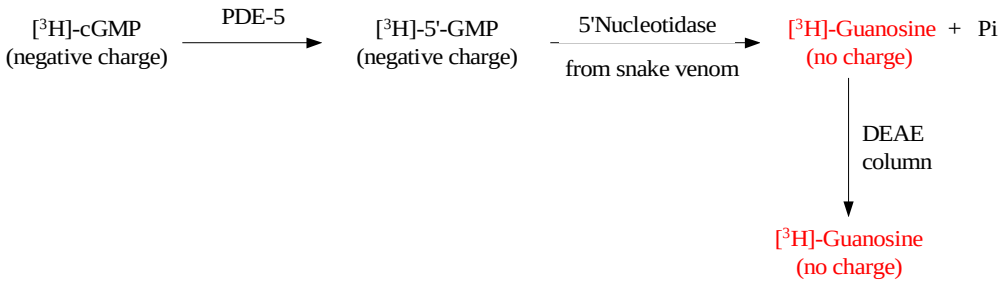


Figure 2.3 Concentration-response curve for the absorbance at 734 nm for $ABTS^{\cdot+}$ as a function of concentration of standard Trolox solution. (Five separately prepared stock standard solutions ±SD.)

2.4.4 Phosphodiesterase-5 Assay

The radioactive method is used in this assay. Cyclic guanosine monophosphate (cGMP) is labeled with tritrium [^{3}H] at 8 position of proton of guanine ring. The enzymatic reaction is based on two steps procedure. Phosphodiesterase-5 (PDE-5) cleaves cyclic guanosine monophosphate (cGMP) to leave 5′-guanosine monophophate (5′-GMP). This reaction is terminated by boiling for 1 minute and cooling. 5′-nucleotidase from snake venom, then breaks 5'AMP into guanosine and phosphate. However, both cGMP and 5′-GMP contain negative charge

while guanosine does not. Therefore, guanosine can be separated from other radiolabled compounds using ionic exchange resin.



Scheme 2.6 Quantitative determination of cGMP phosphodiesterase activity by SPA radio assay method.

2.4.4.1 Chemicals preparation

1. Bovine serum albumin (BSA) *(Sigma, St. Louis, MO, USA)*
2. cGMP *(Sigma, St. Louis, MO, USA)*
3. [8-^{3}H]cGMP *(GE Healthcare, Piscataway, NJ, USA)*
4. DEAE resin *(Sigma, St. Louis, MO, USA)*
5. Dimethylsulfoxide *(Merck, Germany)*
6. Dithiothreitol (DTT) *(Sigma, St. Louis, MO, USA)*
7. Ethylenediaminetetraacetic acid (EDTA) *(Sigma, St. Louis, MO, USA)*
8. EGTA *(Sigma, St. Louis, MO, USA)*
9. Imidazole *(Sigma, St. Louis, MO, USA)*
10. Liquid scintillant (Ultima Gold) *(PerkinElmer, USA)*
11. Magnesium acetate *(JT Baker, NJ, USA)*
12. Magnesium chloride hexahydrate *(Sigma, St. Louis, MO, USA)*
13. Phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride (PMSF) *(Boehringer Mannheim, Indianapolis, IN, USA)*
14. 3-(N-morpholino)propanesulfonic acid (MOPS) *(Sigma, St. Louis, MO, USA)*
15. Snake venom *(Sigma, St. Louis, MO, USA)*
16. Sodium chloride *(Fisher Scientific, USA)*
17. Tris (hydroxymethyl) aminomethane *(Sigma, St. Louis, MO, USA)*

2.4.4.2 Instruments

Liquid Scintillation Analyzer Packard 1600TR (Packard, USA)

2.4.4.3 Preparation of PDE-5

Fresh mice lung or (~50 mg) was cut into small pieces and homogenized in 1 ml Buffer P which containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM DTT and 1 mM PMSF. The supernatant was obtained by centrifugation at 4°C for 20 minutes. This supernatant was used for assay. For studies of the inhibition of the activity of PDE-5, the percentage of hydrolysis was in the range of 20% - 30%.

2.4.4.4 Sample preparation

The tested sample was dissolved in DMSO not exceed 5% of the final concentration and diluted with D_2O to make a concentration of 500 µM. The samples were screened at a final concentration of 100 µM. All determinations are tested on duplicate.

Compounds which give %PDE-5 inhibition more than 80% were further studied on IC_{50}. Each sample was diluted serially with dH_2O to obtain 6 different concentrations (10^3 to 10^{-2} µM). The IC_{50} values, corresponding to the inhibitor concentration that caused 50% inhibitory activity, were analyzed using the software package Prism (Graph Pad Inc, San Diego, USA). All determinations are tested on duplicate.

2.4.4.5 PDEs inhibitory activity assay

The radioactive-PDE-5 assay was followed by the protocol of Beavo et al. Briefly, 25 µl of Buffer C which containing 100 mM Tris-HCl (pH 7.5), 100 mM imidazole, 15 mM $MgCl_2$ and 1 mg/ml BSA was mixed with 25 µl of 10 mM EGTA, 25 µl of sample and 25 µl of PDE-5 that give %hydrolysis not more than 30%. For initial reaction, 25 µl of 5µM cGMP (containing 100,000 count per minuter (CPM) of [^{3}H] cGMP plus cold cGMP) was added and incubated at 30°C for 10 min. The enzymatic reaction was stopped by heating at 100°C for 1 min and cooled on ice. After that, 10 µl of 2.5 mg/ml of snake venom was added and incubated at 30°C for 5 min. Then the final reaction mixture was diluted with 125 µl of low salt buffer (20 mM Tris-HCl, pH 7.5) and transferred to column which packing with 600 µl of

swollen DEAE ion exchange resin. [^{3}H] guanosine was eluted from resin with 250 µl of low salt buffer, 4 times. The filtrate was added with 5 ml of scintillation, mixed and counted on liquid scintillation counter. The percentage of inhibition was calculated from equation (2) below.

$$\%hydrolysis = \left(1 - \left(\frac{CPM_{control/sample} - CPM_{background}}{CPM_{totalcount} - CPM_{background}}\right)\right) x100 \text{ ----------(1)}$$

$CPM_{control\ /\ sample}$ represents radioactive value of control or sample obtained from the assay (with enzyme).

$CPM_{background}$ represents radioactive value of the mixture of only buffer and substrate obtained from the assay (without enzyme).

$CPM_{total\ count}$ represents radioactive value of the mixture of 2 ml of low salt buffer and 25 µl of [^{3}H]cGMP.

$$\%Inhibition = \left(1 - \left(\frac{\%hydrolysis_{sample}}{\%hydrolysis_{control}}\right)\right) X100 \text{ ----------(2)}$$

CHAPTER III

RESULTS AND DISCUSSION

According to the impressive preliminary biological screening results of the methanol extract from the heartwood of *A. luz*onensis, this plant was chosen for further investigating their biologically active principles.

The heartwood of *A. luzonensis* was extracted with appropriate solvents according to the procedure described in Chapter II. All extracts, hexane, CH_2Cl_2 and BuOH extracts were then preliminarily tested for their anticholinesterase activities using TLC method. The results are shown in Tables 3.1.

Table 3.1 The anticholinesterase activities of the extracts from the heartwood of *A. luzonensis*

Extracts	Inhibitory activity against	
	Acetylcholinesterase	Butyrylcholinesterase
Hexane	+	+
CH_2Cl_2	++	+++
BuOH	+++	+++

Note: +++ = strong activity (suddenly detected), ++ = moderate activity (detected at 0-5 minute), + = weak activity (detected at 5-10 minute), - = no activity (not detected)

From the results in Tables 3.1, the BuOH extract displayed the most significant anticholinesterase activities and the CH_2Cl_2 extract was showed moderate activities. Therefore both extracts were further studied to discover the active chemical constituents.

3.1 Separation chemical constituents

3.1.1 Separation of the BuOH extract

A part of the BuOH extract (85.3 g) as a dark brown material was fractioned using Diaion CC eluting with deionized (DI) water, methanol in DI water, methanol, acetone in methanol and finally acetone, respectively. Each fraction was collected approximately 500 mL and then concentrated to a small volume. The fractions were combined monitoring by TLC results to obtain seven fractions, BA to BG. The results of separation are shown in Table 3.2. All fractions were then examined for anticholinesterase activity by TLC method. The results showed that fractions BC and BF possessed the active spots while the rest fractions had no activity.

Table 3.2 The results of fractionation by Diaion CC of the BuOH extract

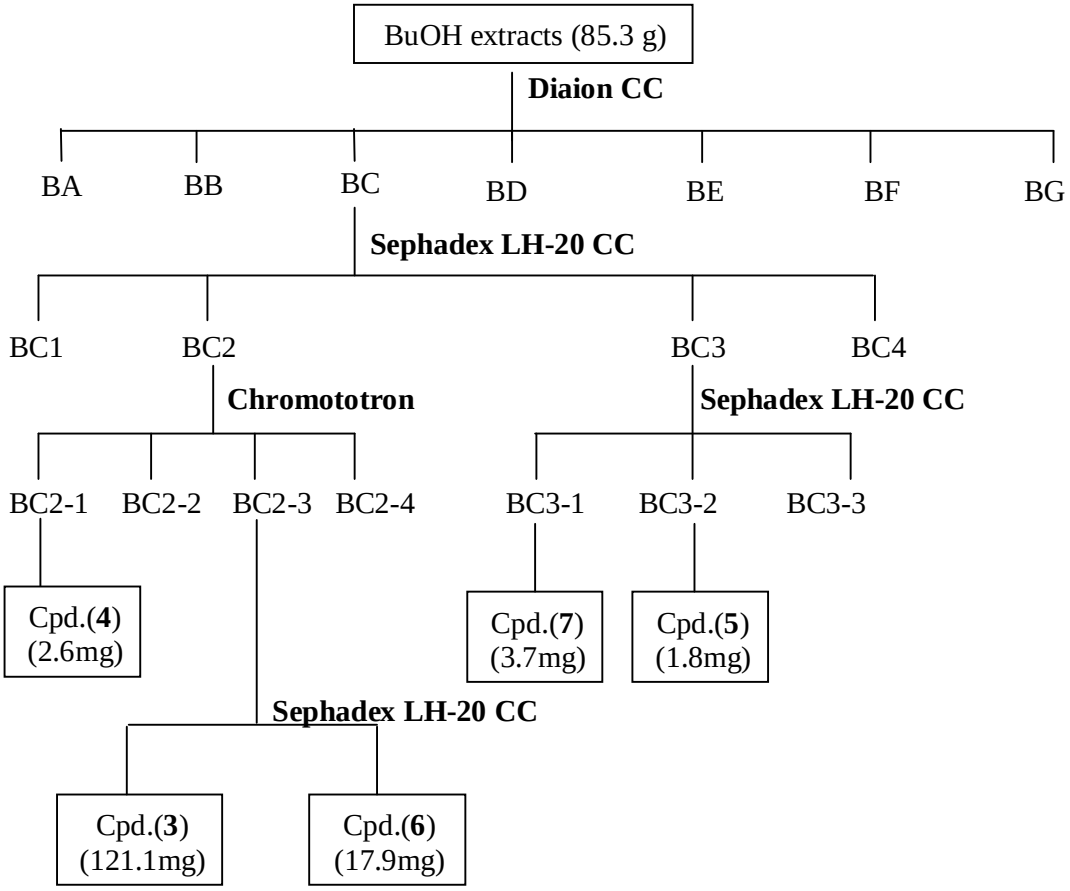
Fraction	Solvent system	Remarks	Weight (g)
BA	100% H_2O-50%MeOH in H_2O	brown syrup	4.8
BB	50%MeOH in H_2O -100%MeOH	brown syrup	3.2
BC	100%MeOH	brown yellow solid	9.7
BD	5-35%acetone in MeOH	brown syrup	1.5
BE	35-50%acetone in MeOH	brown syrup	1.4
BF	50% acetone in MeOH-100%acetone	brown yellow solid	58.5
BG	100%acetone	dark brown	1.1

3.1.1.1 Separation of fraction BC

Fraction BC was subjected to a sephadex LH-20 column eluting with $CHCl_3$: MeOH 1:1 to give four fractions, BC1 to BC4. In addition, fraction BC2 was subjected to a chromatotron chromatography eluting with $CHCl_3$: MeOH 1:1 to give four fractions, BC2-1 to BC2-4. Compound (**4**) was afforded from fraction BC2-1 after recrytallization with hexane and ethyl acetate as yellow solid (2.6 mg)**.** Fraction BC2-3 was reseparated by a sephadex LH-20 column eluting with $CHCl_3$: MeOH 1:1 to furnish compounds (**3**) (121.1 mg) and compound (**6**) (17.9 mg).

BC3 was separated by a sephadex LH-20 column eluting with $CHCl_3$: MeOH 1:1 to furnish fractions BC3-1 to BC3-3**.** Fraction BC3-1 was applied to preparative TLC eluting with $CHCl_3$: MeOH 4:1 to afford compounds (**7**) (3.7 mg). Fraction

BC3-2 was re-crystallization with hexane and ethyl acetate to afford compound (**5**) as yellow solid (1.8 mg)**.** The separation diagram is shown in Scheme 3.1.



* Cpd = compound

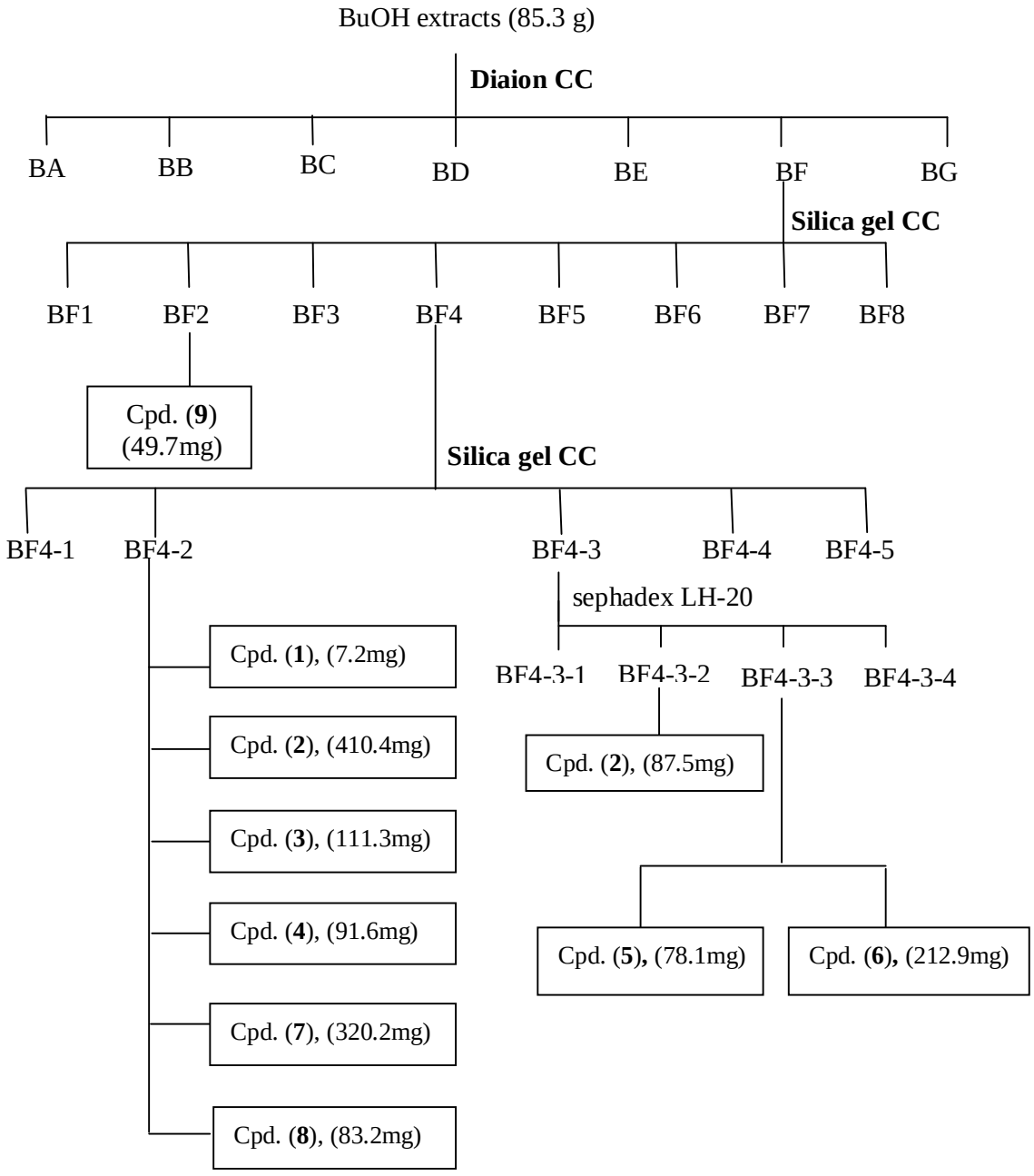
Scheme 3.1 Separation diagram of fraction BC

3.1.1.2 Separation of fraction BF

Fraction BF was repeatedly subjected to a silica gel column using mixtures of n-hexane, EtOAC and MeOH of increasing polarity as eluent to obtain eight fractions, BF1 to BF8. Fraction BF2 was rechromatographed on a sephadex LH-20 column eluting with $CHCl_3$: MeOH 1:1 to furnish compound (**9**) (49.7 mg).

Fraction BF4 was further separated by column chromatography over silica gel (EtOAc-Hexane 1:9 to MeOH-EtOAc 1:9) to give five fractions, BF4-1 to BF4-5. Fraction BF4-2 was purified on a sephadex LH-20 column eluted with MeOH to yield six compounds, compounds (**1**) (7.2 mg), (**2**) (410.4 mg), (**3**) (111.3 mg), (**4**) (91.6 mg), (**8**) (83.2 mg) and (**7**) (320.2 mg). Fraction BF4-3 was subjected to a sephadex

LH-20 column eluting with MeOH to give four fractions, BF4-3-1 to BF4-3-5. Compound (**2**) was obtained from recrytallization fraction BF4-3-2 with hexane and ethyl acetate as yellow powder (87.5 mg)**.** In addition, Fraction BF4-3-3 was purified by sephadex LH-20 column eluting with $CHCl_3$: MeOH 1:1 to furnish compounds (**5**) (78.1 mg) and (**6**) (212.9 mg). The separation diagram of fraction BF is shown in Scheme 3.2.



Scheme 3.2 Separation diagram of fraction BF

3.1.2 Separation of the CH_2Cl_2 extract

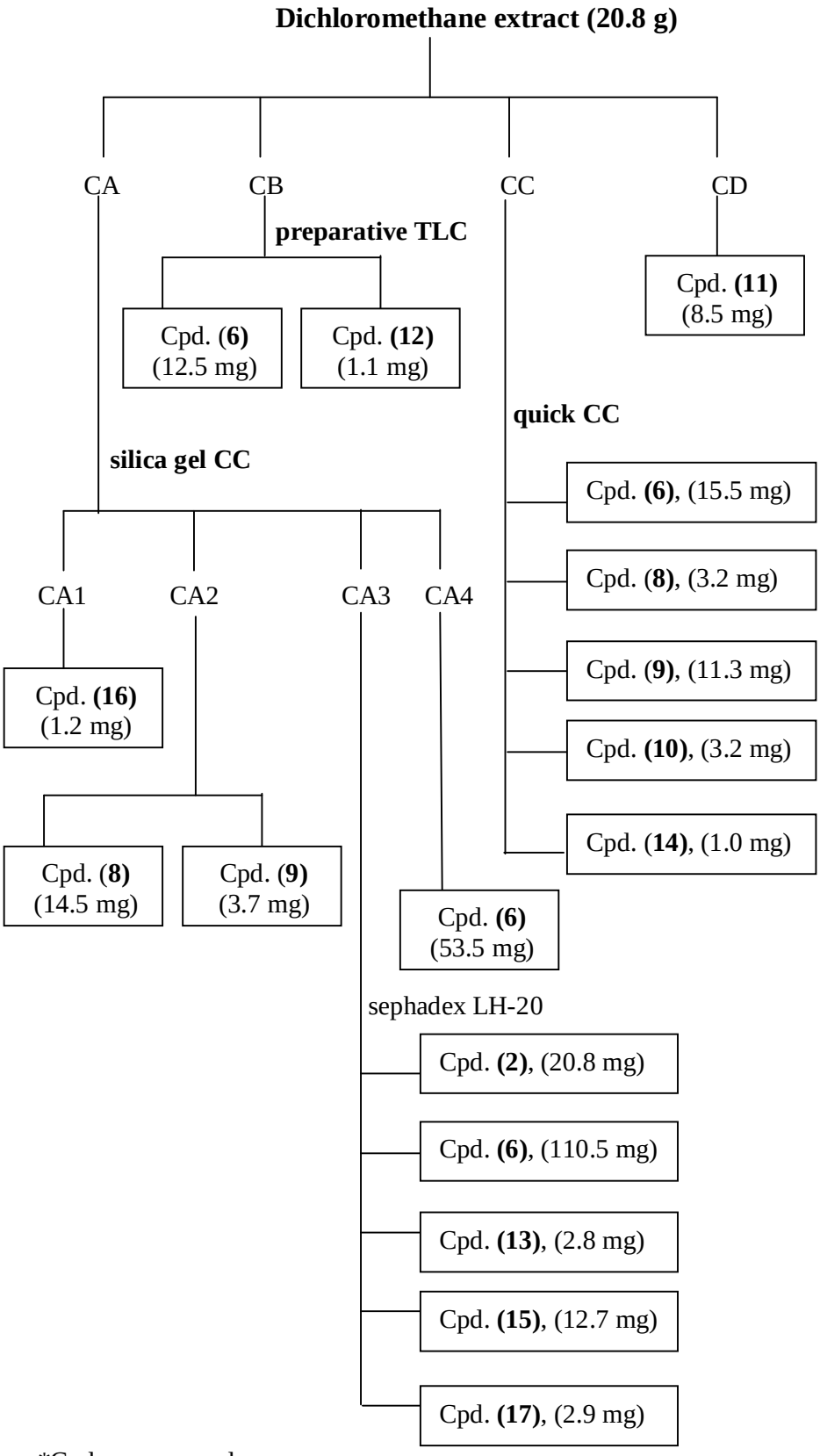
A part of CH_2Cl_2 extract (20.8 g) as a yellow material was fractioned using Quick column chromatography eluting by gradient with an increase polarity of hexane, ethyl acetate, and methanol, respectively. Each fraction was collected approximately 300 mL and then concentrated to a small volume. The fractions were combined monitoring by TLC results to obtain four fractions, CA to CD. The results of fractionation are shown in Table 3.3

Table 3.3 The results of fractionation by Quick CC of the CH_2Cl_2 extract

Fraction	Solvent system	Remarks	Weight (g)
CA	100% hexane-25% EtOAc in hexane	yellow oil	8.7
CB	35-50% EtOAc in hexane	yellow solid	3.4
CC	60-80% EtOAc in hexane	dark brown syrup	4.5
CD	100% EtOAc-20% MeOH in EtOAc	dark brown syrup	1.7

Fraction CA was applied to a silica gel column eluting with a step gradient (hexane-EtOAc 9:1 to MeOH-EtOAc 2:8) to give four fractions, CA1 to CA4. Fraction CA1 was rechromatographed on a sephadex LH-20 column eluting with $CHCl_3$: MeOH 1:1 to yield compound (**16**) (1.2 mg). Recrystallization of fraction CA2 with hexane and ethyl acetate afforded compounds (**8**) (14.5 mg) and (**9**) (3.7 mg). Fraction CA3 was reseparated on a sephadex LH-20 column eluting with $CHCl_3$: MeOH 1:1 yielding compounds (**2**) (20.8 mg)**,** (**6**) (110.5 mg)**,** (**13**) (2.8 mg)**,** (**15**) (12.7 mg) and (**17**) (2.9 mg). Fraction CA4 was recrytallized with hexane and ethyl acetate to yield compound (**6**) (53.5 mg).

Fraction CB was further separated by preparative TLC using hexane–EtOAc (4:1) to yield compounds (**6**) (12.5 mg) and (**12**) (1.1 mg). Fraction CC was separated by quick column chromatography eluting by gradient with an increase polarity of hexane, ethyl acetate, and methanol respectively to furnish compounds (**6**) (15.5 mg)**,** (**8**) (3.2 mg)**,** (**9**) (11.3 mg)**,** (**10**) (3.2 mg) and (**14**) (1.0 mg). Fraction CD was applied to preparative TLC, eluting with hexane-EtOAc (8:2) to afford compound (**11**) (8.5 mg). The separation diagram of the CH_2Cl_2 is shown in Scheme 3.3.



Scheme 3.3 Separation diagram of fraction CA-CD

3.2 Structural elucidation of isolated compounds

Seventeen compounds were isolated from the heartwood of *A. Luzonensis*. Compounds (**1**) – (**9**) were afforded from the BuOH extract and compounds (**10**) - (**17**) were obtained from the CH_2Cl_2 extract. Their structures were established by spectroscopic methods and comparing their data with those of literature reports.

3.2.1 Structural elucidation of compounds (1)-(2), (5)-(10), (12) and (14)-(15)

The heartwood of *A. Luzonensis* was investigated its chemical constituents by Kitaoka *et al* in 1998 [6] and Gonda *et al* in 2000 [4]. The xanthones and flavonoids were reported as the major compounds in this plant. Thus, the ^{1}H-and ^{13}C-NMR data of compounds (**1**)-(**17**) were preliminary compared with those of published reports. The study revealed that compounds (**1**), (**8**)-(**10**) and (**14**) were xanthones namely 1,3,5-trihydroxy-4-(3-hydroxy-3-methylbutyl)xanthone (**1**), 1,3,6-trihydroxy-5-methoxy-xanthone (**8**), 1,3,5-trihydroxy-4-prenylxanthone(**9**), 1,3,5,6-tetrahydroxyxanthone (**10**) and 3,6-dihydroxy-1,5-dimethoxyxanthone (**14**), respectively. Compounds (**2**) and (**12**) were isoflavones namely orobol and biochanin A, respectively whereas, compounds (**5**)-(**7**) and (**15**) were flavonoids as kaempferol, quercetin, taxifolin and 8-prenylnaringenin, respectively.

The structures of known compounds were shown in Figure 3.1, the NMR spectra were shown in appendix and the comparison of spectral data is summarized in appendix.

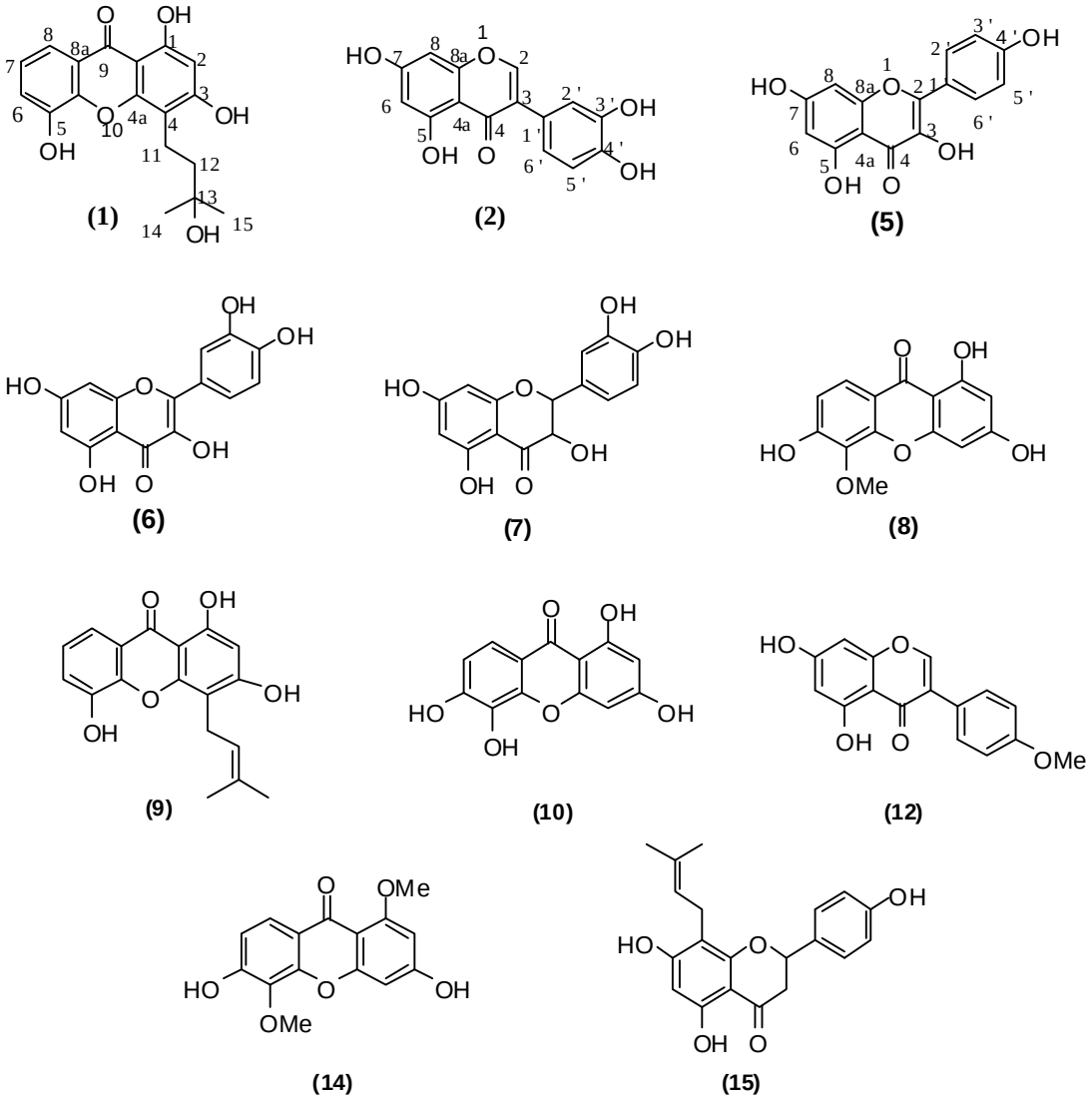


Figure 3.1 The structures of compounds **(1)-(2), (5)-(10), (12) and (14)-(15)**

Table 3.4 The NMR data of compound (**1**) (acetone-d_6) and 1,3,5-trihydroxy-4-(3-hydroxy-3-methylbutyl)xanthone (DMSO-d_6)

Position	d_H (int., mult., *J* in Hz)		d_C	
	1,3,5-trihydroxy-4-(3- hydroxy-3-methylbutyl) xanthone [4]	**compound** (**1**)	**1,3,5-trihydroxy-4-(3- hydroxy-3-methylbutyl) xanthone** [4]	**compound** (**1**)
1	-	-	160.1	163.0
2	6.27 (1H, *s*)	6.34 (1H, s)	97.6	99.5
3	-	-	164.2	164.6
4	-	-	107.6	109.8
4a	-	-	154.2	156.6
5	-	-	146.3	148.1
6	7.28(1H, *dd*, 7.9, 1.8)	7.28 (2H, m)	120.1	122.2
7	7.24(1H, *dd*, 7.3, 7.9)	-	127.7	125.8
8	7.53(1H, *dd*, 7.3, 1.8)	7.53(1H, *dd*, 7.3, 1.8)	114.3	117.0
8a	-	-	120.6	122.9
9	-	-	180.0	182.8
9a	-	-	101.7	104.8
10a	-	-	144.9	146.9
11	2.79(2H, *dd*, 8.6,4.9)	3.02 (2H, m)	17.2	18.5
12	1.61(2H, *dd*, 8.6,4.9)	1.79(2H, m)	42.6	43.7
13	-	-	69.2	72.6
14	1.21 (6H, s)	1.33 (6H, s)	28.9	30.3
15			28.9	30.3
5-OH	12.83 (1H,s)	12.78 (1H,s)	-	-

Table 3.5 The NMR data of compound (**2**) (acetone-d_6) and orobol (CD_3OD)

Position	d_H **(int., mult., *J* in Hz)**		d_C	
	orobol [25]	**compound** (**2**)	**orbol** [26]	**compound** (**2**)
2	8.15 (1H, s)	8.15 (1H, s)	155.9	153.5
3	-	-	132.7	123.2
4	-	-	178.1	145.3
5	-	-	162.1	163.0
6	6.28 (1H, *d*, 1.9)	6.28 (1H, *d*, 2.0)	98.9	98.9
7	-	-	162.9	164.0
8	6.41 (1H, *d*,1.9)	6.41 (1H, *d*, 2.0)	94.2	93.5
9	-	-	162.1	158.1
10	-	-	102.9	105.3
1′	-	-	122.3	120.6
2′	7.14 (1H, *d*, 2.2)	7.14 (1H, *d*,1.6)	116.1	116.3
3′	-	-	144.2	144.7
4′	-	-	148.3	145.3
5′	6.87 (1H, *d*, 8.4)	6.87 (1H, *d*, 8.4)	115.9	115.0
6′	6.94 (1H, *dd*, 8.4, 2.2)	6.94 (1H, *dd*, 8.0, 2.0)	126.9	122.7
5-OH	13.06	13.06 (1H, s)	-	-

Table 3.6 The NMR data of compound (**5**) (acetone-d_6) and kaempferol (CD_3OD)

Position	d_H **(int., mult., *J* in Hz)**		d_C	
	kaempferol [27]	**compound (5)**	**kaempferol** [28]	**compound (5)**
2	-	-	146.9	147.6
3	-	-	136.5	137.6
4	-	-	176.5	177.6
5	-	-	157.7	158.7
6	6.27 (1H, *d*, 2.0)	6.27 (1H, *d*, 1.6)	98.9	100.1
7	-	-	165.0	166.8
8	6.54 (1H, *d*, 2.0)	6.54 (1H, *d*, 1.6)	-	-
9	-	-	160.1	161.5
10	-	-	103.9	104.9
1	-	-	123.1	124.0
3′	7.03 (2H, *d*, 8.8)	7.02 (2H, *d*, 8.8)	116.1	117.3
5′				
2′	8.16 (2H, *d* ,8.8)	8.16 (2H, *d*, 8.8)	130.3	131.4
6′				
5-OH	-	12.06 (1H, s)	-	-

Table 3.7 The NMR data of compound (**6**) (acetone-d_6) and quercetin (acetone-d_6)

Position	$d_{\mathbf{H}}$ **(int., mult., *J* in Hz)**		$d_{\mathbf{C}}$	
	quercetin [28]	**compound (6)**	**quercetin** [28]	**compound (6)**
2	-	-	146.21	147.9
3	-	-	137.21	137.7
4	-	-	177.33	177.5
5	-	-	162.5	163.3
6	6.27 (1H, br, s)	6.26 (1H, *d*, 2.0)	99.25	100.1
7	-	-	165.34	165.9
8	6.53 (1H, br, s)	6.52 (1H, *d*, 2.0)	94.4	95.4
9	-	-	158.22	158.7
10	-	-	104.52	105.1
1′	-	-	124.15	124.7
3′	-	-	116.22	117.2
5′	6.99 (1H, *d*, 8.0)	6.99 (1H, *d*, 8.4)		
2′	7.81 (1H, br,s)	7.83 (1H, *d*, 2.0)	121.67	122.4
6′	7.70 (1H, *d*, 8.0)	7.70 (1H, *dd*, 8.4, 2.0)		
5-OH	-	12.18 (1H, s)	-	-

Table 3.8 The NMR data of compound (**7**) (acetone-d_6) and taxifolin (CD_3OD)

Position	d_H **(int., mult., *J* in Hz)**		d_C	
	taxifolin [29]	**compound (7)**	**taxifolin** [29]	**compound (7)**
2	4.9 (*d*, 11)	4.99 (1H, *d*, 11.6)	85.0	85.4
3	4.5 (*d*, 11)	4.58 (1H, *d*, 11.2)	73.6	74.1
4	-	-	198.3	199.2
5	-	-	165.2	165.9
6	5.91 (*d*,2)	5.91 (1H, *d*, 2.0)	97.3	97.9
7	-	-	168.6	168.8
8	5.87 (*d*, 2)	5.95 (1H, *d*, 2.0)	96.3	96.9
9	-	-	164.4	165.1
10	-	-	101.8	102.5
1′	-	-	129.8	130.7
3′	-	-	146.2	146.7
5′	6.80 (*d*, 8)	6.83 (1H, *d*, 8.4)	115.8	116.7
2′	6.96 (*d*, 2)	7.03 (1H, *d*, 1.6)	116.0	116.8
6′	6.84 (*dd*, 8, 2)	6.88 (1H, *dd*, 8.4, 2.0)	120.9	121.8
4′	-	-	147.1	147.5
3-OH	-	4.66 (1H, *br*, s)	-	-

Table 3.9 The NMR data of compound (**8**) (acetone-d_6) and 1,3,6-trihydroxy-5-methoxyxanthone (DMSO-d_6)

Position	d_H **(int., mult., *J* in Hz)**		d_C	
	1,3,6-trihydroxy-5-methoxy xanthone [30]	**compound (8)**	**1,3,6-trihydroxy-5-methoxy xanthone** [31]	**compound (8)**
1	-	-	162.9	163.8
2	6.21 (1H, *d*, 1.3)	6.25 (1H, *d*, 0.8)	98.2	98.1
3	-	-	165.6	165.1
4	6.21 (1H, *d*, 1.3)	6.25 (1H, *d*, 0.8)	94.2	94.1
4a	-	-	157.1	156.1
5	-	-	134.6	134.5
6	-	-	157.7	157.3
7	6.96 (1H, *d*, 8.0)	6.99 (1H, *d*, 8.8)	114.2	114.2
8	7.78 (1H, *d*, 8.0)	7.83 (1H, *d*, 8.8)	121.1	120.9
8a	-	-	113.4	112.9
9	-	-	179.8	179.2
9a	-	-	102.2	101.4
10a	-	-	150.6	150.5
5-OH	13.02(1H, s)	13.09(1H, s)	-	-
5-OMe	3.96 (3H, s)	4.00 (3H, s)	-	-

Table 3.10 The NMR data of compound (**9**) ($CDCl_3$) and 1,3,5-trihydroxy-4-prenylxanthone ($CDCl_3$)

Position	d_H **(int., mult., *J* in Hz)**		d_C	
	1,3,5-trihydroxy -4-prenyl xanthone [32]	**compound (9)**	**1,3,5-trihydroxy -4-prenyl xanthone** [32]	**compound (9)**
1	-	-	162.3	163.4
2	6.35 (1H, s)	6.32 (1H, s)	98.5	23.1
3	-	-	164.0	164.9
4	-	-	107.6	108.7
4a	-	-	155.4	157.3
5	-	-	147.1	148.0
6	7.23 (1H, *t*, 8)	7.26 (1H, *t*, 7.8)	122.1	122.2
7	7.36(1H, *dd*, 8, 1.5)	7.32(1H, *dd*, 7.6, 1.6)	123.4	125.6
8	7.65(1H, *dd*, 8, 1.5)	7.77(1H, *dd*, 8.0, 1.6)	116.2	117.3
8a	-	-	122.2	124.3
9	-	-	181.8	183.9
9a	-	-	103.7	105.6
10a	-	-	146.4	146.7
11	3.58 (2H, *d*, 7.5)	3.57 (2H, *d*, 6.8)	18.0	18.9
12	5.36 (1H, *t*, 7.5)	5.29 (1H, *t*, 6.8)	131.7	132.8
14	1.64 (3H, s)	1.78 (3H, s)	22.1	23.1
15	1.84 (3H, s)	1.88 (3H, s)	25.9	26.9
1-OH	-	5.70 (1H, s)	-	-
3-OH	9.52 (1H, s)	-	-	-
5-OH	12.97 (1H, s)	12.86 (1H, s)	-	-

Table 3.11 The NMR data of compound (**10**) (acetone-d_6) and 1,3,5,6- tetrahydroxy xanthone (acetone-d_6)

Position	d_H (int., mult., *J* in Hz)		d_C	
	1,3,5,6-tetrahydroxy xanthone [33]	**compound (10)**	**1,3,5,6-tetrahydroxy xanthone** [28]	**compound (10)**
1	-	-	165.7	164.8
2	6.22 (1H, *d*, 2.0)	6.23 (1H, *d*, 2.0)	99.8	98.8
3	-	-	166.8	165.9
4	6.22 (1H, *d*, 2.0)	6.23 (1H, *d*, 2.0)	95.7	94.7
4a	-	-	153.1	152.4
5	-	-	134.2	133.4
6	-	-	159.7	158.8
7	6.96 (1H, *d*, 8.7)	6.98 (2H, *d*, 8.8)	115.7	114.7
8	7.61 (1H, *d*, 8.7)	7.62 (2H, *d*, 8.8)	118.4	117,3
8a	-	-	114.6	113.7
9	-	-	182.1	181.1
9a	-	-	104.0	103.
10a	-	-	147.8	145.9
5-OH	13.19 (1H, s)	13.17 (1H, s)	-	-

Table 3.12 The NMR data of compound (**12**) (acetone-d_6) and biochanin A (acetone-d_6)

Position	d_H **(int., mult., *J* in Hz)**		d_C	
	biochanin A [34]	**compound (12)**	**biochanin A** [34]	**compound (12)**
2	8.21 (1H, s)	8.21 (1H, s)	153.1	153.0
3	-	-	119.7	119.6
4	-	-	180.8	182.2
5	-	-	162.1	162.1
6	6.43 (1H, *d*, 2.1)	6.42 (1H, *d*, 2.0)	100.8	99.3
7	-	-	164.4	164.5
8	6.29 (1H, *d*, 2.1)	6.29 (1H, *d*, 2.0)	93.3	94.0
9	-	-	157.2	157.7
10	-	-	104.1	104.3
1′	-	-	123.1	123.4
2′	7.55 (2H, *d*, 8.9)	7.54 (2H, *d*, 8.0)	131.2	130.1
6′			125.9	125.7
3′	7.55 (2H, *d*, 8.9)	7.54 (2H, *d*, 8.0)	117.3	113.9
5′			110.9	110.0
4′	-	-	158.7	158.4
5-OH	13.01 (1H, s)	13.00 (1H, s)	-	-
4′-OMe	3.89 (1H, s)	3.84 (1H, s)	-	-

Table 3.13 The NMR data of compound (**14**) (CD_3OD-d_4) and 3,6-dihydroxy-1,5-dimethoxyxanthone (DMSO-d_6)

Position	d_H (int., mult., *J* in Hz)		d_C	
	3,6-dihydroxy-1,5-dimethoxy Xanthone [4]	**compound (14)**	**3,6-dihydroxy-1,5-dimethoxy xanthone** [4]	**compound (14)**
1	-	-	161.2	161.8
2	6.37 (1H, *d*, 2.1)	6.25 (1H, *d*, 2.0)	96.0	95.8
3	-	-	162.6	163.7
4	6.45 (1H, *d*, 2.1)	6.44 (1H, *d*, 2.1)	95.8	95.2
4a	-	-	159.9	158.9
5	-	-	134.3	134.0
6	-	-	149.9	149.1
7	6.88 (1H, *d*, 8.6)	6.79 (1H, *dd*, 8.8, 1.2)	113.4	113.3
8	7.64 (1H, *d*, 8.6)	7.68 (1H, *d*, 8.8)	122.4	121.1
8a	-	-	116.9	115.9
9	-	-	174.3	172.9
9a	-	-	104.9	104.7
10a	-	-	155.3	155.1
5-OH	13.19 (1H, s)	13.17 (1H, s)	-	-
1′-OMe	-	-	56.1	55.9
5′-OMe	-	-	61.6	60.7

Table 3.14 The NMR data of compound (**15**) (acetone-d_6) and 8-prenylnaringenin (DMSO-d_6)

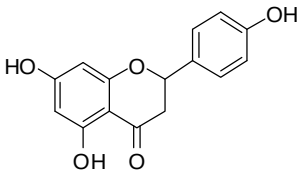
Position	d_H **(int., mult., *J* in Hz)**		d_C	
	8-prenyl-naringenin [19]	**compound (15)**	**8-prenyl-naringenin** [19]	**compound (15)**
2	5.42 (1H, *dd*, 12.5, 2.9)	5.42 (1H, *dd*, 12.5, 2.8)	78.2	79.0
3	2.72 (1H, *dd*, 17.1, 3.0)	2.71 (1H, *dd*, 17.1, 3.2)	42.0	42.7
4	-	-	196.8	196.5
4a	-	-	106.9	106.9
5	-	-	157.6	157.8
6	5.97 (1H, s)	6.02 (1H, s)	95.3	94.3
7	-	-	159.7	159.0
8	5.87 (*d*, 2)	5.95 (1H, *d*, 2.0)	101.8	102.2
8a	-	-	161.2	161.9
1′	-	-	129.3	130.0
3′	6.79 (2H, *d*, 8.5)	6.89 (2H, *d*, 8.4)	115.2	115.2
5′				
2′	7.31 (2H, *d*, 8.5)	7.38 (2H, *d*, 8.4)	128.1	128.1
6′				
4′	-	-	164.4	163.9
1″	3.14 (2H, *d*, .2)	3.17 (1H, *dd*, 17.2, 13.2)	21.3	20.7
2″	5.32 (1H, *t*, 7.2)	5.23 (1H, *m*)	122.7	122.7
4″	1.51 (1H, s)	1.74 (3H, s)	134.8	134.7
5″	3.71 (2H, *d*, 5.1)	3.71 (2H, *d*, 5.1)	17.6	16.9
5-OH	12.11	12.47	-	-
7-OH	10.79	9.60	-	-
4′-OH	9.57	8.55	-	-

3.2.2 Structural elucidation of compounds (**3**), (**4**), (**11**) and (**13**)

The NMR data of compounds (**3**), (**4**), (**11**) and (**13**) were not compatible with those of isolated compounds previously report as chemical constituents of ***A. Luzonensis.*** However, the ^{13}C-NMR spectra of these compounds showed 15 signals without methoxyl group signals as substituents. These compounds were thus assigned to be the flavonoid skeleton, not the xanthone skeleton.

Compound (**3**) was obtained as yellow needle crystal (1.3 mg), m.p. 249.5-250.3˚C and optical rotation $[a]_D^{26.8} -15^\circ$ (*c* 0.05, MeOH). This compound showed a single spot on TLC with R_f value of 0.48 (10% MeOH in $CHCl_3$).

The ^{1}H NMR spectrum of compound (**3**) (Figures 3.15) showed 6 aromatic signals arising from two different aromatic moieties. The two characteristic *meta*-coupled doublets (AB type) at *d* 5.95 and 5.94 (*J* = 1.2 Hz) were attributed to the H-6 and H-8 protons of the flavonoid A-ring structure. Other four signals at *d* 6.89 and 7.39 (2H, *J* = 8.8 Hz of each signal) originated from 2′, 3′, 5′ and 6′ protons of the flavonoid B ring. The presence of the flavanone skeleton was confirmed by observing three double doublets located at *d* 2.72 (H-3b, *J* = 17.2 and 2.8 Hz), 3.18 (H-3a, *J* = 17.2 and 2.8 Hz) and 5.45 (H-2, *J* = 17.2 and 2.8 Hz) in agreement with previous data [36-37]. Thus, compound (**3**) was determined as 5, 7, 4′-trihydroxyflavanone or naringenin. The NMR data of compound (**3**) comparing with those of naringenin were shown in Table 3.15.

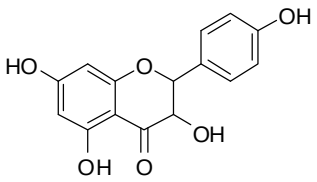


Compound (**3**): naringenin

Compound (**4**) was received as white needle crystal (91.6mg), m.p. 235.5-236.2˚C and optical rotation $[a]_D^{26.1} +14^\circ$ (*c* 0.05, MeOH). This compound showed a single spot on TLC with R_f value of 0.38 (10% MeOH in $CHCl_3$).

The ^{1}H NMR spectrum of compound (**4**) (Figures 3.16) was compatible with those of compound (**3**) except for flavanone skeleton. In the ^{1}H NMR spectrum of compound (**4**), two doublet resonances at *d* 1.62 (H-3, *J* = 11.6 Hz) and 5.05 (H-2, *J* = 11.6 Hz) [36] were characteristic of a dihydroflavonol skeleton with *trans*-

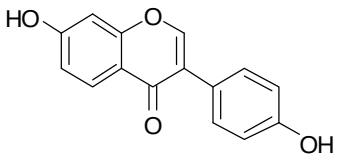
stereochemistry. The ^{13}C NMR data also supported the dihydroflavonol skeleton by observing the C-3 at δ 73.1 which was down field shift compared with those of compound (**3**). Thus, compound (**4**) was identified as (+)-5, 7, 4′-trihydroxy dihydroflavanol or (+)-aromadendrin. The NMR data of compound (**4)** comparing with those of aromadendrin were shown in Table 3.16.



Compound (**4**): aromadendrin

Compound (**11**) was received as white needle crystal (8.5 mg), m.p. 219.5-220.3˚C. This compound showed a single spot on TLC with R_f value of 0.41 (10% MeOH in $CHCl_3$).

The ^1H NMR spectrum of compound (**11**) (Figures 3.15) showed the presence of seven aromatic proton signals located between *d* 6.29 and 7.45. These protons were observed as a set of resonances corresponding to a *p*-substituted phenyl group (A_2B_2 spin system [*d* 7.47 ppm (2H, d, *J* = 8.8 Hz, H-2′, H-6′); 6.87 ppm (2H, d, *J* = 8.8 Hz, H-3′, H-5′)] and a set of resonances corresponding to the ABX spin system of 1,3,4-trisubstituted phenyl group [*d* 6.90 ppm (1H, d, *J* = 2.8 Hz, H-8); 6.99 ppm (1H, dd, *J* = 9.0, 2.0 Hz, H-6); 8.06 ppm (1H, d, *J* = 8.8 Hz, H-5)]. Additional, one more aromatic signal at *d* 8.15 (1H, s) was shown correlation with the carbon signal (Figures 3.17) at *d* 151.1 in the HMQC spectrum. This proton showed the characteristic signal of isoflavonoid skeleton [39]. From the above results, compound (**11**) was defined as 7, 4′-dihydroxyflavone or daidzein. The NMR data of compound (**11)** comparing with those of daidzein were shown in Table 3.17.

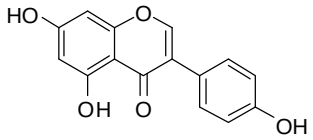


Compound (**11**): daidzein

Compound (**13**) was obtained as yellow needle crystal (2.8 mg) after washing with cold chloroform. Its melting point was 174.7-175.1˚C. This compound showed a single spot on TLC with R_f value of 0.49 (10% MeOH in $CHCl_3$).

The ^{1}H NMR spectrum of compound (**13)** (Figures 3.18) was similarity with those of compound **(11)** except for substituted pattern of A-ring. The two characteristic *meta*-coupled doublets (AB type) at *d* 6.42 and 6.29 (*J* = 2.0 Hz) were attributed to the H-8 and H-6 protons of the isoflavonoid A-ring structure. Thus, compound (**13**) was identified as 5,7,4′-trihydroxy isoflavone or genistein.

The NMR data of compound (**13**) comparing with those of genistein were shown in Table 3.18.



Compound (**13**): genistein

Table 3.15 The NMR data of compound (**3**) (acetone-d_6) and naringenin (CD_3OD)

Position	d_H (int., mult., *J* in Hz)		d_C	
	naringenin [36]	**Compound** (**3**)	**naringenin** [37]	**Compound** (**3**)
1	-	-	128.1	131.6
2	5.43 (*dd*)	5.45 (1H, *dd*, 13.2, 2.8)	79.0	80.9
3a	3.16 (*dd*)	3.18 (1H, *dd*, 17.2, 13.2	42.6	44.4
3b	2.74 (*dd*)	2.72 (1H, *dd*, 17.2, 2.8)		
4	-	-	196.3	198.2
5	-	-	164.4	166.2
6	-	-	95.9	96.8
7	-	-	166.4	168.5
8	5.95 (s)	5.94 (2H, *d*, 1.2)	95.9	97.7
9	-	-	163.5	165.3
10	-	-	102.3	104.1
2′	7.35 (*d*)	7.39 (2H, *d*, 8.8)	129.9	129.9
6′				
3′	6.88 (*dd*)	6.89 (2H, *d*, 8.8)	115.3	117.1
5′				
4′	-	-	157.8	159.7
5-OH	12.15 (s)	12.17 (1H, s)	-	-

Table 3.16 The NMR data of compound (**4**) (CD_3OD) and aromadendrin (acetone- d_6)

Position	d_H (int., mult., *J* in Hz)		d_C	
	aromadendrin [36]	**compound** (**4**)	**aromadendrin** [36]	**compound** (**4**)
1	-	-	128.1	131.6
2	5.06 (d)	5.05 (1H,*d*, 11.6)	79.0	80.9
3	4.62 (d)	4.62 (1H,*d*, 11.6)	42.6	44.4
4	-	-	196.3	198.2
5	-	-	164.4	166.2
6	6.01 (s)	5.95 (1H,*d*, 2.0)	95.9	96.8
7	-	-	166.4	168.5
8	5.95 (s)	5.91 (2H,*d*, 1.6)	95.9	97.7
9	-	-	163.5	165.3
10	-	-	102.3	104.1
2′	7.36 (d)	7.38 (2H,*d*, 8.4)	129.9	129.9
6′				
3′	6.86 (s)	6.86 (2H,*d*, 8.4)	115.3	117.1
5′				
4′	-	-	157.8	159.7
5-OH	11.57 (s)	11.68 (1H, s)	-	-

Table 3.17 The NMR data of compound (**11**) (acetone-d_6) and daidzein (DMSO-d_6)

Position	d_H **(int., mult., *J* in Hz)**		d_C	
	daidzein [39]	**compound** (**11**)	**daidzein** [39]	**compound** (**11**)
2	8.28 (s)	8.15 (1H, s)	152.3	151.1
3	-	-	122.7	122.7
4	-	-	178.6	174.4
5	7.95 (*d*, 8.7)	8.06 (1H, *d*, 8.8)	127.2	126.8
6	6.92 (*dd*, 8.7, 2.1)	6.99 (1H, *dd*, 9.2, 2.4)	115.1	114.4
7	-	-	162.6	162.7
8	6.84 (*d*, 2.1)	6.90 (2H, *d*, 3.2)	102.2	101.7
9	-		157.6	157.6
10	-	-	116.9	116.5
1′	-	-	123.9	123.5
3′	6.79 (*d*, 8.6)	6.87 (2H, *br*, s, 8.8)	130	129.6
5′				
2′	7.36 (*d*, 8.6)	7.47 (2H, *d*, 8.8)	115.1	114.8
6′				
4′	-	-	157.3	157.1
7-OH	-	9.62	-	-
4′-OH	-	8.47	-	-

Table 3.18 The NMR data of compound (**13**) (acetone-d_6) and genistein (acetone-d_6)

Position	d_H **(int., mult., *J* in Hz)**		d_C	
	genistein [40]	**compound (13)**	**genistein** [41]	**compound (13)**
2	8.20 (1H, s)	8.17 (1H, s)	153.1	155.3
3	-	-	121.3	123.9
4	-	-	180.3	182.6
5	-	-	162.2	164.9
6	6.31 (1H, *d*, *J*=2.4 Hz)	6.29 (1H, *d*, *J*=2.0 Hz)	99.0	100.8
7	-	-	164.3	166.0
8	6.84 (*d*, 2.1)	6.90 (2H, *d*, 3.2)	93.7	95.4
9	-		157.7	160.0
10	-	-	104.7	107.1
1′	-	-	122.7	124.9
3′	6.92 (2H, *d*, *J*=8.5 Hz)	6.90 (2H,*d*, *J*=8.4 Hz)	130.0	132.1
5′				
2′	7.00 (2H,*d*, *J*=8.5 Hz)	7.45 (2H,*d*, *J*=8.8 Hz)	115.1	116.9
6′				
4′	-	-	157.5	159.4
5-OH	13.06 (1H, s)	13.03 (1H, *br*, s)	-	-
7-OH	9.87 (1H, *br*,s)	-	-	-
4′-OH	8.62 (1H, *br*,s)	-	-	-

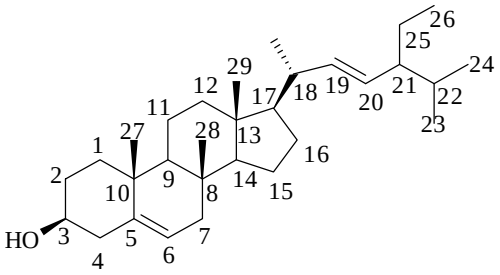
3.2.3 Structural elucidation of compound (16)

Compound (**16**) was isolated as white crystal (1.2 mg), m.p. 175.8-176.2˚C. This compound showed a single spot on TLC with R_f value of 0.45 (10% MeOH in $CHCl_3$). It could not be detected on TLC under UV light, but can be detected as purple-pink spot with 10% H_2SO_4 in ethanol. This indicated that no conjugated double bond was presented in this molecule.

In the ^{1}H-NMR spectrum indicated that compound (**16**) possessed a terpenoid skeleton. The signals at δ_H 4.53-4.65 (m) and 5.29-5.31 (m) were assigned to the olefinic protons. The signals at δ_H 0.46-2.25 (m) was ascribed to the methine, methylene and methyl groups in a molecule. The signal of the methine proton connecting to a hydroxyl group was detected at δ_H 3.60.

In good agreement with ^{1}C-NMR data, the signals of four olefinic carbons could be detected at δ_C 107.7, 123.9, 139.8 and 154.8 in the ^{13}C-NMR ($CDCl_3$) spectrum (Figure 3.19). Another set of signals around δ_C 12.0 to 77.0 revealed an aliphatic part of this compound. In addition, this compound showed the R_f value corresponding to an authentic stigmasterol, the common steroid found in plants.

As a result of its physical properties and spectroscopic data, it could be noticeably concluded that this compound was stigmasterol. The NMR data of compound (**16**) comparing with those of stigmasterol were shown in Table 3.19.



Compound (**16**): stigmasterol

Table 3.19 The NMR data of compound (**16**) (acetone-d_6) and stigmasterol (acetone-d_6)

Position	d_C	
	stigmasterol [42]	**compound (16)**
1	32.9	32.9
2	34.5	34.8
3	79.0	77.0
4	42.0	42.3
5	154.6	154.8
6	124.4	123.9
7	31.2	31.6
8	28.7	28.2
9	42.0	42.3
10	39.6	39.7
11	19.4	19.8
12	31.9	31.8
13	40.8	40.7
14	47.7	47.7
15	21.4	21.8
16	21.3	21.0
17	48.2	48.7
18	18.3	18.8
19	18.2	18.7
20	33.4	33.8
21	17.4	14.1
22	107.1	107.7
23	139.5	139.8
24	47.7	47.7
25	30.6	30.8
26	20.2	20.0
27	20.2	20.0
28	25.4	25.6
29	12.2	12.0

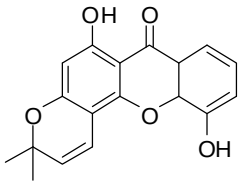
3.2.4 Structural elucidation of compound (17)

Compound (**17**) was obtained as yellow powder (2.9 mg), m.p. 235.2-235.7˚C. This compound showed a single spot on TLC with R_f value of 0.8 (10% MeOH in $CHCl_3$).

In the ^{1}H-NMR spectrum indicated that compound (**17**) showed the occurrence of a xanthone skeleton. The anomeric proton signals at δ_H 5.77 (1H, *d*, *J*=10 Hz) in its ^{1}H-NMR. The AB-system aromatic proton signals at δ_H 7.69 (1H, *dd*, *J*=8, 1.6 Hz) and 7.06 (1H, *d*, *J*=10 Hz) were due to H-2 and H-7, respectively, whereas the aromatic singlet at 6.21 (1H, s) were due to C-4′. The other group of aromatic proton signals at 7.69 (1H, *dd*, *J*=8, 1.6 Hz, H-2), 7.31 (1H, *d*, *J*=8 Hz, H-3) and 1.49 (6H, s, H-2′′) were similar to those of the xanthone.

In good agreement with ^{1}C-NMR data, the signals of the carbonyl carbon (δ_C 182.9), the CH aromatic carbon could be detected at δ_C 117.5, 126.1, 122.9, 100.6, 129.2 and 116.7 in the ^{13}C-NMR (acetone-d_6) spectrum (Figure 3.33), in addition, the aromatic carbons could be detected at δ_C 103.2, 165.1, 157.2, 105.2, 123.3, 147.1 and 80.1. In the CH-COSY experiment the two equivalent methyl protons at δ_H 1.49 correlated with the carbon signal at δ_C 29.4. In addition, this compound showed the R_f value corresponding to an reliable 6-deoxyisojacareubin, the common xanthone found in plants.

As a result of its physical properties and spectroscopic data, it could be noticeably concluded that this compound was 6-deoxyisojacareubin. The NMR data of compound (**17**) comparing with those of 6-deoxyisojacareubin were shown in Table 3.20.



Compound (**17**): 6-deoxyisojacareubin

Table 3.20 The NMR data of compound (**17**) (acetone-d_6) and 6-deoxyisojacareubin (acetone-d_6)

Position	d_H (int., mult., *J* in Hz)		d_C	
	6-deoxyisojacareubin [32]	**compound (17)**	**6-deoxyisoja-careubin** [32]	**compound (17)**
1	7.39 (1H, *dd*, *J*=8, 1.5 Hz)	7.39 (1H, *dd*, *J*=8, 1.5 Hz)	116.5	117.5
2	7.68 (1H, *dd*, *J*= 8, 1.5 Hz)	7.69 (1H, *dd*, *J*=8, 1.6 Hz)	125.1	126.1
3	7.30 (1H, *dd*, *J*=8, 1.5 Hz)	7.31 (1H, *d*, *J*=8 Hz)	122.0	122.9
4	-	-	146.9	147.9
5	-	-	102.1	103.2
5a	-	-	156.1	157.2
6	-	-	164.1	165.1
7	7.05 (1H, *d*, *J*=10 Hz)	7.06 (1H, *d*, *J*=10 Hz)	99.6	100.6
8	-	-	161.7	162.7
8a	-	-	104.1	105.2
9	-	-	181.8	182.9
9a	-	-	122.2	123.3
10a	-	-	146.0	147.1
2′	-	-	79.1	80.1
3′	5.77 (1H, *d*, *J*=10 Hz)	5.77 (1H, *d*, *J*=10 Hz)	128.1	129.2
4′	6.20 (1H, s)	6.21 (1H, s)	115.7	116.7
1″	1.48 (6H, s)	1.49 (6H, s)	28.4	29.4
2″				
4-OH	9.22 (1H, s)	9.23 (1H, s)	-	-
8-OH	13.10 (1H, s)	13.10 (1H, s)	-	-

3.3 Biological activity study of isolated compounds

As aforementioned results, the separation of the heartwood from *A. luzonensis* led to the isolation of seventeen compounds. These compounds were further studied for their bioactivities; cholinesterase inhibitory activities, radical scavenging effect on DPPH radical, ABTS decolorization test and Phosphodiesterase (PDE) inhibitory activity.

3.3.1 Anticholinesterase activity

The cholinesterase inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterse (BChE) of compounds **(1) – (17)** was preliminary evaluated by microplate assay at final concentration of sample 0.1 mg/mL. Since eserine and galanthamin have been reported to inhibit AChE, this compound was used as a positive control in this experiment. The results of activity testing were shown in Table 3.21.

Table 3.21 Cholinesterase inhibitory activity of the isolated compounds at final concentration 0.1 mg/mL

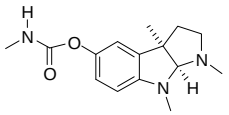
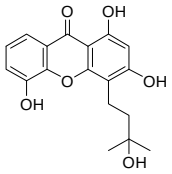
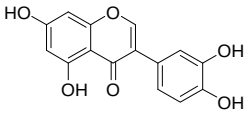
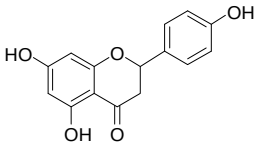
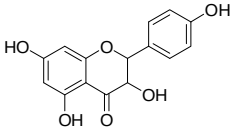
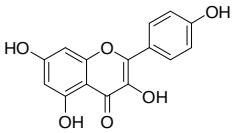
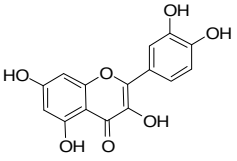
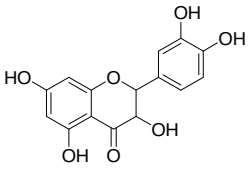
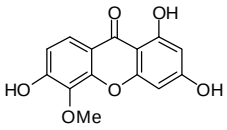
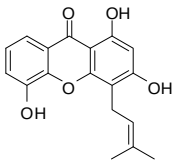
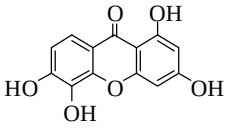
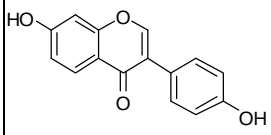
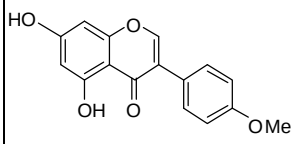
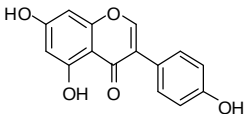
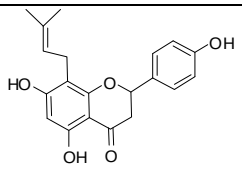
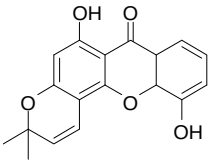
Compound	Structure	%inhibition (mean ± SD)	
		AChE	BChE
Eserine		97.93 ± 2.23	92.17 ± 0.79
(1)		83.40 ± 0.43	84.29 ± 2.12
(2)		78.44 ±0.08	80.85±0.54
(3)		< 20	< 20
(4)		< 20	< 20
(5)		< 20	< 20
(6)		38.8± 0.32	79.2 ± 0.25
(7)		51.62 ± 0.37	< 20

Table 3.21 continued

Compound	Structure	%inhibition (mean ± SD)	
		AChE	**BChE**
(8)		< 20	62.50 ± 0.00
(9)		68.59 ± 0.49	78.37 ± 0.00
(10)		< 20	< 20
(11)		< 20	< 20
(12)		< 20	48.08 ± 3.39
(13)		< 20	42.50± 0.78
(15)		< 20	62.79± 0.42
(17)		< 20	< 20

Compounds (**14**) and (**16**) had not been tested because of sufficient amount.

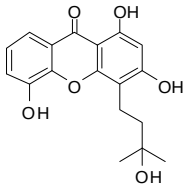
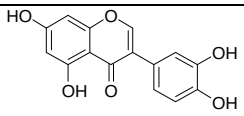
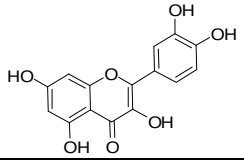
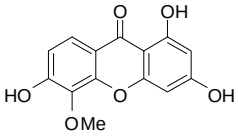
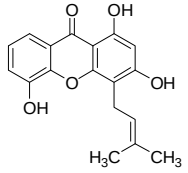
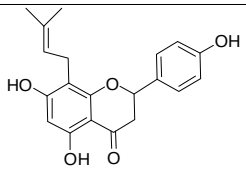
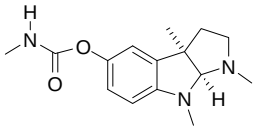
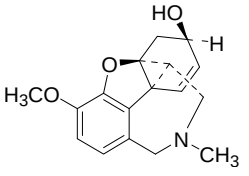
According to the result in Table 3.21, compound (**1**) showed the highest activity against both cholinesterase and followed by compounds (**2**) and (**9**), respectively. Regarding the results, the structural activity relationship of isolated compounds toward AChE and BChE was observed.

The AChE and BChE inhibitory activity of the isolated xanthones, compounds (**1**), (**8**)-(**10**), (**14**) and (**17**), was enhanced by the presence of isopentenyl moiety. The cyclization of isopentenyl chain was clearly reduced the activity. The isopentenyl moiety bearing a hydroxyl group was significant increased the inhibition (compounds (**1**) vs. (**9**)). Additionally, the presence of methoxy group at C-5 was remarkable increased inhibition toward BChE in compound (**8**) comparing with compound (**10**).

Almost all isolated flavonoids had no inhibition against AChE except for compound (**2**), (**6**) and (**7**). A hydroxyl group at C-3′ plays a significant role of the activity (compounds (**2**) vs. (**13**), compounds (**4**) vs. (**7**) and compounds (**6**) vs. (**7**). The methoxy group at C-4′ or the isopentenyl group at C-8 had no effect for this activity which was clearly observed in compounds (**12)** vs (**13**) and compounds (**3**) and (**15**), respectively. In contrast to BChE inhibitory activity, the isopentenyl group was significant enhanced potency.

The quantitative analysis for determination IC_{50} values of those active compounds which had inhibition percentage more than 50%, was then performed using spectrophotometric method as described in Chapter II. The IC_{50} were calculated from the Prism program and tabulated in Table 3.22.

Table 3.22 IC_{50} values of cholinesterase inhibitory activity of isolated compounds.

Compound	Structure	IC_{50} (µM)	
		AChE	BChE
(**1**)		2.36	2.25
(**2**)		2.57	3.66
(**6**)		> 10	1.27
(**8**)		n.t.	> 10
(**9**)		> 10	4.90
(**15**)		n.t.	> 10
Eserine		0.02	0.06
Galanthamin		0.59	1.39

Note: n.t.= not test

According to the results in Table 3.22, compound (**1**) showed the highest inhibition followed by compound (**2**) against acetylcholinesterase and butyrylcholinesterase.

3.3.2 Radical scavenging on DPPH

Flavonoids are a major class of secondary polyphenolic metabolites of wide occurrence in the natural plants. There is a beneficial health effect because of their antioxidant properties and their inhibitory role in various stages of tumor development in animal studies [43]. In order to evaluate the antioxidant activity, almost compounds were tested by spectrophotometric method. The commercial antioxidant compound BHA (3-tert-butyl-4-hydroxyanisole) was used as positive control. Results of this activity are shown in Table 3.23.

Table 3.23 DPPH radical scavenger activity of isolated compounds at final concentration 0.2 mg/mL

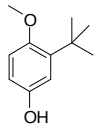
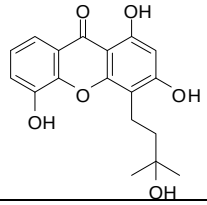
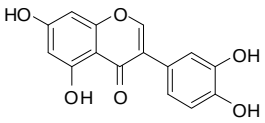
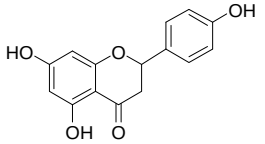
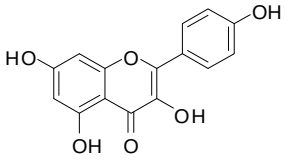
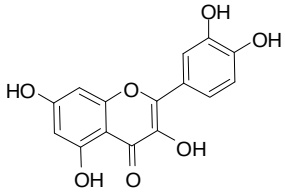
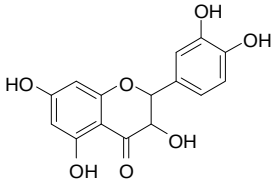
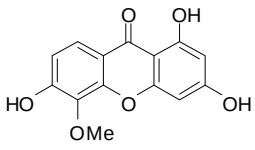
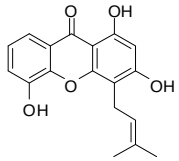
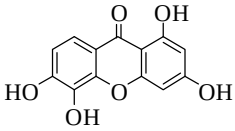
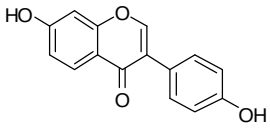
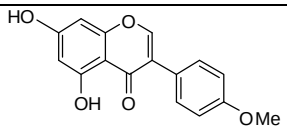
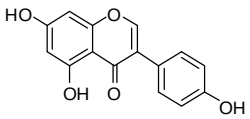
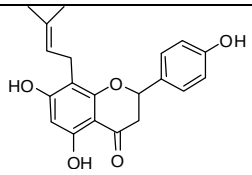
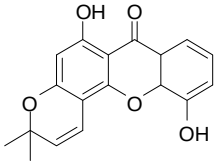
Compound	**Structure**	% **radical scavenging (mean ± SD)**
BHA		94.28 ± 0.03
(1)		14.98 ± 1.89
(2)		89.51 ± 0.21
(3)		12.31 ± 2.07
(5)		94.04 ± 0.18
(6)		93.98 ± 0.33
(7)		93.63 ± 0.49
(**8**)		25.44 ± 1.37

Table 3.23 continued

Compound	Structure	% **radical scavenging (mean ± SD)**
(9)		15.47 ± 3.39
(10)		93.76 ± 0.62
(11)		17.68 ± 1.93
(12)		10.53 ± 0.83
(13)		23.30 ± 1.66
(15)		3.71 ± 0.20
(17)		11.55 ± 1.03

Note: Compounds (**4**), (**14**) and (**16**) had not been tested because of not sufficient amount.

From the results, compounds (**2**), (**5**), (**6**), (**7**) and (**10**) possess high radical scavenging percentages. Their structures were in agreement with the previous studies [44]. The report was concluded the three criteria for effective radical scavenging of flavonoids:

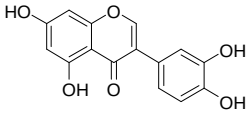
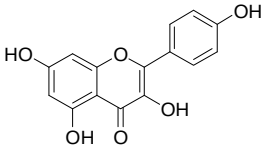
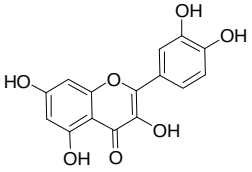
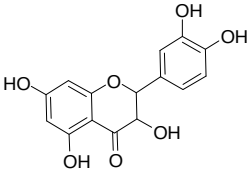
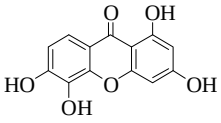
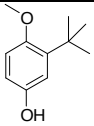
1. The *o*-dihydroxy structure in the B ring, which confers higher stability to the radical form and participates in electron delocalization.
2. The 2,3 double bond in conjugation with a 4-oxo function in the C-ring is responsible for electron delocalization from the B ring. The antioxidant

potency is relates to structure in term of electron delocalization of the aromatic nucleus. Where these compounds react with free radicals, the phenoxyl radicals produced are stabilized by the resonance effect of aromatic nucleus.

3. The 3- and 5-OH groups with 4-oxo function in A and C rings are required for maximum radical scavenging potential.

Compounds (**2**)**,** (**5**)**,** (**6**), (**7**) and (**10**) were further determined their IC_{50} values using spectrophotometric method as described in Chapter II. The IC_{50} values of each substance were calculated from the equation of each curve and are tabulated in Table 3.24.

Table 3.24 IC_{50} values of radical scavenging effect on DPPH radical of the isolated compounds

Compound	**Structure**	**IC_{50} (mM)**
(2)		0.30
(5)		0.34
(6)		0.09
(7)		0.12
(10)		0.17
BHA		0.19

From the results of radical scavenging effect on DPPH radical spectrophotometric assay (Table 3.24), it was found that compound (**6**) showed the highest activity with IC_{50} 0.09 mM which approximately 2-fold higher than that of BHA (IC_{50} 0.19 mM) followed by compounds (**2**), (**7**), (**10**) and (**5**), respectively.

3.3.3 ABTS decolorization method

Furthermore to evaluate the antioxidant activity, compounds (**1**) – (**17**) were tested by spectrophotometric method as described in Chapter II. The commercial antioxidant compound Trolox was used as positive control. Results of this activity were shown in Table 3.25.

From the results of radical scavenging effect on ABTS radical spectrophotometric assay (Table 3.25), it was found that compounds (**6**) and (**10**) showed the highest activity approximately 3 folds higher than Trolox. Compounds (**2**)**,** (**5**)**,** (**7**)**,** (**8**) and (**15**)-(**17**) showed higher activity than Trolox. Compounds (**1**)**,** (**4**)**,** (**9**)**,** (**12**) and (**14**) displayed low activity than standard compound.

According to these results, this is the first report in which compound **(10**) have been isolated from *A. luzonensis* and presented high potent antioxidant activity toward ABTS.

Table 3.25 Antioxidant activities of isolated compounds by the ABTS radical method.

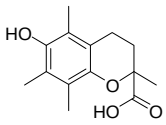
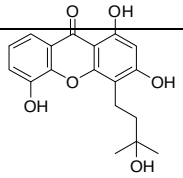
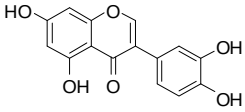
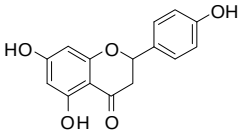
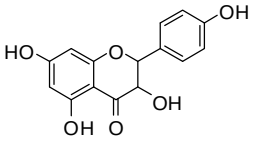
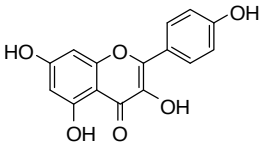
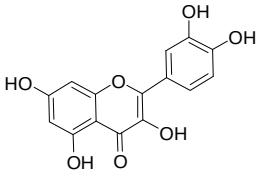
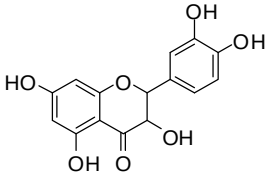
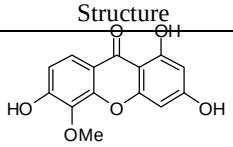
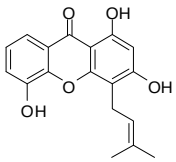
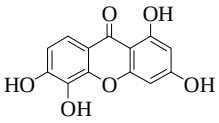
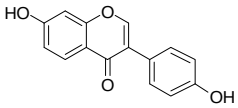
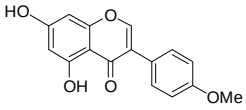
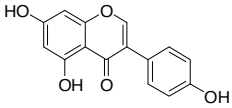
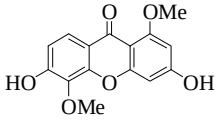
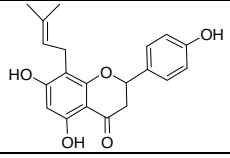
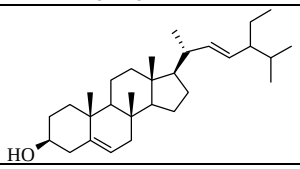
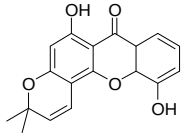
Compound	Structure	TEAC (1 min)	TEAC (6 min)
Trolox		1	1
(1)		0.91	0.68
(2)		1.44	1.46
(3)		n.t.	n.t.
(4)		0.04	0.02
(5)		1.39	1.50
(6)		2.38	2.88
(7)		1.69	1.79

Table 3.25 continued

Compound	Structure	TEAC (1 min)	TEAC (6 min)
(8)		1.23	1.35
(9)		0.91	0.68
(10)		2.45	2.96
(11)		0.004	0.004
(12)		0.003	0.003
(13)		1.24	1.64
(14)		0.04	0.02
(15)		1.39	1.50
(16)		1.44	1.46
(17)		1.69	1.79

3.3.4 Inhibitory activities compounds on *c*GMP phosphodiesterase (PDE)

Ten compounds of BuOH extracts from ***A. luzonensis*** were tested for their cGMP phosphodiesterase inhibition by using SPA radio assay method.

Table 3.26 PDE inhibitory activities of isolated compounds at final concentration 0.1 mM

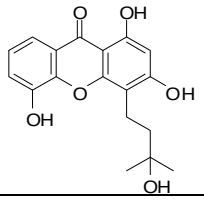
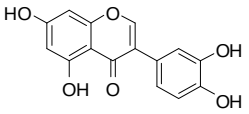
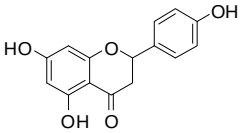
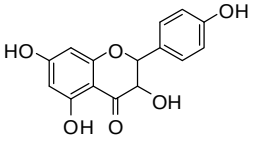
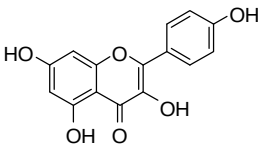
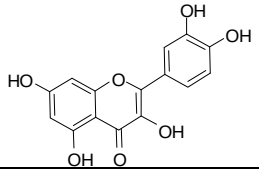
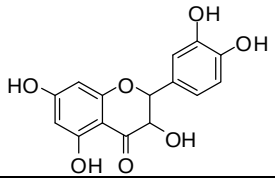
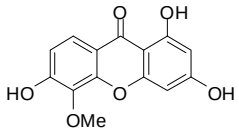
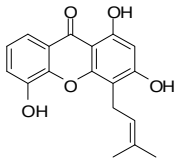
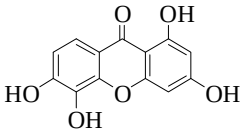
Compound	Structure	% inhibition ± SD
(1)		84.08 ± 0.24
(2)		67.76 ± 0.22
(3)		47.50 ± 0.45
(4)		34.11 ± 0.39
(5)		62.59 ± 0.67
(6)		50.43 ± 0.30
(7)		13.97 ± 0.55

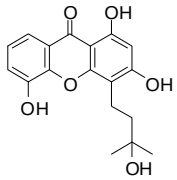
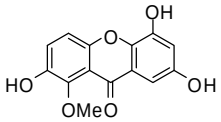
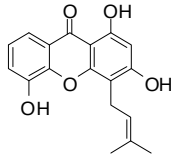
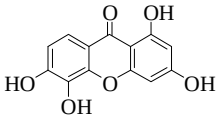
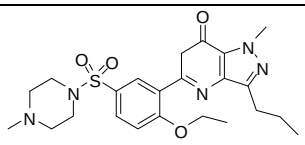
Table 3.26 continued

Compound	Structure	% inhibition ± SD
(8)		76.32 ± 0.18
(9)		97.28 ± 0.08
(10)		72.85 ± 0.00

From the results in Table 3.26, xanthones were exhibited PDE inhibition higher than other flavonoids. Among isolated xanthones, the occurring of isopentenyl moiety in molecule remarkably enhanced the activity (compound (**1**) vs. (**9**)). Moreover, the presence of methoxy group at C-5 was slightly reduced activity (compound (**8**) vs. (**10**)).

Additionally, double bond in C-ring of flavonoid displayed a significant role of the activity (compounds (**6**) vs. (**7**) and compounds (**5**) vs. (**4**)).

Table 3.27 IC_{50} values of PDEs inhibitory activity

Compound	Structure	IC_{50} ± SD (μM)
(1)		3.19 ± 0.15
(8)		34.96 ± 24.12
(9)		3.00 ± 1.68
(10)		26.12 ± 5.88
Sildenafil (Std.)		10 (nM)

From the data in Table 3.27, compounds (**1**) and (**9**) showed the highest activity and followed by (**10**) and (**8**), respectively.

CHAPTER IV

CONCLUSTION

During the course of this research, chemical constituents and biological activities of the heartwood of *A. luzonensis* were thoroughly investigated. Seventeen compounds (**1**) – (**17**) were isolated and elucidated their structures by means of physical properties and spectroscopic evidences. Among of isolated compounds, compounds (**3**), (**4)**, (**11**), (**13**) and **(16)** – (**17)** were first isolated from this plant. Almost all isolated compounds were then evaluated their bioactivitites; cholinesterase inhibitory activity against acetylcholinesterase and butyrylcholinesterase, scavenging effect on DPPH radical, ABTS decolorization and Phosphodiesterase (PDEs) inhibitory activity. The summary of isolated compounds and their bioactivities was accumulated in Table 4.1.

Compounds (**1**) and (**2**) revealed strong activity with IC_{50} 2.36, and 2.57 μM against acetylcholinesterase and 2.25, 3.66 μM against butyrylcholinesterase, respectively. Additionally, compounds (**6**) and (**9**) revealed strong activity with IC_{50} 1.27 and 4.90 μM against butyrylcholinesterase, respectively. Compound (**6**) exhibited the highest scavenging activity toward DPPH radical with IC_{50} 0.09 mM and decolorizing ABTS with TEAC value 2.88. Xanthones (**1**), (**8**), (**9**) and (**10**) exhibited high inhibitory activity against cGMP phosphodiesterase comparing with other flavonoids.

Table 4.1 Isolated compounds from *A. luzonensis* and their bioactivities.

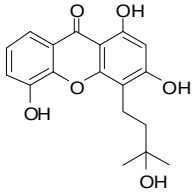
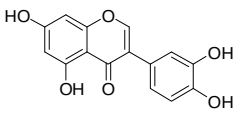
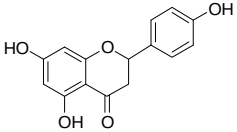
Substance	remark	Weight (mg)/ % Yield	mp.(°C), (mp. of lit.)	% inhibition [a]		DPPH [b] (%rad.)	ABTS (TEAC, 6 min)	PDE-5 [c] (%inhibition)
				AChE	BChE			
 1,3,5-trihydroxy-4-(3-hydroxy-3-methylbutyl)xanthone (**1**)	yellow powder	7.2, 7.85 x 10^{-2}	286.5-287.4, (286-287[45])	83.40 ± 0.43 (IC_{50}=2.36μM)	84.29 ± 2.12 (IC_{50}=2.25 μM	14.98 ± 1.89	0.68	84.08 ± 0.24 (IC_{50}= 3.19 ± 0.15 μM)
 orobol (**2**)	white powder	518.7, 0.68	267.8-268.2 (268[25])	78.44 ±0.08 (IC_{50}=2.57 μM)	80.85±0.54 (IC_{50}=3.66 μM)	89.51 ± 0.21 (IC_{50}=0.3 mM)	1.46	67.76 ± 0.22
 naringenin (**3**), $[a]_D^{26.8} -15^\circ$ (c 0.05, MeOH)	yellow needle crystal	232.4, 0.27	249.5-250.3 (247-254[36])	< 20	< 20	12.31 ± 2.07	n.t.	47.50 ± 0.45

Table 4.1 continued

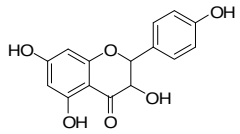
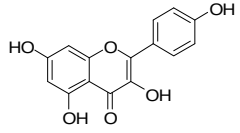
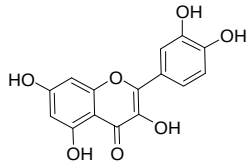
Substance	remark	Weight (mg)/ % Yield	mp.(°C), (mp. of lit.)	% inhibition [a]		DPPH [b] (% rad.)	ABTS (TEAC, 6 min)	PDE-5 [c] (% inhibition)
				AChE	BChE			
 aromadendrin (**4**), $[a]_D^{26.1} +14^\circ$ (c 0.05, MeOH)	white needle crystal	94, 0.11	235.5-236.2 (237-241[36])	< 20	< 20	n.t.	0.02	34.11 ± 0.39
 kaempferol(**5**)	yellow powder	79.9, 0.09	278.3-278.7 (276-278[27])	< 20	< 20	94.04 ± 0.18 (IC_{50}=0.34mM)	1.50	62.59 ± 0.67
 quercetin (**6**)	yellow powder	422.8, 28.01	312.5-312.9 (316[28])	38.8± 0.32	79.2 ± 0.25 (IC_{50}=1.27 µM)	93.98 ± 0.33 (IC_{50}=0.09mM)	2.88	50.43 ± 0.30

Table 4.1 continued

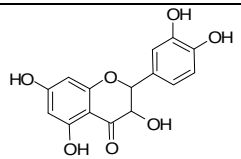
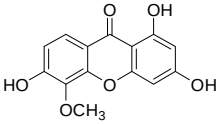
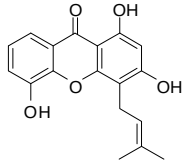
Substance	remark	Weight (mg)/ % Yield	mp.(°C), (mp. of lit.)	% inhibition [a]		DPPH [b] (%rad.)	ABTS (TEAC, 6 min)	PDE-5 [c] (%inhibition)
				AChE	BChE			
 taxifolin (**7**), $[a]_D^{26} + 9^\circ$ (*c* 0.05, MeOH)	yellow powder	323.9, 0.38	240.5-241.2 (240-242[29])	51.62 ± 0.37	< 20	93.63 ± 0.49 (IC_{50}=0.12mM)	1.79	13.97 ± 0.55
 1,3,6-trihydroxy-5-methoxy xanthone (**8**)	white needle crystal	100.9, 0.19	276.7-277.2 (277-278[30])	< 20	62.50 ± 0.00	25.44 ± 1.37	1.35	76.32 ± 0.18 (IC_{50}= 34.96 ± 24.12 µM)
 1,3,5-trihydroxy-4-prenyxanthone (**9**)	white powder	64.7, 0.13	286.2-286.9 (287[32])	68.59 ± 0.49	78.37 ± 0.00 (IC_{50}=4.90µM)	15.47± 3.39	0.68	97.28 ± 0.08 (IC_{50}= 3.00 ± 1.68 µM)

Table 4.1 continued

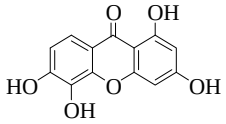
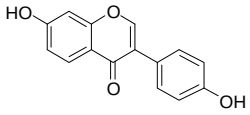
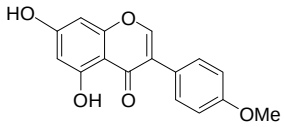
Substance	remark	Weight (mg)/ % Yield	mp.(°C), (mp. of lit.)	% inhibition [a]		DPPH [b] (% rad.)	ABTS (TEAC, 6 min)	PDE-5 [c] (% inhibition)
				AChE	BChE			
 1,3,5,6-tetrahydroxy-xanthen (**10**)	yellow powder	3.2, 0.02	278.2-278.6 (278-279[33])	< 20	< 20	93.76 ± 0.62 (IC_{50}=0.17mM)	2.96	72.85 ± 0.00 (IC_{50}= 26.12 ± 5.88)
 daidzein (**11**)	white needle crystal	8.5, 0.04	219.5-220.3 (221[39])	< 20	< 20	17.68 ± 1.93	0.004	n.t.
 biochanin A (**12**)	yellow powder	1.1, 5.42x10^{-3}	125.2-126.7 (127[34])	< 20	48.08 ± 3.39	10.53 ± 0.83	0.003	n.t.

Table 4.1 continued

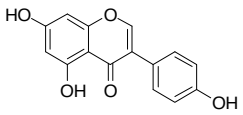
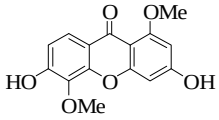
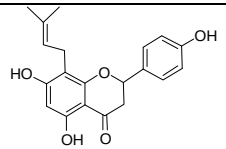
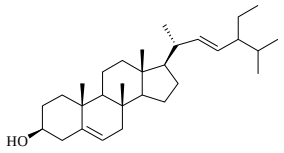
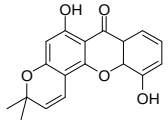
Substance	remark	Weight (mg)/ % Yield	mp.(°C), (mp. of lit.)	% inhibition [a]		DPPH [b] (%rad.)	ABTS (TEAC, 6 min)	PDE-5 [c] (%inhibition)
				AChE	BChE			
 genistein (**13**)	yellow needle crystal	2.8, 0.01	174.7-175.1 (175[40])	< 20	42.50± 0.78	23.30 ± 1.66	1.64	n.t.
 3,6-dihydroxy-1,5-dimethoxy-xanthen (**14**)	brown needle crystal	1.0, 4.93×10^{-3}	275.6-276.3 (275[4])	n.t.	n.t.	n.t.	0.02	n.t.
 8-prenylnaringenin (**15**), $[a]_D^{25.7} +11^\circ$ (*c* 0.05, MeOH)	yellow powder	12.7, 0.06	203.5-204.3 (205[19])	< 20	62.79± 0.42	3.71 ± 0.20	1.50	n.t.

Table 4.1 continued

Substance	remark	Weight (mg)/ % Yield	mp.(°C), (mp. of lit.)	% inhibition [a]		DPPH [b] (% rad.)	ABTS (TEAC, 6 min)	PDE-5 [c] (% inhibition)
				AChE	BChE			
 stigmasterol (**16**)	white needle crystal	1.2, 5.91x10^{-3}	175.8-176.2 (176-177[42])	n.t.	n.t	n.t.	1.46	n.t.
 6-deoxyisojacareubin (**17**)	yellow powder	2.9, 0.01	235.2-235.7 (235-237[32])	< 20	< 20	11.55 ± 1.03	1.79	n.t.

Note: lit. = literature

% rad.= % radical scavenging

n.t.= not test

a = final concentration at 0.1 mg/mL

b = final concentration at 0.2 mg/mL

c = final concentration at 0.1 mM

According to a number of compounds obtained from the heartwood of *A. luzonensis* as well as compounds showed significant activities on a variety of bioassay, it could be clearly seen that the heartwood of *A. luzonensis* could be used as a good source of compound (**6**) (quercetin, 28.01% yields), a significant antioxidant activity toward DPPH, ABTS and against butyrylcholinesterase. Moreover, compounds (**1**) and (**9**) significantly inhibited cholinesterase activity and against cGMP phosphodiesterase. Overall, these compounds are a possible therapeutic candidate to be developed as new drugs in the future.

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APPENDICES

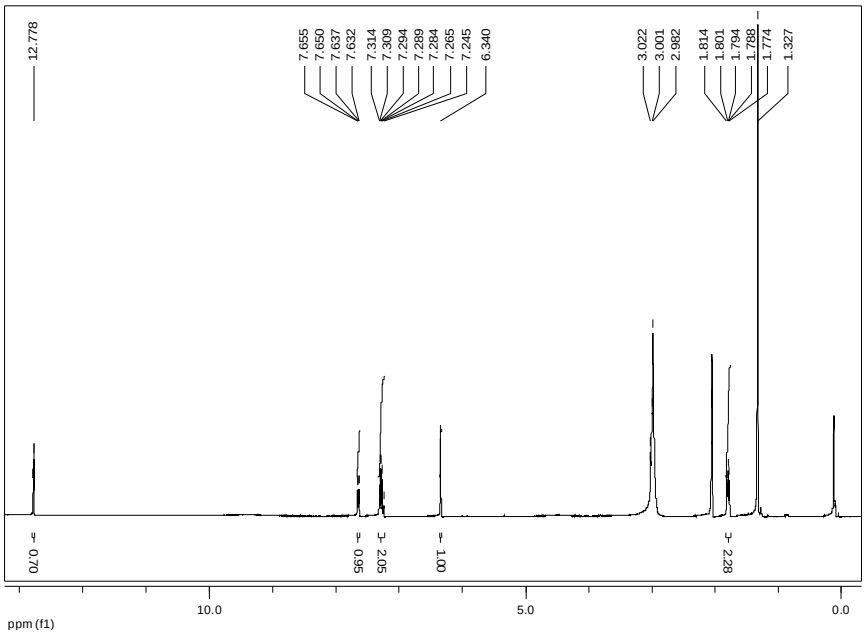

Figure 1 The ^{1}H-NMR spectrum of compound (**1**)

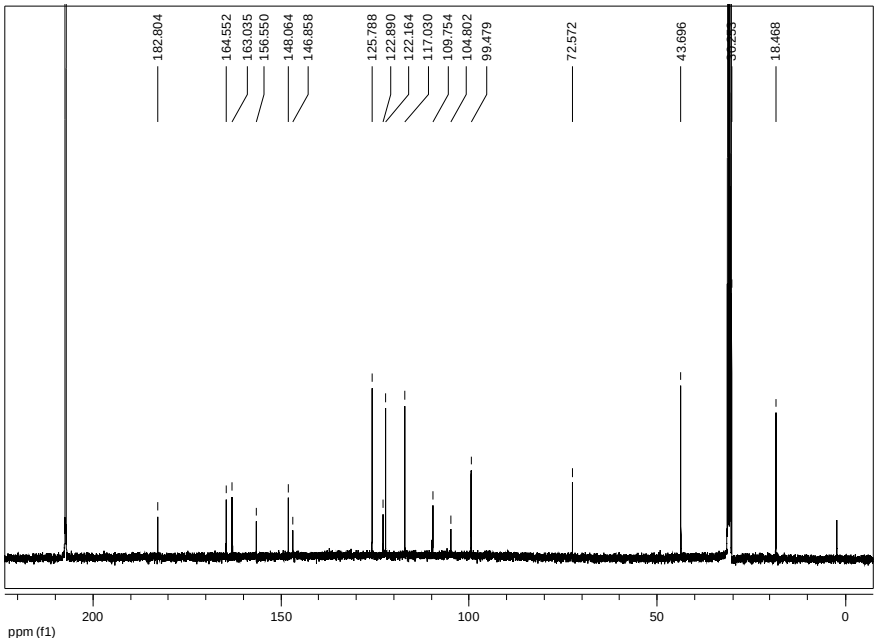

Figure 2 The ^{13}C-NMR spectrum of compound (**1**)

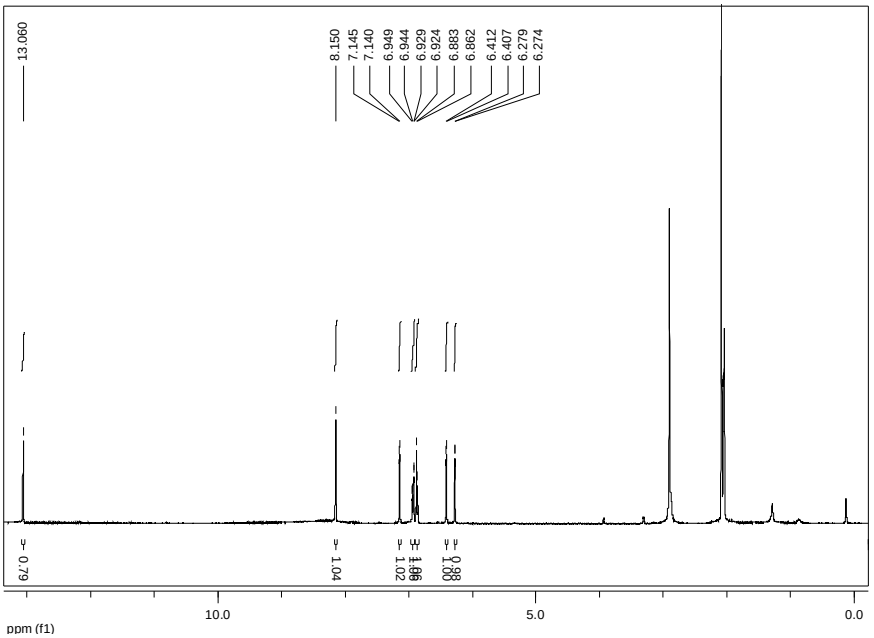

Figure 3 The ^{1}H-NMR spectrum of compound (**2**)

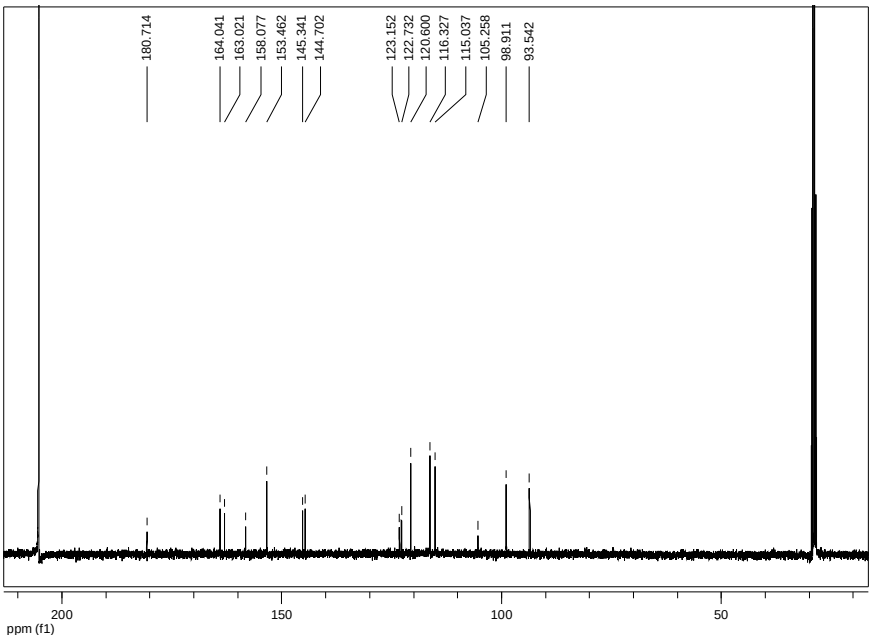

Figure 4 The ^{13}C-NMR spectrum of compound (**2**)

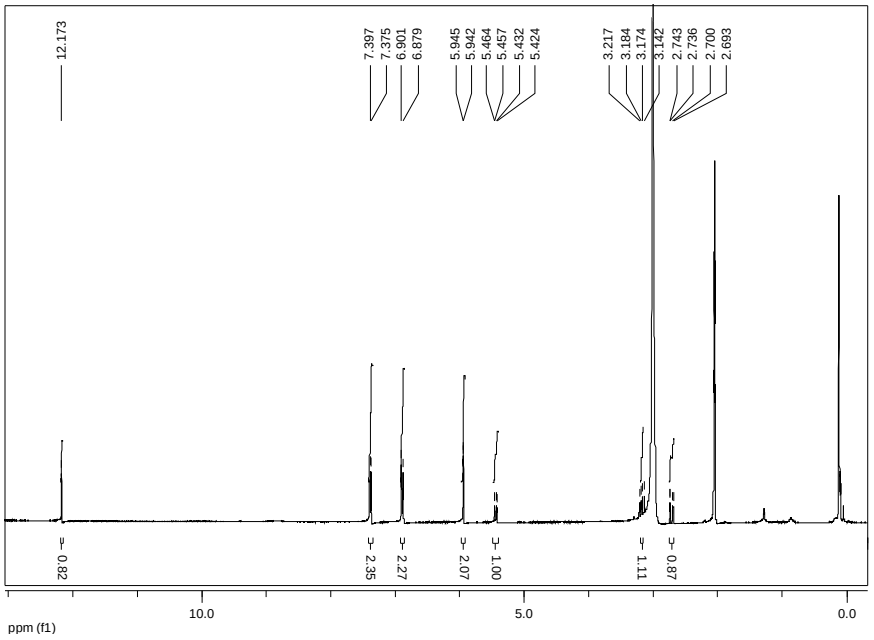

Figure 5 The ^{1}H-NMR spectrum of compound (**3**)

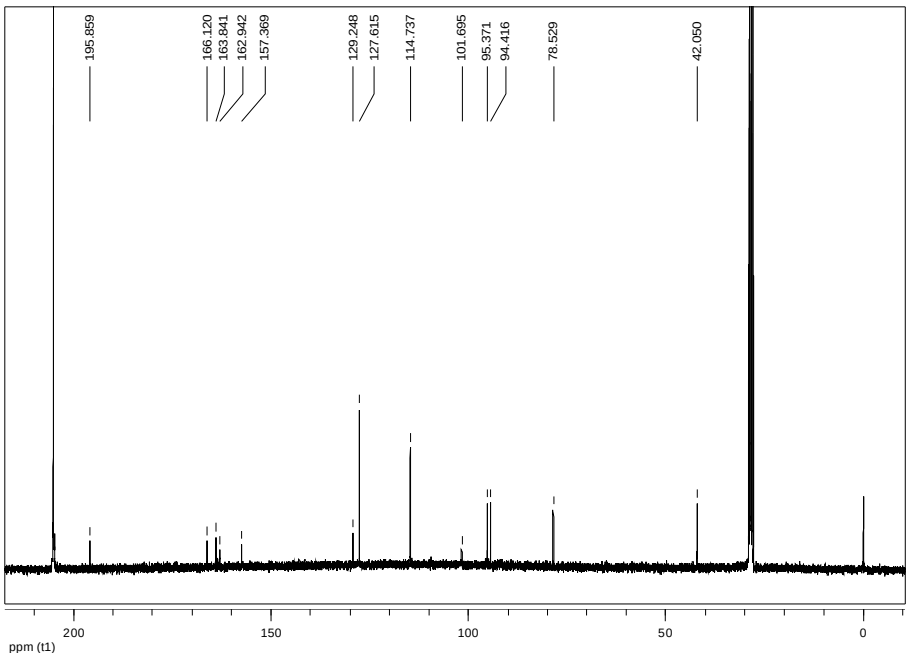

Figure 6 The ^{13}C-NMR spectrum of compound (**3**)

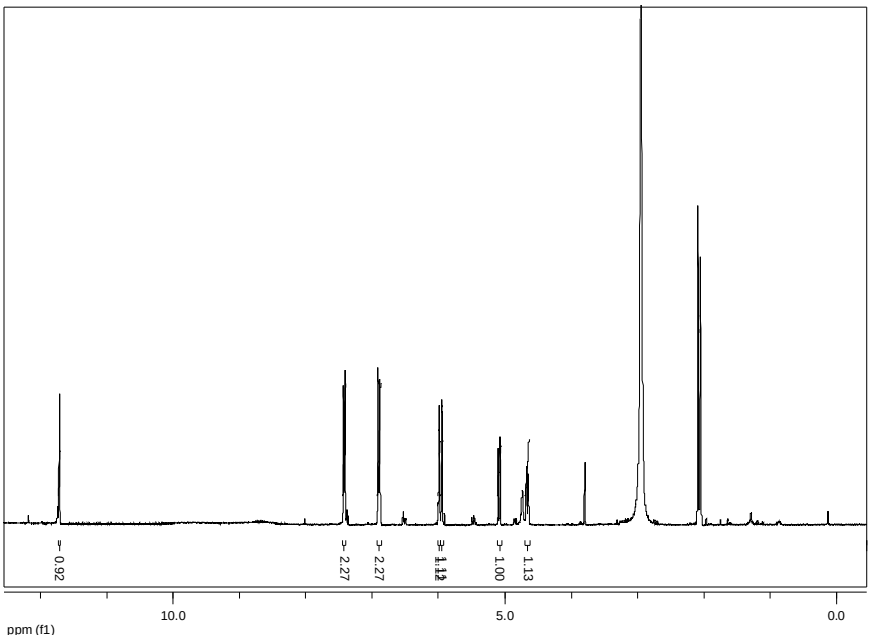

Figure 7 The ^{1}H-NMR spectrum of compound (**4**)

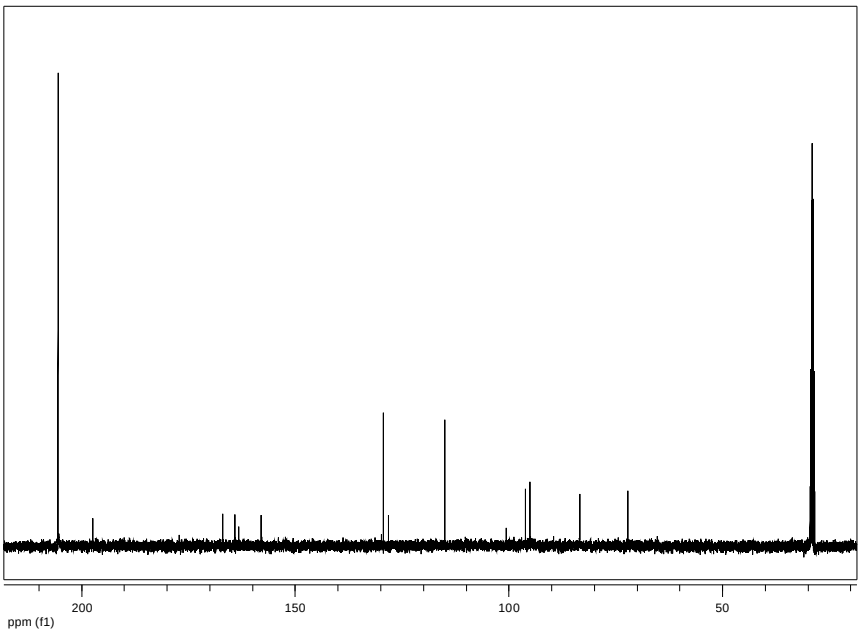

Figure 8 The ^{13}C-NMR spectrum of compound (**4**)

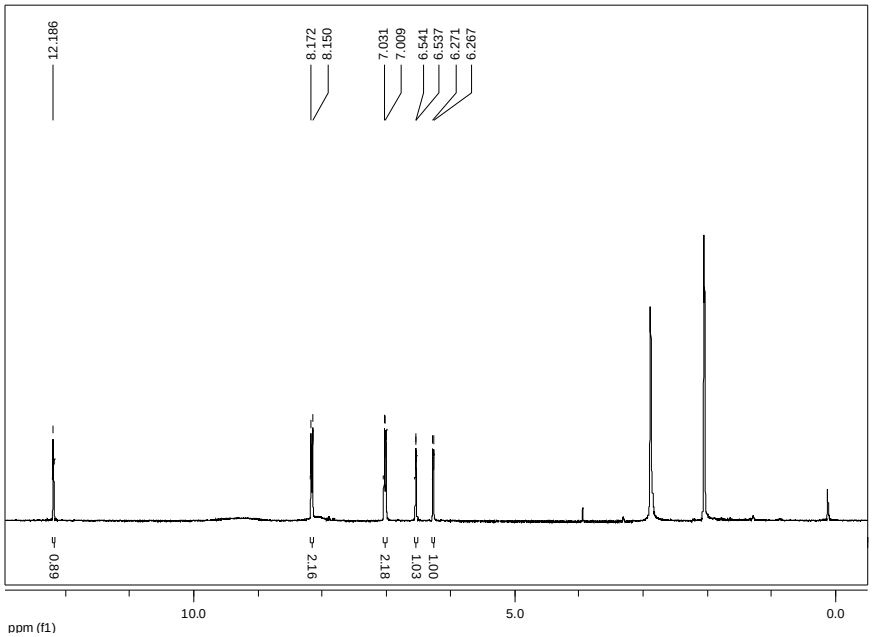

Figure 9 The ^{1}H-NMR spectrum of compound (**5**)

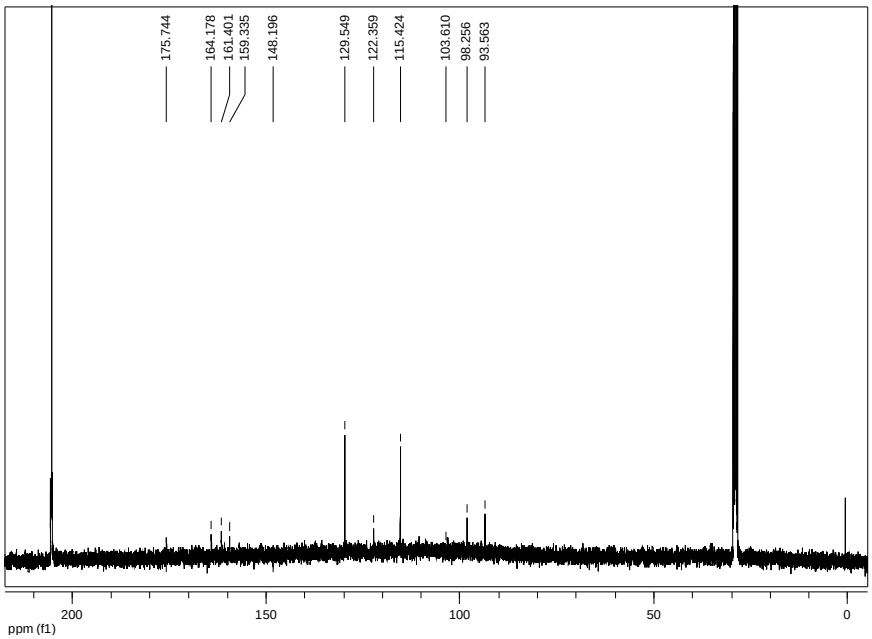

Figure 10 The ^{13}C-NMR spectrum of compound (**5**)

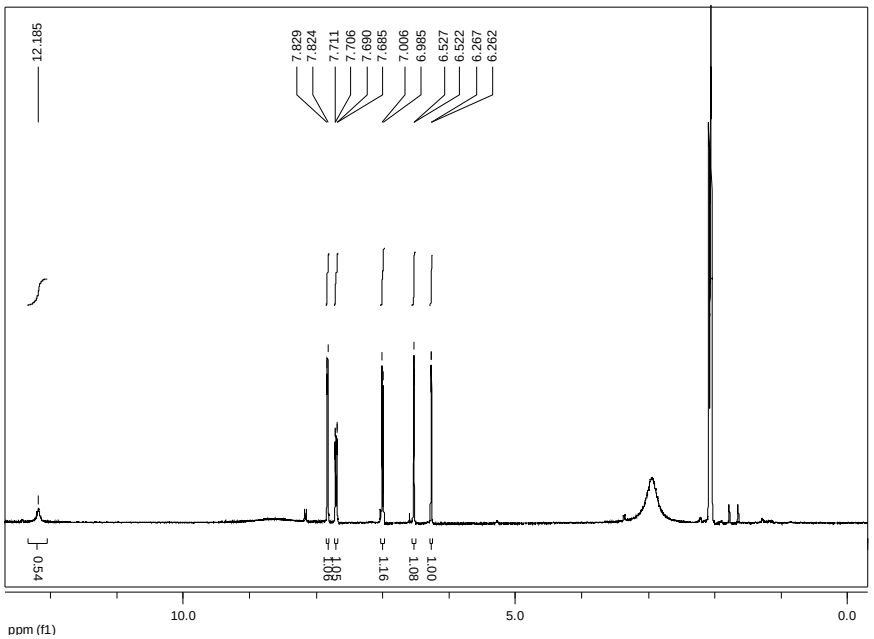

Figure 11 The ^{1}H-NMR spectrum of compound (**6**)

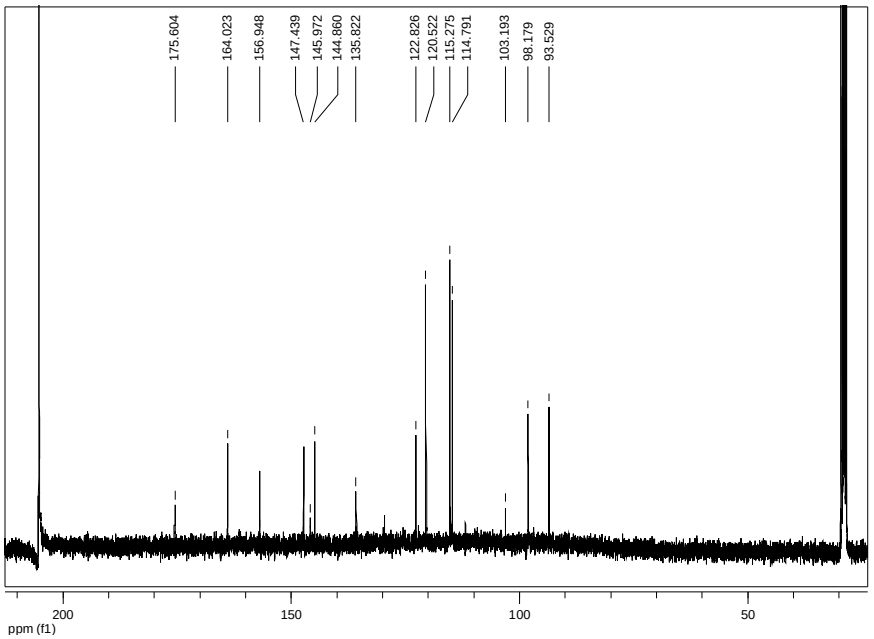

Figure 12 The ^{13}C-NMR spectrum of compound (**6**)

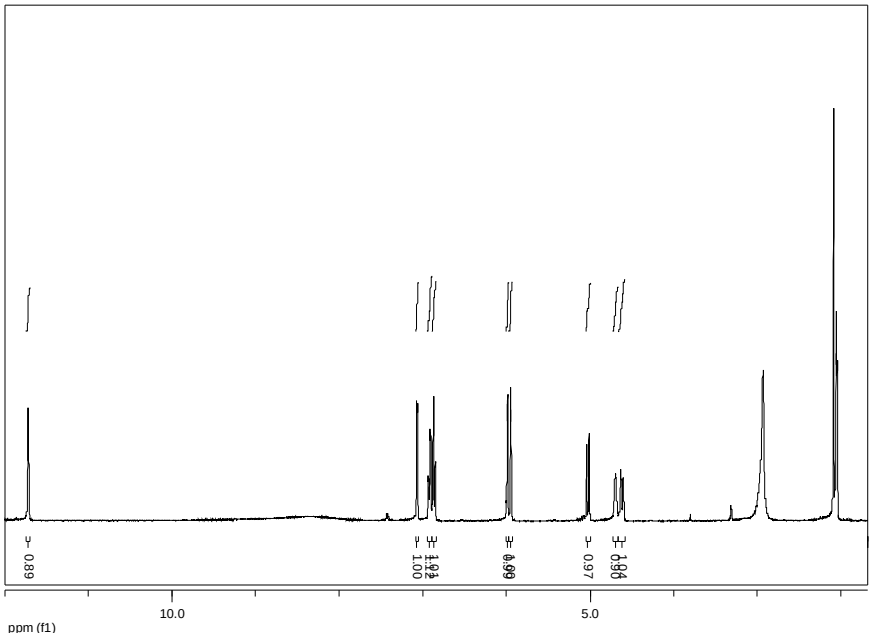

Figure 13 The ^{1}H-NMR spectrum of compound (7)

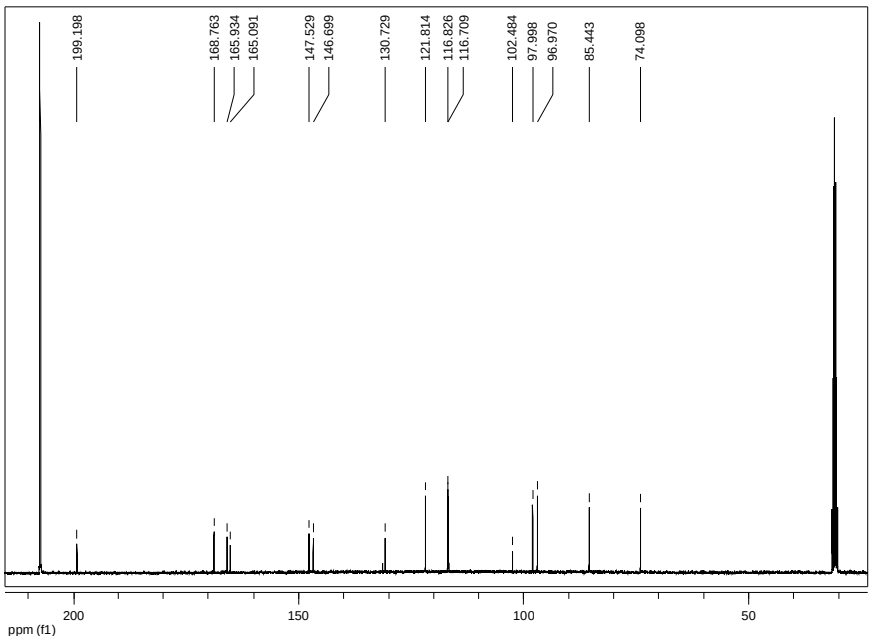

Figure 14 The ^{13}C-NMR spectrum of compound (**7**)

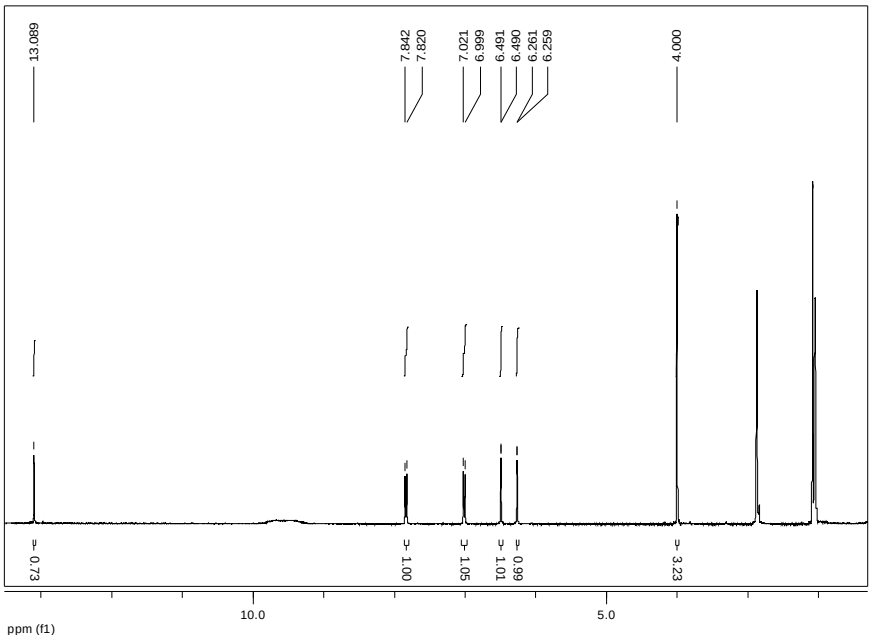

Figure 15 The ^{1}H-NMR spectrum of compound (**8**)

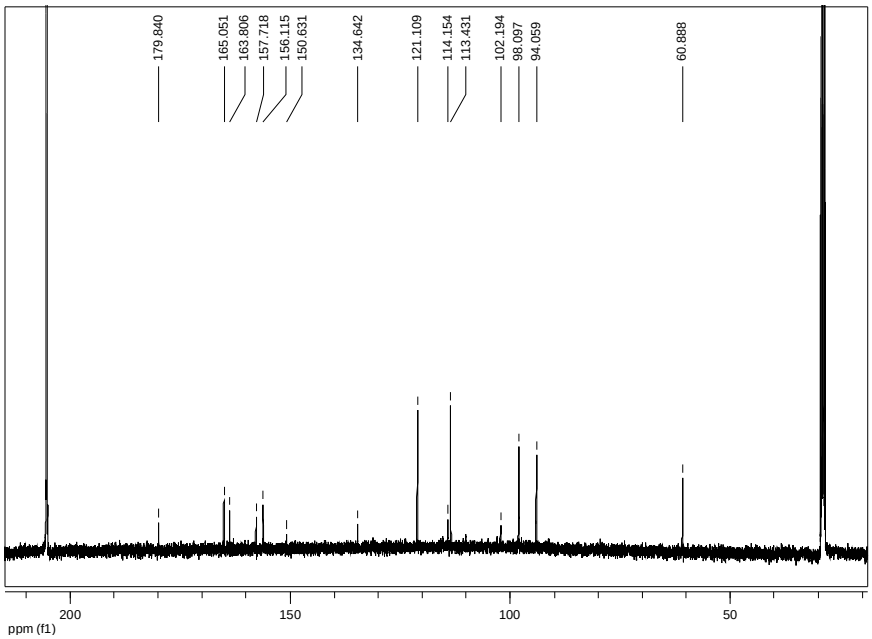

Figure 16 The ^{13}C-NMR spectrum of compound (**8**)

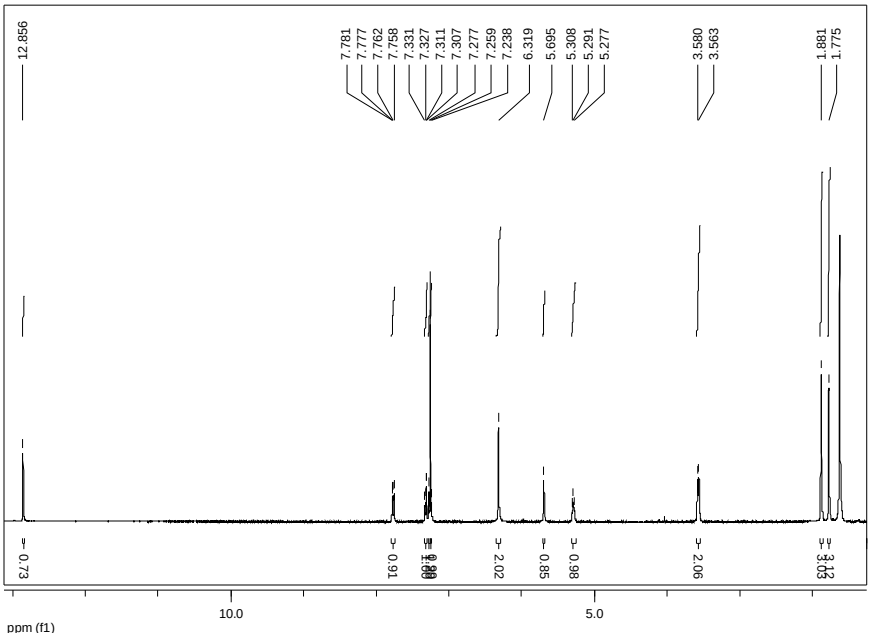

Figure 17 The ^{1}H-NMR spectrum of compound (**9**)

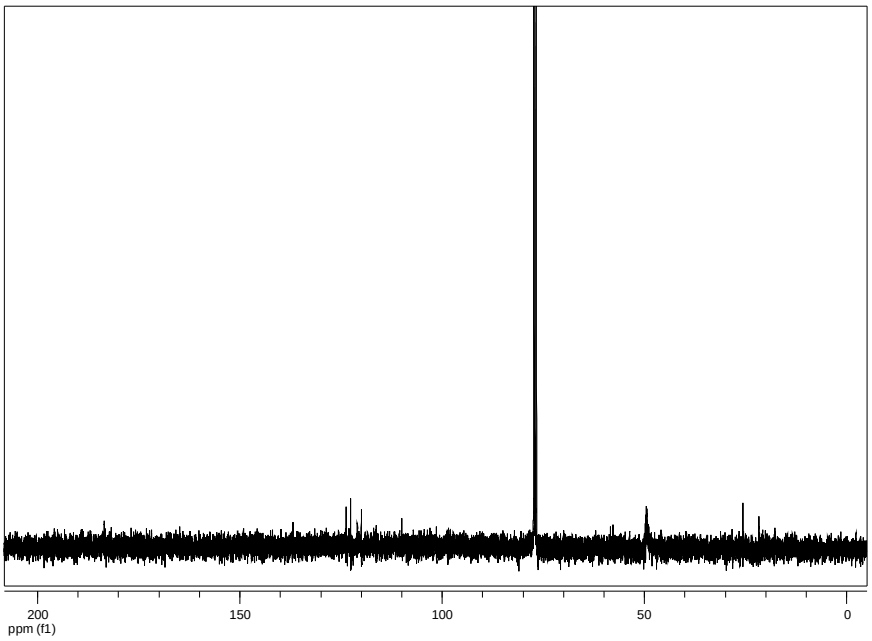

Figure 18 The ^{13}C-NMR spectrum of compound (**9**)

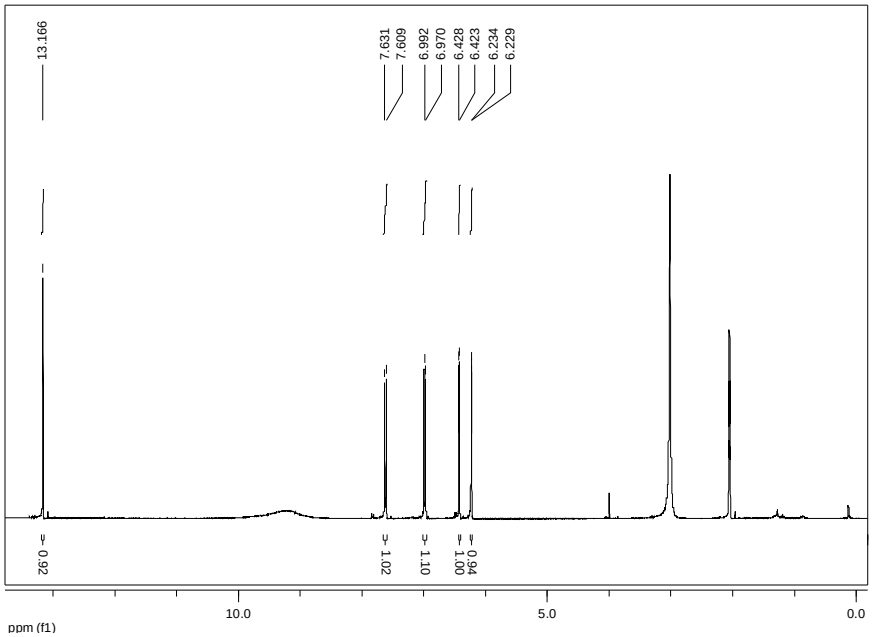

Figure 19 The ^{1}H-NMR spectrum of compound (**10**)

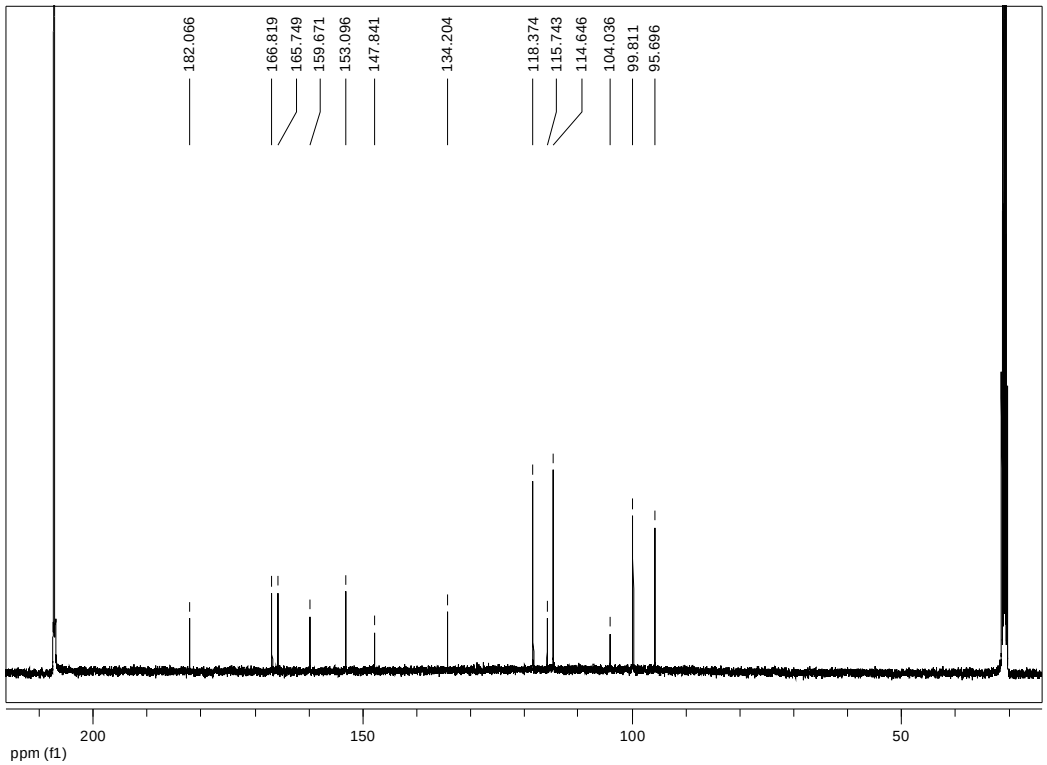

Figure 20 The ^{13}C-NMR spectrum of compound (**10**)

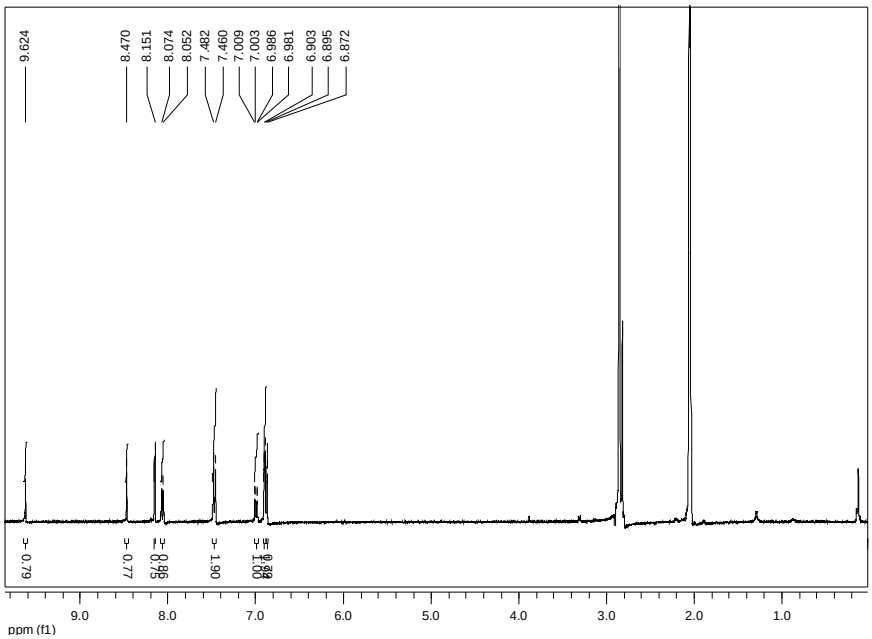

Figure 21 The ^{1}H-NMR spectrum of compound (**11**)

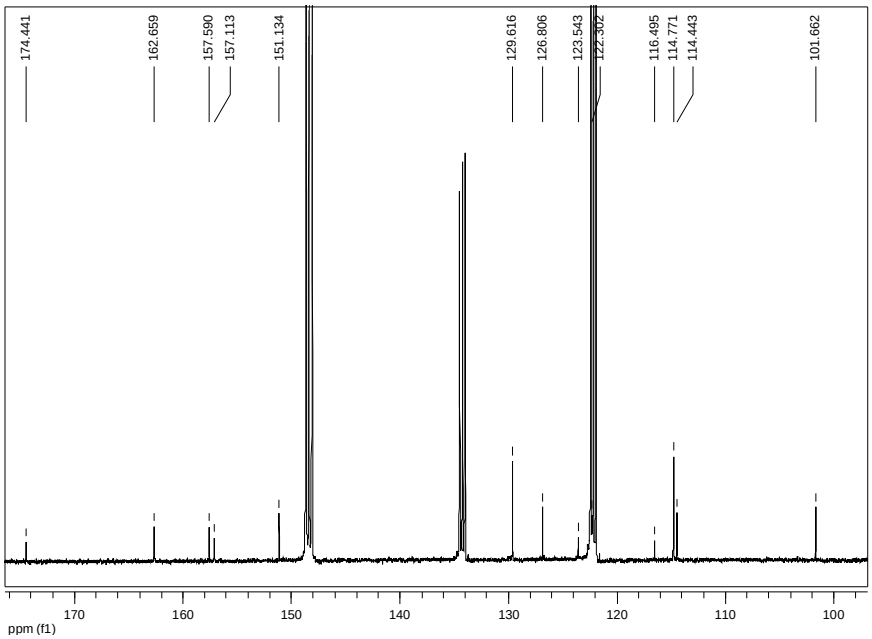

Figure 22 The ^{13}C-NMR spectrum of compound (**11**)

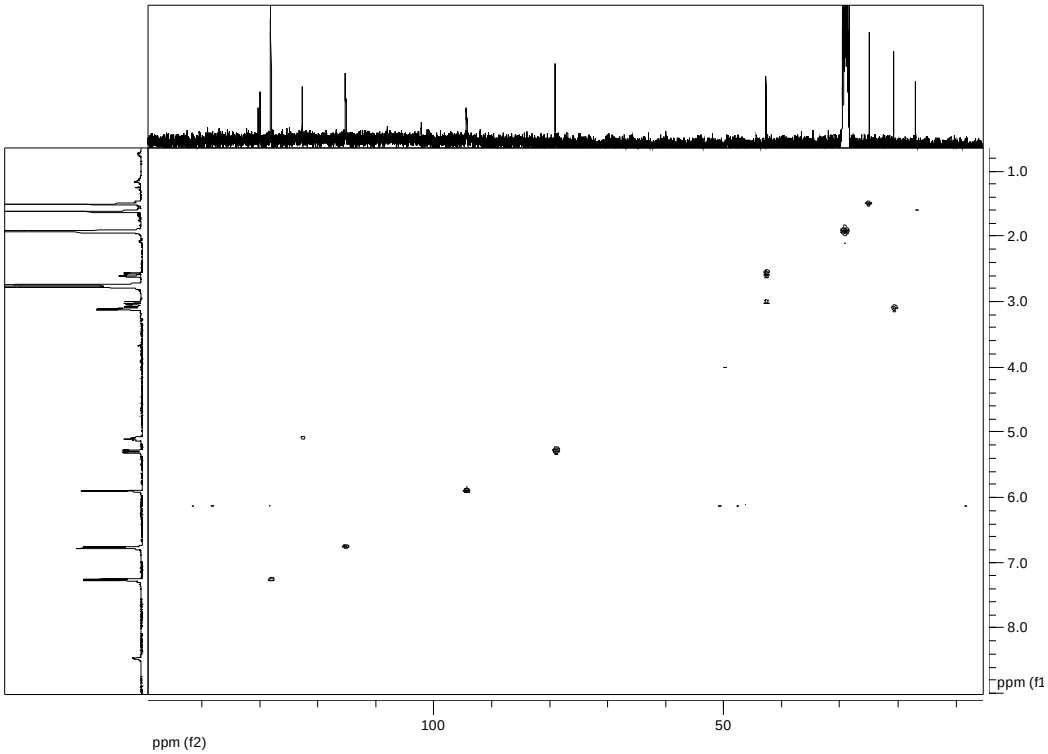

Figure 23 The HSQC spectrum of compound (**11**)

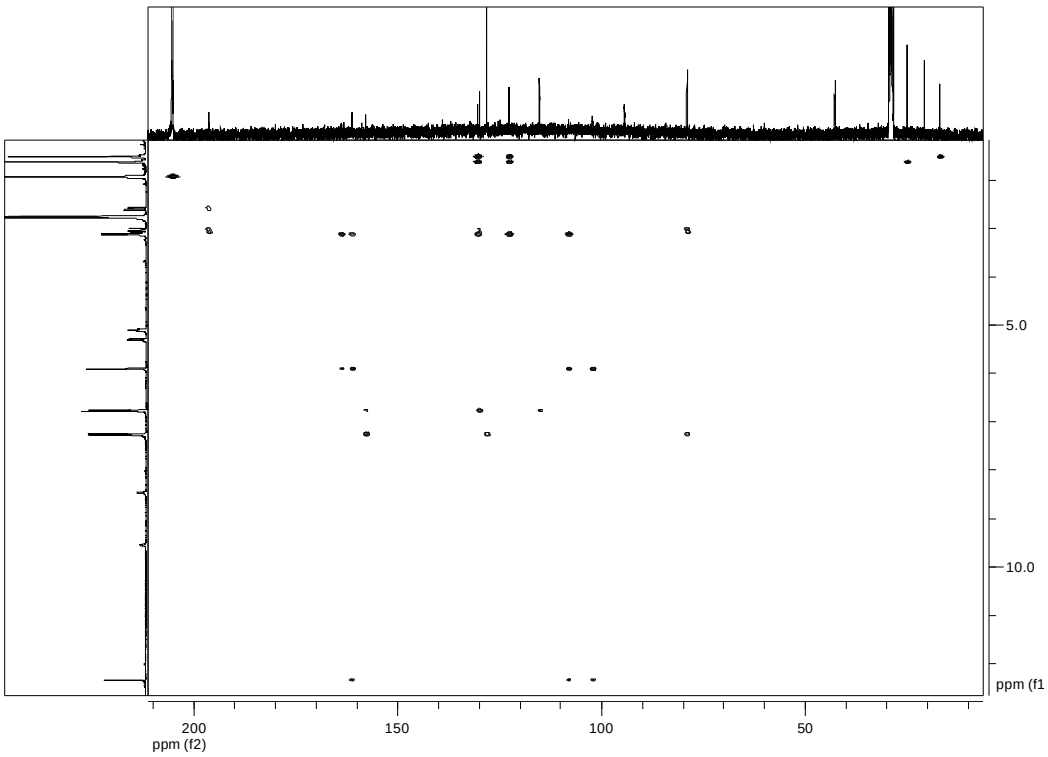

Figure 24 The HMBC spectrum of compound (**11**)

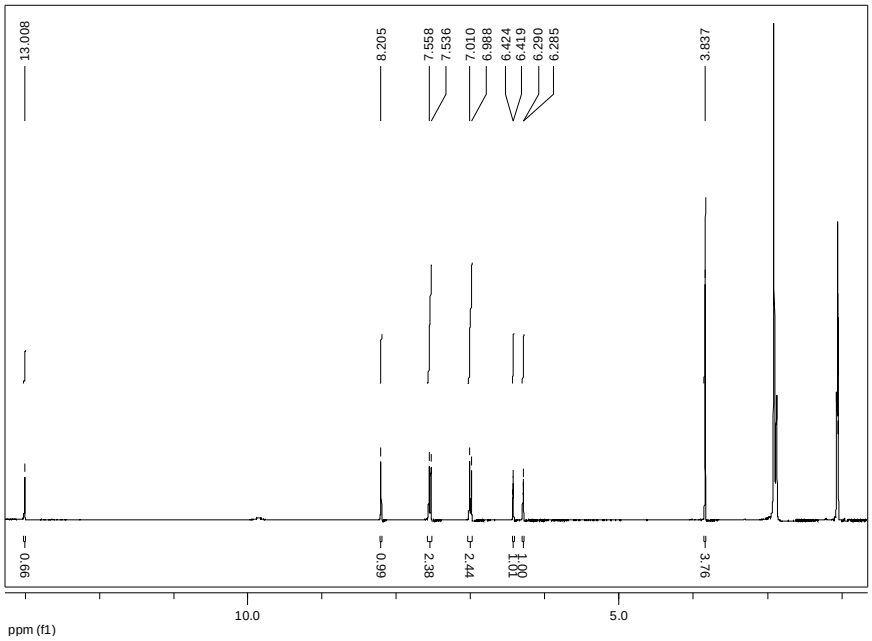

Figure 25 The ^{1}H-NMR spectrum of compound (**12**)

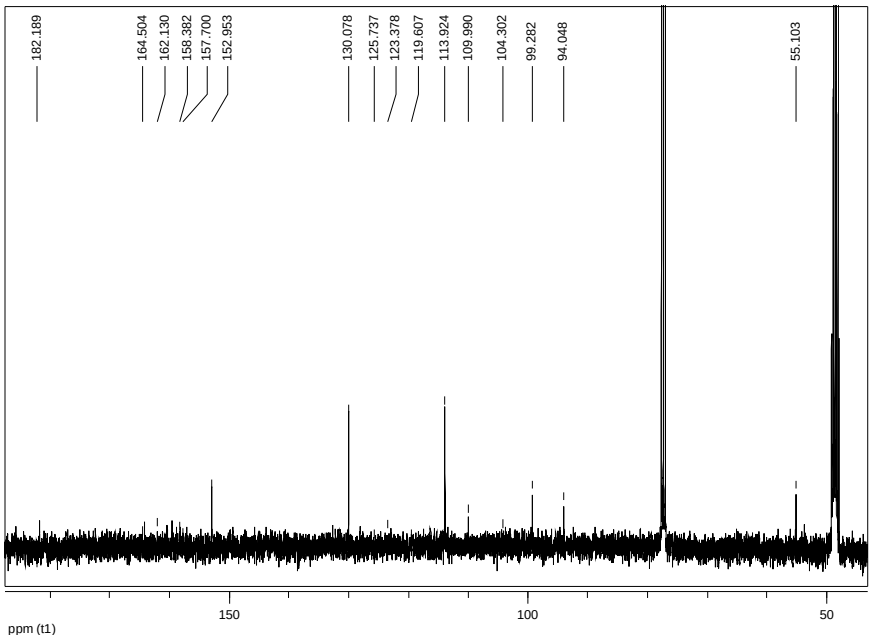

Figure 26 The ^{13}C-NMR spectrum of compound (**12**)

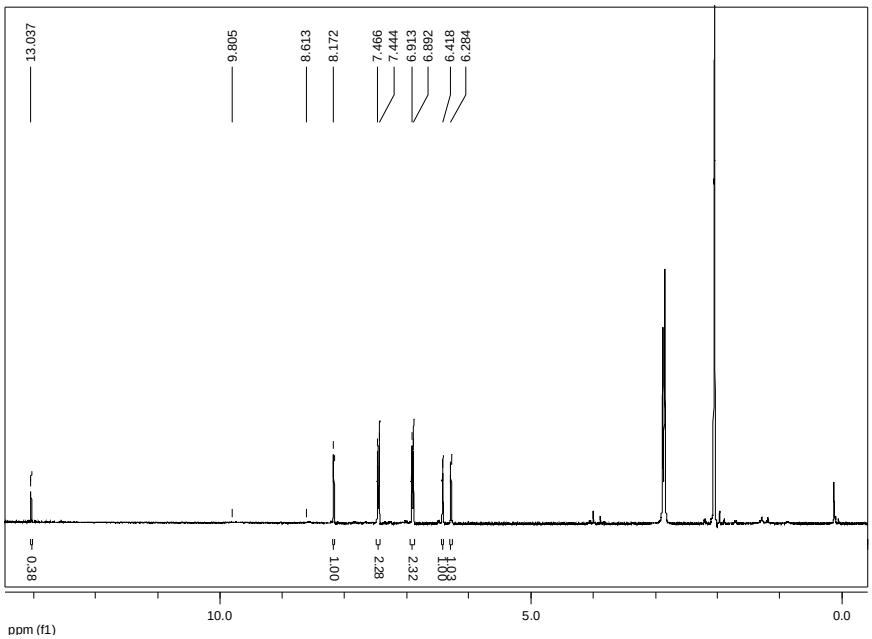

Figure 27 The ^{1}H-NMR spectrum of compound (**13**)

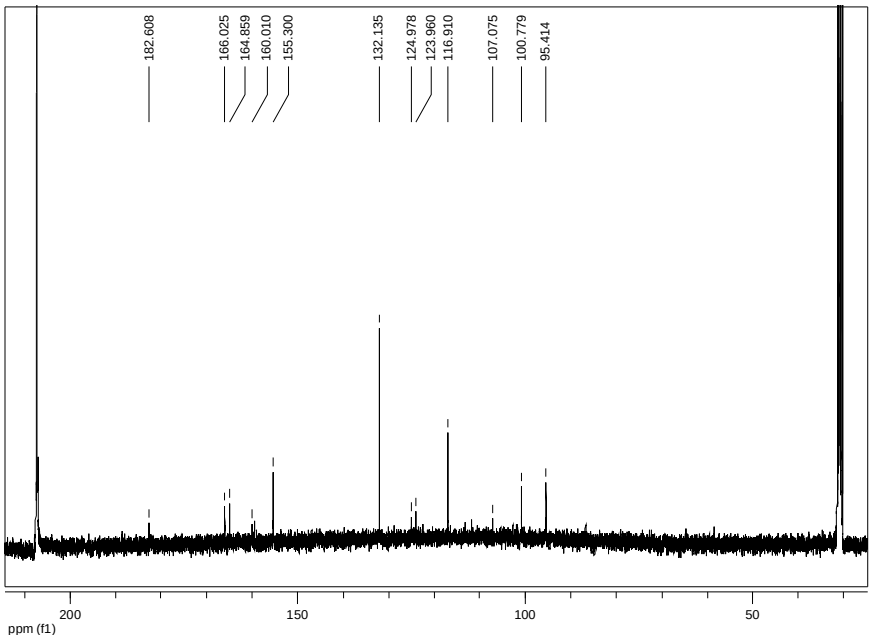

Figure 28 The ^{13}C-NMR spectrum of compound (**13**)

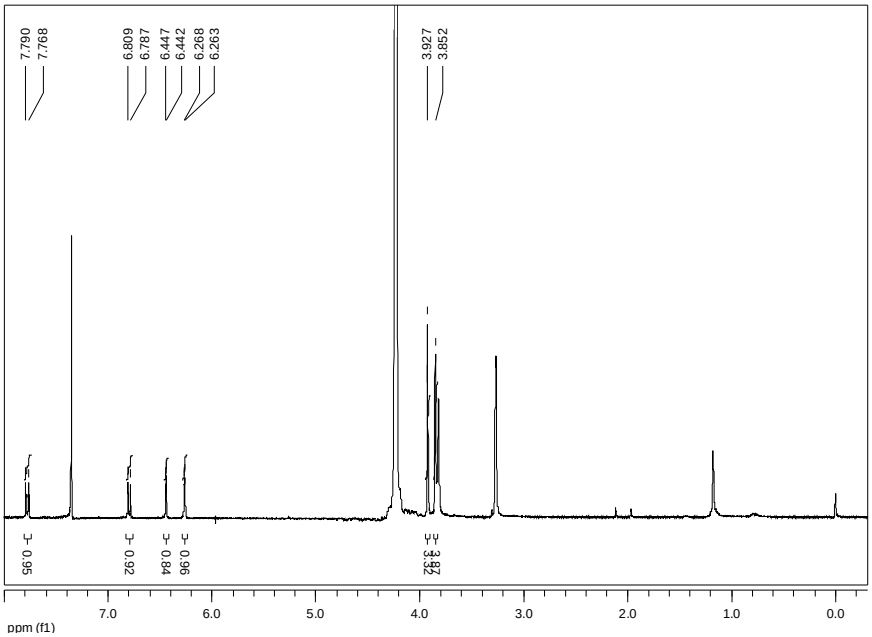

Figure 29 The ^{1}H-NMR spectrum of compound (**14**)

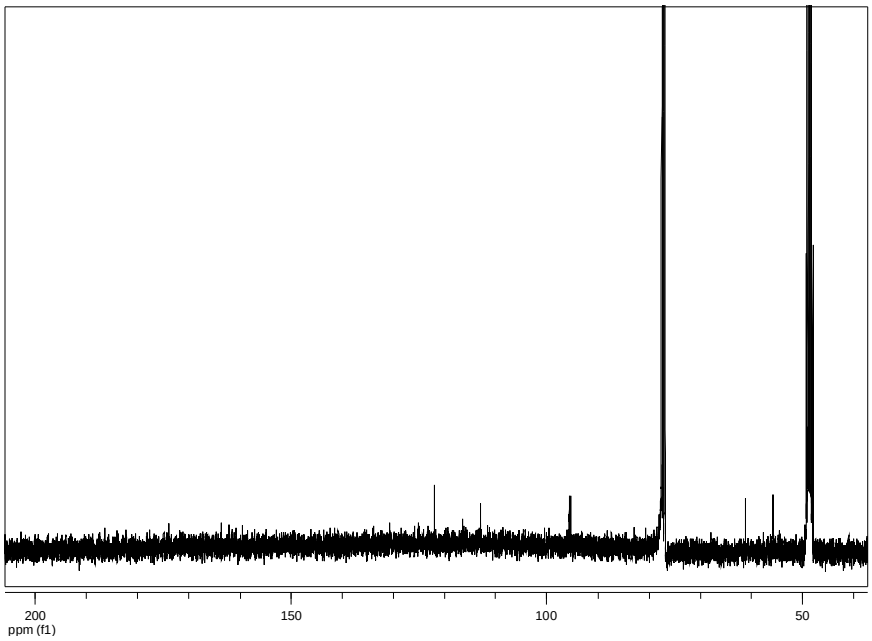

Figure 30 The ^{13}C-NMR spectrum of compound (**14**)

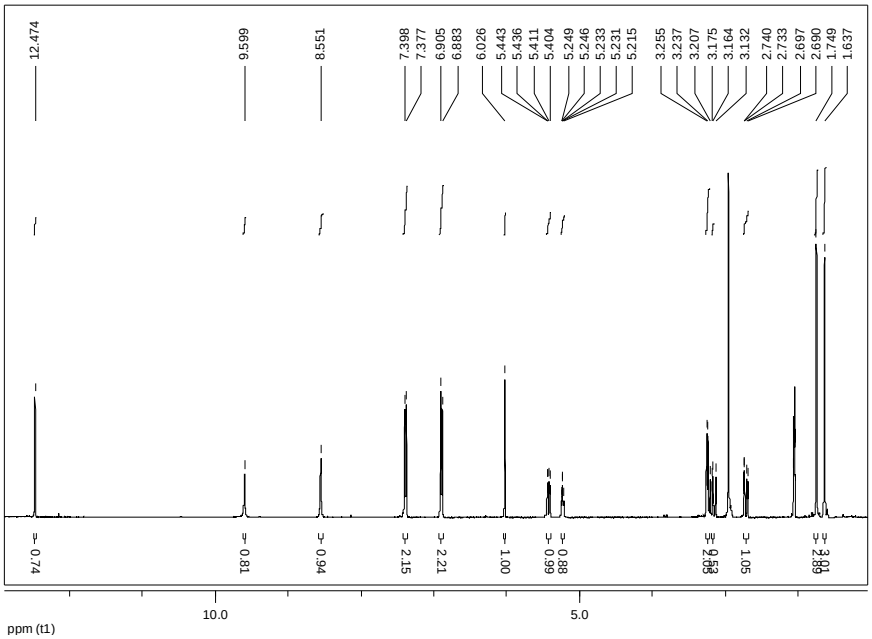

Figure 31 The ^{1}H-NMR spectrum of compound (**15**)

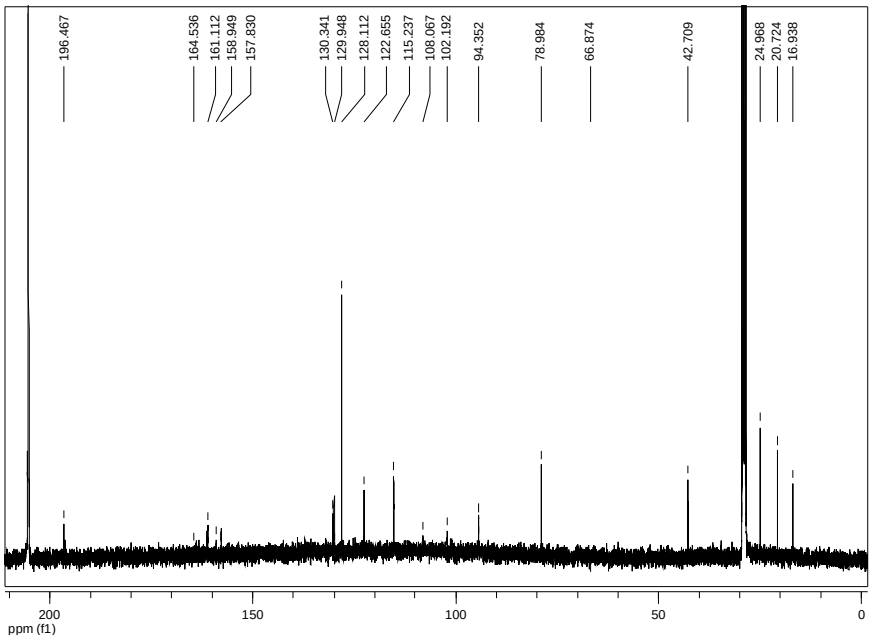

Figure 32 The ^{13}C-NMR spectrum of compound (**15**)

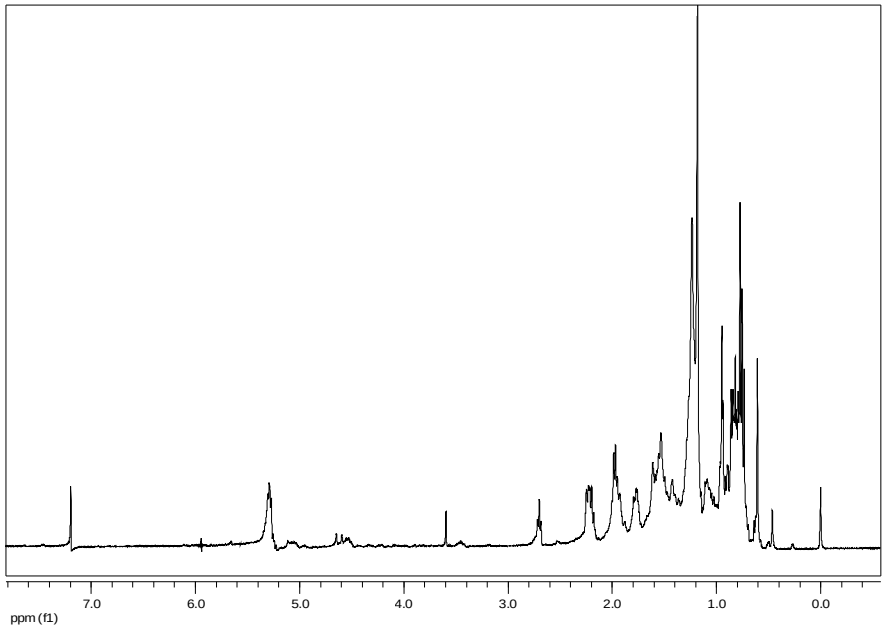

Figure 33 The ^{1}H-NMR spectrum of compound (**16**)

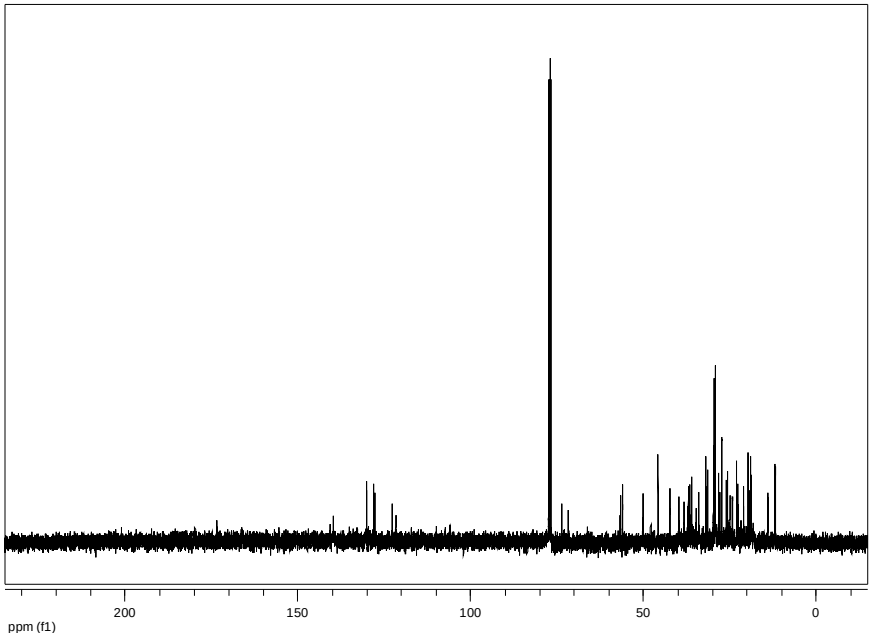

Figure 34 The ^{13}C-NMR spectrum of compound (**16**)

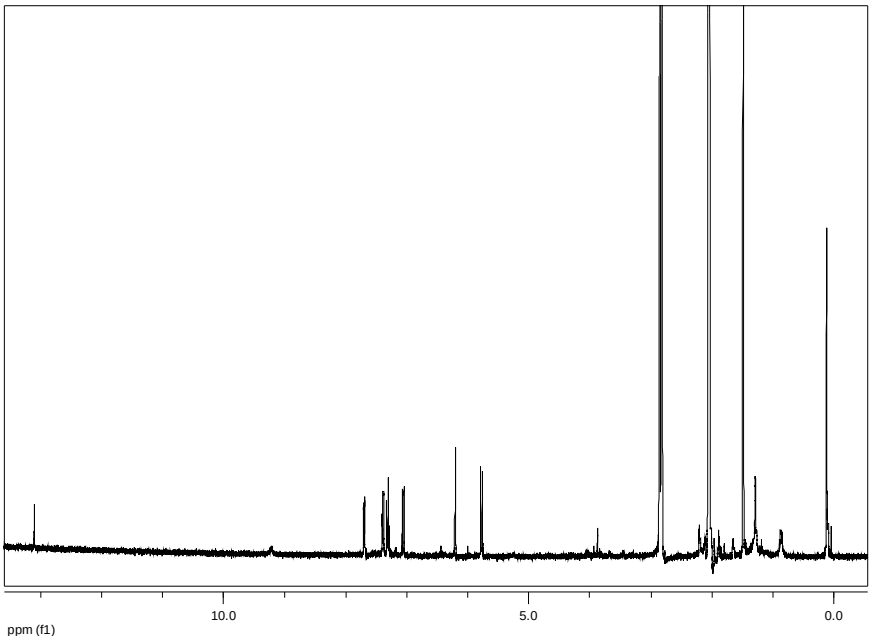

Figure 35 The ^{1}H-NMR spectrum of compound (**17**)

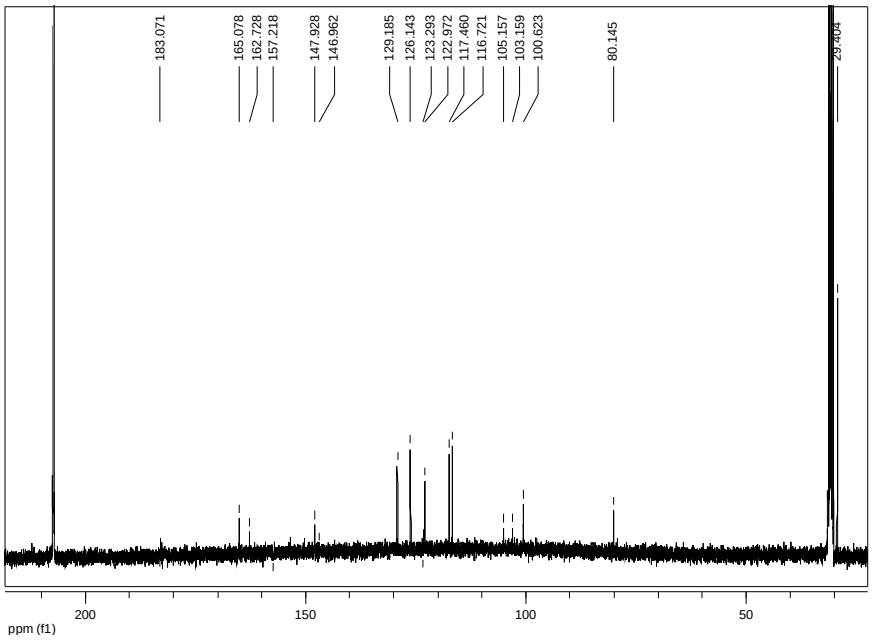

Figure 36 The ^{13}C-NMR spectrum of compound (**17**)

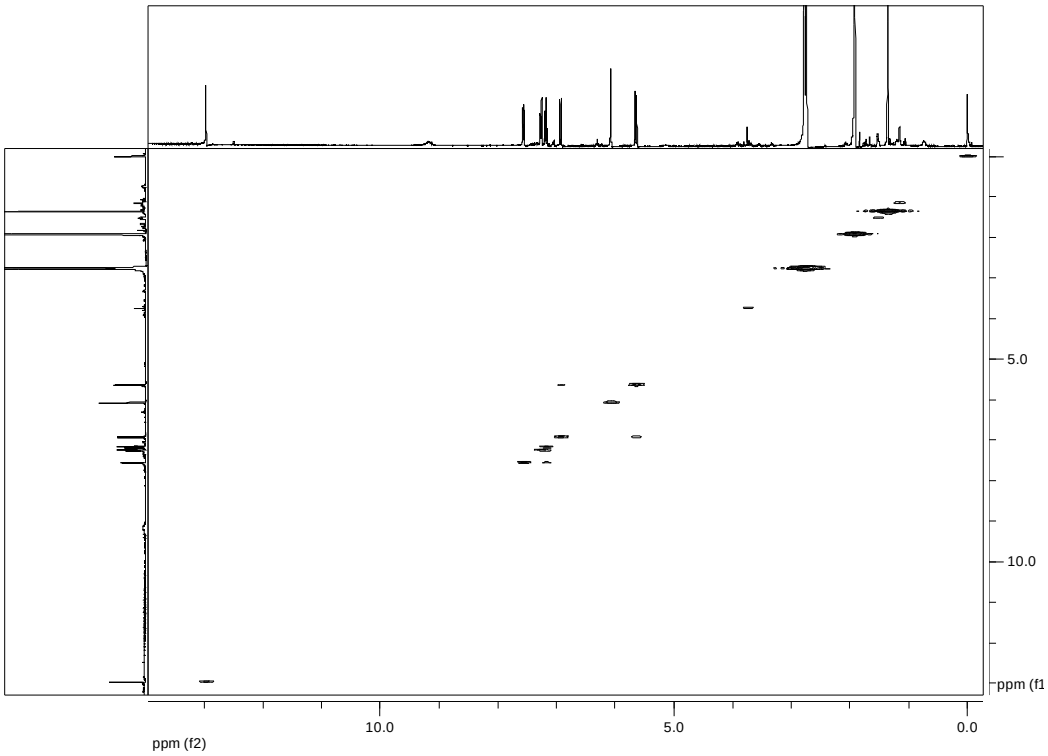

Figure 37 The gCOSY spectrum of compound (**17**)

VITA

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