ISOLATION OF CHEMICAL COMPOUNDS AND ANTIBACTERIAL ACTIVITY FROM THE BARK OF *ALPHONSEA CYLINDRICA*

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ABSTRACT

The objectives of this study are to isolate and determine the chemical compounds from *Alphonsea cylindrica* bark and also to investigate their antibacterial activities. Samples were collected from Kechau Tui, Kuala Lipis, Pahang. The samples were oven dried, ground and serially extracted by maceration technique using hexane, dichloromethane and methanol. Acid base extraction was performed on the dichloromethane extract. Chemical compounds were isolated and purified by means of various chromatographic techniques. Their structures were elucidated with modern spectroscopic techniques including 1D and 2D NMR, MS, IR, UV as well as comparison with literature review. Antibacterial activity on crude extracts and pure compounds was completed using disc diffusion technique. The results of phytochemical study have led to the isolation of seven chemical compounds which were stigmasterol, kinabaline, muniranine, *O*-methylmoschatoline, lysicamine, atherospermidine and *N*-methylouregidione. Compound muniranine was successfully isolated as a new derivative of azafluorenone while stigmasterol, kinabaline, *O*-methylmoschatoline, lysicamine, atherospermidine and *N*-methylouregidione were isolated for the first time from *Alphonsea* species. Furthermore, the results of antibacterial activity showed that dichloromethane extract as well as compounds *O*-methylmoschatoline and lysicamine exhibited zone of inhibition against *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Lysicamine gave minimum inhibition concentration (MIC) between 125 - 250 ug/mL compared with ampicillin, 300 ug/mL. As the conclusion, seven chemical compounds were successfully isolated and identified from *Alphonsea cylindrica* and two of them showed potential as antibacterial agent. For the implication, this study has potential in producing antibacterial agent from plant and supports the traditional use of *Alphonsea sp*. in the treatment of fever and diarrhoea.

PENGASINGAN SEBATIAN KIMIA DAN AKTIVITI ANTIBAKTERIA DARIPADA KULIT BATANG *ALPHONSEA CYLINDRICA*

ABSTRAK

Kajian ini bertujuan mengasing dan mengenal pasti sebatian kimia daripada kulit batang *Alphonsea cylindrica* serta mengkaji aktiviti antibakterianya. Sampel kajian dikumpul dari Kechau Tui, Kuala Lipis, Pahang. Sampel dikeringkan di dalam ketuhar, dikisar dan diekstrak secara bersiri dengan teknik rendaman pelarut heksana, diklorometana dan metanol. Pengekstrakan asid bes dijalankan ke atas ekstrak diklorometana. Sebatian kimia diasing dan ditulenkan melalui pelbagai teknik kromatografi. Struktur sebatian dikenalpasti menggunakan teknik spektroskopi moden iaitu 1D dan 2D RMN, SJ, IM, UL dan juga perbandingan dengan kajian lepas. Aktiviti antibakteria terhadap ekstrak mentah dan sebatian tulen telah dijalankan melalui teknik resapan cakera. Hasil kajian fitokimia telah membawa kepada pengasingan tujuh sebatian kimia iaitu stigmasterol, kinabalina, muniranina, *O*-metilmoskatolina, lisikamina, aterospermidina dan *N*-metiloregidiona. Sebatian muniranina telah berjaya diasingkan sebagai terbitan baharu azafluorinona manakala stigmasterol, kinabalina, *O*-metilmoskatolina, lisikamina, aterospermidina dan *N*-metiloregidiona telah diasingkan untuk pertama kali daripada spesis *Alphonsea*. Seterusnya, dapatan kajian aktiviti antibakteria menunjukkan bahawa ekstrak diklorometana serta sebatian *O*-metilmoskatolina dan lisikamina mempamerkan zon perencatan terhadap *Staphylococcus aureus* dan *Pseudomonas aeruginosa*. Lisikamina memberikan nilai kepekatan perencatan minimum (KPM) antara 125 - 250 ug/mL berbanding ampisillin, 300 ug/mL. Kesimpulannya, tujuh sebatian kimia telah berjaya diasing dan dikenalpasti daripada *Alphonsea cylindrica* dan dua daripadanya menunjukkan potensi sebagai agen antibakteria. Implikasinya, kajian ini berpotensi menghasilkan agen antibakteria daripada tumbuhan bagi menyokong penggunaan spesis *Alphonsea* secara tradisional dalam merawat demam dan cirit-birit.

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LIST OF SCHEMES

ABBREVIATIONS

^{1}H	Proton
^{13}C	13 Carbon
br s	Broad singlet
J	Coupling constant
δ	Delta
°C	Degree celsius
d	Doublet
dd	Doublet of doublet
d_c	Inner diameter of column
g	Gram
λ_{max}	Lambda maximum
m/z	Mass per charge
mg	Miligram
µL	Microlitre
mL	Mililitre
MHz	Megahertz
m	Multiplet
µg/mL	Microgram per millilitre
mg/mL	Miligram per millilitre
nm	Nanometre
±	Plus minus
cm^{-1}	Per centimetre
ppm	Part per million
s	Singlet

t	Triplet
td	Triplet of doublet
CC	Column Chromatography
$CHCl_3$	Chloroform
$CDCl_3$	Deuterated Chloroform
CH_2Cl_2	Dichloromethane
COSY	^1H-^1H Correlation Spectroscopy
DCM	Dichloromethane
DMSO	Dimethylsulphoxide
EIMS	Electron Ionization Mass Spectrum
FDA	Food Drug Administration
FTIR	Fourier Transformation Infra Red
FRIM	Forestry Research Institute Malaysia
GC-MS	Gas Chromatography-Mass Spectrometry
HMQC	Heteronuclear Multiple Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
HREIMS	High Resolution Elecrotrospray Ionization Mass Spectrum
IR	Infrared
LC-MS	Liquid Chromatography-Mass Spectrometry
MS	Mass Spectrometry
MeOH	Methanol
MIC	Minimum Inhibition Concentration
Na_2SO_4	Sodium sulphate
NH_4OH	Ammonium hydroxide solution
NMR	Nuclear Magnetic Resonance
NA	Nutrient Agar
NB	Nutrient Broth

OD	Optical Density
PTLC	Preparative Thin Layer Chromatography
TMS	Tetramethylsilane
TLC	Thin Layer Chromatography
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Introduction

In the eyes of the world, Malaysia is a developing country with tropical rain forests which are rich in the biodiversity of flora and fauna. The forests are also biologically and chemically source of excellent medicine since the plants can synthesize various chemical compounds as defense agents for their survival and growth. According to Jantan (2004), the earliest report on medicinal plant in Malaysia was studied on 205 plants in Sabah. The research reports were mostly on the phytochemical studies, which leading to the discovery of bioactive compounds and are important for drug development.

Most of the world populations use traditional herbal medicine as primary health care (Irchhaiya et al., 2014). Subsequently, studies on the bioactive compounds from plants or phytochemical studies have called lot of attentions globally. Research in Malaysia had focused on medicinal plant such as *Labisia pumila* (kacip fatimah), *Eurycoma longifolia* (tongkat ali), *Clinacanthus nutans* (belalai gajah), *Ficus deltoidea* (mas cotek), *Casia alata* (gelenggang), *Morinda citrifolia* (mengkudu), *Zingiber officinale* (halia), *Melastoma malabatricum* (senduduk), *Phyllanthus niruri* (dukung anak), *Orthosiphon stamineus* (misai kucing) and *Phaleria macrocarpa* (mahkota dewa). They were selected based on the traditional uses and the chemical compounds of the plant (Farizah et al., 2015).

Annonaceae is a flowering plant family and comprises of 130 genera with more than 2000 species (Wiart, 2006). Species from this family are trees, shrubs and climbers that distributed in the tropical and subtropical regions. These species have long been used as traditional medicines to treat diarrhoea, dysentery, fever and rheumatism (Bele at al., 2011; Moghadamtousi et al., 2015). Besides that, Annonaceae plants also used to treat snakebite, respiratory infections, malaria and pneumonia (Okhale et al., 2016; Mustapha, 2013). Scientific studies demonstrated that several species of this family exhibited antiplasmodial (Boyom et al., 2011), antioxidant, antidiabetic (Florence et al., 2014), antinociceptive, anti-inflammatory (Silva et al., 2015), cytotoxic activity (Thuy et al., 2012), insecticidal, antimicrobial (Tan et al., 2015) and anticancer (Piemi et al., 2014). The chemistry of family Annonaceae showed a various group of chemical constituents dominated by alkaloids including aporphinoids, oxoaporphines, phenanthrenes, isoquinolines,

benzylisoquinolines, bisbenzyltetrahydroisoquinolines, protoberberines and tetrahydroprotoberberines (Laboeuf et al., 1982).

Alphonsea cylindrica is a small tree belongs to the family Annonaceae and distributed in lowland forest but not widespread (Hanum et al., 2001). Based on author extensive searches, the chemistry and biological activity of this species has yet to be established and remain to be investigated. In addition, the fruits of *Alphonsea* species were traditionally used as emmanogogue and for diarrhoea and fever treatment (Batugal, 2004). Previous studies reported that *Alphonsea* species have antifungal (Indrani et al., 2015), antioxidant (Narendra, 2009), anticancer, cytotoxic activity (Horgen et al., 2001), anti-inflammatory (Johnson et al. 2013) and antitrypanosomal (Norhayati et al., 2013).

Antibiotic resistance has become a global health issue because of its impact on human death (WHO, 2000). Antibiotic resistance means that the pathogenic bacteria cannot be killed and withstand the effects of antibiotics. When the bacteria become resistant, it has the ability to pass their drug resistant genes to other strains and also to other bacteria. These phenomena showed that this problem can spread easily. In the nut of shell, US Food and Drug Administration (FDA) have encouraged the development of new antibiotics to reduce the bacterial resistance problem. In our study, natural products, particularly from plants, are the most preferred source used to develop new antibiotics.

1.2 Problem statement

Species of *Alphonsea* have been used as traditional medicines to treat bacterial-related diseases such as diarrhoea and fever, as well as emmenagogue. Current phytochemical studies of these species resulted in isolation of alkaloids as a dominant chemical constituent. Moreover, they were demonstrated pronounced biological activities including antimicrobial, anticancer, anti-inflammatory and antioxidant. High content of alkaloids has been indicated in the stem bark of *A. cylindrica* (Teo et al., 1990). However, investigation on chemical constituents including alkaloids of this plant has yet to be established and thus requiring more research.

In current situation, multi-drug resistance bacteria have spread widely and cause the treatment for the infectious disease becomes limited and difficult to solve. Normally the bacterial infection can be treating effectively by using antibiotics. However, when the bacteria become resistance, the antibiotics are no longer effective for the treatment. Antibiotics are sometime also associated with adverse side effects such as hypersensitivity, immuno suppressent and allergic reactions (Hussain et al., 2011).

Therefore, there is a need to study the chemical compounds from natural source such as plant that have antibacterial properties in order to discover the new antibacterial drug. Moreover, natural sources were reported to have fewer side effects, affordable, better patience tolerance and being renewable in nature (Hema et al., 2013).

1.2.1 Significance of study

This is the first phytochemical and biological studies of species *A. cylindrica*. Antibacterial activity of crude extract and chemical compounds isolated from this plant may serve as future references for development of new antibacterial drugs from natural resources.

1.3 Objectives

The objectives of this study were:

1) To extract, isolate and purify chemical compounds from the crude extracts of *A. cylindrica* barks using chromatographic techniques.

2) To identify and elucidate the molecular structures of the isolated compounds using modern spectroscopic techniques.

3) To investigate antibacterial activity of the crude extracts and isolated compounds from barks of *A. cylindrica* against Gram negative and Gram positive bacteria.

CHAPTER 2

LITERATURE REVIEW

2.1 Annonaceae

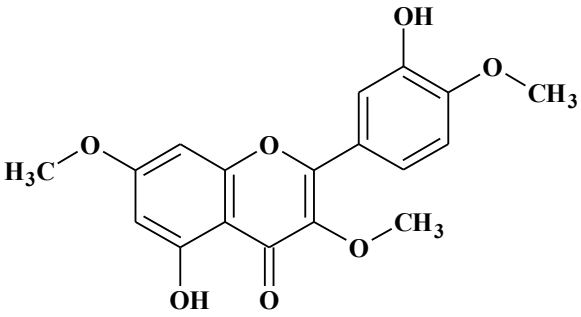
Annonaceae family is a family of flowering plant of trees, shrubs and climbers. This family is called as the custard apple family and locally known as „Mempisang" in Malaysia (Burkill, 1966). This family is also the largest family in Magnoliales that consisted of 130 genera with more than 2000 species. Of these species, as many as 60 species have been used in traditional medicine in Asia and the Pacific (Wiart, 2006). These species are mostly found in tropics region and some species found in temperate regions. According to Sinclair (1955), there are 17 varieties of Annonaceae species in Malaysia. They grow in the lowland forest at below 2000 ft.

Annonaceae plants contain a large number of chemical compounds such as alkaloid, flavonoid and acetogenins (Lúcio et al., 2015; Esquinca et al., 2014). These compounds had shown several pharmacological activities that useful as medicine. Some examples of chemical compounds from Annonaceae and their pharmacological activities are listed in Table 2.1.

Table 2.1

Chemical compounds from Annonaceae plants with their pharmacological activities

No. of compounds	Chemical compounds	Pharmacological activities	Plant source	References
1	5,3′-dihydroxy-3,7,4′-trimethoxyflavone	Anticancer ; [murine lymphocytic leukemia (P388), human colon cancer (Col-2), human breast cancer (MCF-7)]	*Miliussa smithiae*	Naphong et al., 2013
2	Desmosdumotin C	Antioxidant, anti-inflammatory, and anti-*Hpylori*.	*Mitrella kentia*	Sidahmed et al., 2013
3 **4**	Enterocarpam-III Stigmalactam	Cytotoxicity ; [human colon adenocarcinoma (HCT15) cell line.]	*Orophea enterocarpa*	Nayyatip et al., 2012
5 **6**	Pseuduvarine A Pseuduvarine B	Cytotoxicity; [breast cancer cells (MC7) human promyelocyctic leukemia (HL-60) cell lines]	*Pseuduvaria rugosa*	Taha et al., 2011
7 **8**	2′,4′-Dihydroxy-4,6′-dimethoxychalcone *O*-methylmoschatoline	Anti-bacterial; [*B.subtilis, E.aerogenes, E.coli, B.subtilis* and *S.aureus*]	*Ellipeia cuneifolia*	Yusof et al., 2015
9	Anonaine	Vasorelaxant, antibacterial, antifungal, antioxidative, anticancer and antidepressant.	*Michelia alba*	Li et al., 2013



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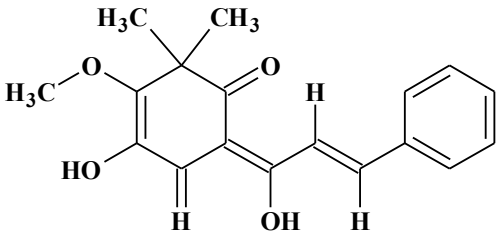

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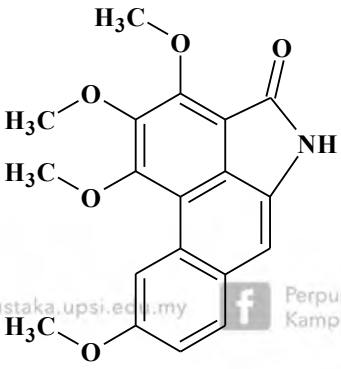

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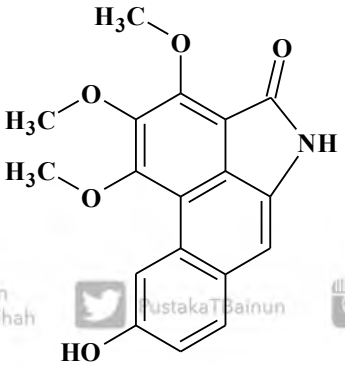

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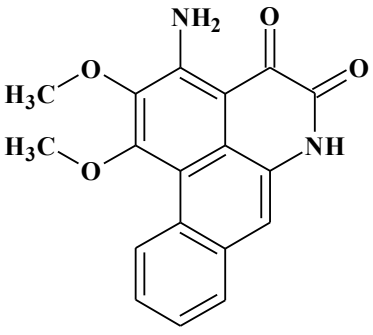

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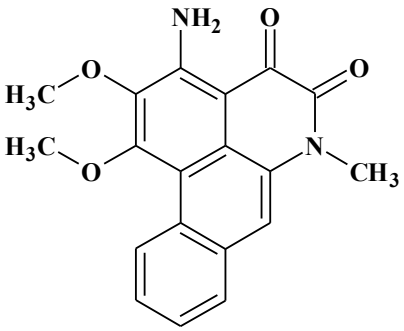

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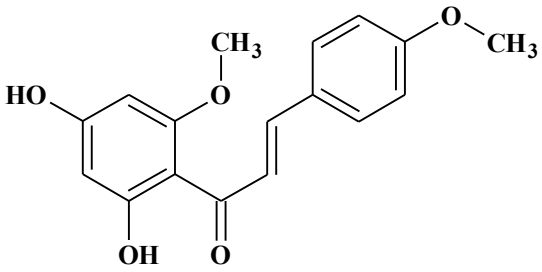

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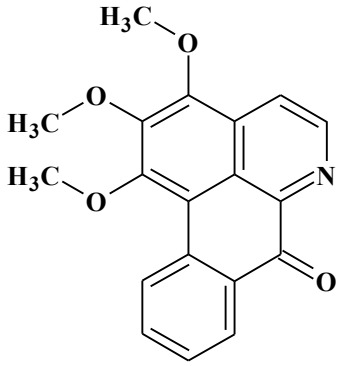

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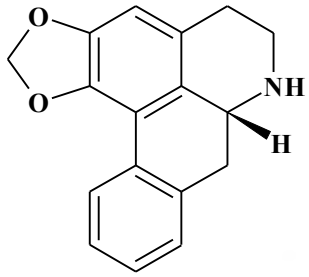

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For the classification, Annonaceae was divided into four subfamilies, Anaxagoreoideae, Ambavioideae, Annonoideae and Malmeoideae. According to Chatrou et al. (2012), subfamilies Annonaideae and Malmeoideae contain 95 % of species in the family. Tribes Miliuseae from subfamily Annonaideae comprises of six genera; *Alphonsea*, *Marsipopetalum*, *Miliusa*, *Phaeanthus*, *Platymitra* and *Orophea*. Most of these genera can be found in Asia. The classifications of Annonaceae were summarized in Figure 2.1 and 2.2.

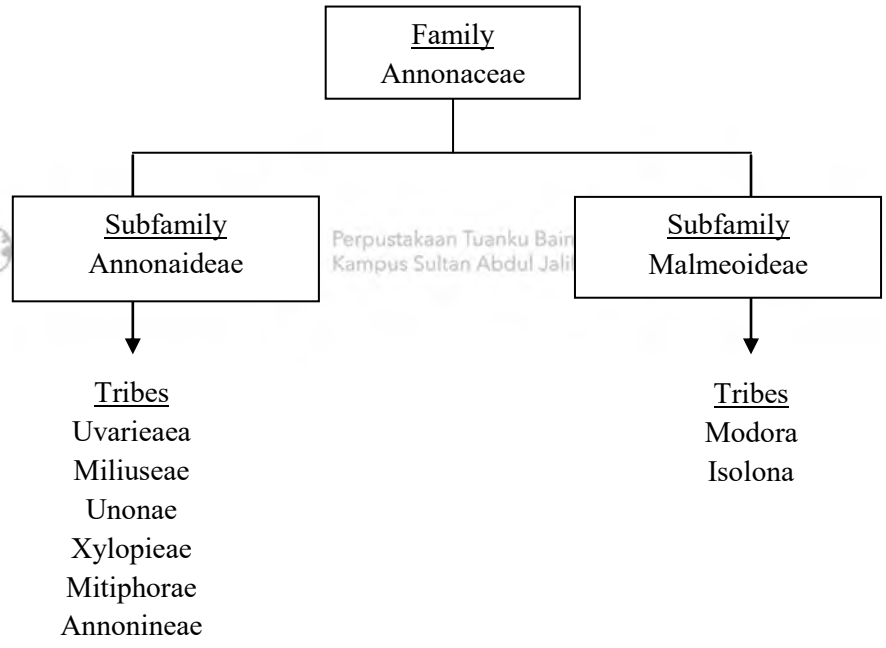


Figure 2.1. Classification of Annonaceae

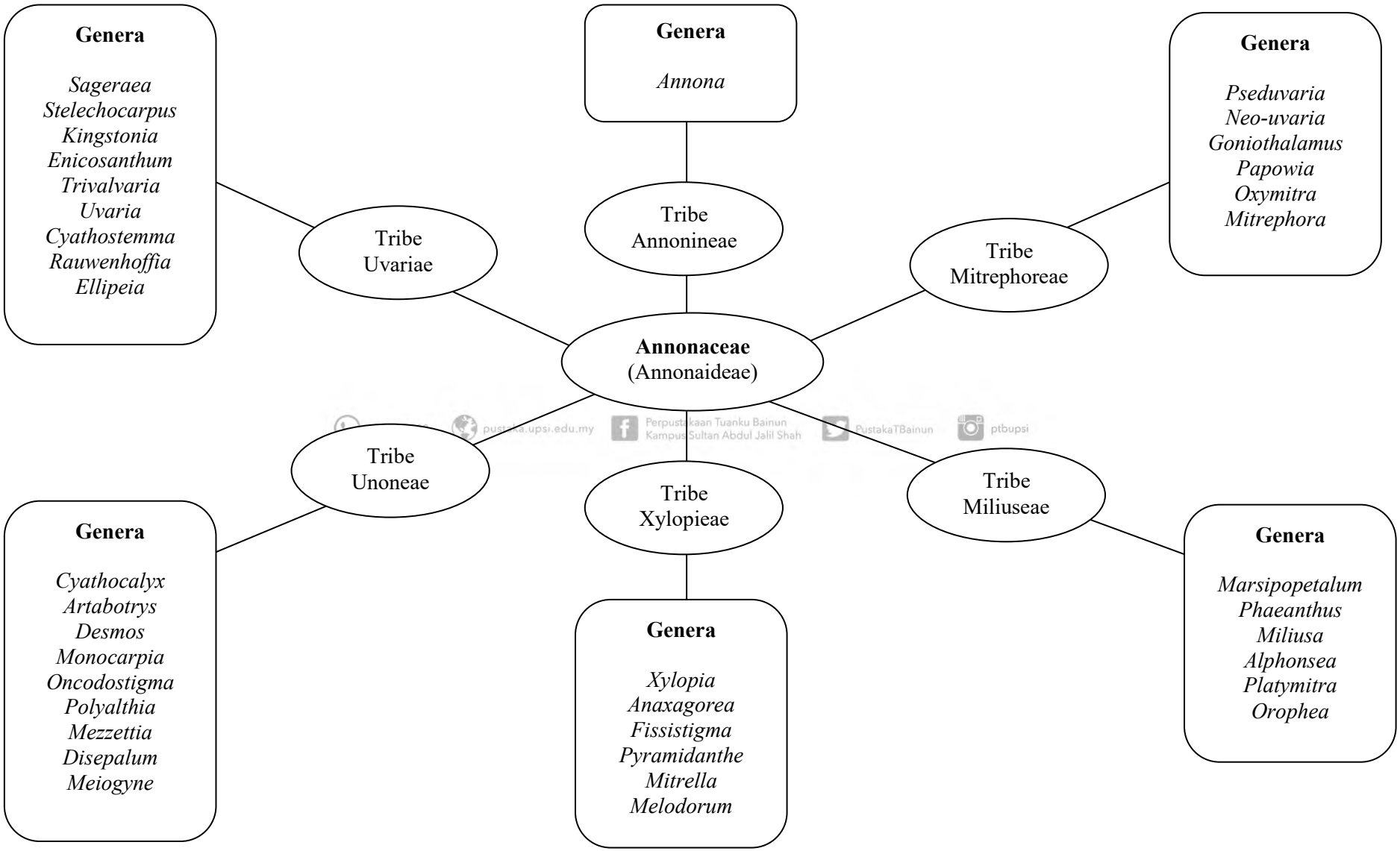


Figure 2.2. Classification of Annonaceae (Subfamily Annonaideae)

2.2 *Alphonsea* sp.

Alphonsea is a small genus of the family Annonaceae. According to The Plant List (2013), as many as 37 *Alphonsea* species have been recognized. Species from this genus are distributed in China and Indo-Malayan (Figure 2.3). In China, *Alphonsea* species were mainly found in Hainan and South Yunnan. Meanwhile in Asia, they can be found in India, Sri Lanka, Myanmar, Thailand, Laos, Vietnam, Cambodia, Malaysia, Indonesia and Papua New Guinea (Srivastava & Mehrotra, 2013). Genus *Alphonsea* were narrowly distributed and only very few species scattered throughout tropical Asia. In Malaysia, eight *Alphonsea* species have been identified, including *A. borneensis* (Turner, 2009)*, A. curtisii, A. elliptica, A. johorensis, A. maingayi, A. rugosa* (Latiff, 2013; FRIM, 2014; Turner & Utteridge, 2015), *A. kingii* and *A. cylindrica* (Turner, 2016). However, *A. hainanensis, A. monogyma* and *A. tsangyuanensis* have been listed as endangered species by the IUCN Red List of Threatened Species (2010).

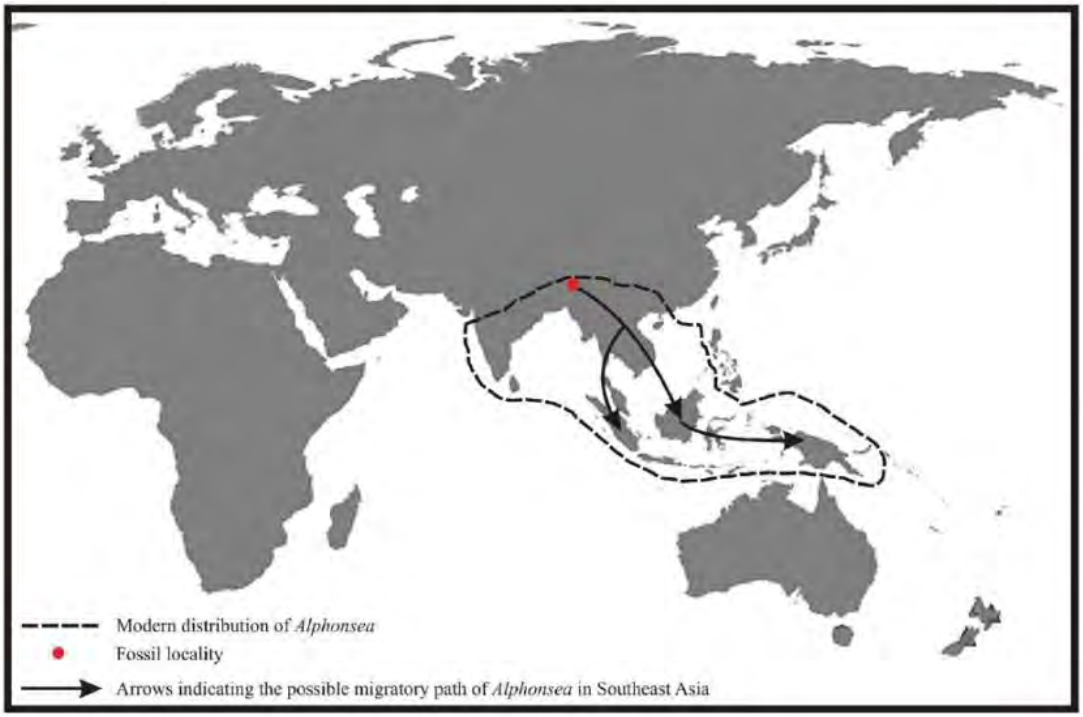


Figure 2.3. Distribution of *Alphonsea* and possible migratory path of *Alphonsea* in Southeast Asia (adapted from Srivastava & Mehrotra, 2013)

2.2.1 Botanical aspect and morphology of *Alphonsea* species

The Genus *Alphonsea* is consisting species of trees or shrubs with about 16 m tall (Turner, 2009). The leaves are elliptic, leathery, shiny, hairy and opposite. Only very few species of *Alphonsea* have flowers. The flowers are mostly bisexual and usually form clusters. In addition, the sepals are much smaller than the petals. The stamens of these plants are mostly numerous. The ovaries are 4 to 24 per carpel, while the sigma inconspicuously capitates with a slit down inner side. The fruits are globose to cylindrical with thick wall, hairy or warty with several seeds per monocarp. Few species of *Alphonsea* species have glabrous fruits, including *A. javanica, A. tonquinensis* and *A. borneensis*. The fruits are nearly sessile to stalked. Some of the species have aromatic barks such as *Alphonsea maingayi* black barks produce strong cucumber odour when cut (Figure 2.4).

(a) (b)

Figure 2.4 (a) Herbarium specimen of *Alphonsea borneensis;* (b) Leaves and trunk of *Alphonsea maingayi*

2.2.2 Traditional uses of *Alphonsea* species

Different parts of *Alphonsea* species have been traditionally used by natives. For example, the timber of the Indian *A. ventricosa* is used in boat making in the Andamas (Burkill, 1966). Moreover, the ripe fruits of *A. ventricosa, A. mollis, A. hainanensis* and *A. lutea* are edible (Kar et al., 2013; Shu et al., 2011). The wood of *A. mollis, A. hainanensis* and *A. monogyma* are used for the construction of carts and agricultural implements. In addition, the flower of *A. monogyma* is also fragrant which is used for perfumery (Shu et al., 2011).

According to Batugal et al. (2004), the boiled fruits of *A. arborea* have been traditionally used to treat diarrhoea and fever as well as emmenagogue or to stimulate menstrual flow. However, the leaves of *A. ventricosa* and *A. javanica* have been reported to contain a poisonous alkaloid known as alphonsein, which is also present in several genera of the family Annonaceae (Burkill, 1966).

2.2.3 Phytochemistry of *Alphonsea* species

There were only a few phytochemical studies on *Alphonsea* species have been reported thus far. A number of chemical compounds including alkaloids, terpenoids and essential oil have been analysed. Most of the phytochemical studies were conducted on the bark, leaves and stem bark of the plants. The chemical compounds isolated from the *Alphonsea* species are summarized in Table 2.2.

Table 2.2

Chemical compounds isolated from Alphonsea species

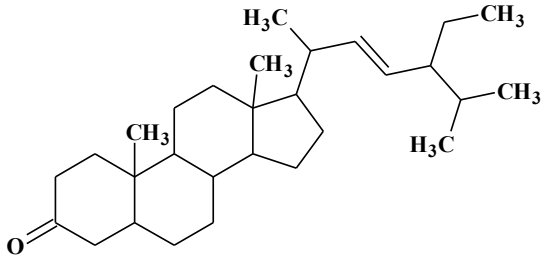
No	Genus *Alphonsea*	Plant part	Chemical compounds	References
1	*A. curtisii*	S	Stigmast-22-ene-3-one (**10**)	Jalil et al., 2015
		S	Stigmast-22-ene-3,6-dione (**11**)	Jalil et al., 2015
		S	Stigmasta-4,6,22-trien-3-one (**12**)	Jalil et al., 2015
2	*A.gaudichaudi-ana*	L	*β*-Caryophyllene (**13**)	Thang et al., 2013
		L	*(E)-β*-Ocimene (**14**)	Thang et al., 2013
		L	g-Eudesmol (**15**)	Thang et al., 2013
		L	Bicyclogermacrene (**16**)	Thang et al., 2013
		L	Bycycloelemene (**17**)	Thang et al., 2013
		L	Guaiol (**18**)	Thang et al., 2013
		L	Viridiflorol (**19**)	Thang et al., 2013
3	*A. javanica*	-	(+)-Altholactone (**20**)	Johnson et al., 2013
4	*A. mollis*	B	(2*R*,3*R*)-2,3-Dihydro-2-(4-hydroxy-3-methoxy phenyl)-3-methyl-5-(*E*)-propenylbenzofuran (**21**)	Xie et al., 1994
		B	Conocarpan (**22**)	Xie et al., 1994
		-	8-Hydroxy-5-methoxyliriodenine (**23**)	Bently 2001
		SB	Oxostephanine (**24**)	Xie et al., 1989
		SB	Liriodenine (**25**)	Xie et al., 1989
		B	Mollisine (**26**)	Xie et al., 1994
		SB	5-Hydroxy -2,6-dimethoxyonychine (**27**)	Xie et al., 1989
5	*A. monogyma*	-	Guaiol (**18**)	Yang et al., 1999
		-	Liriodenine (**25**)	Yang et al., 1999
		-	5-Hydroxy -6,7-dimethoxyonychine (**28**)	Xie & Yang, 1999
		-	Cyathocaline (**29**)	Yang et al., 1999
		-	Darienine (**30**)	Yang et al., 1999
		Br. & S	5-Hydroxy-6,7-dimethoxyonychine *N*-oxide (**31**)	Yang et al., 2000
		-	Isooncodine (**32**)	Yang et al., 1999
6	*A. philastreana*	L	*β*-Caryophyllene (**13**)	Thang et al., 2013
		L	g-Eudesmol (**15**)	Thang et al., 2013
		L	Bicyclogermacrene (**16**)	Thang et al., 2013
		L	Bycycloelemene (**17**)	Thang et al., 2013
		L	Guaiol (**18**)	Thang et al., 2013
		L	*α*-Humulene (**33**)	Thang et al., 2013
		L	*β*-Pinene (**34**)	Thang et al., 2013
7	*A. sclerocarpa*	B	Anonaine (**9**)	Tadić et al., 1987
		B & L	Guaiol (**18**)	Tadić et al., 1987
		B & L	Liriodenine (**25**)	Tadić et al., 1987
		B	Candicine (**35**)	Tadić et al., 1987
		L	Crotsparine (**36**)	Tadić et al., 1987
		L	Isoboldine (**37**)	Tadić et al., 1987
		L	Laurotetanine (**38**)	Tadić et al., 1987
		L	Petalinemethine (**39**)	Tadić et al., 1987

(continued)

Table 2.2 *(Continued)*

No	Genus *Alphonsea*	Plant part	Chemical compounds	References
		B	Stepholidine (**40**)	Tadić et al., 1987
		B	Magnoflorine (**41**)	Tadić et al., 1987
		B	Phenethyltrimethylammonium (**42**)	Tadić et al., 1987
		L	Sparsiflorine (**43**)	Tadić et al., 1987
		B	Stepharine (**44**)	Tadić et al., 1987
		B	Norushinsunine (**45**)	Tadić et al., 1987
		B	Ushinsunine (**46**)	Tadić et al., 1987
8	*A. ventricosa*	-	2-(8,10-Heptadecadiinyl)furan (**47**)	Gopinath et al., 1976
		-	2-(12t-Heptadecaen-8,10-diinyl)furan (**48**)	Gopinath et al., 1976
		-	1,2-Dihydroxy-12,14-heneicosadiin-4-on (**49**)	Gopinath et al., 1976
		L	Glaucine (**50**)	Mahanta et al., 1976
		L	Norglaucine (**51**)	Mahanta et al., 1976

Note: L= Leaves; B= Bark; Br.= Branches; S= Stem; SB= Stem bark



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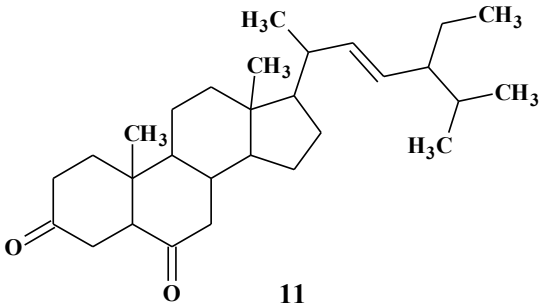

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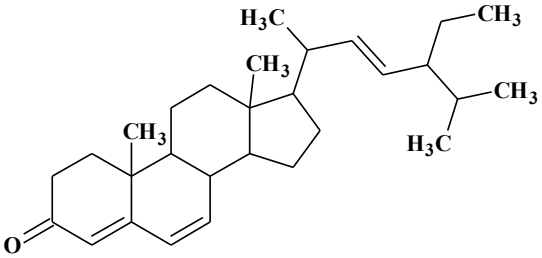

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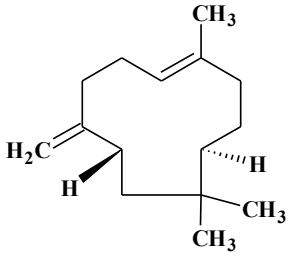

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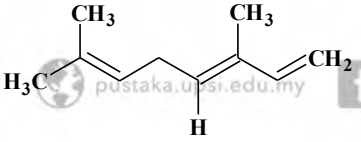

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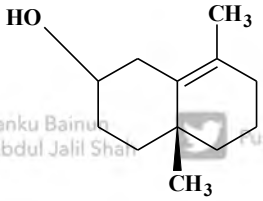

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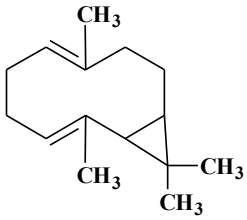

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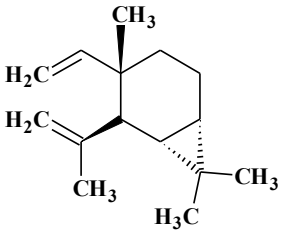

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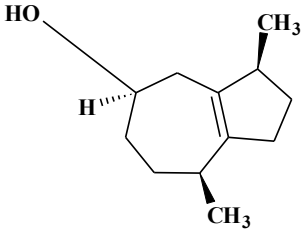

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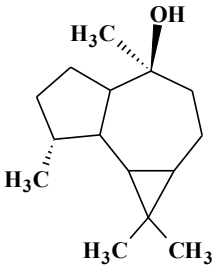

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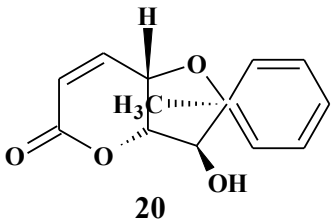

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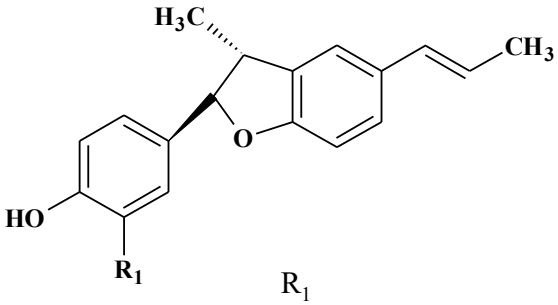
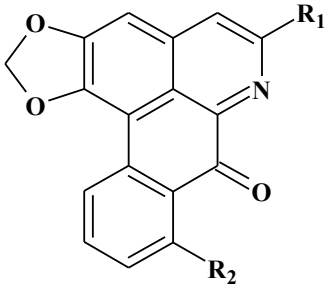
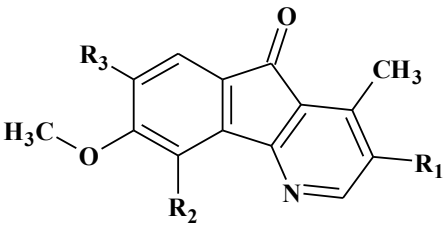


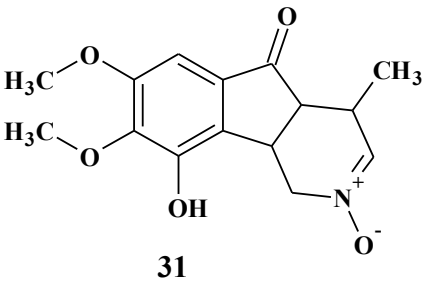
	R_1
21	CH_3
22	H



	R_1	R_2
23	OCH_3	OH
24	H	OCH_3
25	H	H
26	OCH_3	OCH_3



	R_1	R_2	R_3
27	OCH_3	OH	H
28	H	OH	OCH_3
29	H	OH	OH
30	H	OCH_3	OH



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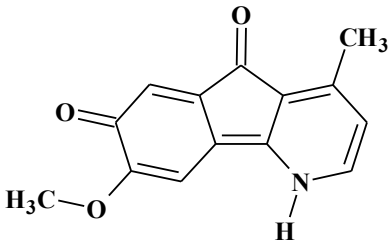

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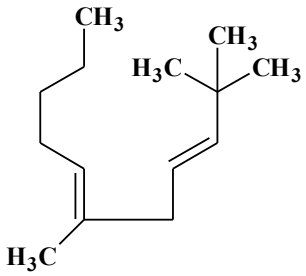

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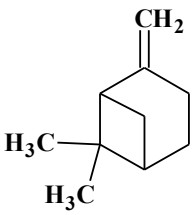

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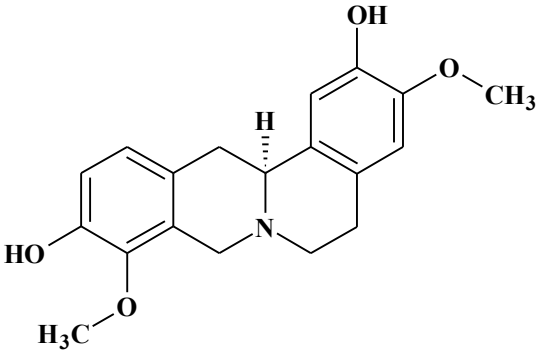

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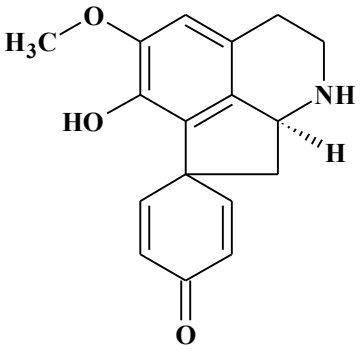

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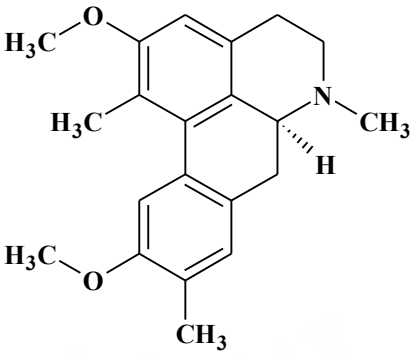

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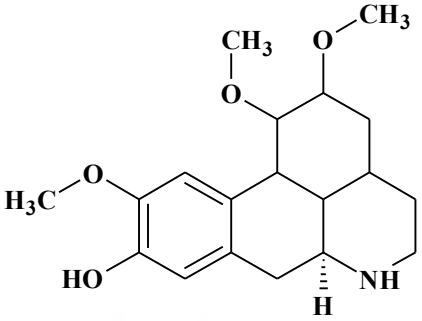

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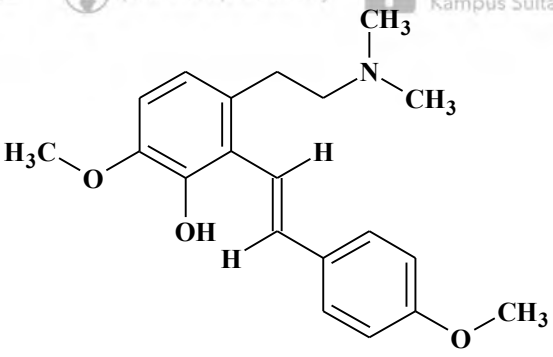

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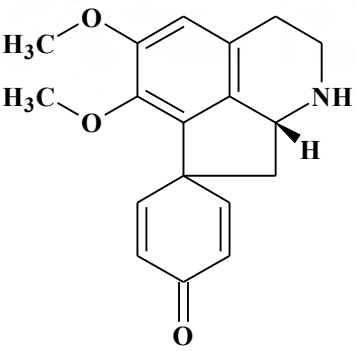

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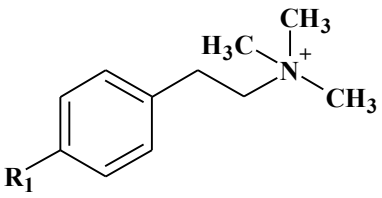
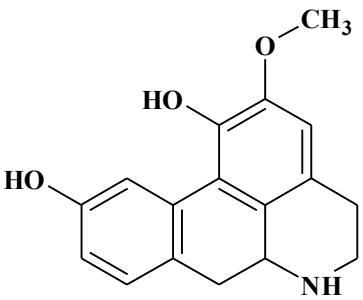


R_1

41 H

42 CH_3



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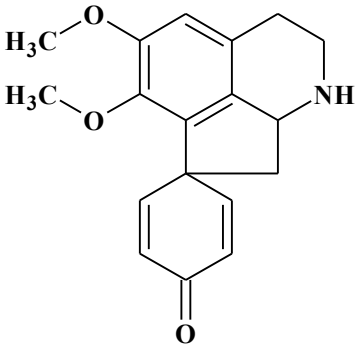

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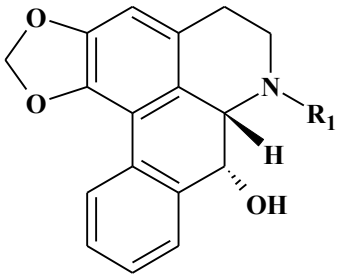
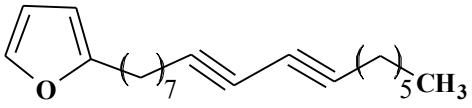


R_1

45 H

46 CH_3



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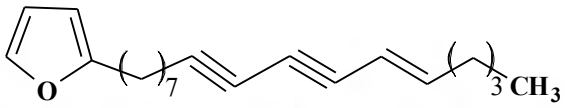

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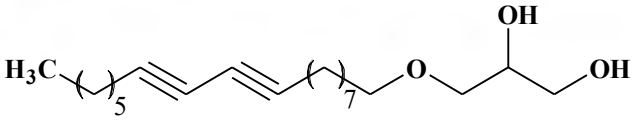

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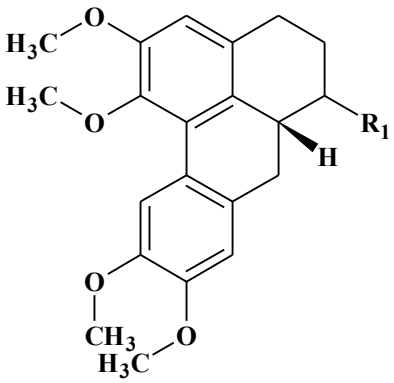


R_1

50 CH_3

51 H

2.2.4 Biological activities of *Alphonsea* species

Based on extensive searches, very few investigations on biological activities of *Alphonsea* species have been conducted thus far. According to Horgen et al. (2001), the root extract of an unnamed *Alphonsea* species, has been reported to exhibit toxicity towards brine shrimps at a concentration of 100 µg/mL and was cytotoxic against human epidermoid carcinoma and murine lymphoid neoplasm cancer cell lines with $IC_{50} \leq 20\mu g/mL$.

Besides that, the ethanolic extract of *A. sclerocarpa* bark measured by DPPH and hydroxyl radicals showed a remarkable antioxidant activity as compared to positive controls, Vitamin E and catechin (Narendra, 2009). In addition, the extract of *A. sclerocarpa* exhibited fungal inhibition against dandruff-associated fungi, *Aspergillus flavus,* when compared with local herbs shampoo (Indrani et al., 2015). According to Johnson et al. (2013), the methanol extract of Indonesian *A. javanica* also possessed anti-inflammatory activity, while the methanol extract of *A.maingayii* leaves and stem showed antitrypanosomal with $IC_{50} > 12.5$ µg/mL (Norhayati et al., 2013).

2.3 *Alphonsea cylindrica* King.

In Malaysia, *A. cylindrica* King. is found in lowland forest, but not widespread and is locally known as „mempisang"(Hanum et al., 2001). *A. cylindrica* can grow up to 20 m tall and 0.18 m in diameter. The outer bark of this plant is greyish or brownish when mature, while the inner bark is pale yellow or brown in colour. The bark is also fissured to crack. The leaves are 6.5-11.5 cm X 3-5 cm in size, elliptic and alternate simple (Figure 2.5 (a)). This plant has shining dark green colour on the upside leaves, while pale green on the downside. The secondary nerves of the leaves have 10-15 pairs joining near the margin, tertiary nerves and then reticulate forming a network. The fruits are globose to cylindrical and hairy (Figure 2.5 (b)). Based on extensive search, the phytochemical and pharmacological aspects of *A. cylindrica* have yet to be published and thus requiring more research.

(a) (b)

Figure 2.5. (a) Herbarium specimen of *A. cylindrica;* (b) Fruits of *A. cylindrica*

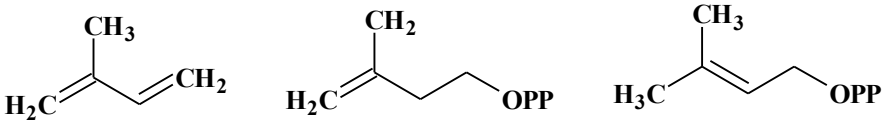
2.4 General chemical aspects

The studies of chemical compounds from plants usually refer to phytochemical study. Phytochemical is grouped into two main categories; primary compounds and secondary compounds. Primary compounds in plant occur naturally for the plant growth and development (Sivarajan, 1991). Meanwhile secondary compounds are derived by unique biosynthetic pathways from primary metabolites and intermediates (Krishnaiah et al., 2009). In addition, secondary compounds are bioactive compounds that have pharmacological or toxicological effects in man and animals which can provide opportunities for new drug discoveries (Bernhorf, 2010). Based on the review, terpenoids, alkaloids and essential oil were isolated from genus *Alphonsea.* Therefore, general chemical aspects for terpenoids and alkaloids are briefly discussed in this chapter.

2.4.1 Terpenoids

Terpenoids are one of the natural products produced by plants, insects, animals and microorganisms. The term terpenes originate from turpentine, the so-called “resin of pine trees”. Terpenoid is a general name for terpenoids and terpenes, more strictly, terpenoid is a terpene with hydroxyl, carbonyl or carboxyl function. These compounds are the most important constituents in essential oils of plants with over than 20 000 known structures (Breitmaier, 2006). According to Nassar et al. (2010) terpenoids had been defined as the secondary metabolites with molecular structures containing carbon backbones made up of isoprene (2-methylbuta-1, 3-diene) units

(**52**). Instead of isoprene, terpenes also can form from isopentyl pyrophosphate (IPP) (**53**) and dimethylallyl phosphate (DMAPP) (**54**) which are synthesized from acetyl-CoA in melavonic acid pathway.



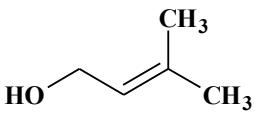
52 **53** **54**

The classification of terpenoids is based on the number of isoprene unit. An isoprene unit contains five carbons and eight hydrogens (C_5H_8). Then, the carbon skeleton of terpene builds from the isoprene unit, which is also called isoprene rule found by Ruzicka and Wallach (Breitmaier, 2006). In addition, isoprene units are typically linked in a head-to-tail fashion in terpenes. Table 2.3 summarized the classification of terpenes.

Table 2.3

The classification of terpenes based on the number of C_5 isoprene units in their structures (Kabera et al., 2014)

Isoprene units, n	Carbon atom	Name	Example
1	C_5	Hemiterpenes	Prenol (**55**)
2	C_{10}	Monoterpenes	Limonene (**56**)
3	C_{15}	Sesquiterpenes	δ-Codinene (**57**)
4	C_{20}	Diterpenes	Phytol (**58**)
5	C_{25}	Sesterterpenes	Geranylfarnesol (**59**)
6	C_{30}	Triterpenes	Squalene (**60**)
8	C_{40}	Tetraterpene	β-Carotene (**61**)
9-30000	$C_{>40}$	Polyterpenes	Rubber (**62**)



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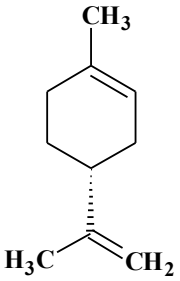

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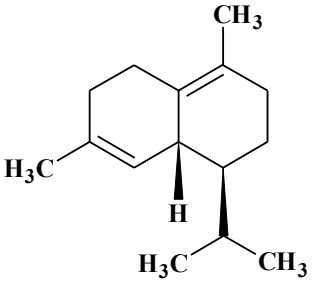

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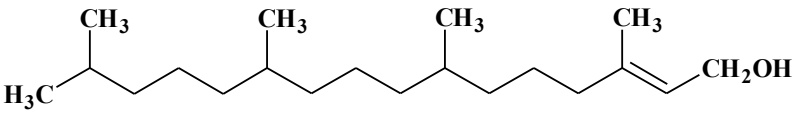

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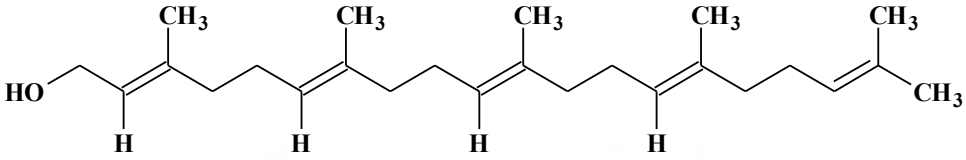

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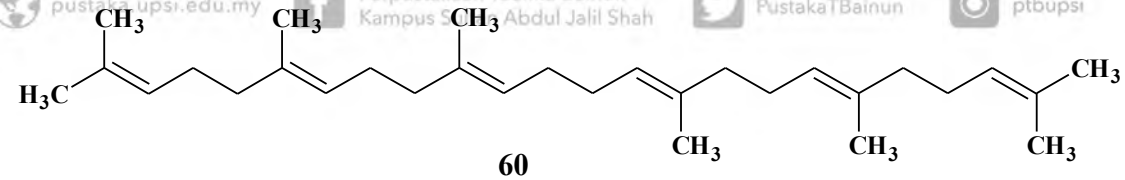

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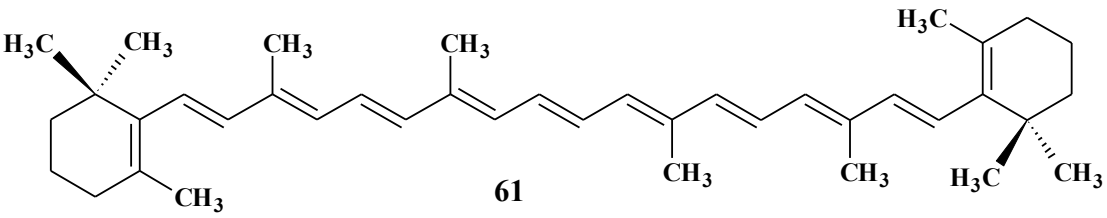

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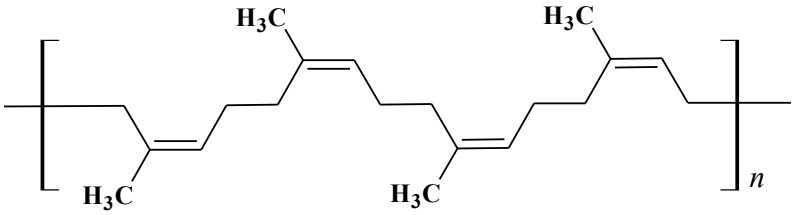

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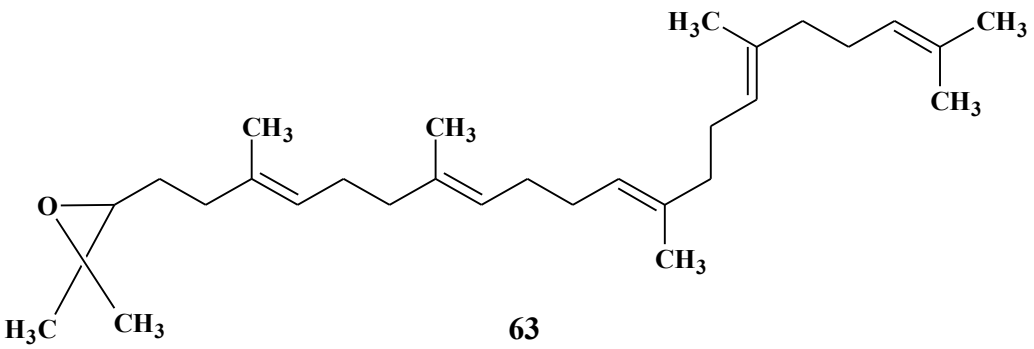
Terpenoids is the largest class of plant metabolites since more than 36 000 terpenoids compounds have been identified. These compounds show significant pharmacological activities such as antiviral (Bajpai et al., 2016), antimicrobial (Cherdtrakulkiat et al., 2015), antimalarial (Onguéné et al., 2013), anti-inflammatory (Han & Bakovic, 2015) and anti-osteoarthritic (Gabay et al., 2010).

a. Triterpenes

Triterpenoid are highly diverse group in natural products and widely distributed in plants. Triterpenes are chemical compounds derived from six isoprene units. The molecular formula is $C_{30}H_{48}$. Squalene (**60**) was a C_{30} precursor that first isolated from shark liver (Bruneton, 1995). According to Michaudel (2013), triterpenes can be found in their free form or bound to glycosides. Moreover, biosynthesis of triterpene derived from mevalonic acid pathway (MVA), produce IPP (**53**) and DMPP (**54**) as the final product. From the mevalonic pathway, squalene is formed by the condensation of two units of farnesyl pyrophosphate. After that, squalene monooxygenase oxidize squalene to 2, 3-oxidosqualene (**63**) and then this intermediate catalyzed by oxidosqualene cyclase to form triterpenes (Sawai & Saito, 2011).



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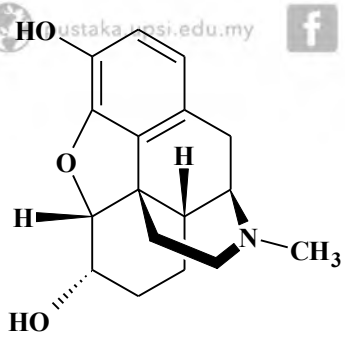
2.4.2 Alkaloids

Alkaloids are defined as natural compounds that have a basic character and contain at least one nitrogen atom in a heterocyclic ring and have biological activity. According to Dictionary of Organic Chemistry (Daintith, 2004), alkaloids contain oxygen and nitrogen atom and most are poisonous. However, they are numbers of important drugs such as morphine (**64**), codeine (**65**), caffeine (**66**), cocaine (**67**) and nicotine (**68**). Alkaloids had been found in 15-30% of all flowering plants and commonly found in certain family of plant; Annonaceae, Fabaceae, Liliaceae, Ranunculaceae, Apocynaceae, Solanaceae, and Papaveraceae (Pengelly, 2004).



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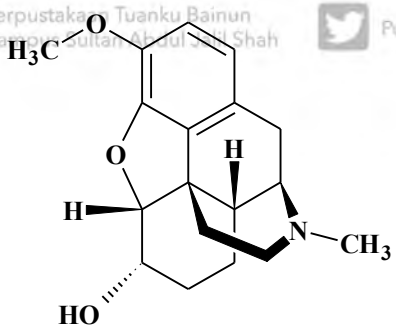

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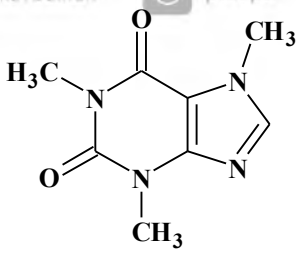

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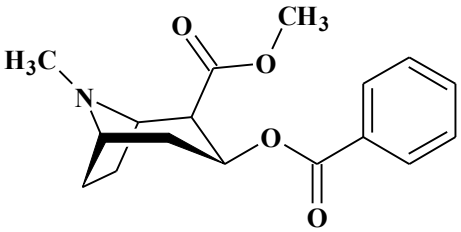

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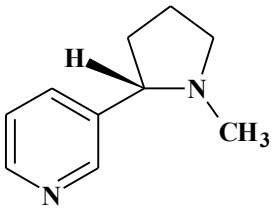

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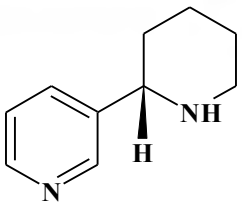
Alkaloids have been classified into three classes based on the biogenetic pathway; true alkaloids, protoalkaloid and pseudoalkaloid (Aniszewski, 2007). True alkaloid derived from amino acid and have nitrogen atom in a heterocyclic ring, for example anabasine (**69**), while protoalkaloid also derived from amino acid but do not have nitrogen atom in a heterocyclic ring, e.g. epinephrine (**70**). For pseudoalkaloid, the compound was not derived from amino acid but have nitrogen atom in a heterocyclic ring. These groups may terpene-like, steroid-like or purine-like e.g. caffein (**66**). The true alkaloids can be classified into different types of skeletons; (i) pyrrolidine, (ii) pyridine-piperidine, (iii) isoquinoline, (iv) quinoline, (v) indole and (vi) eryrtrina. In this review only several groups including aporphine, oxoaporphines and azafluorenone are discussed briefly.



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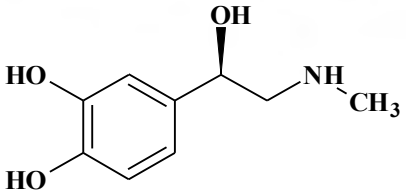

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a. Aporphine

According to Lúcio et al., (2015), aporphine types of alkaloid represent the prominent group in family Annonaceae. This type of alkaloid had been isolated from more than 20 plant families and gives high interest to the researcher to study their biological activity (Udvardy et al., 2014). The general backbone skeleton of aporphine is shown in Figure 2.6. In addition, about 800 alkaloids which consist of isoquinolines, protoberines, aporphines and others had been isolated from different genera of Annonaceae (Lúcio et al., 2015).

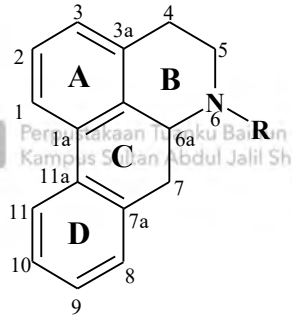


Figure 2.6. The general backbone skeleton of aporphine

b. Oxoaporphines

Oxoaporphine alkaloids represent as the most highly oxidized state of the aporphine skeleton. This type of alkaloid usually have colour such as yellow, orange or orange red because of the high degree of aromaticity of the compounds. Besides that, oxoaporphine were also found to accompany aporphines alkaloid in most plants while the numbering system of the compound structure is same as that of the aporphine. In oxoaporphine structure, C-7 was substituted with carbonyl group (Stévigny et al., 2005; Bentley, 1965) as shown in Figure 2.7. Scheme 2.1 showed the biogenetic pathway of aporphine to an oxoaporphine.

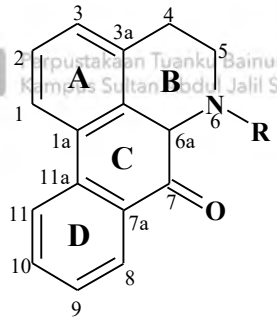
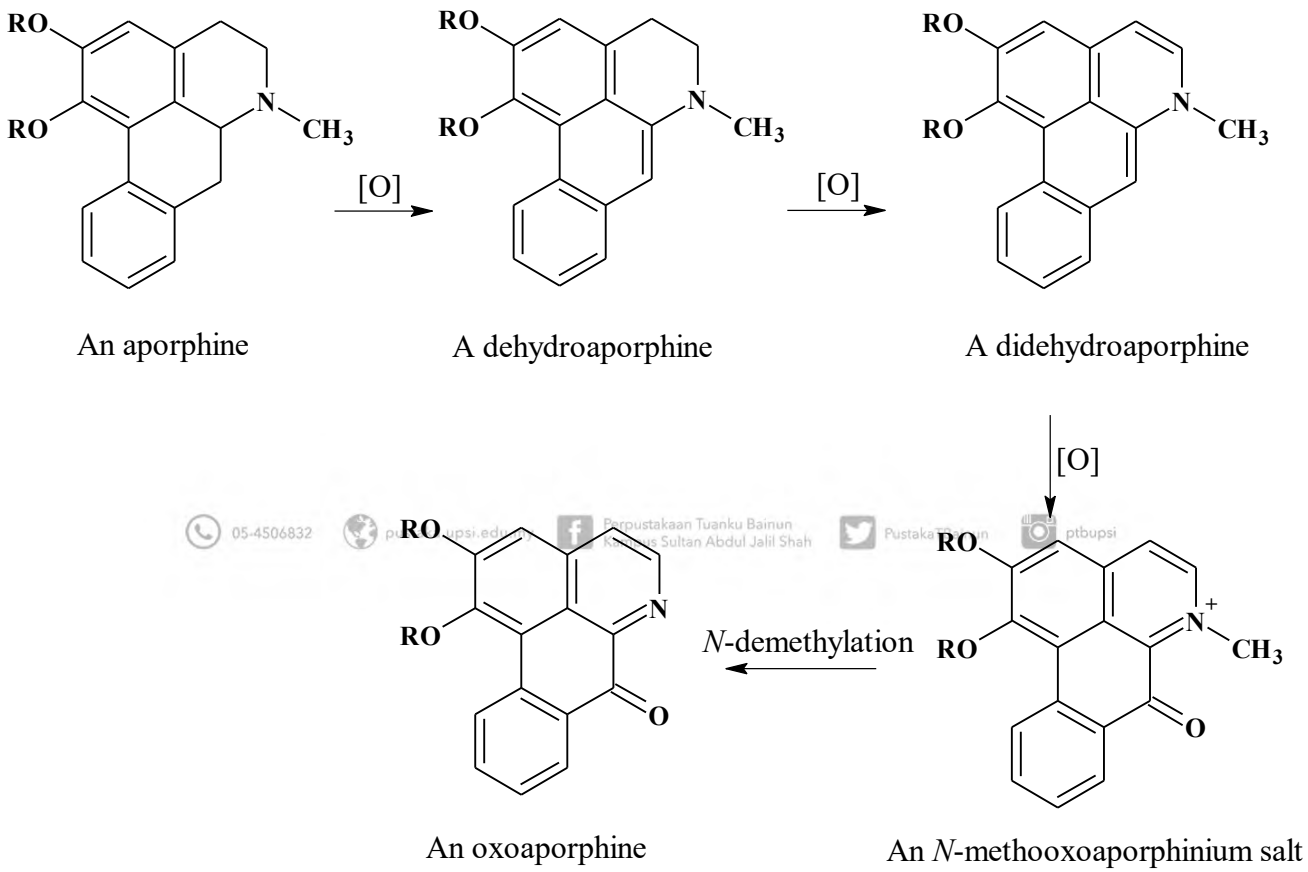


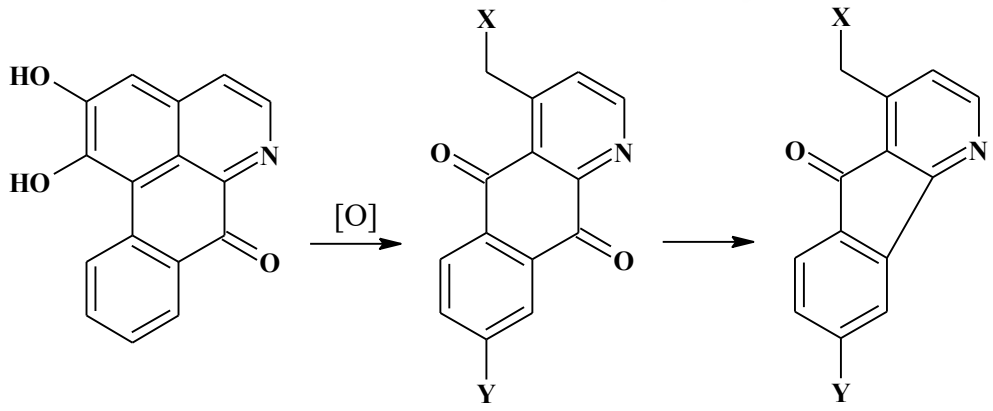
Figure 2.7. The skeleton of oxoaporphine



*Scheme 2.1.*The biogenetic pathway to an oxoaporphine

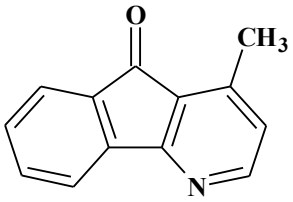
c. Azafluorenone

In Annonaceae, azafluorenone alkaloid is one of the alkaloid types that have been isolated. Azafluorenones are fused tricyclic compounds that are pyridine analogs to fluorenones. The first identified azafluorenone alkaloid was onychine (4-methyl-1-azafluoren-9-one) (**71**). Onychine would be derived from the liriodenine, lysicamine or other 1, 2-dioxygenated oxoaporphine via the azaantraquinone cleistopholine (Scheme 2.2). It was postulated that one, two or three oxygen atoms may be introduced at different positions of the benzene ring; either at the azaantraquinone stage or once the azafluorenone skeleton has been formed (Arango et al., 1987). From literature, onychine (**71**) and 7-methoxyonychine (**72**) have been isolated from the roots of *Polyalthia debilis* by using solvent chloroform in the extraction (Prachayasittikul, 2009).



1,2-dioxygenated oxoaporphine Azaantraquinone Azafluorenone

Scheme 2.2. The derivation of onychine



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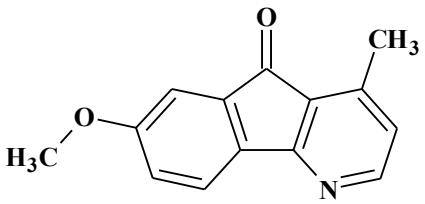

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2.5 Isolation and elucidation of chemical compounds

Isolation of chemical compounds from plant extract or fraction is a process of separation in phytochemical analysis. According to Sarker et al. (2006), the isolation process was done based on the solubility (hydrophobicity or hydrophilicity), acid-base properties, charge, stability and molecular size of the chemical compounds. Chromatography is the most widely used techniques in the isolation process.

Chromatographic techniques can be classified into two categories; classical and modern (Sarker et al., 2006). The classical chromatographic techniques are thin layer chromatography (TLC), preparative thin-layer chromatography (PTLC), open-column chromatography (CC) and flash chromatography (FC). The most widely used modern chromatographic techniques are high-performance thin-layer chromatography (HPTLC), vacuum liquid chromatography (VLC), chromatotron and high-performance liquid chromatography (HPLC). In this study, TLC, PTLC and CC were used in the isolation of chemical compounds from *A. cylindrica* extracts. These chromatographic techniques usually involve two phases which are stationary phase and mobile phase (Houghton & Raman, 1998). In order to isolate the pure chemical compounds, the suitable solvent polarity and its ratio are important. After obtaining

the pure chemical compounds, the end points are the elucidation and identification of the chemical compounds.

For the known chemical compound, the comparison spectroscopic data with the literature data of standard compound can be done. However, the unknown or new compounds need comprehensive analysis to identify the chemical structures. Nowadays, various spectroscopic methods are available to obtain the information of the chemical structures. The spectroscopic methods that are widely used in organic chemistry are nuclear magnetic resonance (NMR), mass spectrometry (MS), infrared spectroscopy (IR), ultraviolet-visible spectroscopy (UV-vis) and x-ray crystallography (Balci, 2005).

2.6 Antibacterial analysis

Antibacterial analysis is important and become a high interest among the researchers because of the increasing numbers of bacterial infections and antibiotic-resistance bacteria. Moreover, these matters have become the world problem including health, economic and social problem (Chudobova et al., 2014).

2.6.1 Antibiotic resistance and antibacterial agents

In United States, at least 2 million people acquire serious infections with bacteria that are resistant to antibiotic, and about 23 000 people die each year because of antibiotic resistance (Frieden, 2013). Antibiotic resistance means that the bacteria are not longer killed by the formerly use antibiotic. Therefore, it is important for the professionals in the field to search for new antibacterial agents that are more effective in killing the pathogenic bacteria and help the infected patients.

According to Sheldon (2005), there are three types of antibacterial agents that are natural, synthetic and semi-synthetic. Antibacterial agents are also known as antibiotic. The sources of natural antibiotics are endophytes, plants, animals, soil microorganisms and marine organisms, while synthetic antibiotics are antibiotics that produced by chemical method. In addition, some natural antibiotics can be chemically modified to produce new antibiotics or called semi-synthetic antibiotics. Ekor (2014) had stated that more than 80% of the world‟s population depend on traditional medicinal plants as the best source to obtain a variety of therapeutic agent. Therefore,

such plant which had been traditionally used in herbal remedies should be investigated for their medicinal properties.

2.6.2 Natural products as antibacterial agents

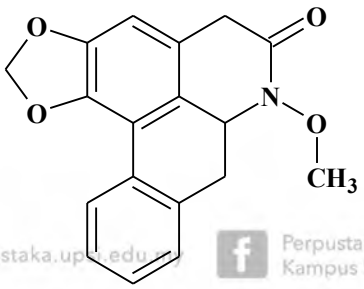
Researches on new therapeutic agents especially from the natural resources are necessary to help patients infected by the bacteria. Therefore, there are many scientific studies that had been done on the medicinal plants based on their traditional use to treat disease caused by pathogenic bacteria (Bussmann et al., 2010). The investigation usually starts with the preliminary study of the plant extract before continuing with the isolation and purification of the bioactive compound with antibacterial properties.

a. Alkaloid as antibacterial agents

A number of alkaloids have been reported to have antibacterial activity. Alkaloids posses a nitrogen atom with an unshared pair of electrons which make this compound a proton-accepting nitrogen atom and the proton donating amine hydrogen atom (Cushnie et al., 2014). Therefore, alkaloids ready to form hydrogen bond with proteins, enzymes and receptors. However, the degree of antimicrobial activity was influenced by the substitution groups (Verpoorte, 1998).

Artamonteirine (**73**), a type of aporphinoid compound isolated from the stem bark of *Artabotrys monteiroae* had been tested for antibacterial and showed positive

result against *Staphylococcus aureus* with efficacy comparable to the standard antibiotic ampicillin. This compound have substitution groups, methylenedioxy and alkoxyamido moieties, which might attribute to the antibacterial activity (Nyandoro et al., 2013). Another alkaloid compound, (*S*)-3-methoxy-nordomesticine (**74**) isolated from the stem of *Ocotea macrophylla* had shown antibacterial properties against *S. aureus* and *Enterococcus faecalis* (Pabon & Cuca, 2010).



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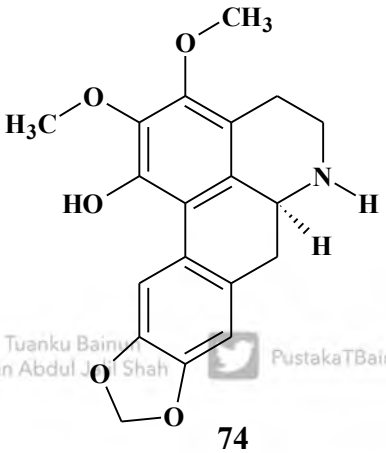

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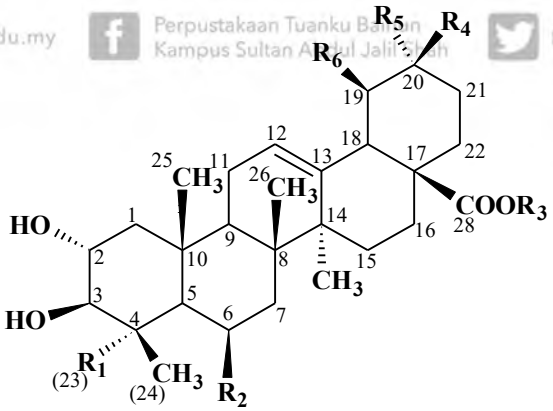
In the study on antibacterial mechanism of action by isoquinoline compounds, it was reported that these compounds distract the cell division and inhibit the nucleic acid synthesis. This type of compound can bind to the active site of enzyme and inhibit the enzyme activity, which is important for the cell development. Besides that, alkaloids also have been reported as the excellent DNA intercalator with a target on RNA polymerase, gyrase, topoisomerase IV and nucleic acid (Cushnie et al., 2014).

b. Terpenoid as antibacterial agent

Previous research reported that terpenoid also have potential as antibacterial agent (Djoukeng et al., 2005). Structure-activity relationship (SAR) on terpenoid showed that hydroxylation of this compound at the suitable position may contribute to antibacterial activity. For example, the present of hydroxyl group at C-23 of arjulonic acid (**75**) and asiatic acid (**76**) give the antibacterial activity. However, when the hydroxyl group was absent at C-23 in 2-hydroxyoleanolic acid (**77**) and 2-hydroxyursolic acid (**78**) which has been substituted with methyl group, the compound loss the antibacterial activity. Mechanism of action by terpenoid was speculated to involve in the membrane disruption.

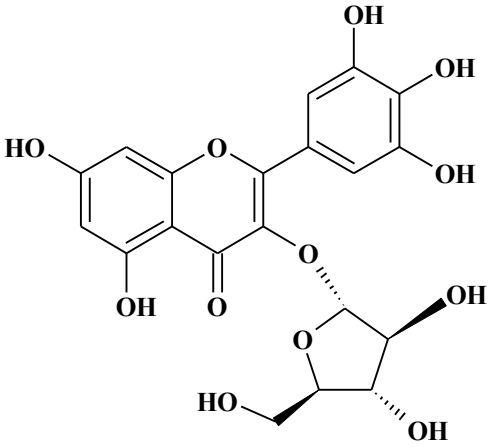


	R_1	R_2	R_3	R_4	R_5	R_6
75	CH_2OH	H	H	CH_3	CH_3	H
76	CH_2OH	H	H	CH_3	H	CH_3
77	CH_3	H	H	CH_3	CH_3	H
78	CH_3	H	H	H	CH_3	CH_3

c. Flavonoid as antibacterial agent

Flavonoids are group of heterocyclic compound and water soluble. Based on previous research, the present of hydroxyl and lipophylic group at the perfect position of flavonoid ring plays important role in the antibacterial activity. However, methoxylation and fluorination may decrease the antibacterial activity of the flavonoid compounds (Cushnie et al., 2005; Cushnie et al., 2011). These compounds are important in the disruption of the nucleic acid synthesis, inhibition of the cytoplasmic membrane, alteration of the membrane permeability and reduce the pathogenicity of the bacteria (Xie at al., 2015).

According to Okoth et al. (2013), myricetin-3-O-*α*-arabinofuranoside (betmidin) (**79**) was found to show the best antibacterial activity against Gram positive bacteria such as *S. aureus*, *S. sciuri* and *S. xylosus*, followed by the dihydrolanneaflavonol (**80**). The best antibacterial activity showed by compound (**79**) might be caused by arabinose moiety at 3-*O*-position and hydroxyl group; while the methoxyl group in dihydrolanneaflavonol (**80**) cause the decrease effect of antibacterial activity.



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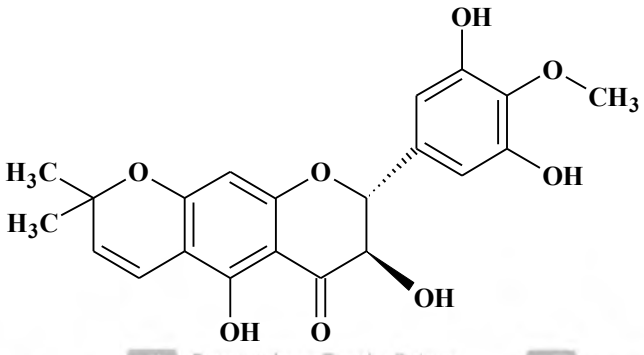

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CHAPTER 3

MATERIALS AND METHODS

3.1 Plant material

The barks of *Alphonsea cylindrica* King. were collected from Kechau Tui, Kuala Lipis, Pahang in March 2007. The plant sample was identified and collected by the phytochemical group of Chemistry Department, University of Malaya, Kuala Lumpur. The voucher specimen of *A. cylindrica* (KL 5379) was deposited at the Chemistry Department, University of Malaya, Kuala Lumpur.

3.2 Phytochemical study of *A. cylindrica* bark

3.2.1 Instruments and apparatus

Instruments used for extraction and isolation were including the electronic balance (Shimadzu, Kyoto, Japan), rotary evaporator (BÜCHI, Flawil, Switzerland), oven (Memmert, Schwabach, Germany), ultrasonic cleaner (Wise Clean, Wertheim, Germany) and ultra visible light detector (UVGL-58, New Hampshire, USA).

Apparatus used were conical flask (Pyrex, Tewksbury, USA), round flask 50 - 500 mL (Pyrex, Tewksbury, USA), beaker 25 - 5000 mL (Pyrex, Tewksbury, USA), glass road, filter funnel, separating funnel 500 - 1000 mL (Pyrex, Tewksbury, USA), measuring cylinder 10 - 250 mL (Pyrex, Tewksbury, USA), scintillation vials 20 mL, test tubes (Pyrex, Tewksbury, USA), Pasteur pipette, thin layer chromatography (TLC) tank, column chromatography with various inner diameter (d_c) 2.0, 2.5 and 4.5 cm (Favorit, Selangor, Malaysia), and 20 x 20 cm preparative thin layer chromatography (PTLC) glass.

3.2.2 Chemical reagents

The industrial grade solvents of hexane (C_6H_{14}), dichloromethane (CH_2Cl_2), ethyl acetate ($C_4H_8O_2$) and methanol (CH_3OH) were used in the extraction, isolation and purification process. These solvents were purified by distillation before used.

Other chemicals such as chloroform ($CHCl_3$), sulphuric acid (H_2SO_4), hydrochloric acid (HCl), mercuric(II) chloride ($HgCl_2$), bismuth(III) nitrate ($Bi(NO_3)_3.5H_2O$), potassium iodide (KI), glacial acetic acid ($C_2H_4O_2$), ammonia solution (NH_4OH), *p*-Anisaldehyde, and sodium sulphate (Na_2SO_4) used were of analytical grade purchased from Merck (Darmstadt, Germany).

The chemical reagent used to test the acidic condition in this study was as follow:

Mayer's Reagent

Solution A : Mercuric(II) chloride (1.4 g) was dissolved in distilled water (60 mL).

Solution B : Potassium iodide (5.0g) was dissolved in distilled water (10 mL).

Reagent solution : Solutions A and B were mixed and made up to 150 mL with distilled water.

Mayer's test : A positive result was indicated by the formation of white precipitate under acidic condition.

The spray reagents used for detection and visualization of the separated compounds on the thin layer chromatography were including:

Dragendorff's reagent

Solution A : Bismuth(III) nitrate (0.85 g) was dissolved in a mixture of glacial acetic acid (10 mL) and distilled water (40 mL).

Solution B : Potassium iodide (8.0 g) was dissolved in distilled water (20 mL).

Stock solution : Solution A and B were mixed with equal volumes.

Reagent solution : The stock solution (20 mL) was diluted in a mixture of acetic acid (20 mL) and distilled water (60 mL).

Dragendorff's test : The formation of orange, yellow or red spots on the TLC plate indicated the presence of alkaloids in the sample.

***p*-Anisaldehyde/sulphuric acid reagent**

p-Anisaldehyde/sulphuric acid : *p*-Anisaldehyde solution (0.5 mL) was added with glacial acetic acid (10 mL) followed by methanol (85 mL) and concentrated sulphuric acid (5 mL).

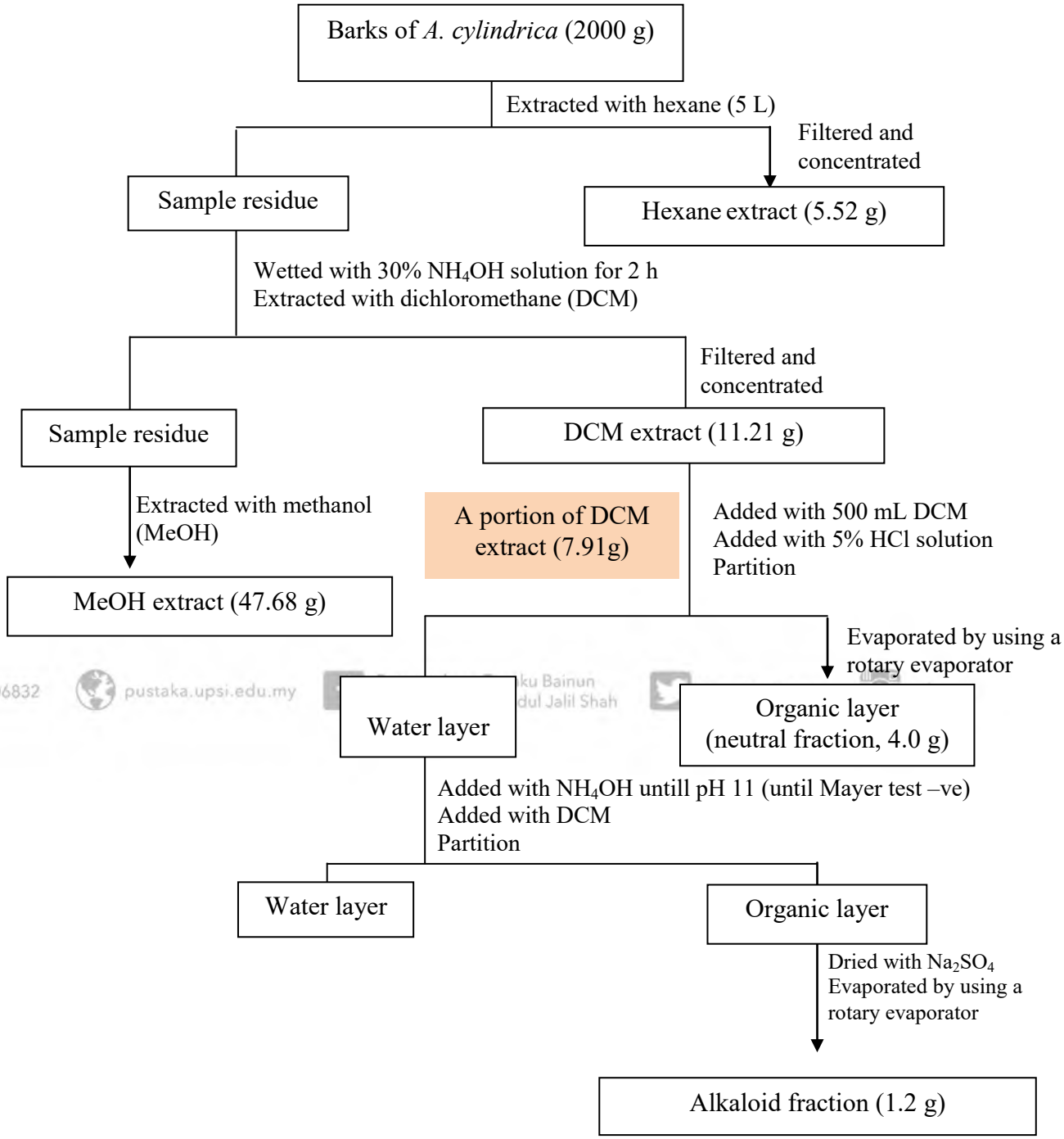
p-Anisaldehyde/sulphuric acid test : Visualisation was made after derivatisation with *p*-Anisaldehyde/sulphuric acid reagent and heating the plate at 100-105°C. Phenols, terpenes, sugars and steroids turned to violet, blue, red, grey or green.

3.2.3 Extraction method

The barks of *A. cylindrica* were dried and ground into powdered form by using an electrical grinder (Kuao Fung, Taiwan, China). The dried powdered bark of *A. cylindrica* (2000 g) was defatted using hexane (5 L) for 72 h at room temperature to yield residue of hexane. The hexane extract (5.52 g) was then evaporated under reduced pressure by using a rotary evaporator (BÜCHI, Flawil, Switzerland) and kept at 4°C until further investigation.

Furthermore, the dried residue of sample was wetted with 30% ammonia solution and left for 2 hours in order to aggregate the nitrogen-containing compounds in the plant. Sample was re-extracted with dichloromethane (DCM, 5 L) for 72 h, and the filtrate was concentrated under reduced pressure. This extract (7.91 g) was redissolved in DCM (500 mL) and subjected to acid/base extraction by using a separating funnel. An aliquot of DCM extract was further acidified by the addition of 5% hydrochloric acid (HCl) solution (3 L) and shaken vigorously. The organic layer (neutral fraction) was separated from the water layer (ionic and water soluble fraction) and evaporated under reduced pressure. Meanwhile, the water layer was further basified with 30% ammonium hydroxide solution (NH_4OH) until pH 11. This fraction was further extracted with DCM until a negative Mayer test obtained. Finally, the DCM extract was dried with sodium sulphate (Na_2SO_4) and evaporated to dryness to give an alkaloid fraction (1.2 g).

On the other hand, the residue of sample was re-extracted with methanol (5 L) for 72 h at room temperature. The methanol extract was filtered and concentrated under reduced pressure. This extract (47.68 g) was stored in a refrigerator at 4 °C until further analyses. The extraction method of *A. cylindrica* barks in this study is summarized in *Scheme 3.1*.



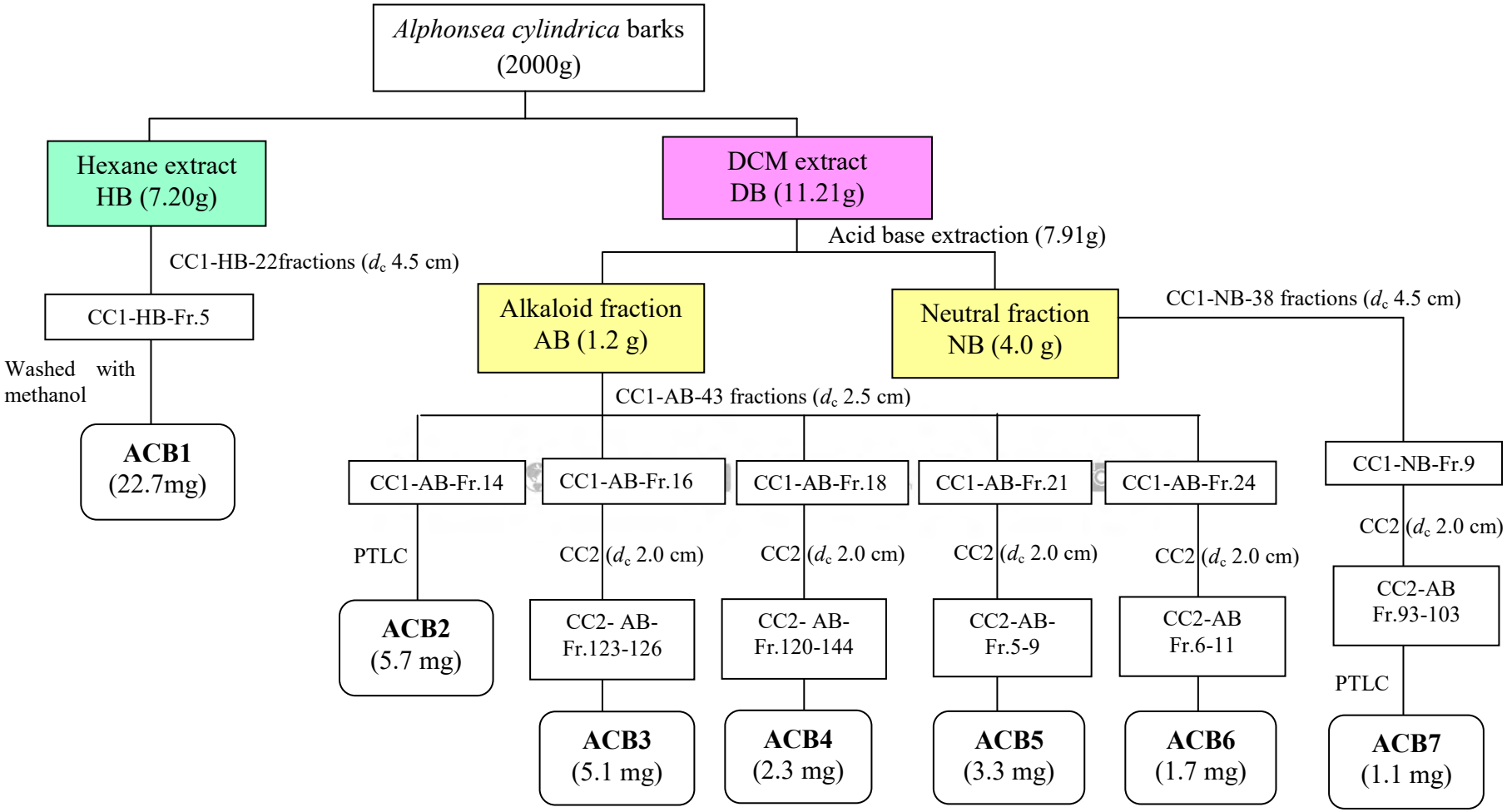
Scheme 3.1. Extraction process of *Alphonsea cylindrica* King. bark

3.2.4 Isolation and purification

In this study, three types of chromatographic techniques were applied for isolation and purification of chemical compounds from *A. cylindrica* barks, including Thin Layer Chromatography (TLC), Column Chromatography (CC) and Preparative Thin Layer Chromatography (PTLC). TLC was performed on Kieselgel 60 F_{254} 20 X 20 cm (Merck 1.05554.0001) aluminium TLC sheets and CC was carried out on silica gel 60, 230-400 mesh ASTM (Merck 1.09385.1000). Meanwhile, PTLC was performed on 20 X 20 cm glass plate containing silica gel 60 F_{254} with gypsum (Merck 1.07749.1000). UV light model UVGL-58 was used to observe spots on the TLC and PTLC plates. The spots were visualized under ultraviolet light of 254 and 365 nm.

Hexane extract, alkaloid fraction and neutral fraction were subjected to isolation and purification of compounds from *A. cylindrica* barks. Extract and fractions were separated by using column chromatography over silica gel based on gradient elution method. Further purification was subjected to exhaustive CC, TLC and PTLC to obtain pure compounds.

During the purification process, it is necessary to analysed the eluted fractions by TLC for their chemical composition. Each of developed TLC plates were analysed under ultraviolet light and daylight, and after derivatization with Dragendorff's and *p*-Anisaldehyde/H_2SO_4 reagents. Overview of isolation and purification of compounds from *A. cylindrica* barks is displayed in *Scheme 3.2*.



Scheme 3.2. Isolation and purification of chemical compounds from *Alphonsea cylindrica* bark

3.2.4.1 Chemical compounds from hexane extract (HB)

The hexane extract (7.20 g) was introduced to first column chromatography (CC1-HB, inner diameter, d_c 4.5 cm) over silica gel (300.0 g) with 100% hexane, hexane/ethyl acetate mixture, ethyl acetate, ethyl acetate/methanol and methanol as eluting solvents. Eluents were collected for approximately 200 mL per fraction to yield 35 fractions. These fractions were grouped into 22 fractions based on TLC analysis using 100% hexane and hexane: ethyl acetate (8:2) as developing solvents.

Fraction (CC1-HB-Fr.5, 471.8 mg) from the first column chromatography was purified using trituration with methanol. The yellowish solution was removed out and the colourless solution was evaporated at room temperature to form white amorphous powder. Based on TLC analysis, the latter was obtained as a pure compound and labelled as **ACB1** (22.7 mg).

3.2.4.2 Chemical compounds from alkaloid fraction (AB)

The dried alkaloid fraction (1.2 g) was subjected onto silica gel column chromatography (CC1-AB, 60.0 g, d_c 2.5 cm) and separated gradiently with dichloromethane (100%), mixtures of dichloromethane/methanol (99:1, 98:2, 96:4, 94:6, 92:8, 90:10, 80:20 and 50:50) and methanol (100%). Eluents were collected approximately 20 mL per fraction and 335 fractions were obtained. These fractions were combined into 43 fractions based on TLC analysis, eluted with a mixture of

CH_2Cl_2: MeOH (99:1, 98:2, 96:4 and 94:6). Fractions with similar pattern were combined into 43 fractions based on TLC analysis.

Fraction 14 from CC1-AB (CC1-AB-Fr.14, 17.5 mg) was separated using the preparative TLC with solvent system chloroform: ethyl acetate (9:1) treated with ammonia vapour. Yellow band of a compound was collected by scratching the silica throughout the band. This compound was further washed with dichloromethane, which saturated in ammonia to give compound **ACB2** (5.7 mg).

Fraction 16 from CC1-AB (CC1-AB-Fr.16, 42.8 mg) was purified using silica gel column chromatography (CC2-AB, 30.0 g, d_c 2.0 cm). The fraction was fractionated gradiently with dichloromethane (100%), mixtures of dichloromethane/ethyl acetate (95:5, 90:10, 85:15, 80:20, 70:30 and 50:50), ethyl acetate (100%), mixtures of ethyl acetate/methanol (50:50) and methanol (100%) and yielded 127 sub-fractions of 20 mL each. Compound **ACB3** (5.1 mg) was purified from sub-fractions 123 to 126.

Fraction 18 from CC1-AB (CC1-AB-Fr.18, 30.4 mg) was further fractionated onto column chromatography (CC2-AB, d_c 2.0 cm) using silica gel (31.0 g) as the stationary phase. Gradient elution from chloroform (100%), mixtures of chloroform/ethyl acetate (90:10, 80:20, 70:30 and 50:50), ethyl acetate (100%), mixture of ethyl acetate/methanol (50:50) and methanol (100%) was performed. As many as 148 sub-fractions (10 mL each) were successfully collected and analysed with TLC. Sub-fractions 120 - 144 were combined to yield a pure compound **ACB4** (2.3 mg).

Fraction CC1-AB-Fr.21 (18.8 mg) was subjected onto column chromatography (CC2-AB, silica gel, 19.0 g, d_c 2.0 cm) and fractionated using solvent system of 100% chloroform, mixture of chloroform/ethyl acetate (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 60:40 and 50:50), ethyl acetate (100%), mixture of ethyl acetate/methanol (50:50) and methanol (100%) to afford 61 sub-fractions (approximately 20 mL per fraction). After TLC analysis, sub-fractions with similar TLC profile were combined into 16 fractions. Compound **ACB5** (3.3 mg) was precipitated from fractions 5 to 9.

Fraction CC1-AB-Fr.24 (15.6 mg) was further fractionated using column chromatography (CC2-AB, silica gel, 11.0 g, d_c 2.0 cm), eluted with dichloromethane (100%), mixtures of dichloromethane/ethyl acetate (90:10, 80:20, 75:25, 70:30, 65:35, 60:40, 55, 45, 50:50, 45:55, 40:60, 35:65), ethyl acetate (100%), mixture of ethyl acetate/methanol (50:50) and methanol (100%) to yield 48 sub-fractions. Compound **ACB6** (1.7 mg) was precipitated from sub-fractions 6 to 11.

3.2.4.3 Chemical compounds from neutral fraction (NB)

Neutral fraction (4.0 g) was subjected onto silica gel column chromatography (CC1-NB, d_c 4.5 cm in diameter) and eluted with hexane (100%), mixtures of hexane/dichloromethane (50:50 and 30:70), dichloromethane (100%), mixtures of dichloromethane/methanol (99:1, 98:2, 97:3, 96:4, 95:5, 90:10 and 50:50) and methanol (100%). Eluents were collected for 250 mL each and 38 fractions were obtained. After TLC analysis, fractions were grouped into 11 fractions.

Fraction 9 from CC1-NB (CC1-NB-Fr.9, 1.0 g) was subjected onto silica gel column chromatography (CC2-NB, 20.0 g, d_c 2.0 cm) and fractionated gradiently using hexane (100%), mixtures of hexane/dichloromethane (50:50, 30:70, 20:80, 10:90), dichloromethane (100%), mixtures of dichloromethane/methanol (99:1, 98:2, 97:3, 96:4, 95:5, 90:10) and methanol (100%). Eluents were collected for 20 ml each and 109 sub-fractions were successfully obtained. Sub-fractions 93 - 103 were combined and separated isocratically using PTLC with mobile phase dichloromethane: ethyl acetate (98:2) and ammonia vapour. The yellow band of a compound was scratched and washed with dichloromethane saturated in ammonia to give compound **ACB7** (1.1 mg).

3.3 Structure elucidation of the isolated compounds

The isolated compounds were elucidated using modern spectroscopic techniques, including nuclear magnetic resonance spectroscopy (NMR), mass spectroscopy (MS), Fourier transform infrared spectroscopy (ATR-FTIR), and ultraviolet-visible spectroscopy (UV).

Nuclear Magnetic Resonance (NMR) analyses, including one-dimensional (1D-NMR) and two-dimensional (2D-NMR) assignments were carried out using JEOL ECX (500 MHz). 1D-NMR assignments were including the measurements of proton (^{1}H) and carbon (^{13}C). Meanwhile, 2D-NMR assignments were including the measurements of HMQC, HMBC, COSY and NOESY correlations. Deuterated chloroform ($CDCl_3$) purchased from Merck (Darmstadt, Germany) was used as a

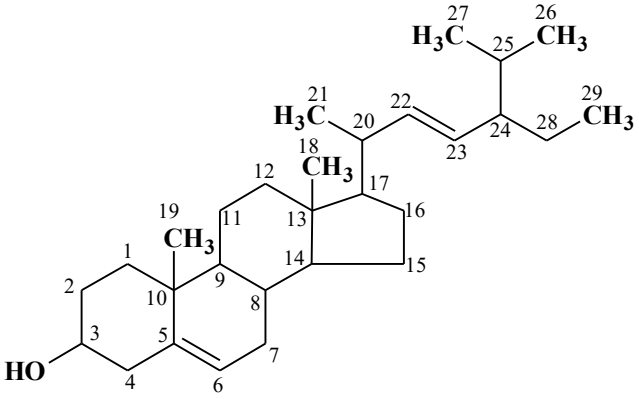
solvent for all analyses with 1% tetramethylsilane (TMS) as internal standard. NMR data and structure of isolated compounds were analysed using Advanced Chemistry Development (ACD) NMR Processor Academic Edition (Toronto, ON, Canada) and ACD/Labs Chemsketch Version 12.0 (Toronto, ON, Canada) softwares.

The molecular weight of the compounds was determined using Gas chromatography/Mass Spectrometry Agilent Technologies GCMS-5975C VL MSD spectrometer (Santa Clara, California, USA) at UPSI Tanjung Malim and Liquid Chromatography-Tandem Mass Ion Trap Agilent Technology 1200 Series (Santa Clara, California, USA) at UiTM Puncak Alam. Thermo Scientific Nicolet ATR-FTIR model 6700 spectrophotometer (Waltham, Massachusetts, USA) was used to determine the presence of certain functional groups in a molecule, while the π-electron systems, conjugated unsaturations, aromatic compounds and conjugated non-bonding electron systems were identified using an Agilent Technology UV Visible CARY 60 spectrophotometer.

3.3.1 Physicochemical and spectroscopic data of the isolated compounds

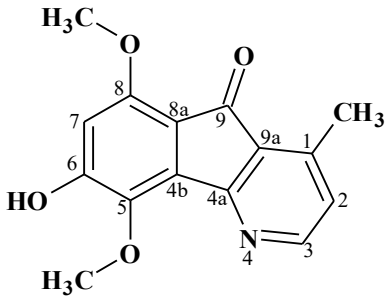
Seven compounds were successfully isolated from the barks of *A. cylindrica*. These compounds were identified by modern spectroscopic techniques and comparison with those reported in the literatures.

a. ACB1



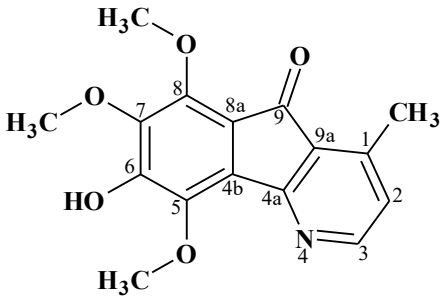
Stigmasterol (**81**); 22.7 mg; white amorphous powder; UV (MeOH) λ_{max} nm: 249; FTIR (ATR) υ_{max}, cm^{-1}: 3329, 2932, 1457, 1366 and 1048; positive EI-MS: *m/z* 412.4 $[M]^+$, ($C_{29}H_{48}O$, MW 412.691); ^{1}H-NMR ($CDCl_3$ with 1% v/v TMS, 500 MHz) δ (ppm): 5.35 (1H, *m*, H-6), 5.15 (1H, *dd*, *J* = 15.0, 8.6 Hz, H-22), 5.01 (1H, *dd*, *J* = 15.0, 8.6 Hz, H-23), 3.52 (1H, *m*, H-3), 2.31, 2.27 (1H each, *m*, H-4), 2.06 (1H, *m*, H-20), 2.01, 1.20 (1H each, *m*, H-12), 1.97, 1.51 (1H each, *m*, H-7), 1.87, 1.08 (1H each, *m*, H-1), 1.83, 1.50 (1H each, *m*, H-2), 1.72, 1.27 (1H each, *m*, H-16), 1.57, 1.51 (1H each, *m*, H-11), 1.56, 1.05 (1H each, *m*, H-15), 1.55 (1H, *m*, H-25), 1.53 (1H, *m*, H-24), 1.49 (1H, *m*, H-8), 1.47, 1.23 (1H each, *m*, H-28), 1.13 (1H, *m*, H-17), 1.02 (1H, *d*, *J* = 6.8 Hz, H-21), 1.01 (3H, *s*, H-19), 0.98 (1H, *m*, H-14), 0.92 (1H, *m*, H-9), 0.86 (3H, *d*, *J* = 6.8 Hz, H-26), 0.84 (3H, *d*, *J* = 6.8 Hz, H-27), 0.80 (3H, *m*, H-29) and 0.69 (3H, *s*, H-18); ^{13}C-NMR ($CDCl_3$ with 1% v/v TMS, 125 MHz) δ (ppm): 140.8 (C-5), 138.4 (C-22), 129.3 (C-23), 121.8 (C-6), 71.9 (C-3), 56.9 (C-14), 56.0 (C-17), 51.3 (C-24), 50.2 (C-9), 42.4 (C-4), 42.3 (C-13), 40.6 (C-20), 39.7 (C-12), 37.3 (C-1), 36.2 (C-10), 32.0 (C-7, C-8, C-25), 31.7 (C-2), 29.0 (C-16), 25.5 (C-28), 24.5 (C-15), 21.3 (C-21, C-26), 21.2 (C-11), 19.5 (C-19), 19.1 (C-27), 12.4 (C-29) and 12.1 (C-18).

b. ACB2



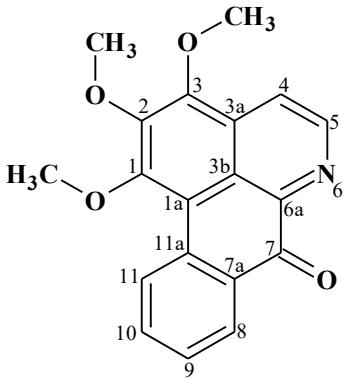
Kinabaline (**82**); 5.7 mg; yellow amorphous; UV (MeOH) λ_{max} nm: 268, 298; FTIR (ATR) υ_{max}, cm^{-1}: 3308, 2918, 1660; positive EI-MS: *m/z* 271.1 $[M]^+$, ($C_{15}H_{13}NO_4$, MW 271.268); ^{1}H-NMR ($CDCl_3$ with 1% v/v TMS, 500 MHz) δ (ppm): 8.81 (1H, *br s*, OH-6), 8.50 (1H, *d*, *J* = 5.0 Hz, H-3), 6.94 (1H, *d*, *J* = 5.0 Hz, H-2), 6.32 (1H, *s*, H-7), 3.99 (3H, *s*, OCH_3-8), 3.91 (3H, *s*, OCH_3-5) and 2.61 (3H, *s*, CH_3-1); ^{13}C-NMR ($CDCl_3$ with 1% v/v TMS, 125 MHz) δ (ppm): 194.0 (C-9), 162.5 (C-5), 163.4 (C-4a), 155.8 (C-6), 152.8 (C-3), 146.9 (C-1), 140.0 (C-8), 131.7 (C-8a), 127.7 (C-9a), 125.2 (C-2), 111.0 (C-4b), 101.8 (C-7), 62.0 (OCH_3-8), 56.7 (OCH_3-5) and 17.3 (CH_3-1).

c. ACB3



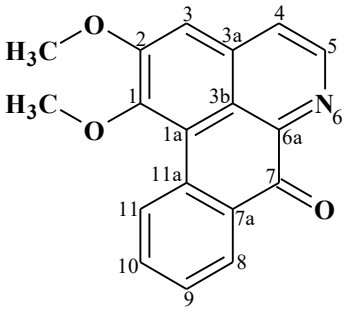
Muniranine (**83**); 5.1 mg; yellow amorphous; UV (MeOH) λ_{max} nm: 250, 269; FTIR (ATR) υ_{max}, cm^{-1}: 3305, 2932, 1676; negative HR-ESI-MS: *m/z* 300.0869 $[M-H]^+$ ($C_{16}H_{15}NO_5$, MW 301.294); ^{1}H-NMR ($CDCl_3$ with 1% v/v TMS, 500 MHz) δ (ppm): 8.71 (1H, *br s*, OH-8), 8.45 (1H, *d*, *J* = 5.0 Hz, H-3), 6.89 (1H, *d*, *J* = 5.0 Hz, H-2), 4.04 (3H, *s*, OCH_3-8), 4.01 (3H, *s*, OCH_3-5), 3.98 (3H, *s*, OCH_3-7) and 2.59 (3H, *s*, CH_3-1); ^{13}C-NMR ($CDCl_3$ with 1% v/v TMS, 125 MHz) δ (ppm): 194.8 (C-9), 164.0 (C-4a), 154.7 (C-5), 153.1 (C-3), 148.8 (C-6), 147.2 (C-1), 144.0 (C-8), 143.8 (C-7), 127.9 (C-8a), 127.0 (C-9a), 124.7 (C-2), 113.8 (C-4b), 62.2 (OCH_3-8), 61.8 (OCH_3-5), 61.5 (OCH_3-7) and 17.4 (CH_3-1).

d. ACB4



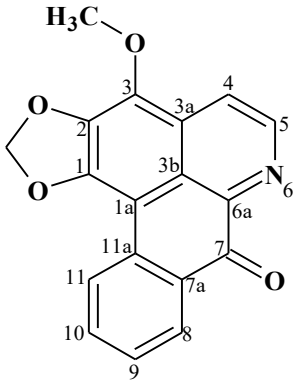
***O*-methylmoschatoline** (**8**); 2.3 mg; orange amorphous; UV (MeOH) λ_{max} nm: 250; FTIR (ATR) υ_{max}, cm^{-1}: 2948, 1655, 1577, 1463, 1389; positive EI-MS: *m/z* 321.1 $[M]^+$, ($C_{19}H_{15}NO_4$, MW 321.327); ^{1}H-NMR ($CDCl_3$ with 1% v/v TMS, 500 MHz) δ (ppm): 9.11 (1H, *d*, *J* = 8.5 Hz, H-11), 8.96 (1H, *d*, *J* = 5.5 Hz, H-5), 8.57 (1H, *d*, *J* = 7.5 Hz, H-8), 8.22 (1H, *d*, *J* = 5.0 Hz, H-4), 7.76 (1H, *t*, H-10), 7.55 (1H, *t*, H-9), 4.19 (3H, *s*, OCH_3-3), 4.10 (3H, *s*, OCH_3-2) and 4.08 (3H, *s*, OCH_3-1); ^{13}C-NMR ($CDCl_3$ with 1% v/v TMS, 125 MHz) δ (ppm): 182.7 (C-7), 156.6 (C-1), 148.5 (C-3), 147.4 (C-2), 145.5 (C-6a), 144.5 (C-5), 134.6 (C-11a), 134.5 (C-10), 131.5 (C-7a), 131.2 (C-3b), 129.0 (C-8), 128.2 (C-9), 127.7 (C-11), 122.9 (C-3a), 119.3 (C-4), 115.7 (C-1a), 61.9 (OCH_3-3), 61.6 (OCH_3-2) and 61.1 (OCH_3-1).

e. ACB5



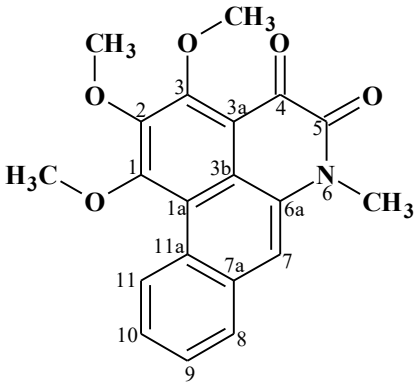
Lysicamine (**84**); 3.3 mg; yellow amorphous powder; UV (MeOH) λ_{max} nm: 240 and 270; FTIR (ATR) υ_{max}, cm^{-1}: 1665; positive EI-MS: *m/z* 291.1 $[M]^+$, ($C_{18}H_{13}NO_3$, MW 291.301); ^{1}H-NMR ($CDCl_3$ with 1% v/v TMS, 500 MHz) δ (ppm): 9.17 (1H, *d*, *J* = 7.5Hz, H-11), 8.89 (1H, *d*, *J* = 5.5 Hz, H-5) 8.58 (1H, *d*, *J* = 7.5 Hz, H-8), 7.79 (1H, *d*, *J* = 5.0Hz, H-4), 7.76 (1H, *t*, H-10), 7.58 (1H, *t*, H-9), 7.21 (1H, *s*, H-3), 4.09 (3H, *s*, OCH_3-1) and 4.01 (3H, *s*, OCH_3-2). ^{13}C-NMR ($CDCl_3$ with 1% v/v TMS, 125 MHz) δ (ppm): 182.8 (C-7), 156.9 (C-1), 152.1 (C-2), 145.4 (C-6a), 145.1 (C-5), 135.6 (C-3a), 134.43 (C-11a), 134.38 (C-10), 132.1 (C-7a), 129.0 (C-8), 128.5 (C-11), 128.9 (C-9), 123.7 (C-4), 122.2 (C-3b), 119.9 (C-1a), 106.5 (C-3), 60.7 (OCH_3-2) and 56.3 (OCH_3-1).

f. ACB6



Atherospermidine (**85**); 1.7 mg; orange amorphous powder; UV (MeOH) λ_{max} nm: 250, 270; FTIR (ATR) υ_{max}, cm^{-1}: 2915, 1666 and 962; positive HR-ESI-MS: *m/z* 306.0762 $[M+H]^+$, ($C_{18}H_{11}NO_4$, MW 305.284); ^{1}H-NMR ($CDCl_3$ with 1% v/v TMS, 500 MHz) δ (ppm): 8.88 (1H, *d*, *J* = 5.0 Hz, H-5), 8.51 (1H, *dd*, *J* = 7.5, 1.2 Hz, H-8), 8.47 (1H, *d*, *J* = 8.0 Hz, H-11), 8.11 (1H, *d*, *J* = 5.0 Hz, H-4), 7.67 (1H, *td*, *J* = 8.0, 1.2 Hz, H-10), 7.49 (1H, *td*, *J* = 8.0, 1.2 Hz, H-9), 6.32 (2H, *s*, 1-OCH_2-2) and 4.28 (3H, *s*, OCH_3-3); ^{13}C-NMR ($CDCl_3$ with 1% v/v TMS, 125 MHz) δ (ppm): 182.4 (C-7), 149.8 (C-1), 144.7 (C-6a), 144.0 (C-5), 136.7 (C-3), 136.3 (C-2), 134.0 (C-10), 133.2 (C-11a), 130.9 (C-3a), 130.5 (C-7a), 128.7 (C-8), 127.7 (C-9), 126.7 (C-11), 122.8 (C-3b), 119.4 (C-4), 102.6 (C-1a), 102.4 (1-OCH_2-2) and 60.2 (OCH_3-3).

g. ACB7



***N*-methylouregidione (86)**; 1.1 mg; yellow amorphous powder; UV (MeOH) λ_{max} nm: 245; FTIR (ATR) υ_{max}, cm^{-1}:1691, 1663; positive HR-ESI-MS: *m/z* 374.1034 $[M+Na]^+$, ($C_{20}H_{17}NO_5$, MW 351.353); ^{1}H-NMR ($CDCl_3$ with 1% v/v TMS, 500 MHz) δ (ppm): 9.49 (1H, *m*, H-11), 7.93 (1H, *m*, H-8), 7.67 (2H, *m*, H-9, H-10), 7.63 (1H, *s*, H-7), 4.18 (3H, *s*, OCH_3-3), 4.13 (3H, *s*, OCH_3-2), 4.09 (3H, *s*, OCH_3-1) and 3.87 (3H, *s*, *N*-CH_3); ^{13}C-NMR ($CDCl_3$ with 1% v/v TMS, 125 MHz) δ (ppm): 170.4 (C-4), 160.2 (C-3), 158.4 (C-1), 157.0 (C-5), 147.3 (C-2), 131.7 (C-7a), 128.8 (C-8), 127.63 (C-6a), 127.59 (C-9), 127.3 (C-10), 127.0 (C-11), 121.4 (C-11a), 121.3 (C-3b), 117.0 (C-3a), 115.2 (C-1a), 114.5 (C-7), 62.2 (OCH_3-1), 61.8 (OCH_3-2), 61.3 (OCH_3-3) and 31.0 (*N*-CH_3).

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3.4 Antibacterial Analysis

Antibacterial activity test was measured using agar disc diffusion and broth dilution methods. Hexane, dichloromethane and methanol extracts as well as seven compounds isolated from *A. cylindrica* barks were screened for their antibacterial activity by using agar disc diffusion method. Samples showing inhibition zone in the agar disc diffusion assay were further tested to determine their minimum inhibition concentration (MIC) using broth dilution method.

3.4.1 Instruments and apparatus

Instruments used were electronic balance (Shimadzu, Kyoto, Japan), ultrasonic cleaner (Wise Clean, Wertheim, Germany), vortex mixer, autoclave (HVE-50, Tokyo, Japan), laminar flow clean benches (ESCO, Changi South Street, Singapore), incubator (Memmert Universal, Schwabach, Germany) and UV-Visible spectrophotometer (BIOMATE 3S, Madison, USA).

Apparatus used were Schott duran bottle 2000 mL (Mainz, Germany), beaker 50-2000 mL (Pyrex, Tewksbury, USA), scintillation vials 20 mL, universal bottle, Petri dish, cotton swab, forceps, filter paper discs (Whatmann, Maidstone, UK), micropipette 2-20 µL and 100 - 1000 µL (Eppendorf, Hamburg, Germany), pipette tips (GEB, Zhejiang, China), cuvettes 1.5 mL and microtubes 1.5 mL.

3.4.2 Chemical reagents

Solvent used were 95% ethanol and methanol from analytical grade purchased from Merck (Darmstadt, Germany). Other chemical reagents were nutrient agar (NA) and nutrient broth (NB) purchased from Fluka Analytical (Steinheim, Germany) while ampicillin sodium salt was purchased from Sigma-Aldrich (St. Louis, USA).

The compositions of NA medium are meat extract (1 g/L), yeast extract (2 g/L), peptone (5 g/L), sodium chloride (5 g/L) and agar (15 g/L). Meanwhile, the compositions of NB medium are meat peptone (4.3 g/L), casein peptone (4.3 g/L), and sodium chloride (6.4 g/L). The final pH for both of the medium is 7.4 ± 0.2 and prepared according to the standard preparation protocols.

3.4.3 Preparation of microorganisms

Bacteria cultures were obtained from the culture collection, Department of Biology, Universiti Pendidikan Sultan Idris, Perak. The tested bacteria used in the screening for antibacterial properties were Gram negative bacteria*, Pseudomonas aeruginosa* and Gram positive bacteria, *Staphylococcus aureus.* The bacteria from stock cultures were streaked onto NA plates by using sterile wire loop. The wire loop was sterilized by flaming. Then, the cultures were incubated overnight for 18 to 24 h at 37 °C. Single colony was then subcultured into 5 mL of NB. The tested bacteria were incubated for another 18 h at 37 °C with shaking (160 rpm) by using an incubator shaker.

3.4.4 Antibacterial disc diffusion assay

In this study, three extracts from hexane, dichloromethane and methanol were dissolved in methanol and prepared to obtain 200 mg/mL concentration as stock solutions. Working solutions for the plant extracts were obtained by diluting the stock solution with required volume of methanol. The concentrations tested for extracts were 200, 100, 75, 50 and 25 mg/mL.

An aliquot (10 µL) of the prepared concentration of each extract and chemical compounds were applied to a 6 mm filter paper disc (Amel et al., 2012). Methanol was used as negative control and ampicillin as positive control (1 mg/mL). Subsequently, the discs were left about 30 minutes to dry in the laminar flow to avoid contamination (Odebode et al., 2004).

Next, 1000 µL of the prepared test bacteria from Section 3.4.4 were streaked on the NA plate by using sterile cotton swab and left about 15 minutes to dry in the laminar flow. Then, the discs were placed on the streaked NA plate with suitable distance from each other. The discs were pressed down gently by using sterile forceps to ensure complete contact with agar surface. Prior to incubation at 37 °C overnight in the incubator (Memmert Universal, Schwabach, Germany), the plates were sealed with parafilm and inverted. Observations were recorded after overnight incubation. Antibacterial activities were interpreted from the diameters of inhibition zone around the disc and assayed in triplicate (Niederstebruch & Sixt, 2013).

3.4.5 Determination of minimum inhibition concentration (MIC) (EUCAST, 2003)

Andrews (2001) defined MIC as the lowest concentration of sample that will inhibit the visible growth of microorganisms. In this study, MIC was performed on extracts and isolated compounds showing zone of inhibition in disc diffusion assay. MICs were determined by a broth micro dilution method with slight modification of the method described by EUCAST (2003). The samples were prepared to several desired concentrations (1500, 1400, 1300, 1200, 1100, 1000, 900, 800, 700, 600, 500, 400, 300, 250, 200, 125, 100, 62.5, 50, 31.25, 25, 12.5, 6.25, 3.125 and 1.5625 µg/mL) in methanol. Then, 50 µL of each dilution were introduced into micro tubes in 50 µL NB that were already inoculated with tested microorganism. After well mixing, the inoculated micro tubes were placed in the incubator at 37 ^{o}C overnight. The MIC of the selected samples were determined by measuring the optical density (OD) at 620 nm, the amount of bacteria growth in each micro tube is compared with the uninoculated or negative growth control. Sterile nutrient broth was used as blank. The OD was obtained from BIOMATE 3S UV-visible spectrophotometer and performed in triplicate. Graphs of the bacterial optical density measured at 620 nm versus concentrations of sample were plotted.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Plant material

In this study, the plant sample was in powder form and brown in colour. It is important to make the sample in small pieces or powder form so that, the extraction process become more effective. The small particle of sample will increase the surface area and facilitate the penetration of solvent into the plant cells. Therefore, the yield of extraction can be improved and the chemical compounds can be extracted easily (Khoddami et al., 2013; Sarker et al., 2006). In addition, the samples had been dried by using oven under controlled temperature at about 40 °C. It is important to make sure that the temperature is not too high in order to avoid the degradation of chemical

compounds in which they are heat sensitive. The drying process is also necessary to reduce the moisture content and prevent the growth of microorganisms (Rocha et al., 2011). Hence, the samples can be preserved for a long period.

4.2 Phytochemical studies of *A. cylindrica*

Natural product present in the living organisms such as plant giving a huge diversity of chemical compounds. The study of plant chemical compounds also referred as phytochemical study involving extraction, isolation and identification of the chemical compounds (Banu & Catherine, 2015).

4.2.1 Extraction

Extraction is a process of separating the desired compound from plant material by using particular solvents (Ramaan, 2006). In this study, plant material was serially extracted by increasing polarity of solvents. The solvent used influence the extracted compound, because the solvent will solubilise compounds with the same polarity following the principle of “like dissolve like” (Ncube et al., 2008). Hexane, dichloromethane and methanol are representing as non-polar, semi-polar and polar solvents, respectively. These solvents were used in the maceration technique by soaking the sample in a glass conical flask at room temperature and wrapped tightly with aluminium foil to avoid evaporation. The sample was soaked for three days with occasional stirring and repeated for thrice with fresh solvent to ensure exhaustive

extraction. Maceration was preferred compared to other modern techniques due to some advantages such as applicable, convenient and simple (Azwanida, 2015).

Acid base extraction is known as a technique that uses liquid-liquid extraction to separate the acidic, basic and neutral compounds from a mixture (Pavia, 2005). The liquids have to be immiscible, in order to form two separable layers. In this process, the charged/ionic compounds (salts) are soluble in water (a polar solvent), while uncharged compounds are soluble in organic solvents such as dichloromethane.

At first, the sample was wetted with ammonia solution to aggregate the nitrogen containing compounds (alkaloids) before extracted with dichloromethane. After that, acid solution was added to the dichloromethane extract, which supposedly contained organic base and organic neutral compounds. Protons of the acid solution were allowed to interact with organic base compounds to form ionic and water-soluble compounds. Meanwhile, the neutral compounds will remain in the dichloromethane extract. In order to get the pure basic compound, a mineral base, ammonia solution was added to take the proton away. After removing the proton, the organic compounds became uncharged and not soluble in water, but soluble in dichloromethane solvent. Then, sodium sulphate was added as inert drying agent to remove the traces of water from the dichloromethane solution, and evaporated to gain the alkaloid fraction. The dichloromethane extract which contain neutral compounds, was evaporated and dried to obtain the pure neutral fraction (Schaller, 2013).

4.2.2 Yield of extraction

The yield of the dried crude extracts obtained is recorded in Table 4.1. From the table, it showed that methanol extraction (2.38%) produced the highest yield as compared to hexane (0.36%) and dichloromethane (0.56%). Results from extraction showed that *A. cylindrica* barks contain polar compound more than non-polar and semi-polar compounds. After acid base extraction of the dichloromethane extract (7.91 g), alkaloid fraction (15.17%) produced lower yield when compared with the neutral fraction (50.57%) indicating that the dichloromethane extract contains less amount of alkaloid compounds.

Table 4.1

The yield of extraction A. cylindrica bark

No.	Crude/ Fraction	Code	Yield (g)	Percentage of yield (%)
1	Hexane	HB	7.20	0.36
2	Dichloromethane	DB	11.21	0.56
3	Methanol	MB	47.68	2.38
4	Alkaloid	AB	1.20	15.17
5	Neutral	NB	4.00	50.57

4.2.3 Isolation and purification

A total of seven compounds were successfully isolated and purified from the barks of *A. cylindrica* (Table 4.2).

The compound (**ACB1**) from hexane extract did not show any spot after sprayed with Dragendorff‟s reagent. However, compound **ACB1** appeared as violet spot on TLC after derivatised with *p*-Anisaldehyde/sulphuric acid. Therefore, **ACB1** was recognized as terpenoid based on the TLC characteristic.

Five compounds (**ACB2**-**ACB6**) from the alkaloid fraction and one compound (**ACB7**) from the neutral fraction gave positive results as alkaloid compounds when tested with Dragendorff‟s reagent, which was indicated by orange, yellow or red spots on TLC. Furthermore, the compounds also can be visualised under UV light. According to Cordell (1998), the family Annonaceae is known as the source of variety of alkaloids. The isolation of six alkaloid compounds had revealed that *A. cylindrica*, a species from family Annonaceae can be the source of alkaloid compounds.

Table 4.2

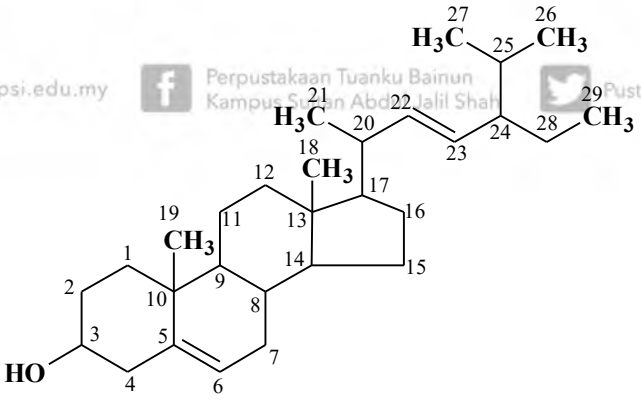
The isolated compounds from A. cylindrica bark

Code of compound	Extract/fraction	Weight (mg)
ACB 1	Hexane crude extract	22.7
ACB 2	Alkaloid fraction	5.7
ACB 3	Alkaloid fraction	5.1
ACB 4	Alkaloid fraction	2.3
ACB 5	Alkaloid fraction	3.3
ACB 6	Alkaloid fraction	1.7
ACB 7	Neutral fraction	1.1

4.3 Structure elucidation of compounds from *A. cylindrica* bark

Seven compounds were successfully isolated from the barks of *A. cylindrica,* including a triterpenoid (**ACB1**), azafluorenone alkaloids (**ACB2** and **ACB3**), oxoaporphine alkaloids (**ACB4**, **ACB5** and **ACB6**) and 4, 5-dioxoaporphine alkaloid (**ACB7**). **ACB3** was identified as a new derivative of azafluorenone alkaloid, while **ACB1**, **ACB2**, **ACB4**, **ACB5**, **ACB6** and **ACB7** were isolated for the first time from *Alphonsea* species.

4.3.1 Terpenoid ACB1: Stigmasterol (81)



81

Compound **ACB1** (22.7 mg) was obtained from the hexane extract as white amorphous and dissolved in CH_2Cl_2. For TLC observation, this compound did not appear under long UV (365 nm) and short UV (254 nm), but appeared as violet spot after derivatisation with *p*-Anisaldehyde/sulphuric acid reagent. The molecular formula of **ACB1** was $C_{29}H_{48}O$ as established from GC-MS spectrum in accordance with the appearance of significant peak at *m/z* 412.4 $[M]^+$ by EI-MS (Figure 4.1).

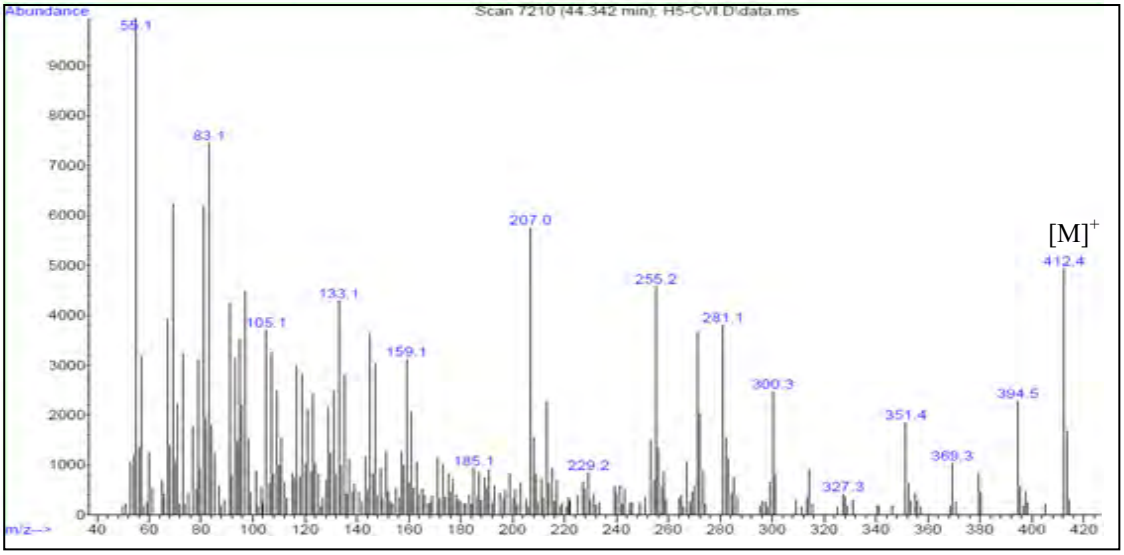


Figure 4.1 GCMS spectrum of **ACB1**

UV spectrum of **ACB1** in methanol displayed maximum absorption peaks at 249 nm (Siripong et al., 1992). The IR spectrum exhibited a strong band hydroxyl (O-H) group at 3339 cm^{-1}. Absorptions at 2932 and 2862 cm^{-1} revealed the stretching of aliphatic alkane (C-H) group, while absorption at 1646 cm^{-1} indicated the presence of alkene (C=C) group. In addition, bands at 1458 and 1048 cm^{-1} were corresponded for vibrations of bending of CH_2 and cycloalkane respectively (Kamboj & Saluja, 2011). The IR spectrum for **ACB1** is showed in Figure 4.2.

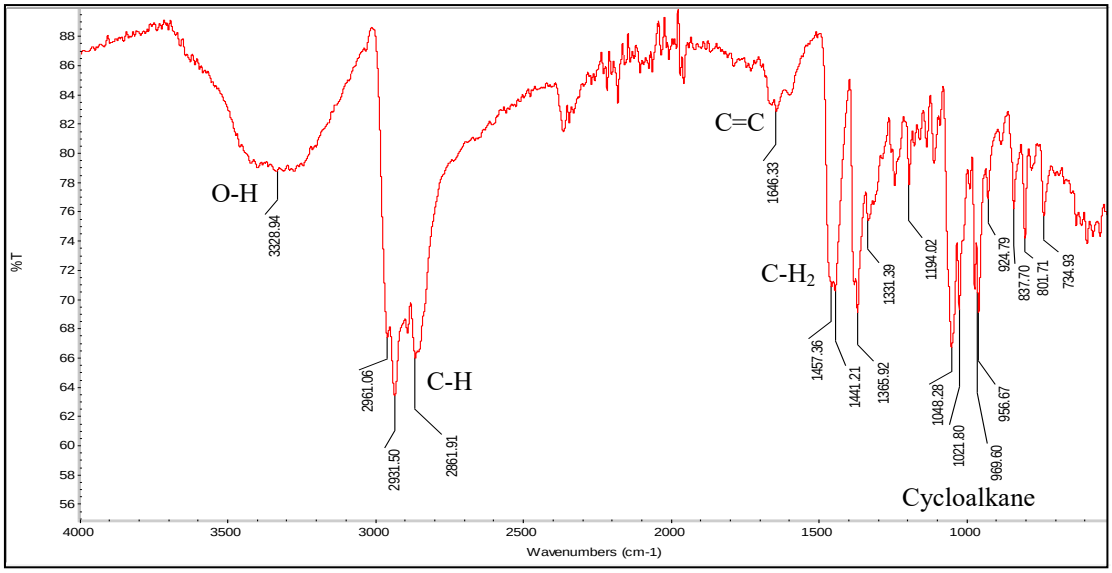


Figure 4.2 ATR-FTIR of **ACB1**

The ^{1}H NMR spectrum of **ACB1** can be referred in Figure 4.3. The spectrum exhibited 48 protons with the presence of a hydroxyl group at the same carbon of proton at δ 3.52 as multiplet signal and two methyl groups at δ 0.69 and 1.01 as singlet signal corresponding to H-18 and H-19, respectively. There were also methyl groups observed at δ 1.07, 0.86 and 0.84, which were attributable to H-21, H-26 and H-27, respectively. Meanwhile, a multiplet signal of methyl group at δ 0.80 was assigned as H-29. Multiplet signals at δ 2.31 and 1.72 were assigned to H-4 and H-16, respectively. The olefinic proton at H-6 was observed at δ 5.35 as multiplet, while two *exo*-olefinic protons at H-22 and H-23 were observed at δ 5.15 (J = 15.0, 8.6 Hz) and 5.01 (J = 15.0, 8.6 Hz) as double of doublet. The coupling constants values (J = 15.0, 8.6 Hz) revealed that these *exo*-olefinic protons coupled each other with *trans* vicinal position (Pavia et al., 2001). Some of proton signals at the shielding region in ^{1}H NMR spectrum were also overlapping which cause the top of proton peaks revealed unclear.

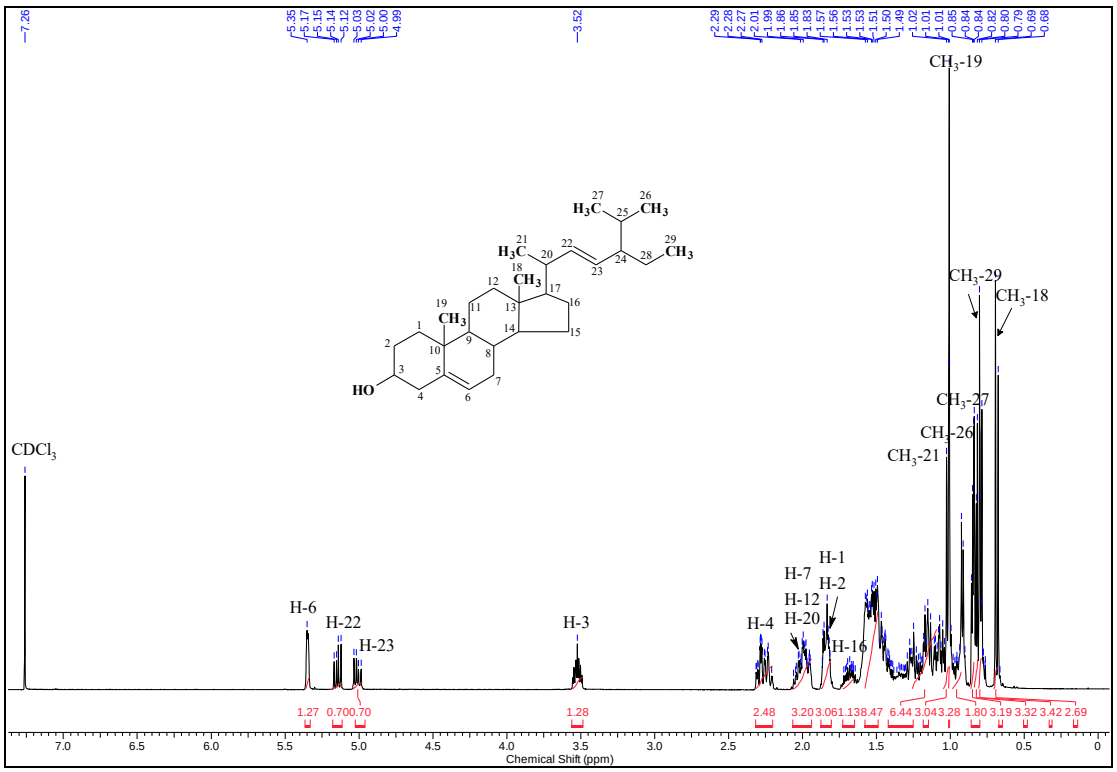


Figure 4.3. ^{1}H NMR spectrum of **ACB1**

The ^{13}C NMR spectrum (Figure 4.4) of **ACB1** showed twenty nine signals consisting of six methyl carbons (CH_3) at δ 12.1, 19.5, 21.2, 21.2, 19.1 and 12.4, which assigned to C-18, C-19, C-21, C-26, C-27 and C-29, respectively. Methyl carbons for C-21 and C-26 was overlapped each other. Three quartenary carbons (C) exhibited at δ 140.8, 36.2 and 42.3 were assigned to C-5, C-10 and C-13, respectively. Nine methylene carbons (CH_2) observed at δ 37.3, 31.7, 42.4, 32.0, 21.3, 39.7, 24.5, 29.0, and 25.5 were assigned to C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16 and C-28, respectively. In addition, four olefinic carbons appeared at δ 140.8, 121.8, 138.4 and 129.3 were assigned to C-5, C-6, C-22 and C-23, respectively. Meanwhile, an isolated signal at δ 71.9 attributed to C-3 due to the presence of a hydroxyl group. Data analysis of compound **ACB1** as presented in Table 4.1 was in close agreement to stigmasterol data reported by Malebo et al. (2013).

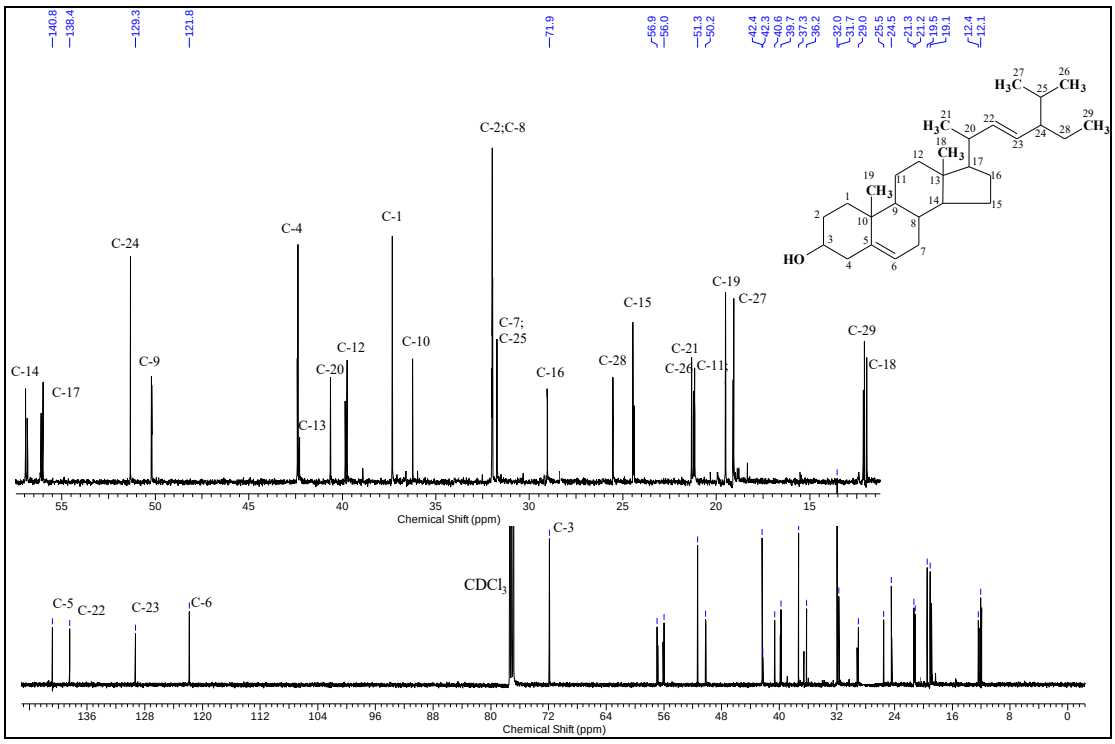


Figure 4.4. ^{13}C NMR spectrum of **ACB1**

In 2D NMR experiments; COSY, HMQC and HMBC data analyses help to confirm the assignments of carbon and proton in the molecule. Based on COSY spectrum in Figure 4.5, there were correlations between H-6/H-7, H-22/H-20, H-22/H-23, H-3/H-2 and H-3/H-4. The direct correlation (HMQC) and long correlation (HMBC) of protons and carbons in the molecule is displayed in Figures 4.6 and 4.7, respectively. In addition, Figure 4.8 shows the HMBC and COSY correlations of **ACB1**.

Stigmasterol (**81**) is a phytosteroid compound, which is usually found in plant membrane cell and important for the function and structure of the membrane. This compound has been reported as antimicrobial, antioxidant, cytotoxic and antitrypanosomal (Cherdtrakulkiat et al., 2015; Malebo et al., 2013).

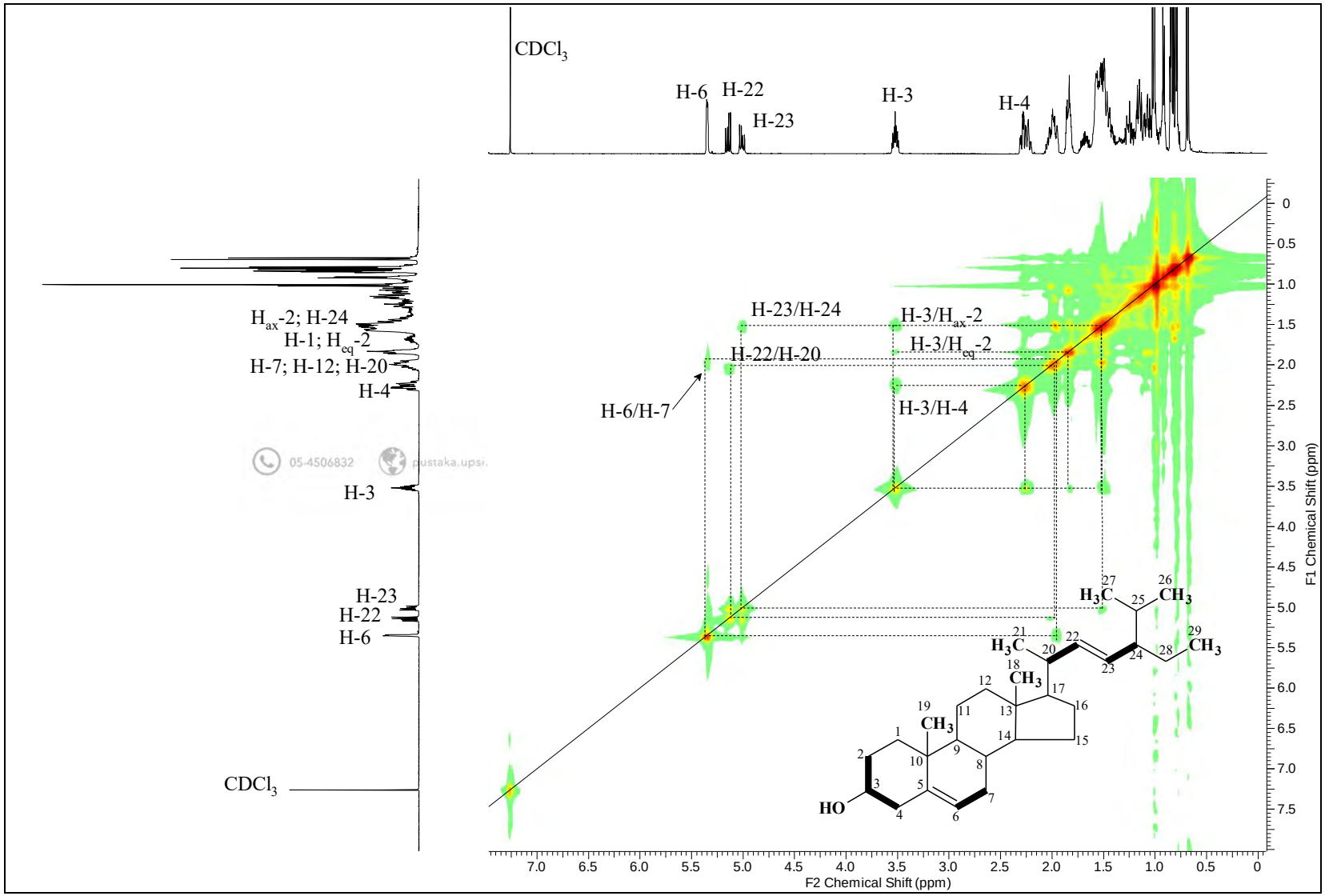


Figure 4.5. COSY spectrum of **ACB1**

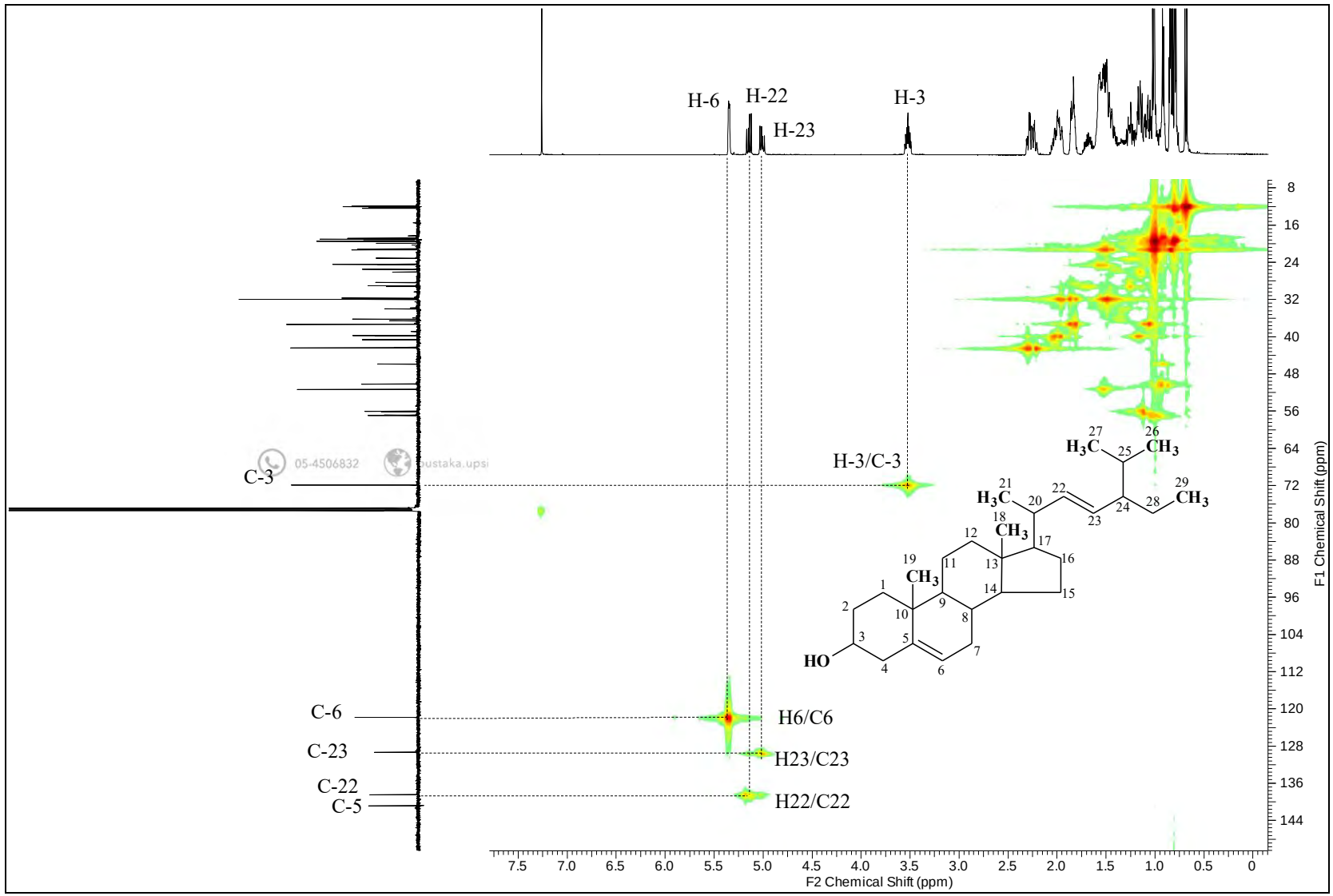


Figure 4.6. HMQC spectrum of **ACB1**

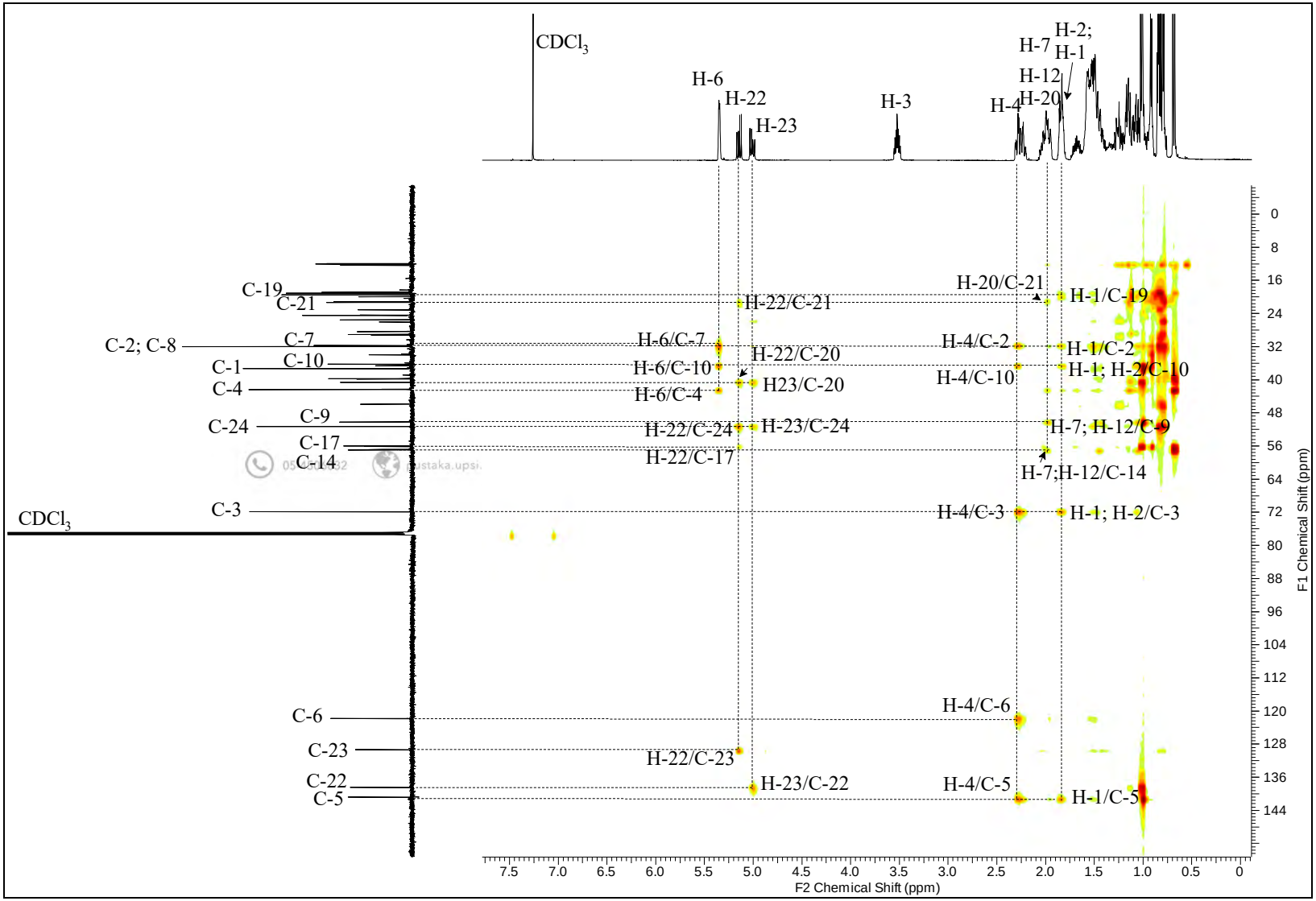


Figure 4.7. HMBC spectrum of **ACB1**

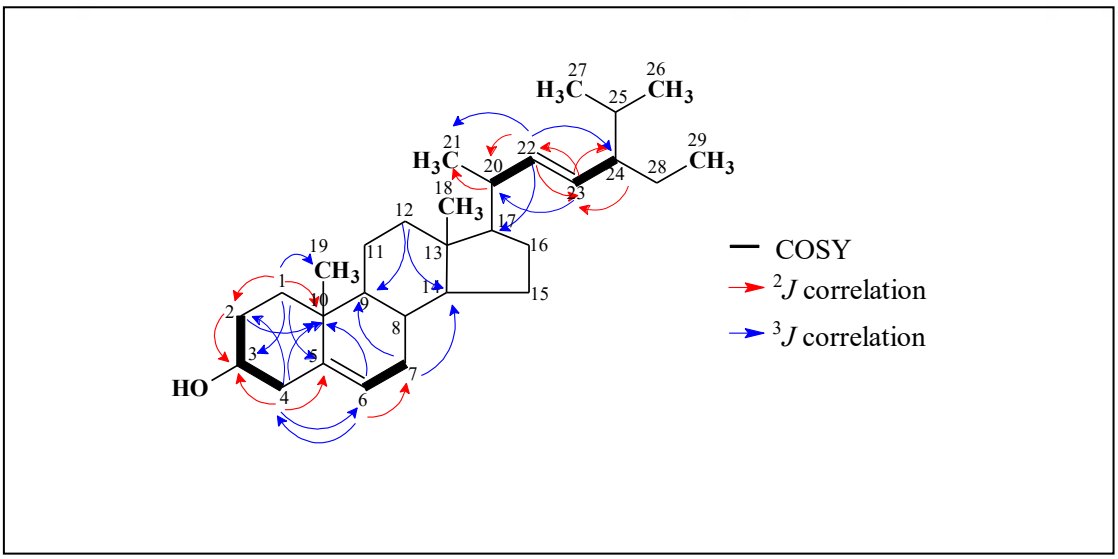


Figure 4.8. Selected HMBC and COSY of **ACB1**

Table 4.3

Spectral data of ***ACB1*** *(CDCl$_3$, 500 MHz) and Stigmasterol* ***(81)***

	ACB1				Stigmasterol (81)	
No.	**δ^{13}C (ppm)**	**δ^{1}H (ppm)**	**HMBC**	**COSY**	**δ^{13}C (ppm)**	**δ^{1}H (ppm)**
1	37.3	1.87 (*m*, 1H)	C-19, C-2, C-3, C-10, C-5		37.4	1.86 (*m*, 1H-$_{eq}$)
		1.08 (*m*, 1H)				1.05 (*m*, 1H-$_{ax}$)
2	31.7	1.83 (*m*, 1H)	C-3,C-10	H-3	31.8	1.84 (*m*, 1H-$_{eq}$)
		1.50 (*m*, 1H)				1.51 (*m*, 1H-$_{ax}$)
3	71.9	3.52 (*m*, 1H)		H-2, H-4	71.9	3.52 (*m*, 1H)
4	42.4	2.31 (*m*, 1H)	C-2, C-10, C-3,	H-3	42.4	2.27 (*m*, 1H-$_{eq}$)
		2.27 (*m*, 1H)	C-6, C-5			2.24 (*m*, 1H-$_{ax}$)
5	140.8	-			140.9	-
6	121.8	5.35 (*m*, 1H)	C-7, C-10, C-4	H-7	121.9	5.35 (*m*,1H)
7	32.0	1.97 (*m*, 1H)	C-9, C-14	H-6	32.0	1.96 (*m*, 1H-$_{eq}$)
		1.51 (*m*, 1H)				1.49 (*m*,1H-$_{ax}$)
8	32.0	1.49 (*m*, 1H)			32.0	1.45 (*m*, 1H-$_{eq}$)
9	50.2	0.96 (*m*, 1H)			50.3	0.95 (*m*, 1H)
10	36.2	-			36.7	-
11	21.2	1.57 (*m*, 1H)			21.2	1.60 (*m*, 1H-$_{eq}$)
		1.51 (*m*, 1H)				1.50 (*m*, 1H-$_{ax}$)
12	39.7	2.01 (*m*, 1H)	C-9, C-14		39.8	2.00 (*m*, 1H-$_{eq}$)
		1.20 (*m*, 1H)				1.20 (*m*, 1H-$_{ax}$)
13	42.3	-			42.4	-
14	56.9	0.98 (*m*, 1H)			57.0	1.01 (*m*, 1H-$_{ax}$)

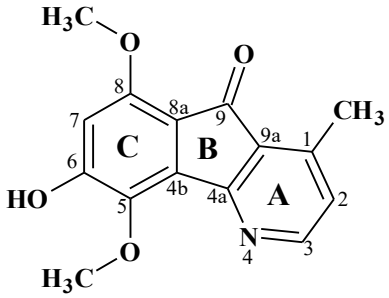
(continued)

Table 4.3 *(Continued)*

	ACB1				Stigmasterol (81)	
No.	**$\delta^{13}C$ (ppm)**	**$\delta^{1}H$ (ppm)**	**HMBC**	**COSY**	**$\delta^{13}C$ (ppm)**	**$\delta^{1}H$ (ppm)**
15	24.5	1.56 (*m*, 1H) 1.05 (*m*, 1H)			24.5	1.55 (*m*, 1H-$_{eq}$) 1.04 (*m*, 1H-$_{ax}$)
16	29.0	1.72 (*m*, 1H) 1.27 (*m*, 1H)			29.1	1.71 (*m*, 1H-$_{eq}$) 1.27 (*m*, 1H-$_{ax}$)
17	56.0	1.13 (*m*, 1H)			56.1	1.15 (*m*, 1H-$_{ax}$)
18	12.1	0.69 (*s*, 3H)			12.2	0.69 (*s*, 3H)
19	19.5	1.01 (*s*, 3H)			19.5	1.01 (*s*, 3H)
20	40.6	2.06 (*m*, 1H)	C-21, C-14	H-22	40.7	2.04 (*m*, 1H)
21	21.3	1.02 (*d*, *J* = 6.8 Hz, 3H)			21.4	1.04 (*d*, 3H)
22	138.4	5.15 (*dd*, *J* = 8.6, 15.0 Hz, H)	C-24, C-20, C-21, C-17, C-23	H-20, H-23	138.5	5.18 (*dd*, 1H)
23	129.3	5.01 (*dd*, *J* = 8.6, 15.0 Hz, H)	C-20, C-24, C-22		129.4	5.03 (*dd*, 1H)
24	51.3	1.53 (*m*, 1H)			51.4	1.51 (*m,* 1H)
25	32.0	1.55 (*m*, 1H)			32.0	1.56 (*m*, 1H)
26	21.2	0.86 (*d*, *J* = 6.3 Hz, 3H)			21.2	0.87 (*d,* 3H)
27	19.1	0.84 (*d*, *J* = 6.8 Hz, 3H)			19.1	0.79 (*d*, 3H)
28	25.5	1.47 (*m*, H) 1.23 (*m*, H)			25.6	1.43 (*m,* 1H-$_{eq}$) 1.18 (*m*, 1H-$_{ax}$)
29	12.4	0.80 (*m*, 3H)			12.4	0.81 (*t*, 3H)

Note: *Measured at 125 (^{13}C) and 500 (^{1}H) in $CDCl_3$, δ TMS = 0 (Malebo et al., 2013)

4.3.2 Alkaloid ACB2: Kinabaline (82)



82

Compound **ACB2** (5.7 mg) was isolated as yellow amorphous from the alkaloid fraction and dissolved in CH_2Cl_2. This compound appeared as yellow spot under long UV (365 nm) and as a dark spot under short UV (254 nm), and remained as a yellow spot before and after derivatisation with Dragendorff's reagent. The UV spectrum for this compound in methanol showed maximum absorption at 268 and 298 nm identifying an onychine skeleton (Xie & Yang, 1999). The IR spectrum of this compound showed strong absorptions at 3308 cm^{-1} and 1660 cm^{-1} attributed to O-H and C=O functional groups, respectively (Pavia et al., 2001). Its molecular formula of $C_{15}H_{13}NO_4$ was supported by its molecular ion peak at *m/z* 271.1 $[M]^+$ analysed using EI-MS technique (Figure 4.9).

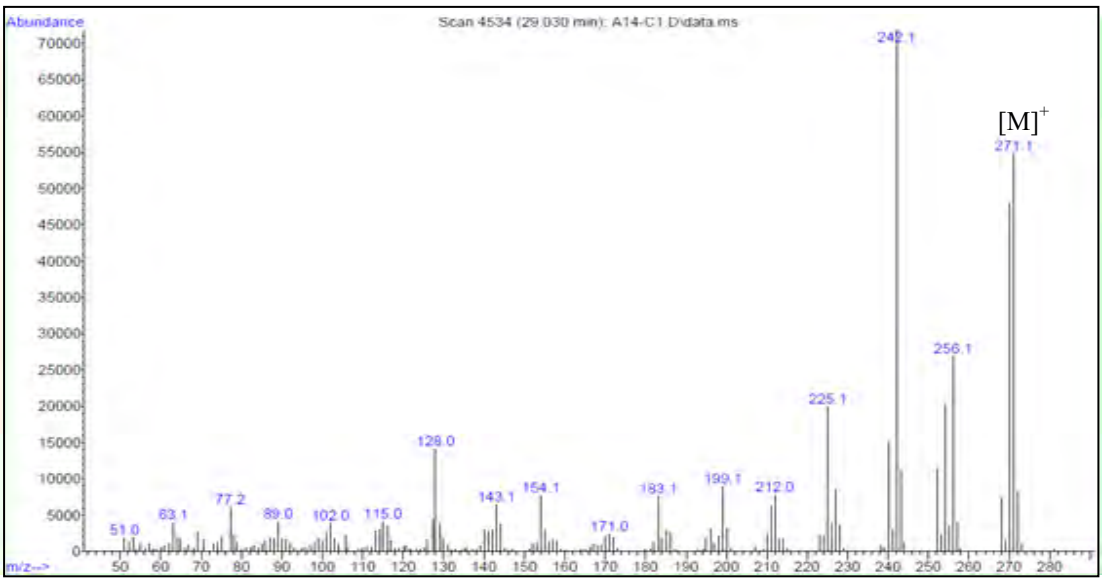


Figure 4.9. GCMS spectrum of **ACB2**

The ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of **ACB2** (Figure 4.10) displayed resonances of one singlet of a methyl group at δ 2.61 (CH_3-1), two singlets of methoxyl groups at δ 3.91 (OCH_3-5) and δ 3.99 (OCH_3-8), one singlet of an aromatic proton of ring **C** at δ 6.32 (H-7), two doublets of aromatic protons of ring **A** at δ 6.94 (H-2, J = 5.0 Hz) and δ 8.50 (H-3, J = 5.0 Hz) and one broad singlet of a hydroxyl group at δ 8.81 (OH-6).

The ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of **ACB2** as shown in Figure 4.11 revealed fifteen carbon signals consisting of three methine carbons; C-7, C-2 and C-3 resonated at δ 101.8, 125.2 and 152.8, respectively. Eight quaternary carbons at δ 111.0, 127.7, 131.7, 140.0, 146.9, 155.8, 162.5 and 163.4 were designated to C-4b, C-9a, C-8a, C-8, C-1, C-6, C-5 and C-4a. One carbonyl carbon observed at δ 193.9 was assigned to C-9, while two methoxyl carbon signals at δ 62.0 and 56.7 were referred to OCH_3-8 and OCH_3-5, respectively. In addition, one methyl carbon positioned at C-1 was appeared at δ 17.3.

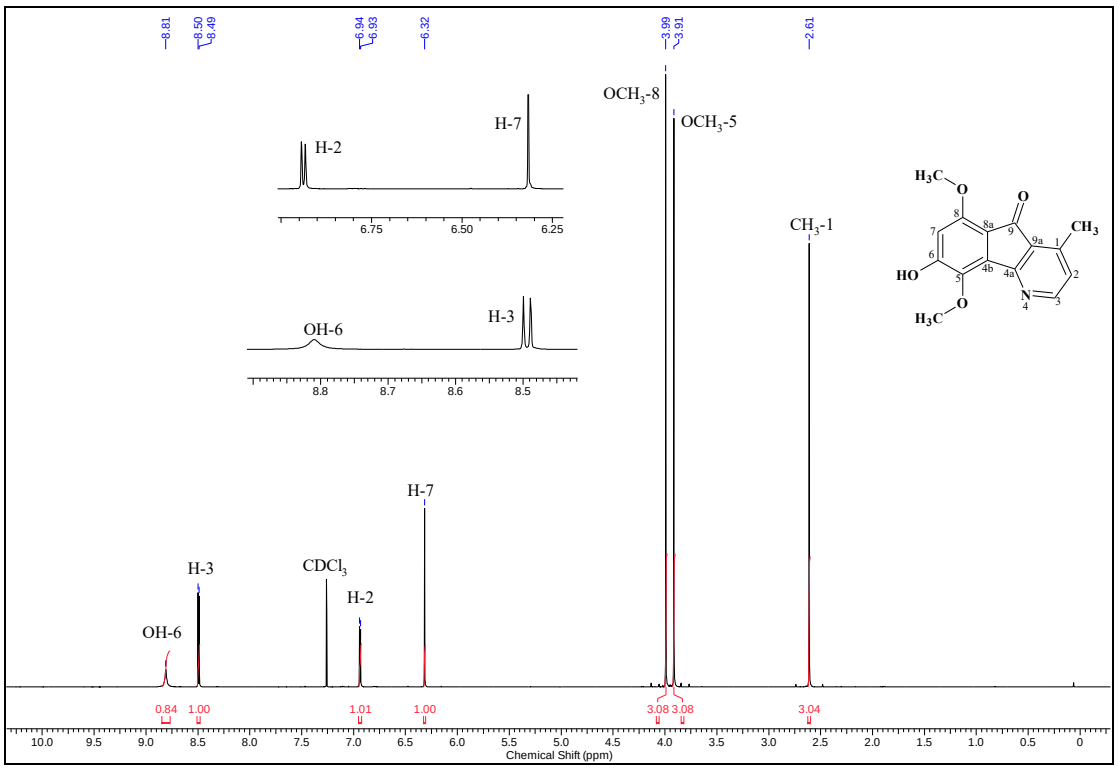


Figure 4.10. ^{1}H NMR spectrum of **ACB2**

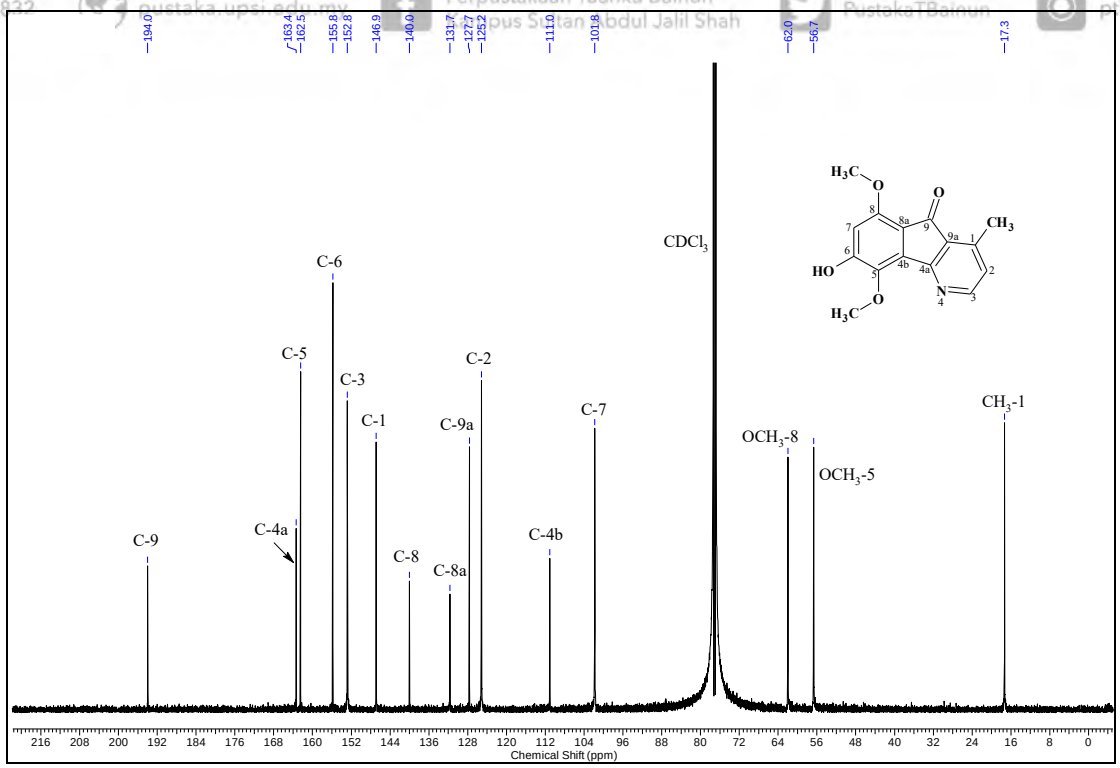
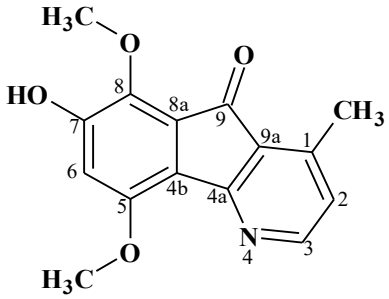


Figure 4.11. ^{13}C NMR spectrum of **ACB2**

Furthermore, the evidence from 2D NMR including COSY, HMQC and long-range coupling in HMBC supported the assignments of protons and carbons in the molecule. In COSY data analysis (Figure 4.12), there was a correlation between H-2 and H-3. All direct proton-carbon correlations were assigned by following HMQC experiment as referred in Figure 4.13. From HMBC spectrum in Figure 4.14, the position of methoxyl groups were further confirmed due to the cross peak between OCH_3-8 with C-8 (δ 140.0) and OCH_3-5 with C-5 (δ 162.5). In addition, proton OH-6 showed an HMBC correlation to C-6 (δ 155.8) in Figure 4.15. The HMBC and COSY correlations are summarized in Figure 4.16.

From the data analyses, the ^{1}H NMR chemical shifts were found to be identical with those reported for 6-hydroxy-5,8-dimethoxyonychine or other name as kinabaline (**82**). This compound was previously isolated from the trunk bark of *Meiogyne virgata,* which collected from Mount Kinabalu, Sabah (Tadic et al., 1987). The ^{13}C NMR chemical shifts were also compared with the 5,8-dimethoxy-7-hydroxy-1-methyl-4-azafluoren-9-one (**87**) (Yoshida et al., 2013) in which the C-6 of this compound displayed a higher field resonance (δ 101.5) when compared to C-6 (δ 155.8) in **ACB2** due to the presence of a hydroxyl group.

The comparison of data analysis of compound **ACB2** and values of kinabaline (**82**) is tabulated in Table 4.4. On these evidences, compound **ACB2** was determined as kinabaline (**82**) and according to the current knowledge, the biological activity of this compound on particular assays has not been reported thus far.



87

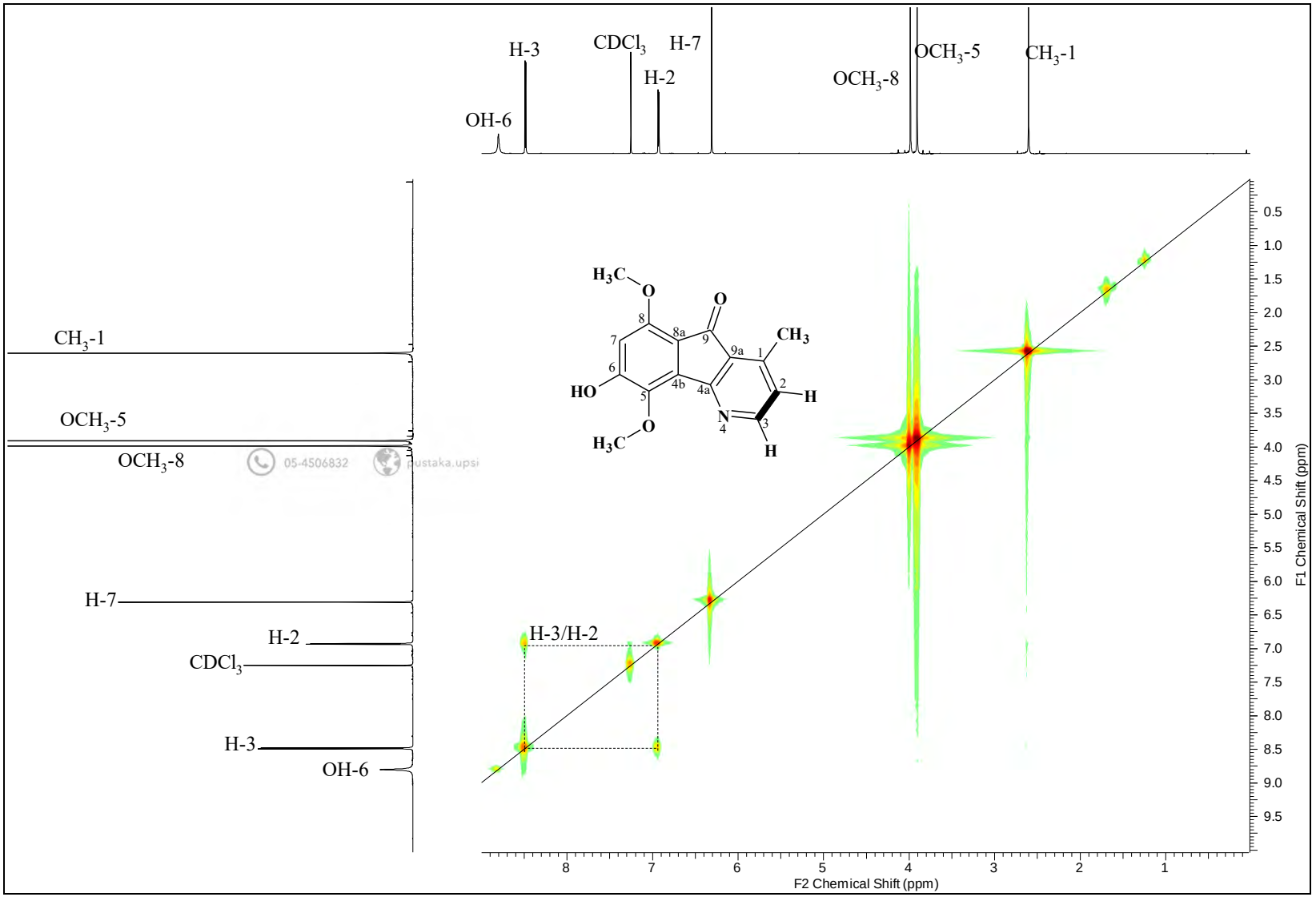


Figure 4.12. COSY spectrum of **ACB2**

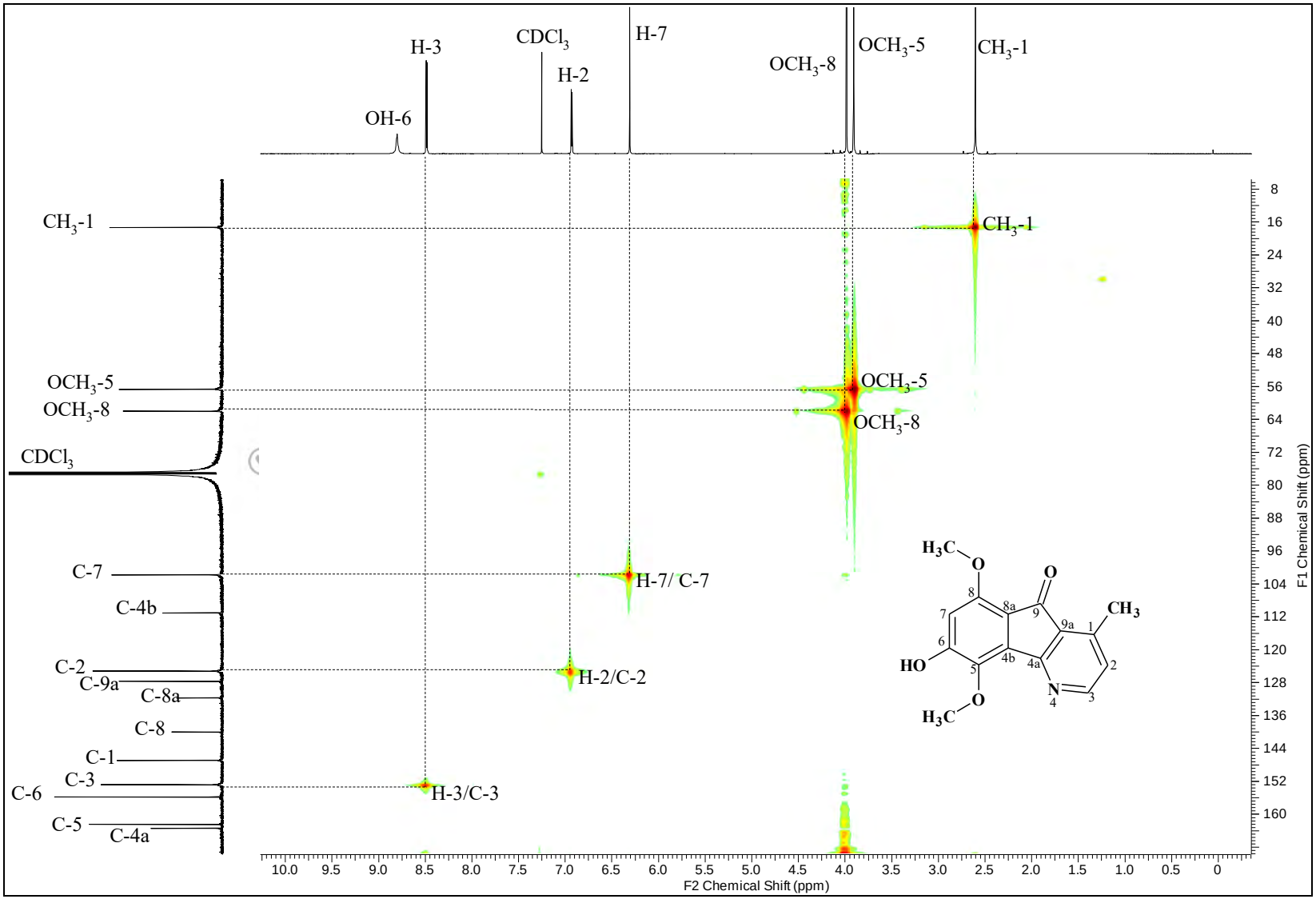


Figure 4.13. HMQC spectrum of **ACB2**

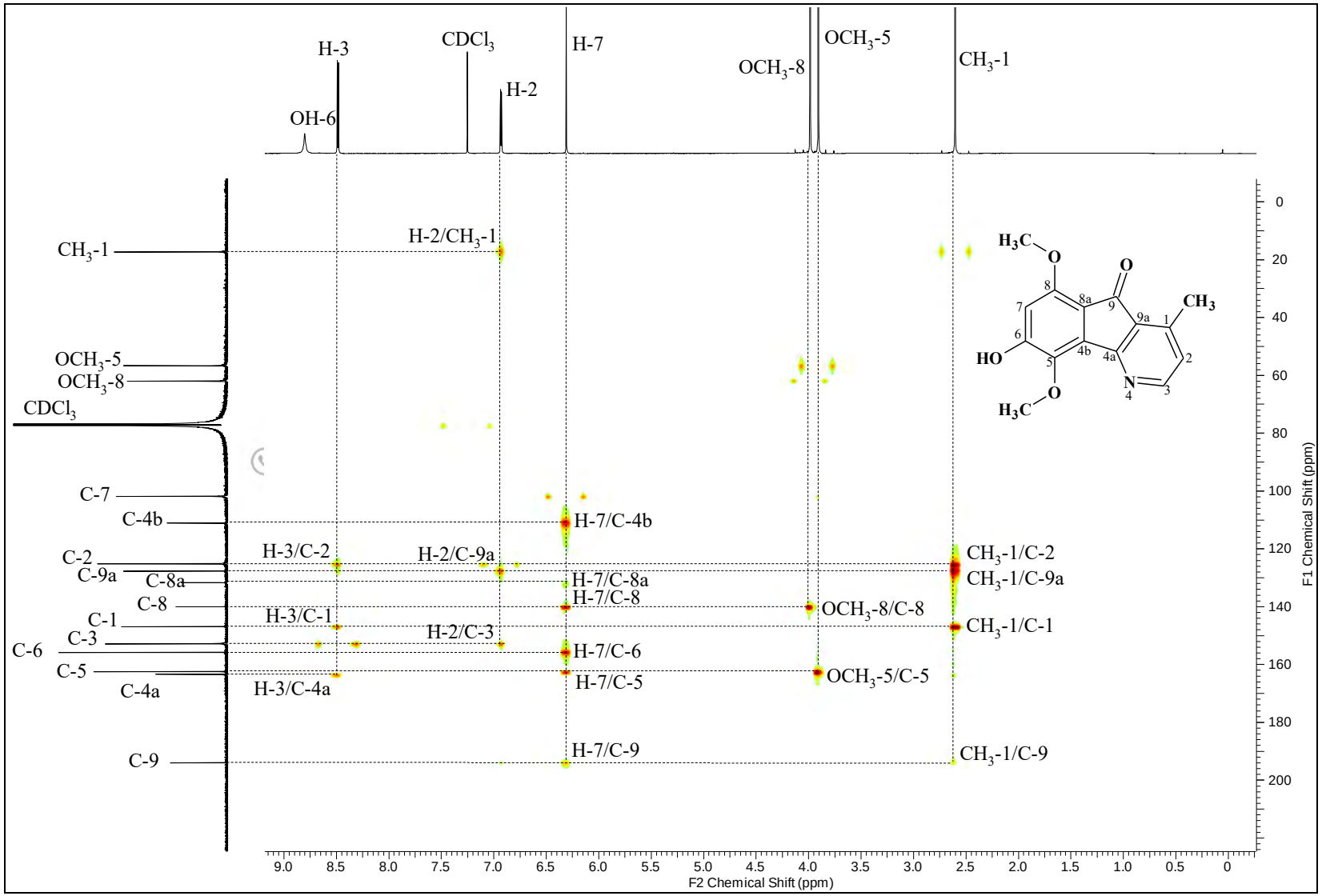


Figure 4.14. HMBC spectrum of **ACB2**

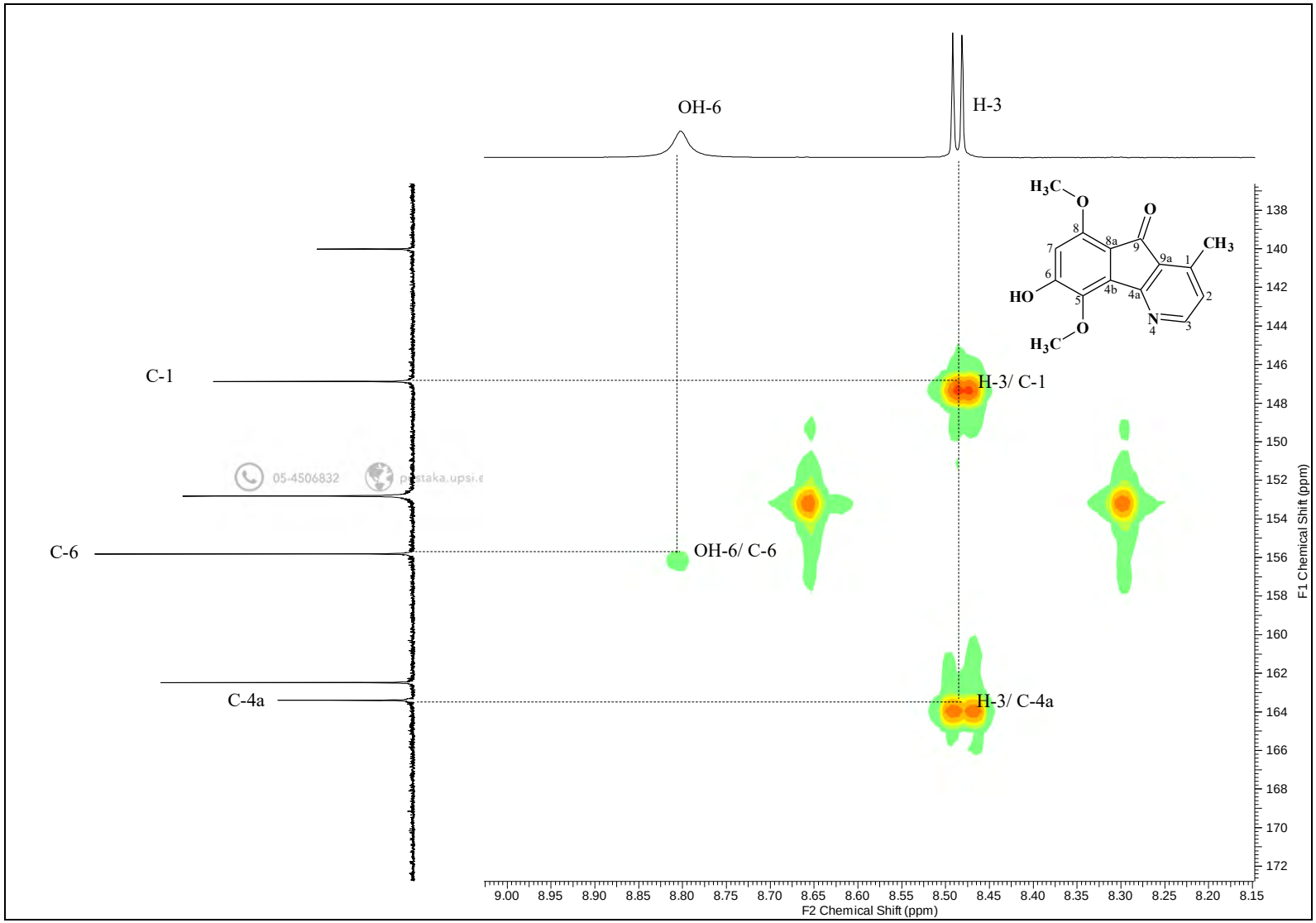


Figure 4.15. Expansion HMBC spectrum of **ACB2**

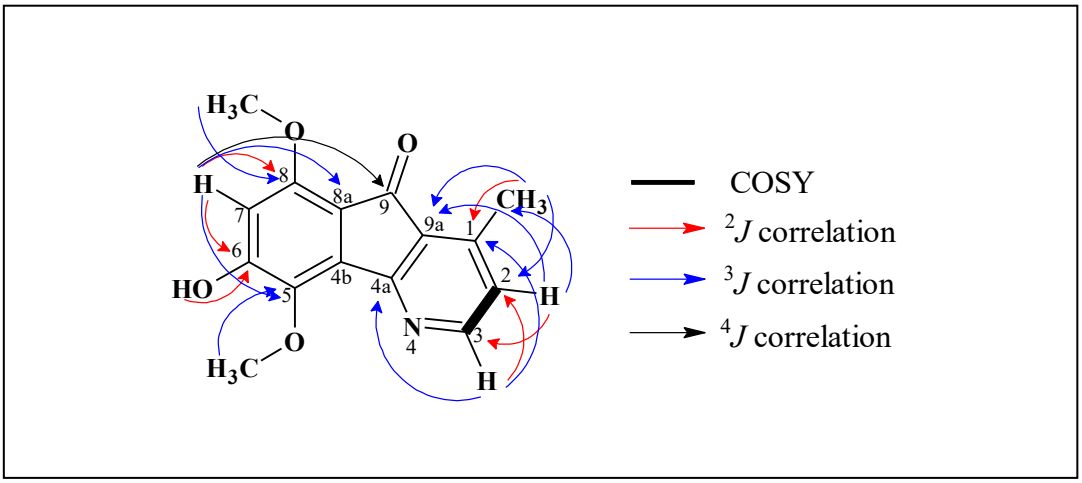


*Figure 4.16.*COSY, ^{1}H and ^{13}C correlations observed in HMBC spectrum of **ACB2**

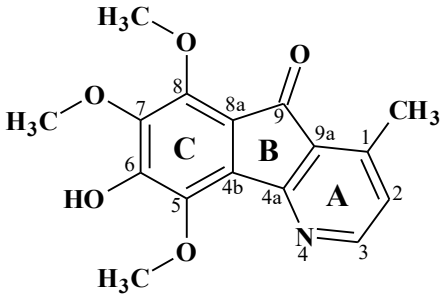
Table 4.4

*Spectral data of **ACB2** (500 MHz, $CDCl_3$), ^1H NMR data of kinabaline (**82**) and ^{13}C NMR data of 5,8-dimethoxy-7-hydroxy-1-methyl-4-azafluoren-9-one (**87**)*

Position	ACB2					Kinabaline (82)	(87)
	$\delta^{13}C$ (ppm)	**δ^1H (ppm) *J* (Hz)**	**HMQC**	**HMBC**	**COSY**	***δ^1H (ppm) *J* (Hz)**	****$\delta^{13}C$ (ppm)**
1	146.9						149.2
2	125.2	6.94 (*d*, 5.0Hz, 1H)	H-2	CH_3-1, C-9a,C-3	H-3	6.95 (*d*, 5.0 Hz, 1H)	124.6
3	152.8	8.50 (*d*, 5.0Hz, 1H)	H-3	C-2,C-1, C-4a	H-2	8.51 (*d*, 5.0 Hz, 1H)	148.6
4a	163.4						164.0
4b	111.0						119.2
5	162.5						156.4
6	155.8						101.5
7	101.8	6.32 (*s*, 1H)	H-7	C-4b, C-8a, C-8, C-6, C-5		6.34 (*s*, 1H)	147.2
8	140.0						142.5
8a	131.7						129.6
9	194.0						190.7
9a	127.7						126.0
OCH_3-8	62.0	3.99 (*s*, 3H)	OCH_3-8	C-8		4.02 (*s*, 3H)	61.1
OCH_3-5	56.7	3.91 (*s*, 3H)	OCH_3-5	C-5		3.93 (*s*, 3H)	56.5
CH_3-1	17.3	2.61 (*s*, 3H)	CH_3-1	C-2, C-9a, C-1, C-9		2.63 (*s*, 3H)	17.3
OH-6		8.81 (*br s*,1H)		C-6		n.a	

Note: *Measured at 80.13 MHz (^1H NMR) in $CDCl_3$ (Tadic et al., 1987);
** Measured at 75 MHz (^{13}C NMR) in $CDCl_3$ (Yoshida et al., 2013). n.a = not available

4.3.3 Alkaloid ACB3: Muniranine (83)



83

Compound **ACB3** (5.1 mg) was afforded as orange amorphous and dissolved in CH_2Cl_2. After sprayed with Dragendroff"s reagent, TLC of this compound showed yellow spot, indicating a positive result of a typical of alkaloid compounds. Moreover, the TLC analysis under long UV (365 nm) observed as dark red spot and appeared as dark spot under short UV (254 nm). The LC-MS data revealed a very significant molecular ion peak at *m/z* 300.0869 $[M-H]^+$ measured by HR-ESI-MS and supported the molecular formula of $C_{16}H_{15}NO_5$ (Figure 4.17).

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

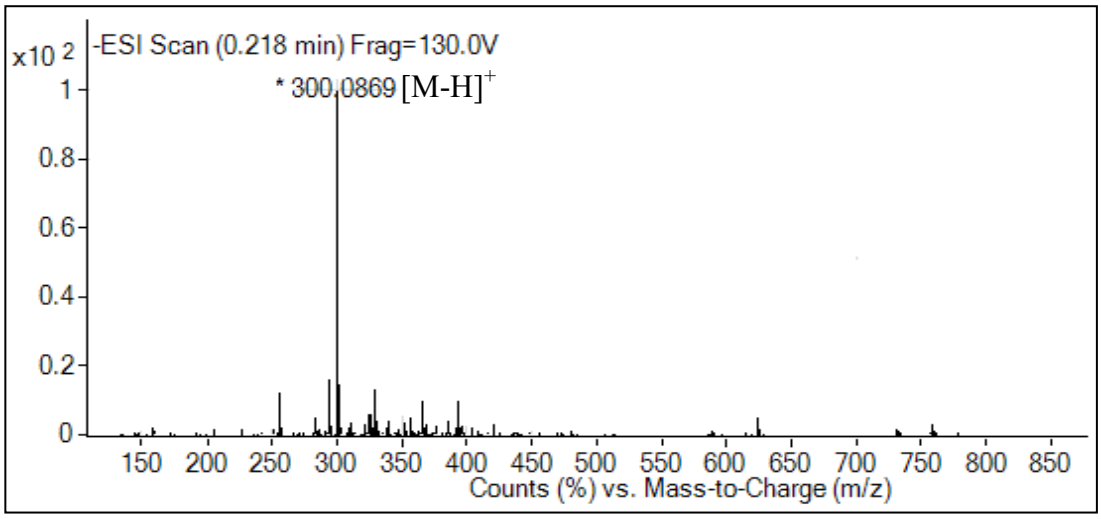


Figure 4.17. LCMS spectrum of **ACB3**

The IR spectrum of this compound (Figure 4.18) exhibited the presence of hydroxyl group at 3305 cm^{-1} and also showed the presence of C-H stretching at 2932 cm^{-1} (Pavia et al. 2001). Absorption peak at 1676 cm^{-1} displayed the existence of C=O group. Moreover, The UV spectrum displayed absorption at 250 and 269 nm in accordance with an azafluorenone skeleton (Arango et al., 1987).

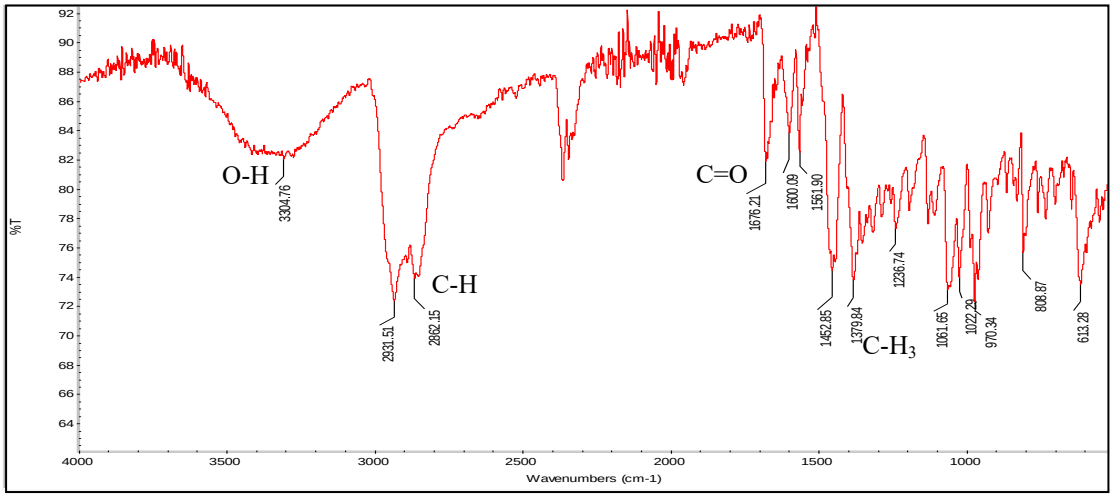


Figure 4.18. ATR-FTIR of **ACB3**

In ^{1}H NMR spectrum of **ACB3** in Figure 4.19, showed resonance of one singlet for methyl of ring **A** (H-1; δ 2.59) and three singlets of methoxyl of ring **C** (OCH_3-5; δ 4.01, OCH_3-7; δ 3.98, OCH_3-8; δ 4.04). Two doublets of aromatic protons of ring **A** (H-2; δ 6.89, J = 5.0 Hz and H-3; δ 8.45, J = 5.0 Hz) with *ortho* position and one broad singlet of hydroxyl group of ring **C** (OH-6; δ 8.71) also identified in the spectrum. In addition, the methoxyl proton at OCH_3-5 is more deshielded than the methoxyl proton at OCH_3-7 is due to the proximity of pyridine ring (Tadić et al., 1988).

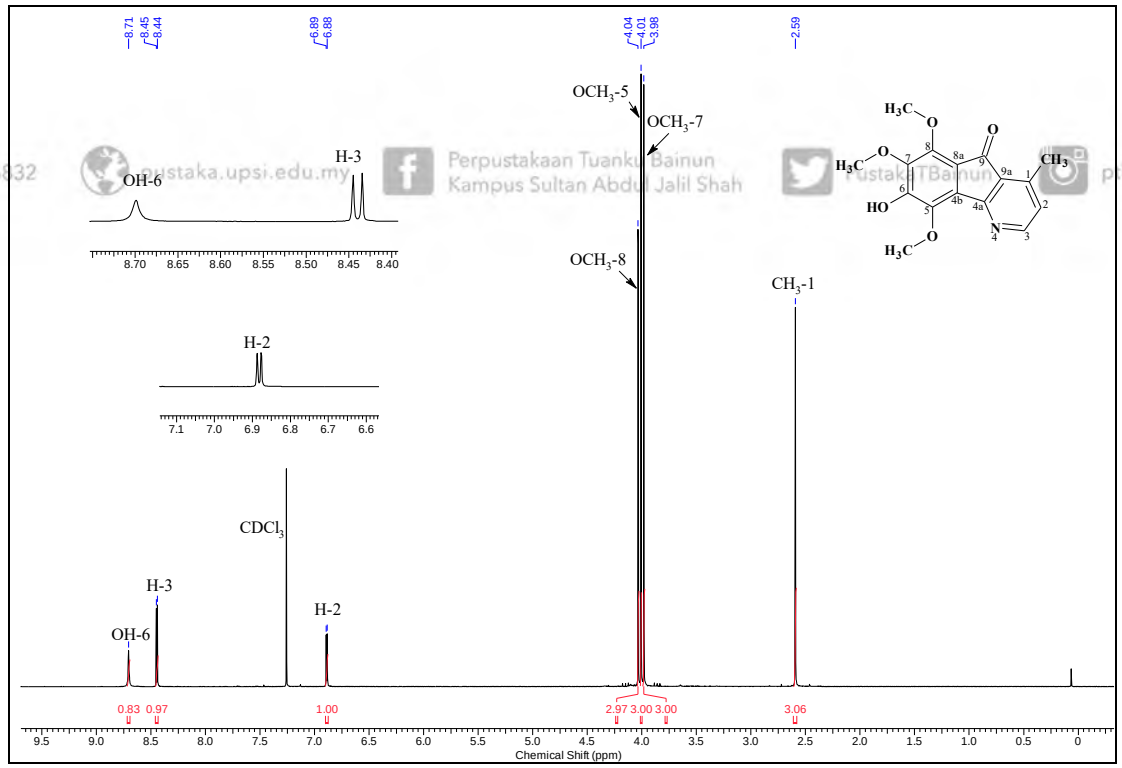


Figure 4.19. ^{1}H NMR spectrum of **ACB3**

The ^{13}C NMR spectrum of **ACB3** (Figure 4.20) revealed sixteen signals of carbons. Two methine carbons (δ 124.7 and 153.0) were assigned to C-2 and C-3, respectively. Meanwhile, nine quaternary carbons at δ 113.8, 127.0, 127.9, 143.8, 144.0, 147.2, 148.8, 154.6 and 164.0 were designated to C-4b, C-9a, C-8a, C-7, C-8, C-1, C-6, C-5 and C-4a, respectively. The presence of a carbonyl (δ 194.8) and three methoxyl carbons (δ 61.5, 61.8 and 62.2) was identified in the spectrum and assigned to C-9, OCH_3-7, OCH_3-5 and OCH_3-8 respectively. In addition, one methyl group attached to C-1 appeared at δ 17.4.

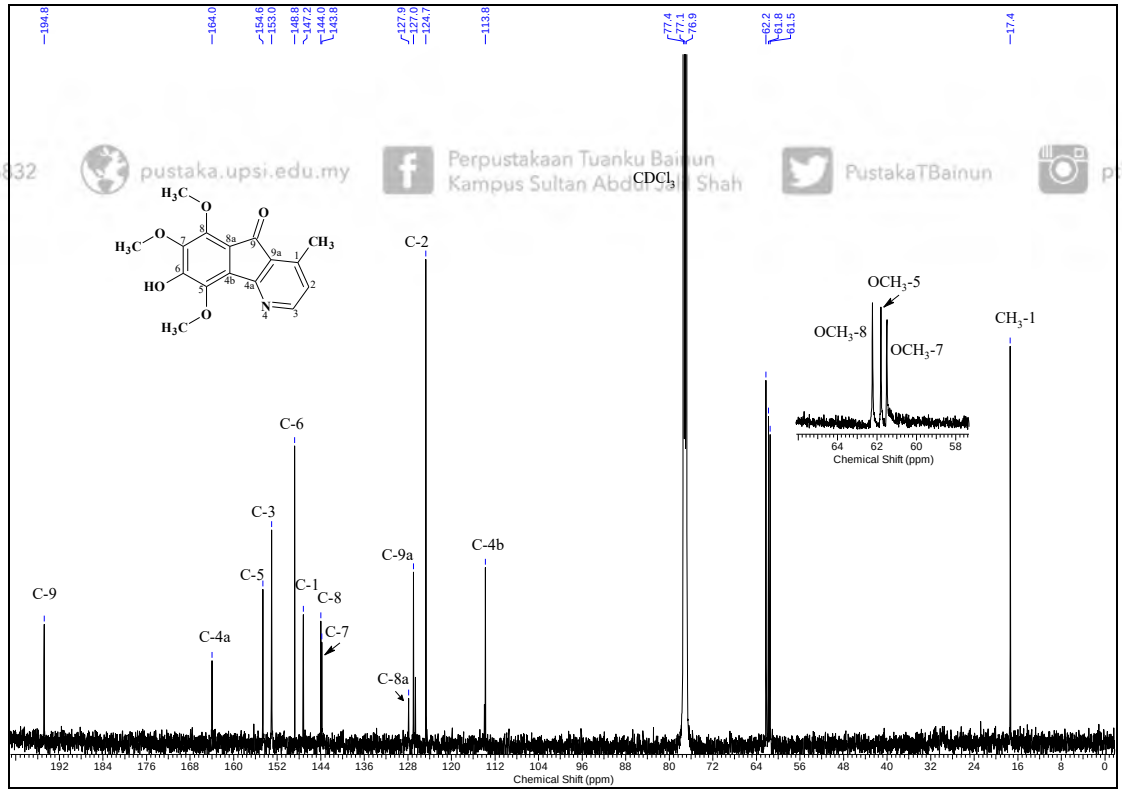


Figure 4.20. ^{13}C NMR spectrum of **ACB3**

In COSY spectrum (Figure 4.21), there was a correlation between proton H-2 and H-3 while NOESY spectrum (Figure 4.22) revealed a strong correlation between OH-6 with OCH_3-7, and CH_3-1 with H-2 and H-3. The HMQC spectrum of **ACB3** is shown in Figure 4.23. The structure of **ACB3** was further confirmed by the HMBC spectrum as shown in Figure 4.24.

The HMBC spectrum showed $^3J_{CH}$ correlations between OCH_3-5 with C-5 (δ 154.7), OCH_3-7with C-7 (δ 143.8) and C-8 (δ 144.0), and OCH_3-8 with C-7 (δ 143.8) and C-8 (δ 144.0). In addition, HMBC spectrum exhibited strong correlations of proton OH-6 (δ 8.71) with carbons at δ 113.8 (C-4b), 143.8 (C-7), 144.0 (C-8), 148.8 (C-6) and 154.7 (C-5), suggesting the position of this group was attached to C-6.

After data analyses, the ^{1}H NMR spectrum was found to exhibit the same pattern as **ACB2**, but **ACB2** showed only two methoxyl resonances. If thus, it seemed that **ACB3** must be a close analogue of **ACB2**, but with three methoxyl groups instead of two. Therefore, compound **ACB3** was identified as muniranine or 6-hydroxy-5,7,8-trimethoxyonychine (**83**), has been successfully isolated as a new derivative of azafluorenone. The ^{13}C data was compared with **ACB2** and summarized in Table 4.5 while Figure 4.25 shows the HMBC and COSY correlations of **ACB3.**

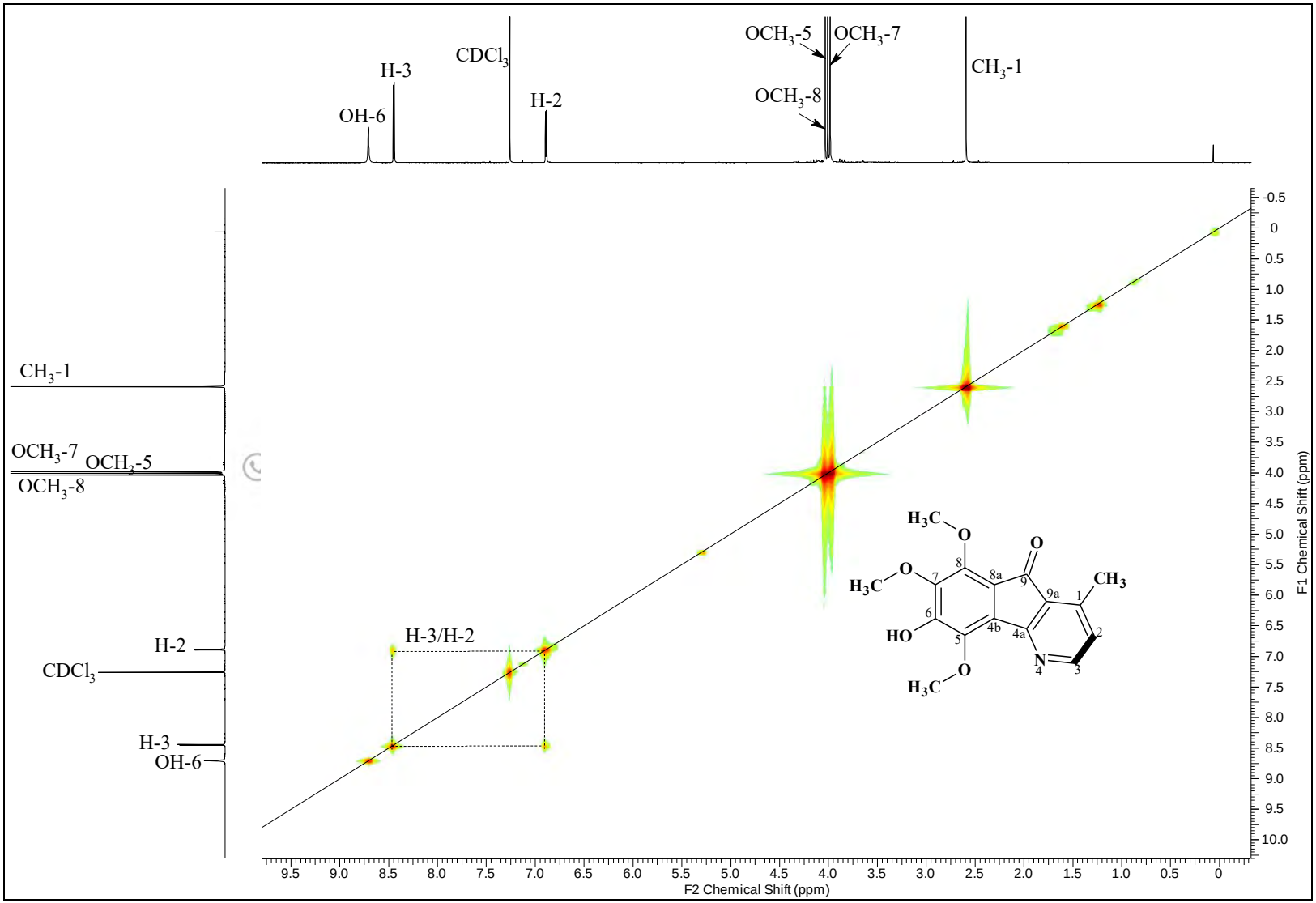


Figure 4.21. COSY spectrum of **ACB3**

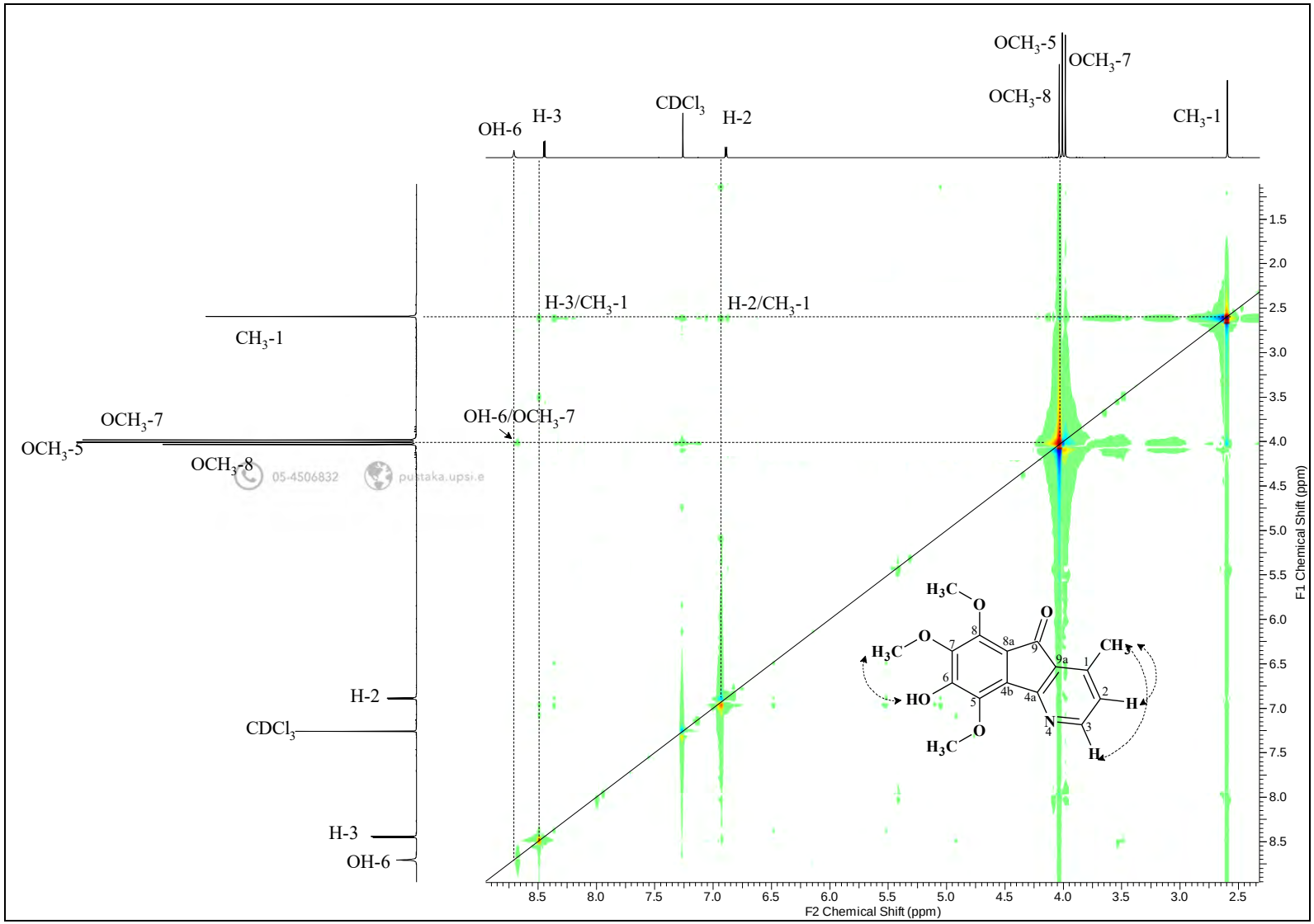


Figure 4.22. NOESY spectrum of **ACB3**

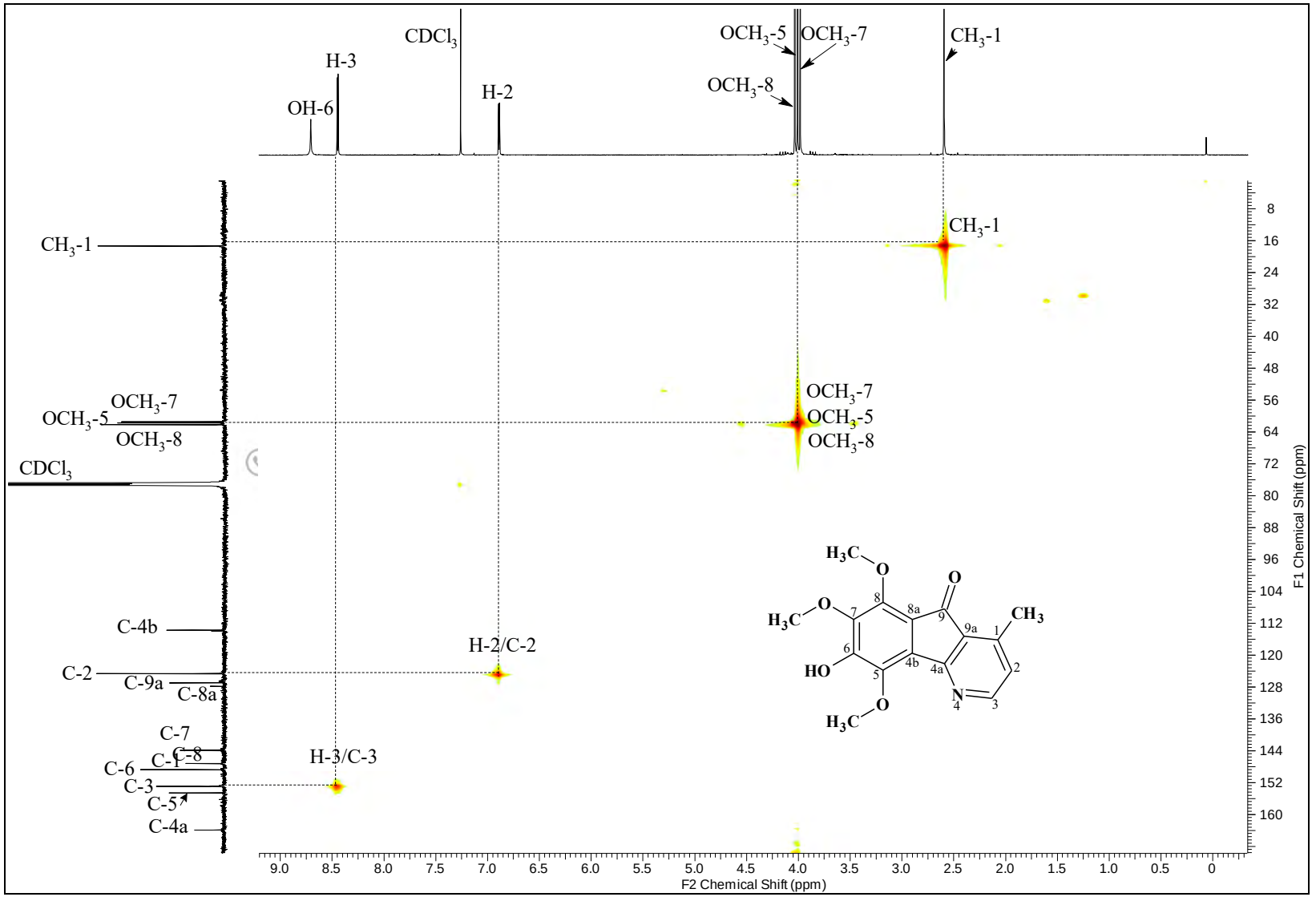


Figure 4.23. HMQC spectrum of **ACB3**

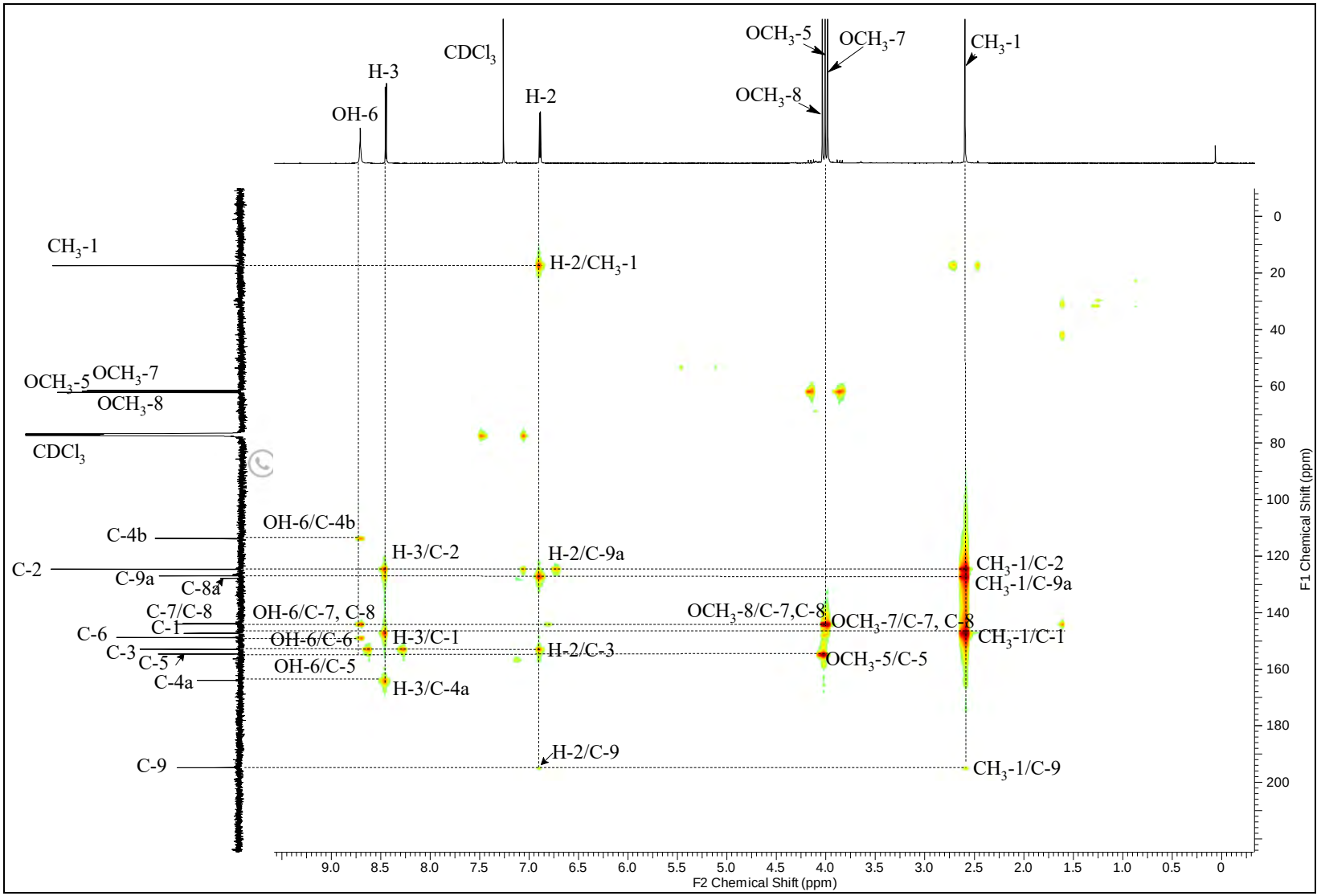


Figure 4.24. HMBC spectrum of **ACB3**

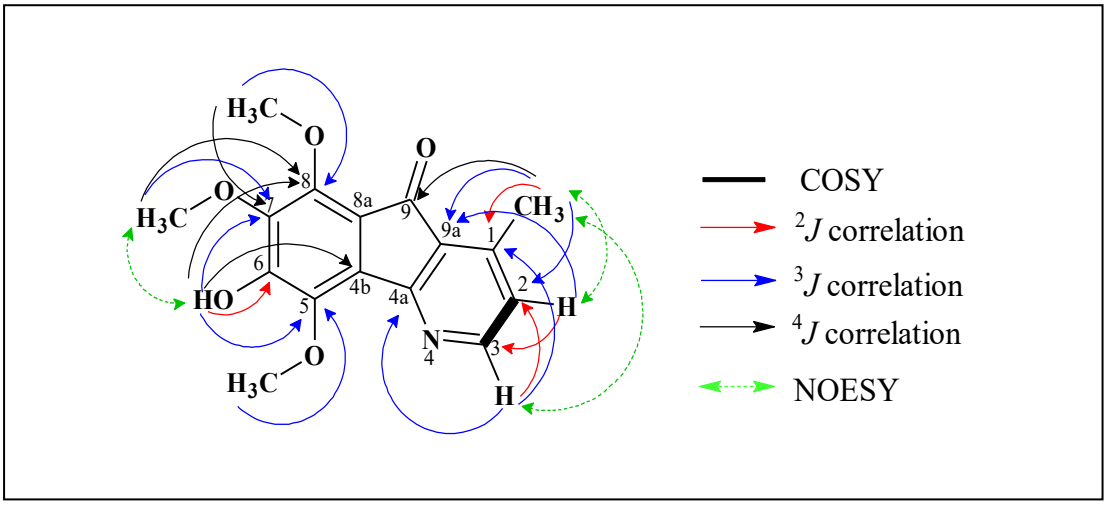


Figure 4.25. COSY, NOESY, ^{1}H and ^{13}C correlations observed in HMBC spectrum of **ACB3**

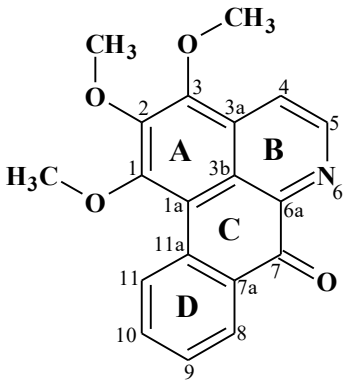
Table 4.5

*Spectral data of **ACB3** (500 MHz, $CDCl_3$) and ^{13}C NMR data of **ACB2***

Position	ACB3 *δ^{13}C (ppm)	ACB2 *δ^{13}C (ppm)	ACB3 *δ^{1}H (ppm) *J* (Hz)	ACB3 HMBC	ACB3 COSY
1	147.2	146.9			
2	124.7	125.2	6.89 (*d*, 5.0 Hz, 1H)	CH_3-1,C-9a,C-3,C-9	H-3
3	153.0	152.8	8.45 (*d*, 5.0 Hz, 1H)	C-2,C-1,C-4a	H-2
4a	164.0	163.4			
4b	113.8	111.0			
5	154.7	162.5			
6	148.8	155.8			
7	144.0	101.8			
8	143.8	140.0			
8a	127.9	131.7			
9	194.8	194.0			
9a	127.0	127.7			
OCH_3-5	61.8	56.7	4.01 (*s*, 3H)	C-5	
OCH_3-7	61.5	-	3.98 (*s*, 3H)	C-7, C-8	
OCH_3-8	62.2	62.0	4.04 (*s*, 3H)	C-7, C-8	
CH_3-1	17.4	17.3	2.59 (*s*, 3H)	C-1, C-2, C-9a, C-9	
OH-6			8.71 (*br s*, 1H)	C-5, C-6, C-7, C-8 C-4b	

Note: *Measured at 125 (^{13}C) and 500 (^{1}H) MHz in $CDCl_3$, δ TMS = 0.

4.3.4 Alkaloid ACB4: *O*-methylmoschatoline (8)



8

ACB4 (2.3 mg) was separated from alkaloid extract and isolated as orange amorphous. It was dissolved in CH_2Cl_2 and appeared as a red spot on TLC after sprayed with Dragendorff's reagent, confirming as an alkaloid compound. When observed under long UV, this compound observed as a red spot and appeared as a dark spot under short UV. The IR spectrum exhibited maximum adsorption for carbonyl group at 1655 cm^{-1} due to the conjugation with the aromatic ring and double bond. Hence, the absorption was lower than the unsaturated aliphatic ketones which absorbed near 1715 cm^{-1} (Coates, 2006). The EI-MS spectrum (Figure 4.26) revealed a strong molecular ion peak at *m/z* 321.1 $[M]^+$, thus giving the possibility of the molecular formula to be $C_{19}H_{15}NO_4$.

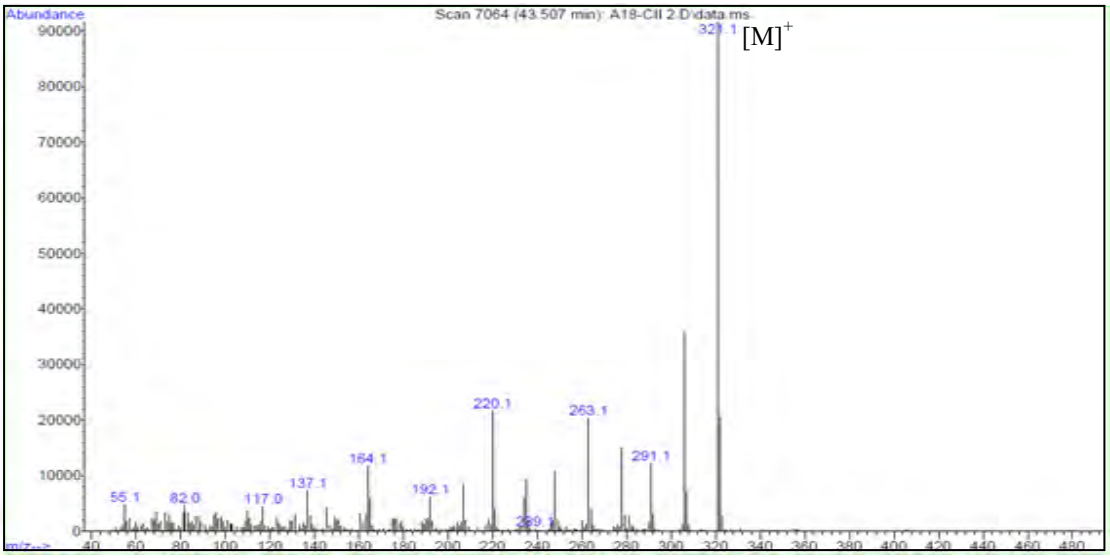


Figure 4.26. GCMS spectrum of **ACB4**

In the ^{1}H NMR spectrum of compound **ACB4** (Figure 4.27), three singlets of methoxyl protons were observed at δ 4.08, 4.10 and 4.19, and assigned to OCH_3-1, OCH_3-2 and OCH_3-3 respectively. There were two doublets of aromatic proton of ring B (H-4; δ 8.21, J = 5.0 Hz and H-5; δ 8.96, J = 5.0 Hz) with *ortho* position based on their coupling constant values. The small value of coupling constant (J) is due to the adjacent of N atom (Pavia et al., 2001). The proton H-5 that is adjacent to the N atom was slightly deshielded, resulted in lower field resonance. Another two doublets of aromatic proton of ring **D** (H-8; δ 8.57, J = 7.5 Hz, and H-11; δ 9.11, J = 8.5 Hz) were found to be positioned at downfield region more than the other aromatic protons, because H-8 experienced deshielding effect from the neighbouring C-7 carbonyl group (Omar et al., 2013). Meanwhile, the more downfield of H-11 was due to the deshielding effect of the facing ring **A** and hydrogen bonding with the methoxyl group of C-1. Another two aromatic protons appeared as triplet at δ 7.55 and 7.72 were attributed to H-9 and H-10, respectively.

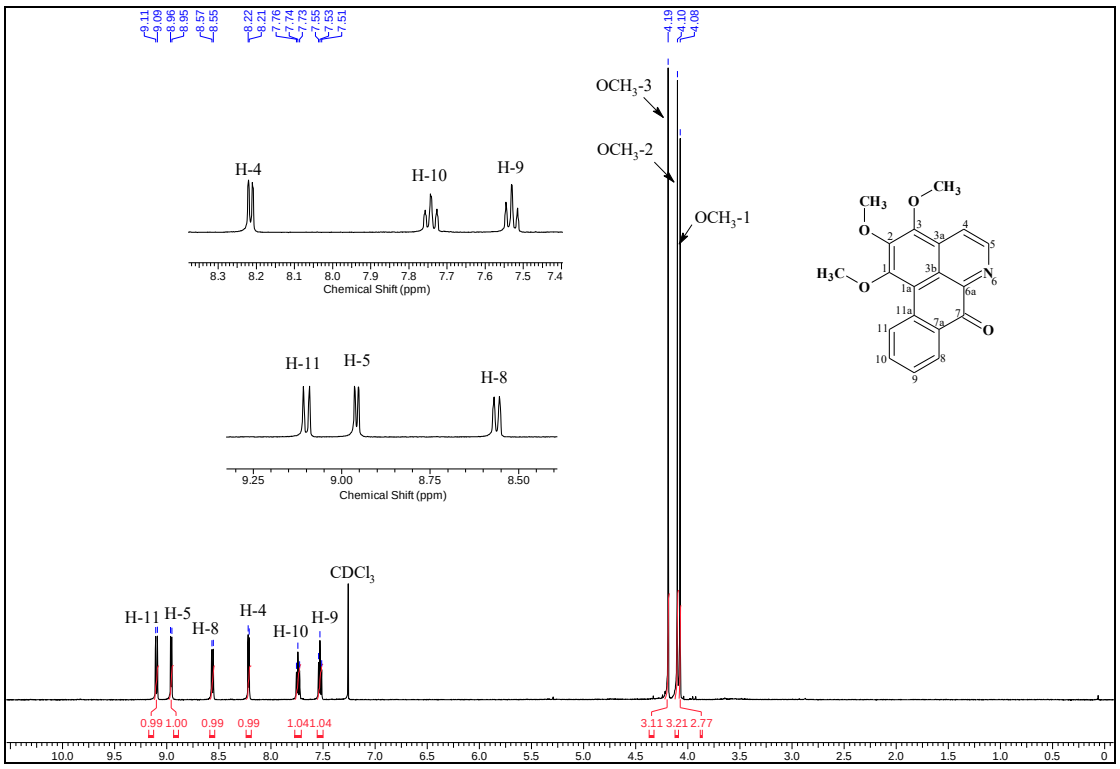


Figure 4.27. ^{1}H NMR spectrum of **ACB4**

Nineteen carbon signals appeared in the ^{13}C NMR spectrum of **ACB4 (**Figure 4.28) and were corresponded to three methoxyl carbons, six methine carbons, nine quaternary carbons and one carbonyl group. Three methoxyl carbons appeared at δ 61.1, 61.6 and 61.9 belong to OCH_3-1, OCH_3-2 and OCH_3-3, respectively. Furthermore, six methine carbons at δ 119.3, 127.7, 128.2, 129.0, 134.5 and 144.5 were assigned to C-4, C-11, C-9, C-8, C-10 and C-5, respectively. In addition, nine quaternary carbons resonated at δ 115.7, 122.9, 131.2, 131.5, 134.6, 145.4, 147.4, 148.5 and 156.6 was designated to C-1a, C-3a, C-3b, C-7a, C-11a, C-6a, C-2, C-3 and C-1, respectively. One carbonyl signal (δ 182.7) of a ketone was identified at C-7.

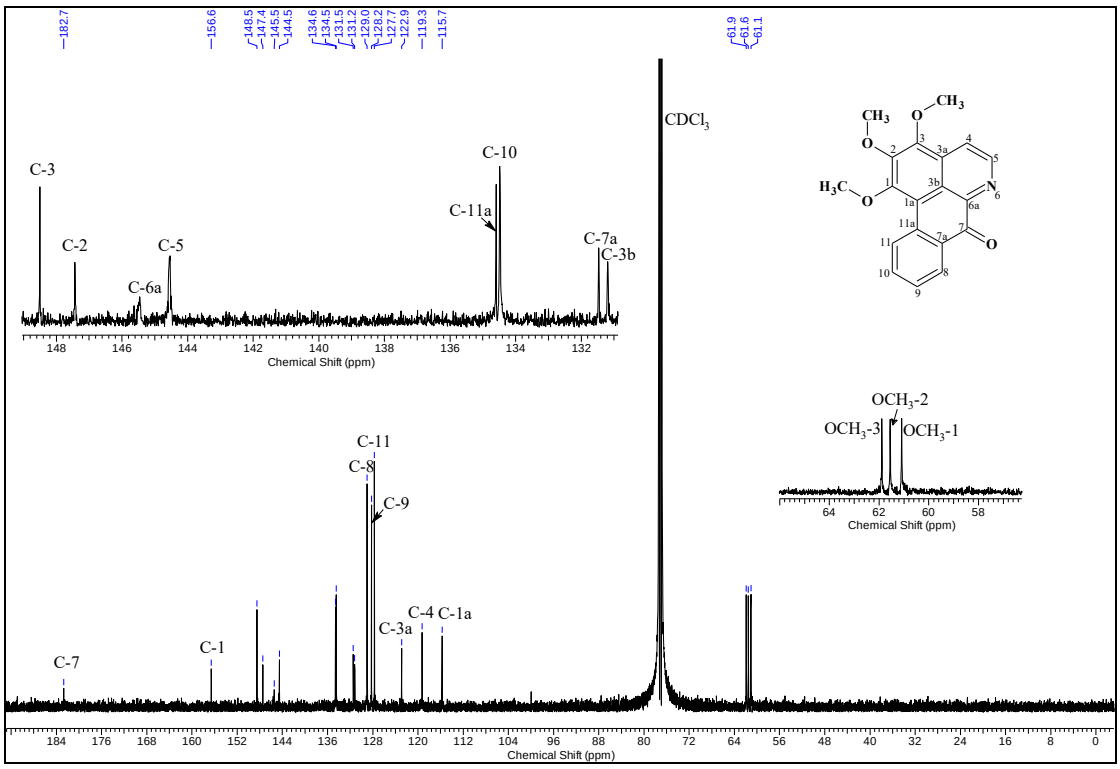


Figure 4.28. ^{13}C NMR spectrum of **ACB4**

The COSY spectrum of **ACB4** (Figure 4.29) showed correlations between H-5 to H-4, H-8 to H-9, and H-10 to H-11. The direct correlations between carbon-hydrogen of **ACB4** were shown in HMQC spectrum (Figure 4.30) and the ^{1}H-^{13}C long range correlation signals were observed in HMBC spectrum (Figure 4.31).

The HMBC spectrum (Figure 4.31) displayed cross peaks between OCH_3-3 with C-3 (δ 148.5), OCH_3-2 with C-2 (δ 147.4) and OCH_3-1 with C-1 (δ 156.6). The HMBC spectrum (Figure 4.32) also showed correlation of H-11 with C-1a, C-9 and C-7a, H-5 with C-4, C-3b and C-6a, H-8 with C-10 and C-7, H-4 with C-3a, C-5, and C-3, H-10 with C-8 and C-11a, and H-9 with C-11 and C-7a. Figure 4.33 illustrates the HMBC and COSY correlations of **ACB4**.

^{1}H and ^{13}C values of compound **ACB4** were found to be in full agreement with those reported for *O*-methylmoschatoline (**8**) from *Ellipeia cuneifolia* (Yusof et al., 2015). Therefore, compound **ACB4** was highly suggested as *O*-methylmoschatoline (**8**). The NMR data of **ACB4** and the values of *O*-methylmoschatoline (**8**) is summarized in Table 4.6.

O-methylmoschatoline (**8**) known as liridine has been isolated from other species of Annonaceae such as *Xylopia parvifolia* (Puvanendran et al., 2010), *Xylopia ferruginea* (Zawawi et al., 2012), *Guatteria blepharophylla* (Costa et al., 2011) and *Fusaea longifolia* (Tavares et al., 2005). Furthermore, this compound isolated from *Unozopsis buchtienii* was found to have activity against *Trypnosoina brucei* which responsible in Chaga‟s disease with an IC_{50} of 6.25 µg/mL (Waechter et al., 1999). Meanwhile, Yusof et al. (2015) isolated *O*-methylmoschatoline (**8**) from *Ellipeia cuneifolia* and reported that this compound exhibited strong growth inhibition against *Bacillus subtillis* and *Staphylococcus aureus*.

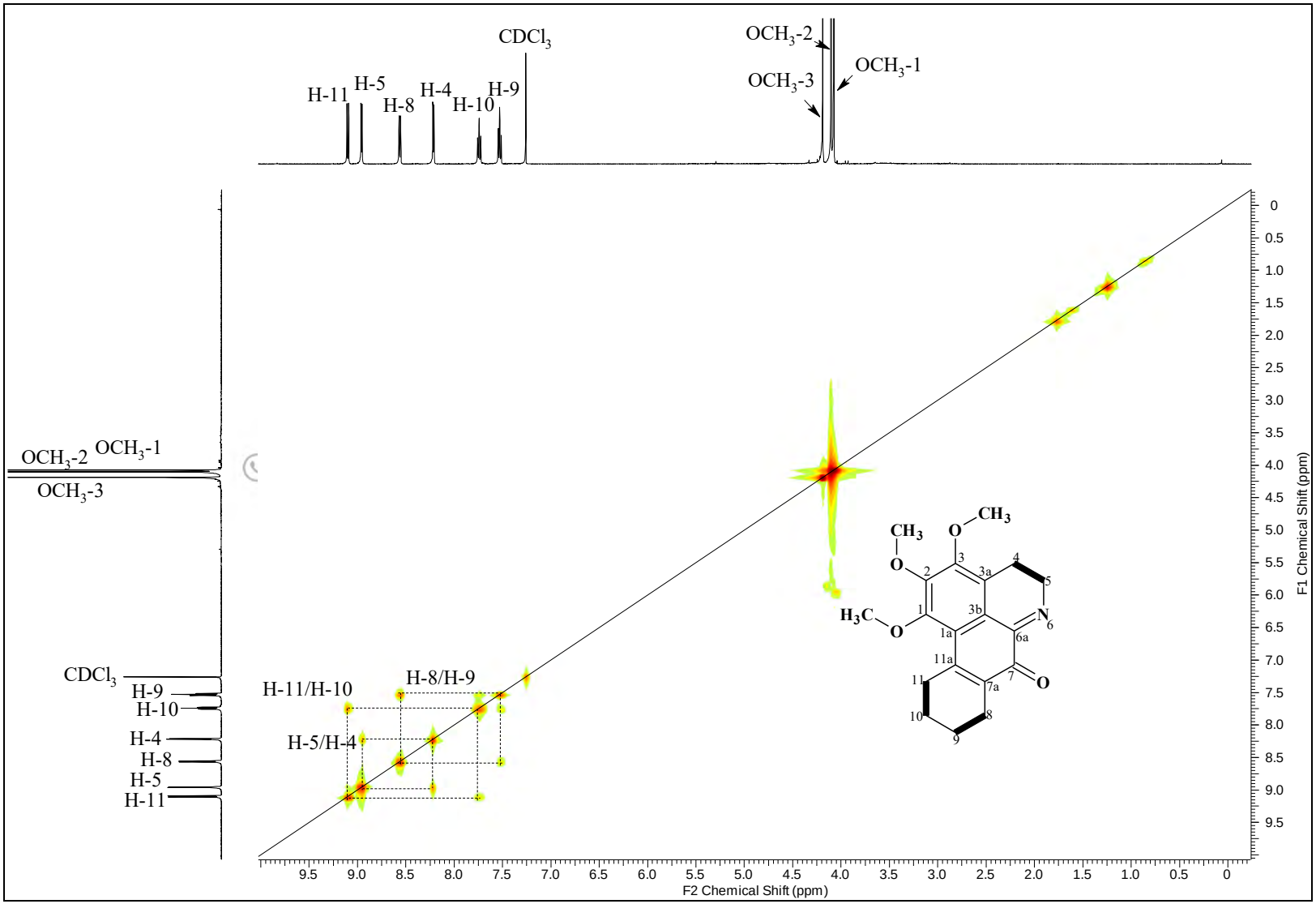


Figure 4.29. COSY spectrum of **ACB4**

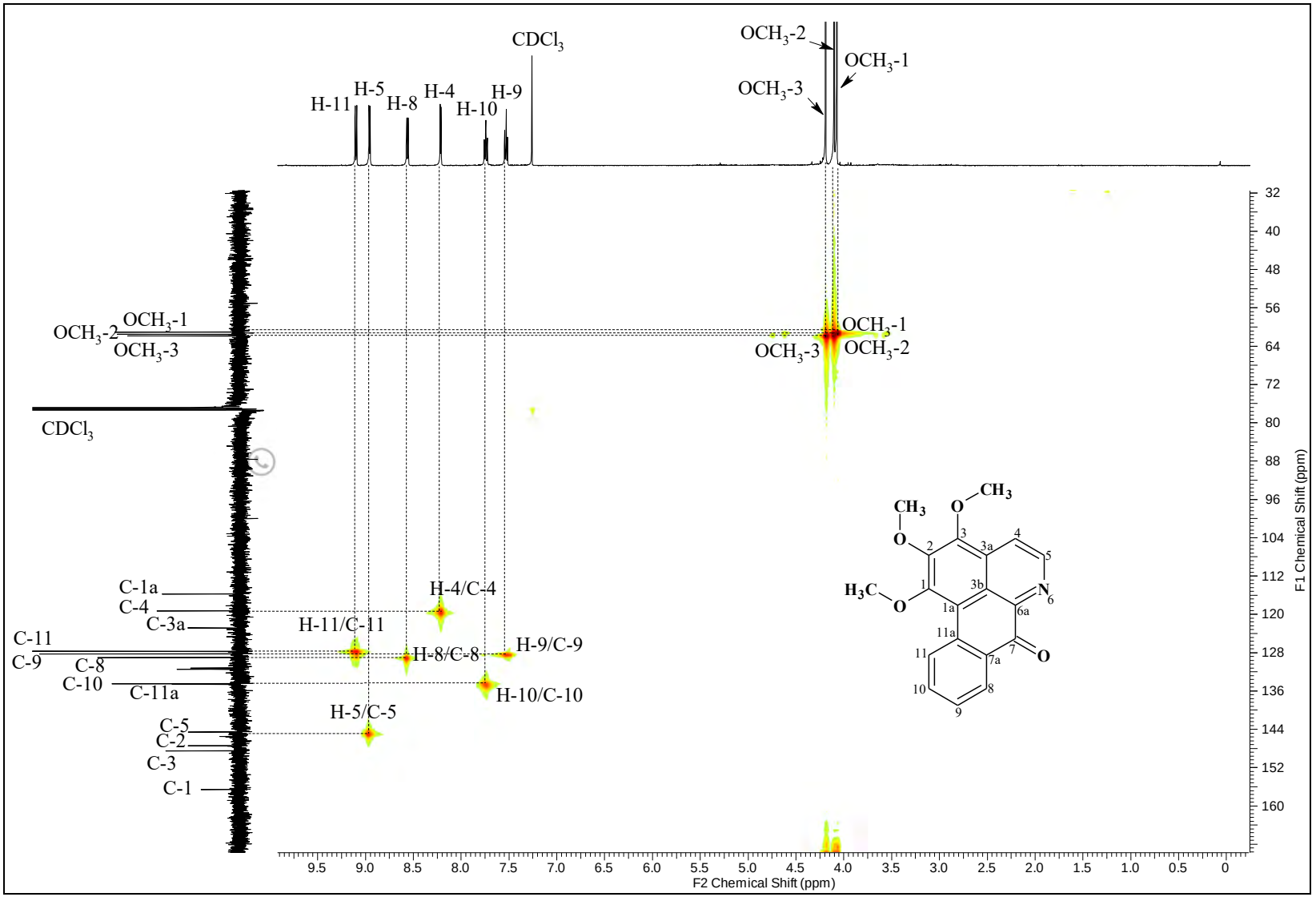


Figure 4.30. HMQC spectrum of **ACB4**

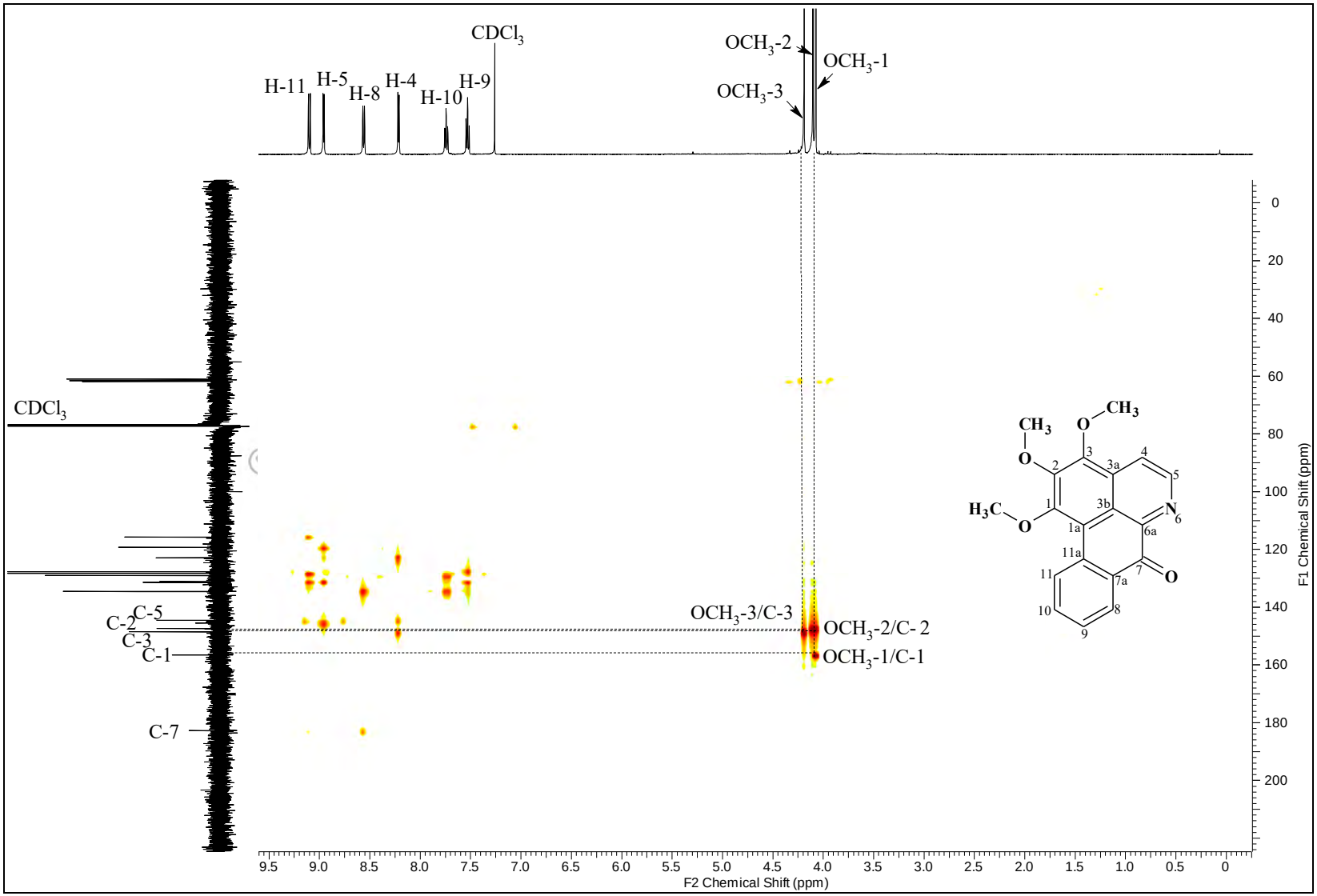


Figure 4.31. HMBC spectrum of **ACB4**

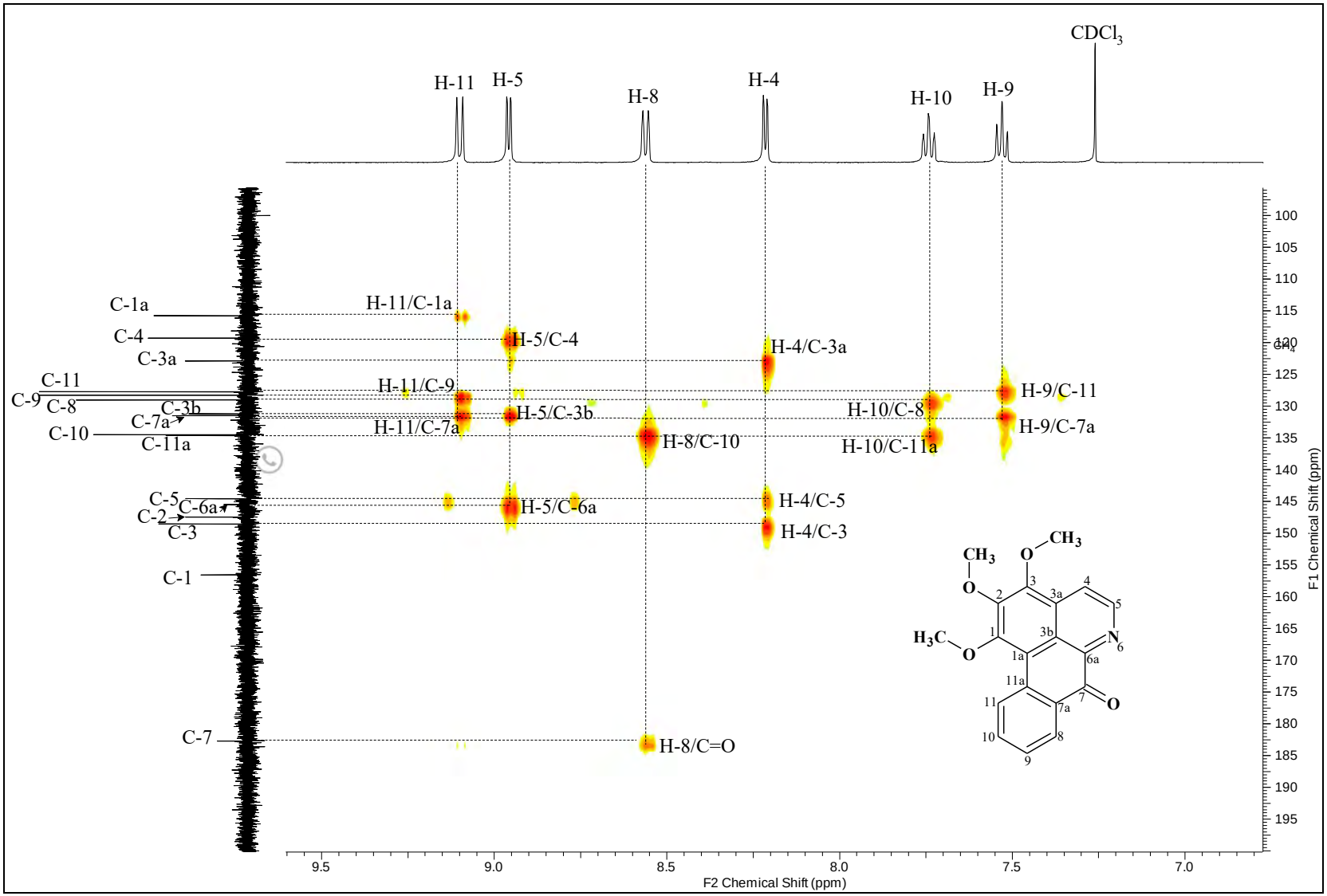


Figure 4.32. Expansion HMBC spectrum of **ACB4**

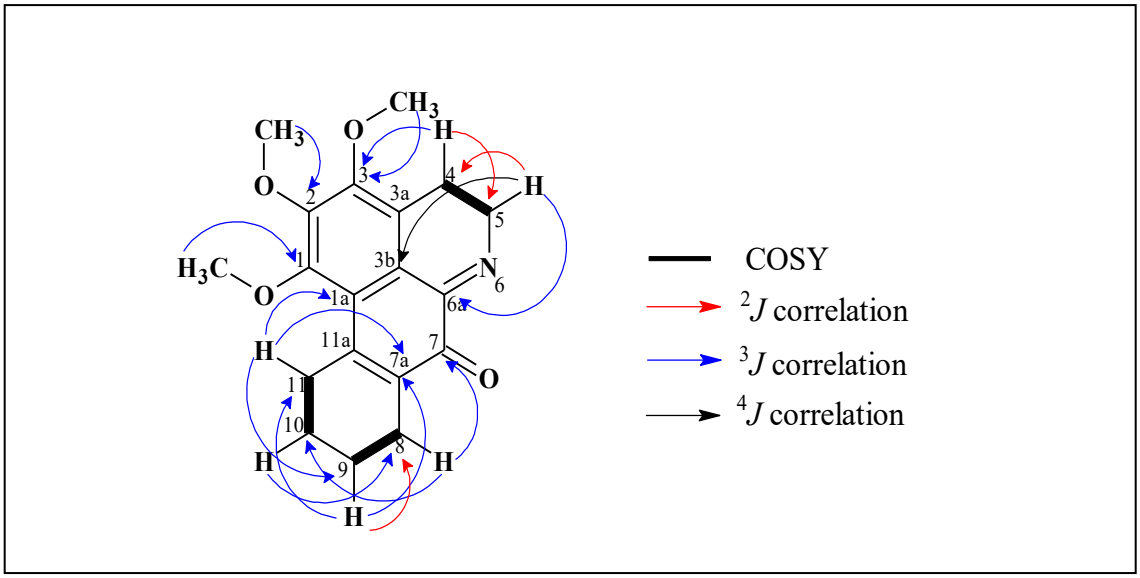


Figure 4.33. COSY, ^{1}H and ^{13}C correlations observed in HMBC spectrum of **ACB4**

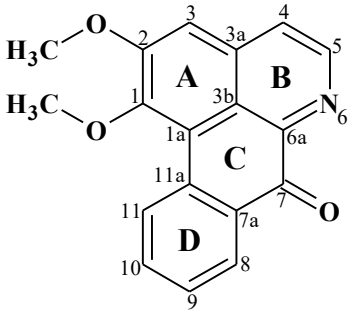
Table 4.6

*NMR spectral data of **ACB4** (500 MHz, $CDCl_3$) and O-methylmoschatoline (**8**)*

Position	ACB4				*O*-methylmoschatoline (8)	
	$\delta^{13}C$ (ppm)	**$\delta^{1}H$ (ppm) *J* (Hz)**	**HMBC**	**COSY**	***$\delta^{13}C$ (ppm)**	***$\delta^{1}H$ (ppm) *J* (Hz)**
1	156.6				156.5	
1a	115.7				115.7	
2	147.4				147.4	
3	148.5				148.5	
3a	122.9				122.8	
3b	131.2				131.1	
4	119.3	8.21 (*d,* 5.0 Hz, 1H)	C-3b, C-5, C-3	H-5	119.2	8.22 (*d*, 5.4 Hz, 1H)
5	144.5	8.96 (*d*, 5.7 Hz, 1H)	C-4, C-3b, C-6a	H-4	144.6	8.97 (*d*, 5.4 Hz, 1H)
6a	145.5				145.5	
7	182.7				182.7	
7a	131.5				131.4	
8	129.0	8.57 (*d*, 7.5 Hz, 1H)	C-10,C-7	H-9	129.0	8.58 (*dd*, 7.7, 1.2 Hz, 1H)
9	128.2	7.55 (*t,* 1H)	C-11, C-7a, C-10	H-8	128.2	7.54 (*t*, 7.8 Hz, 1H)
10	134.5	7.74 (*t,* 1H)	C-8, C-11a	H-11	134.4	7.75 (*dt*, 8.4, 1.2 Hz, 1H)
11	127.7	9.11 (*d*, 8.5 Hz, 1H)	C-1a, C-7a, C-9	H-10	127.7	9.11 (*d*, 8.4 Hz, 1H)
11a	134.6				134.6	
OCH_3-1	61.1	4.08 (*s,* 3H)	C-1		61.0	4.09 (*s,* 3H)
OCH_3-2	61.6	4.10 (*s,* 3H)	C-2		61.5	4.11 (*s,* 3H)
OCH_3-3	61.9	4.19 (*s,* 3H)	C-3		61.8	4.20 (*s,* 3H)

Note: *Measured at 150 (^{13}C) and 600 (^{1}H) in $CDCl_3$, δ TMS = 0 (Yusof et al., 2015)

4.3.5 Alkaloid ACB5: Lysicamine (84)



84

Compound **ACB5** (3.3 mg) was obtained as orange amorphous which dissolved in CH_2Cl_2. This compound also showed orange spot on TLC before and after sprayed with Dragendorff‟s reagent, indicating it to be an alkaloid compound. For TLC observation under UV light, this compound appeared as yellow spot under long UV and dark spot under short UV. The molecular formula of this compound is $C_{18}H_{13}NO_3$ with determination of molecular ion peak at *m/z* 291.1 [M+] measured by using EI-MS technique (Figure 4.34). In addition, the IR spectrum showed maximum adsorption at 1665 cm^{-1} due to the stretching of carbonyl group (C=O) (Omar et al., 2013). The UV spectrum displayed absorption at 240 and 270 nm, which are typical for molecules from oxoaporphine skeleton (Costa et al., 2011).

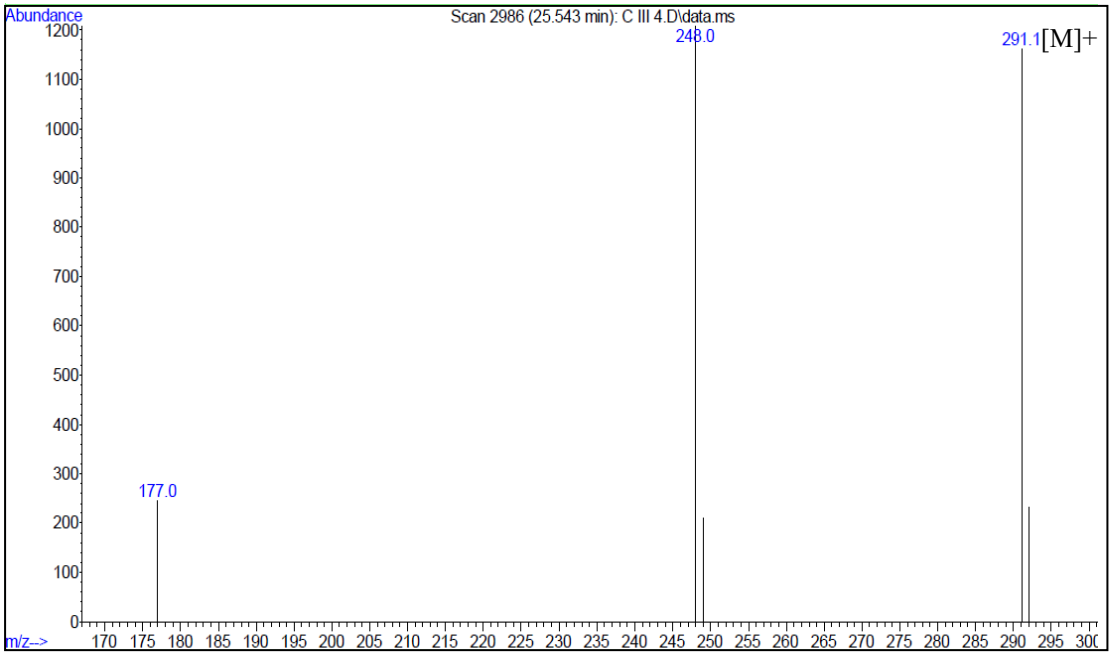


Figure 4.34. GCMS spectrum of **ACB5**

The ^{1}H-NMR spectrum of compound **ACB5** (Figure 4.35) showed two singlets of methoxyl of ring **A** (OCH_3-2; δ 4.01 and OCH_3-1; δ 4.09), a singlet of aromatic proton H-3 (δ 7.21), two triplets of proton of ring **D** (H-9; δ 7.58 and H-10; δ 7.76), two doublets of aromatic proton of ring **A** (H-4; δ 7.79, J = 5.0 Hz and H-5, δ 8.89, J = 5.0 Hz) and another two doublets of aromatic proton of ring **D** (H-8; δ 8.58, J = 7.5 Hz and H-11; δ 9.17, J = 7.5 Hz).

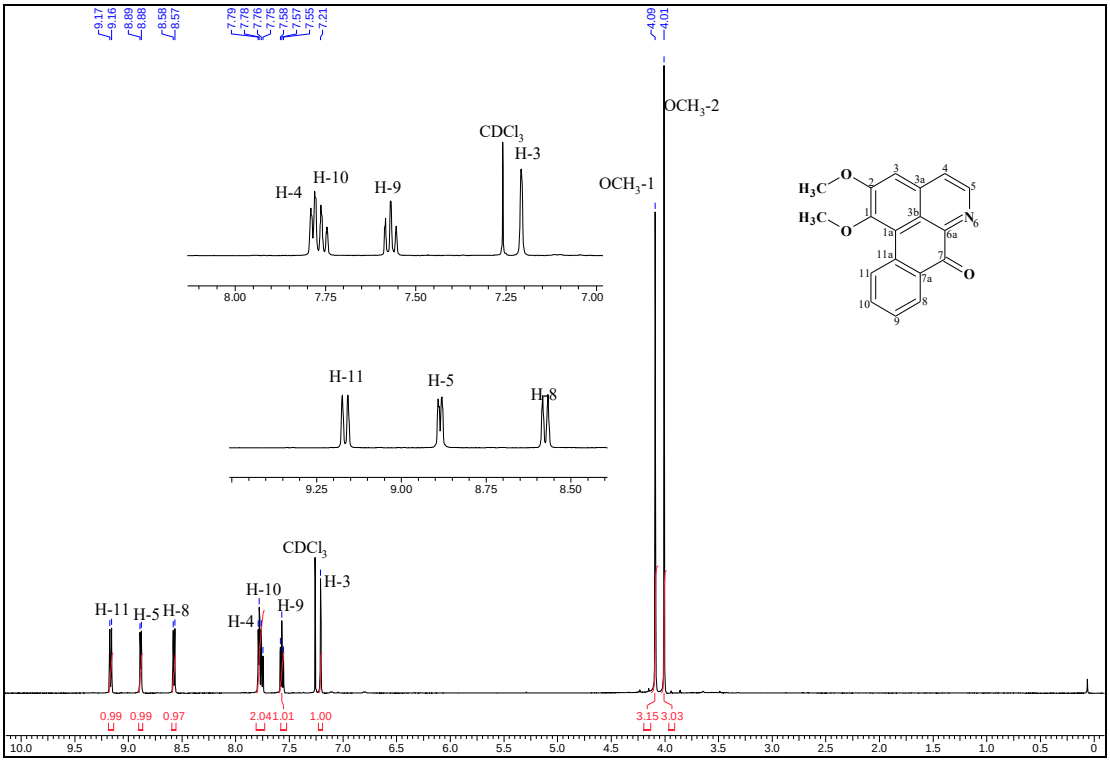


Figure 4.35. ^{1}H NMR spectrum of **ACB5**

The ^{13}C-NMR spectrum (Figure 4.36) showed eighteen carbons signals, including one carbonyl group resonated at δ 182.8 belong to C-7. Two methoxyl carbons appeared at δ 56.3 and 60.7 were assigned to OCH_3-1 and OCH_3-2, respectively. Meanwhile, seven methine aromatic carbons resonated at δ 106.5, 123.7, 145.1, 129.0, 128.5, 134.38 and 128.9 was designated to C-3, C-4, C-5, C-8, C-11, C-10 and C-9, respectively. In addition, eight quaternary carbons assigned as C-1, C-1a, C-2, C-3a, C-3b, C-6a, C-7a and C-11a were identified at chemical shifts of δ 156.9, 152.1, 135.6, 122.2, 145.4, 132.1 and 134.43, respectively. The resonances of the proton and carbon signals of compound **ACB5** were tabulated in Table 4.7.

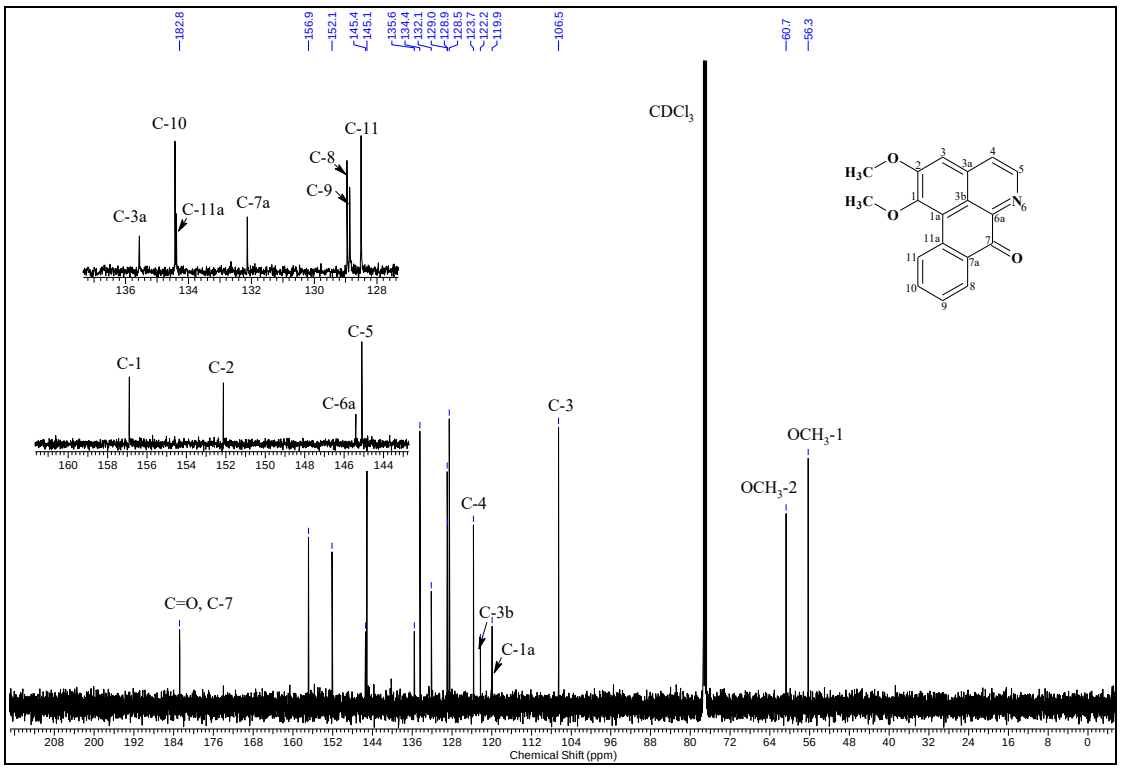


Figure 4.36. ^{13}C NMR spectrum of **ACB5**

Comparison of the data with literature values of known compound from *Cananga odorata* were then supported compound **ACB5** as lysicamine (**84**) (Husain et al. 2012). This compound also known as oxonucuferine and has been isolated from other species of plants, including *Fissistigma glaucescens* (Lo et al., 2000), *Fissistigma latifolium* (Alias et al., 2010; Lan et al., 2011), *Annona pickelii* (Dutra et al., 2012), *Guatteria friesiana* (Costa et al., 2013) and *Tinospora crispa* (Bakhari et al., 2013). Lysicamine (**84**) also had been reported as antimicrobial and antiproliferative agent (Costa et al., 2010; Costa et al., 2011; Omar et al, 2013; Tan et al., 2015).

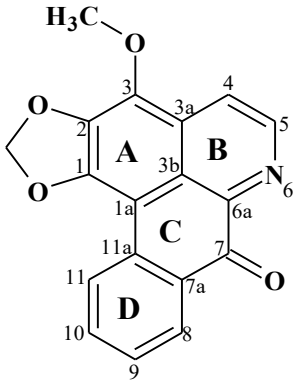
Table 4.7

^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C ($CDCl_3$, 125 MHz) of ***ACB5*** *and Lysicamine* ***(84)***

Position	ACB5		Lysicamine (84)	
	$\delta^{13}C$ (ppm)	**$\delta^{1}H$ (ppm) *J* (Hz)**	***$\delta^{13}C$ (ppm)**	***$\delta^{1}H$ (ppm) *J* (Hz)**
1	156.9		157.5	
1a	119.9		120.0	
2	152.1		152.8	
3	106.5	7.21 (*s*)	106.6	7.24 (s)
3a	135.6		135.5	
3b	122.2		122.3	
4	123.7	7.79 (*d*, 5.0 Hz, 1H)	123.8	7.84 (*d*, 5.0 Hz, 1H)
5	145.1	8.89 (*d*, 5.0 Hz, 1H)	144.9	8.92 (*d*, 5.0 Hz, 1H)
6a	145.4		145.4	
7	182.8		182.9	
7a	132.1		132.2	
8	129.0	8.58 (*d*, 7.5 Hz, 1H)	129.0	8.61 (*dd*, 5.6, 0.7 Hz, 1H)
9	128.9	7.58 (*t*, 1H)	128.6	7.60 (*ddd*, 8.4, 8.4, 1.4 Hz, 1H)
10	134.38	7.76 (*t*, 1H)	134.2	7.78 (*ddd*, 8.4, 8.4, 1.4 Hz, 1H)
11	128.5	9.17 (*d*, 7.5Hz, 1H)	128.4	9.19 (*dd*, 5.5, 0.7Hz, 1H)
11a	134.43		134.4	
OCH_3-1	56.3	4.09 (*s,* 3H)	56.3	4.10 (*s,* 3H)
OCH_3-2	60.7	4.01 (*s,* 3H)	60.2	4.02 (*s,* 3H)

Note: *Measured at 125 MHz (^{13}C) and 500 MHz (^{1}H) in $CDCl_3$, δ TMS = 0 (Husain et al., 2012)

4.3.6 Alkaloid ACB6: Atherospermidine (85)



85

Compound **ACB6** (1.7 mg) was afforded as orange amorphous and dissolved in CH_2Cl_2. The compound showed as orange spot on TLC after sprayed with Dragendorff‟s reagent, confirming a positive result of alkaloid type of compounds. The IR spectrum of **ACB6** showed a strong absorption at 2915 cm^{-1}, indicating the presence of C-H stretching. Absorption band at 1666 cm^{-1} was attributed to carbonyl group (C=O), while band at 962 cm^{-1} revealed the presence of methylenedioxyl functionality (-OCH_2O-) (Pabon & Cuca, 2010). Figure 4.38 shows a molecular ion peak of mass spectrum at *m/z* 306.0762 $[M+H]^+$ measured by using HR-ESI-MS and supported the molecular formula of $C_{18}H_{11}NO_4$ (Figure 4.37). The UV spectrum of **ACB6** showed maximum peak at 250 and 270 nm, revealed the typical adsorption of 1,2-disubstituted oxoaporphine (Sangster & Stuart, 1964).

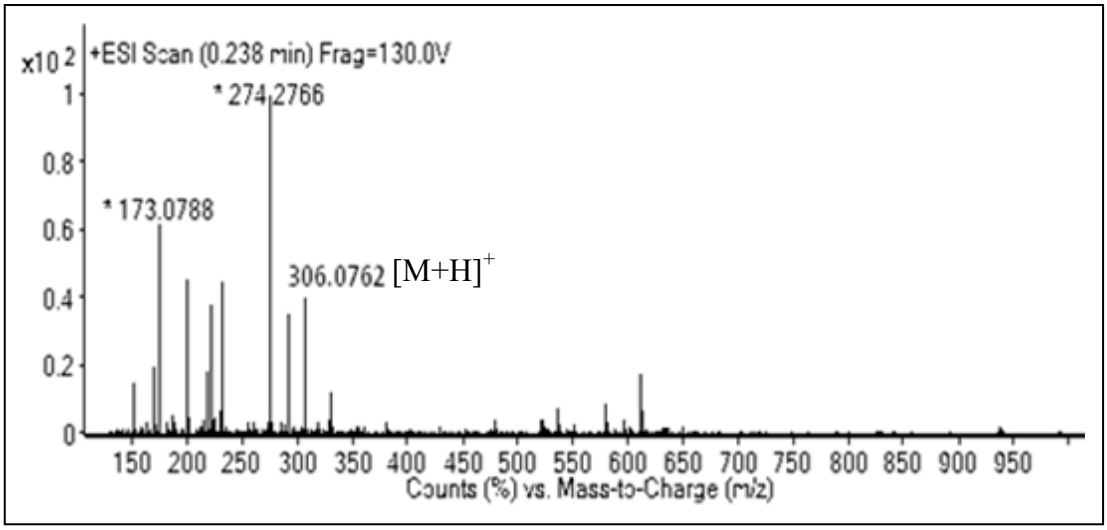


Figure 4.37. LCMS spectrum of **ACB6**

The ^{1}H-NMR spectrum of compound **ACB6** (Figure 4.38) revealed resonances of one singlet of methoxyl (OCH_3-3; δ 4.28), one singlet of methylenedioxy (1-OCH_2O-2; δ 6.32), and two triplet of doublet of protons at δ 7.49 (H-9, J = 8.0 and 1.2 Hz) and at δ 7.67 (H-10, J = 8.0 and 1.2 Hz), respectively. Two doublets of aromatic protons of ring **B** were observed at δ 8.11 (H-4, J = 5.0 Hz) and at δ 8.88 (H-5, J = 5.0 Hz), one doublet of proton at δ 8.47 (H-8, J = 8.0 Hz) and one doublet of doublet of proton H-11 in ring **D** (δ 8.51, J = 7.5 and 1.2 Hz) were identified.

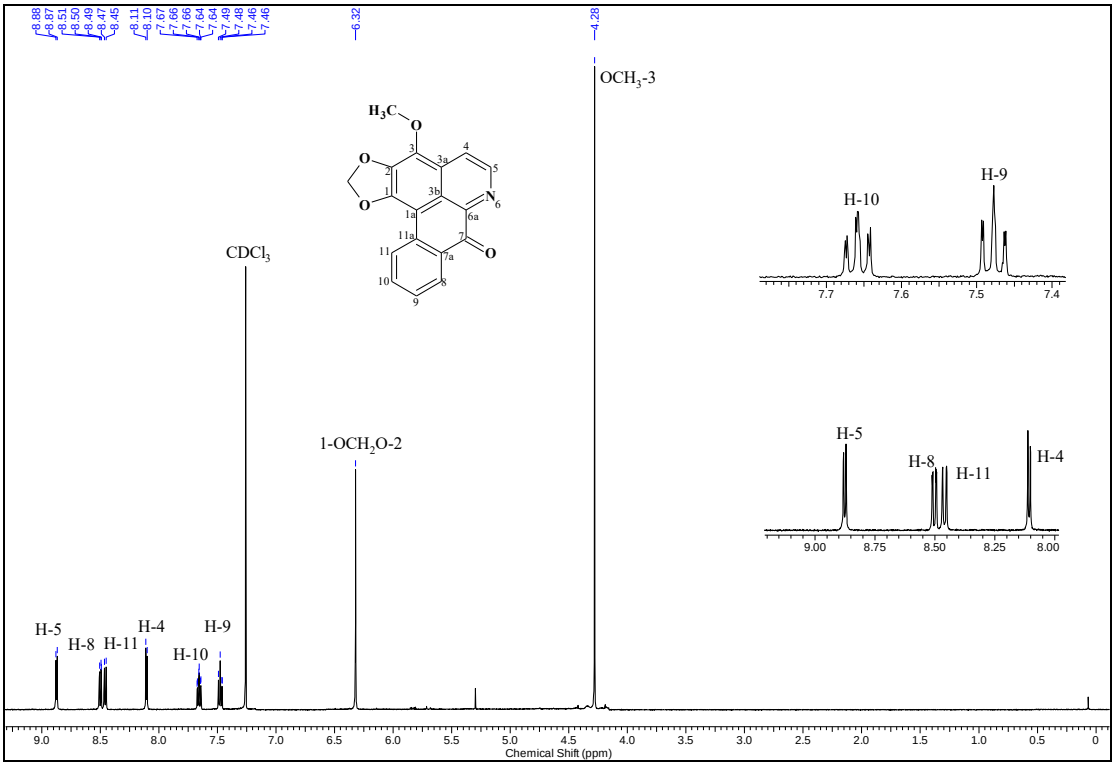


Figure 4.38. ^{1}H NMR spectrum of **ACB6**

Eighteen carbon signals were appeared in the ^{13}C NMR spectrum of **ACB6** (Figure 4.39). One methoxyl carbon was resonated at δ 60.2 and one methylene carbon at δ 102.4. Nine quaternary carbons also observed at C-1a, C-3b, C-7a, C-3a, C-11a, C-2, C-3, C-6a and C-1 were resonated at δ 102.6, 122.8, 130.5, 130.9, 133.2, 136.3, 136.7, 144.7 and 149.8, respectively. Six methine carbons at δ 119.4, 126.7, 127.7, 128.7, 134.0 and 144.0 were assigned to C-4, C-11, C-9, C-8, C-10 and C-5, respectively. One carbonyl was identified in the spectrum at δ 182.4 assigned to C-7 in the structure of **ACB6**.

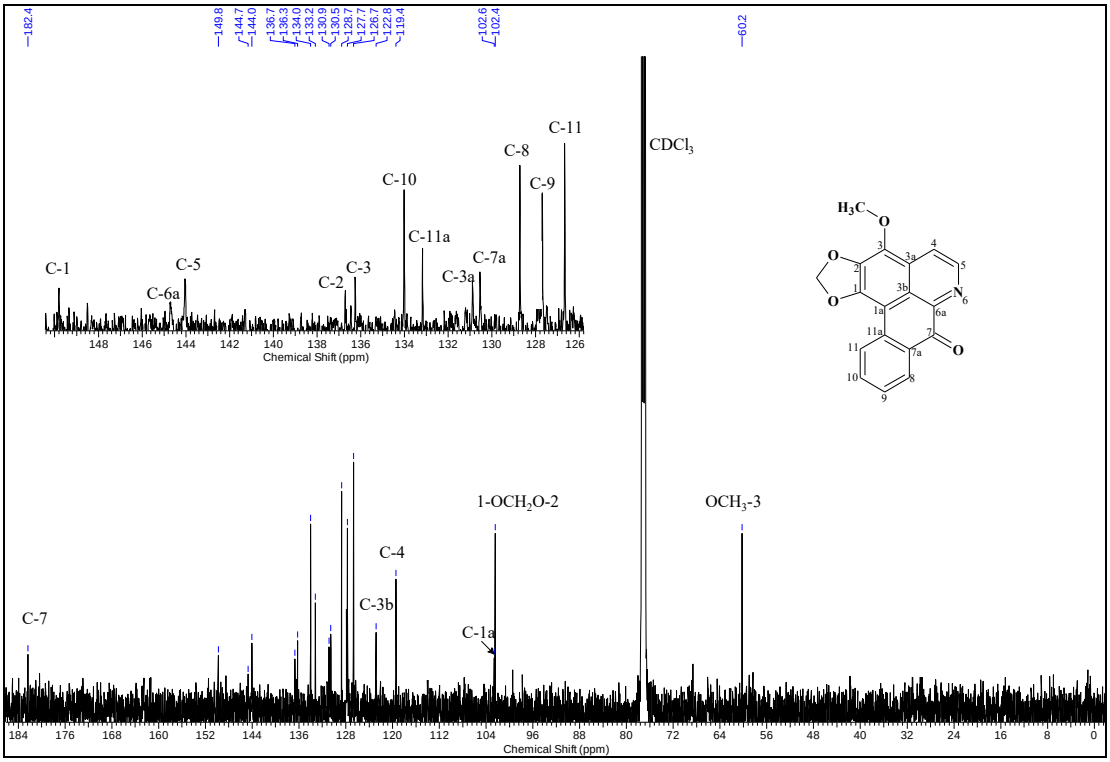


Figure 4.39. ^{13}C NMR spectrum of **ACB6**

Based on the measured spectral data and comparison with literature values, compound **ACB6** showed similarity with the known compound, atherospermidine (**85**), which was previously isolated from *Duguetia furfuracea* (Pérez and Cassels, 2010), *Annona foetida* (Costa et al., 2011), *Fissistigma latifolium* (Lan et al., 2011), *Jatropa curcas* (Gupta et al., 2011) and *Mitrella kentii* (Azizan & Hadi, 2014). Atherospermidine (**85**) is an oxoaporphine alkaloid that had been reported to have DNA damaging activity (Gören et al., 2003; Stévigny et al., 2005) and exhibited citotoxicity against hepatocarcinoma cancer cell line (Hsieh et al., 2001). Table 4.8 summarized the ^{1}H NMR and ^{13}C NMR spectral data of **ACB6** and comparison with literature values of atherospermidine (**85**).

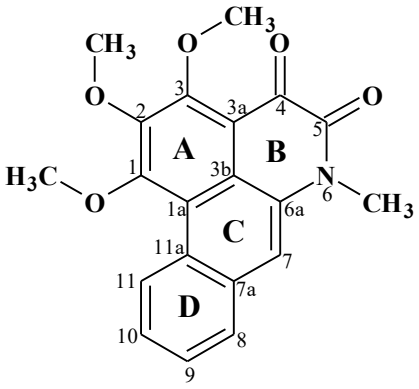
Table 4.8

^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C ($CDCl_3$, 125 MHz) of ***ACB6*** *and Atherospermidine (**85**)*

Position	ACB6		Atherospermidine (85)	
	$\delta^{13}C$ (ppm)	**$\delta^{1}H$ (ppm) *J* (Hz)**	***$\delta^{13}C$ (ppm)**	***$\delta^{1}H$ (ppm) *J* (Hz)**
1	149.8		149.5	
1a	102.6		102.6	
2	136.3		136.3	
3	136.7		136.1	
3a	130.9		130.64	
3b	122.8		122.8	
4	119.4	8.11 (*d*, 5.0 Hz, 1H)	119.5	8.11 (*d*, 5.3 Hz, 1H)
5	144.0	8.88 (*d*, 5.0 Hz, 1H)	144.3	8.88 (*d*, 5.0 Hz, 1H)
6a	144.7		144.9	
7	182.4		182.6	
7a	130.5		130.61	
8	128.7	8.51 (*dd*, 7.5 and 1.2 Hz, 1H)	128.5	8.52 (*dd,* 8.1 and 1.5 Hz, 1H)
9	127.7	7.49 (*td*, 8.0 and 1.2 Hz, 1H)	127.4	7.47 (*ddd*, 8.0, 7.2 and 1.5 Hz, 1H)
10	134.0	7.67 (*td*, 8.0, and 1.2 Hz, 1H)	134.1	7.67 (*ddd,* 8.2, 7.2 and 1.5 Hz, 1H)
11	126.7	8.47 (*d*, 8.0 Hz, 1H)	126.7	8.50 (*dd*, 8.2 and 1.5 Hz, 1H)
11a	133.2		133.1	
1-OCH_2O-2	102.4	6.32 (*s*, 2H)	102.3	6.30 (*s*, 2H)
OCH_3-3	60.2	4.28 (*s*, 3H)	60.2	4.28 (*s*, 3H)

Note: *Measured at 100 (^{13}C) and 400 (^{1}H) in $CDCl_3$, δ TMS = 0 (Costa et al., 2011)

4.3.7 Alkaloid ACB7: *N*-methylouregidione (86)



86

Compound **ACB7** (1.1 mg) was obtained from the neutral fraction as yellow amorphous and dissolved in CH_2Cl_2. For TLC observation under UV light, the compound was appeared as a dark spot under short UV and gave a positive result of alkaloid compounds as an orange spot after sprayed with Dragendorff's reagent. The UV spectrum showed maximum absorption at 245 nm, indicating the presence of 4, 5-dioxoaporphine alkaloid (Desai et al., 1988). The IR spectrum showed maximum absorption of two carbonyl groups at 1691 and 1663 cm^{-1} (Wirasathien et al., 2006). In addition, its molecular formula of $C_{20}H_{17}NO_5$ was supported by its molecular ion peak at m/z 374.1034 $[M+Na]^+$ by HR-ESI-MS technique (Figure 4.40).

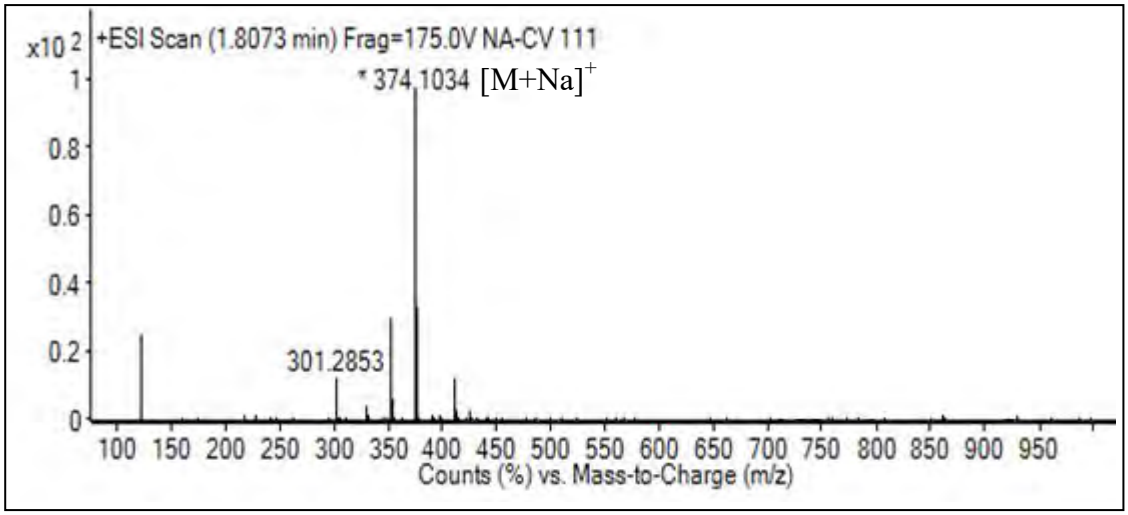


Figure 4.40. LCMS spectrum of **ACB7**

The ^{1}H NMR spectrum of **ACB7** (Figure 4.41) displayed resonances of one singlet of *N*-methyl (δ 3.87), three singlets of methoxyl (OCH_3-3; δ 4.09, OCH_3-2; δ 4.13; OCH_3-1; δ 4.18), one singlet of proton H-7 (δ 7.63) and four multiplets of protons of ring **D**. One multiplet at δ 7.66 was integrated for two protons, which assigned to H-9 and H-10, while another two multiplets of protons assigned to proton H-8 (δ 7.92) and H-11 (δ 9.48).

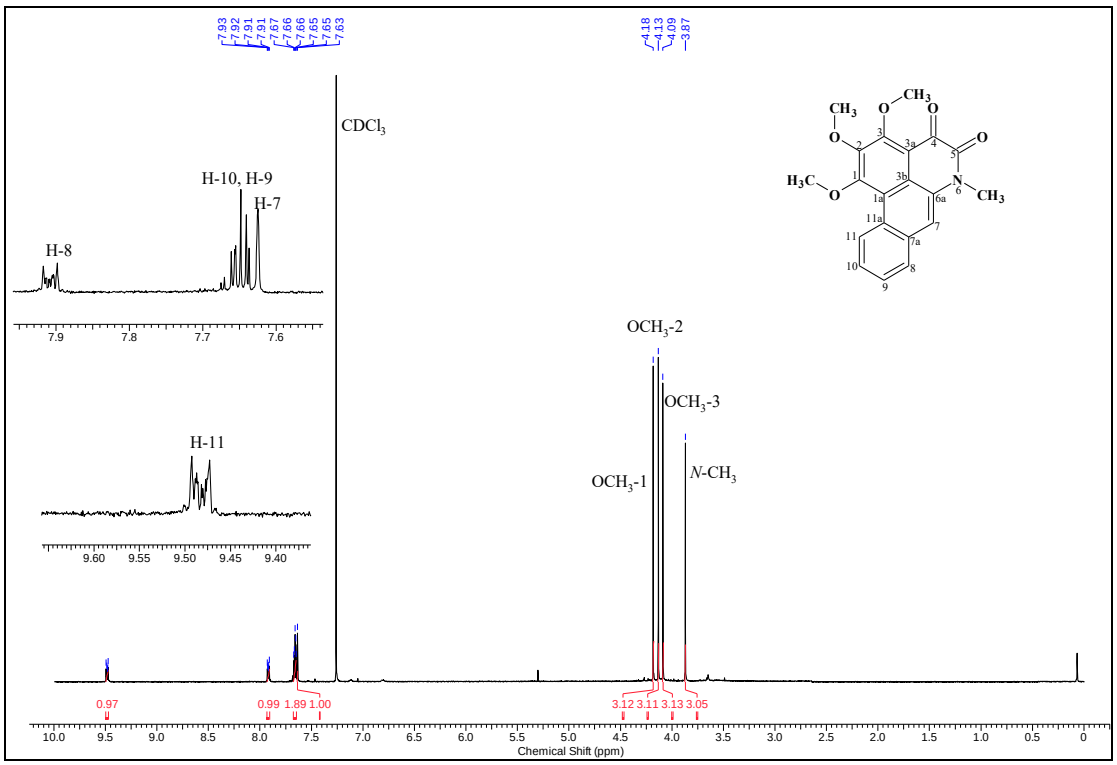


Figure 4.41. ^{1}H NMR spectrum of **ACB7**

The ^{13}C NMR spectrum (Figure 4.42) showed twenty peaks of carbon. A peak at δ 31.0 was assigned to *N*-CH_3, while the three methoxyl signals at δ 61.3, 61.8 and 62.2 assigned as OCH_3-3, OCH_3-2 and OCH_3-1, respectively. The ketone and amide carbonyl carbons in dioxoaporphines usually identified at δ 176 and 153, respectively (Kim et al., 2001). For this compound, the ketone carbonyl was resonated at δ 170.4, while the amide carbonyl at δ 157.0. In addition, C-7 was assigned at δ 114.5.

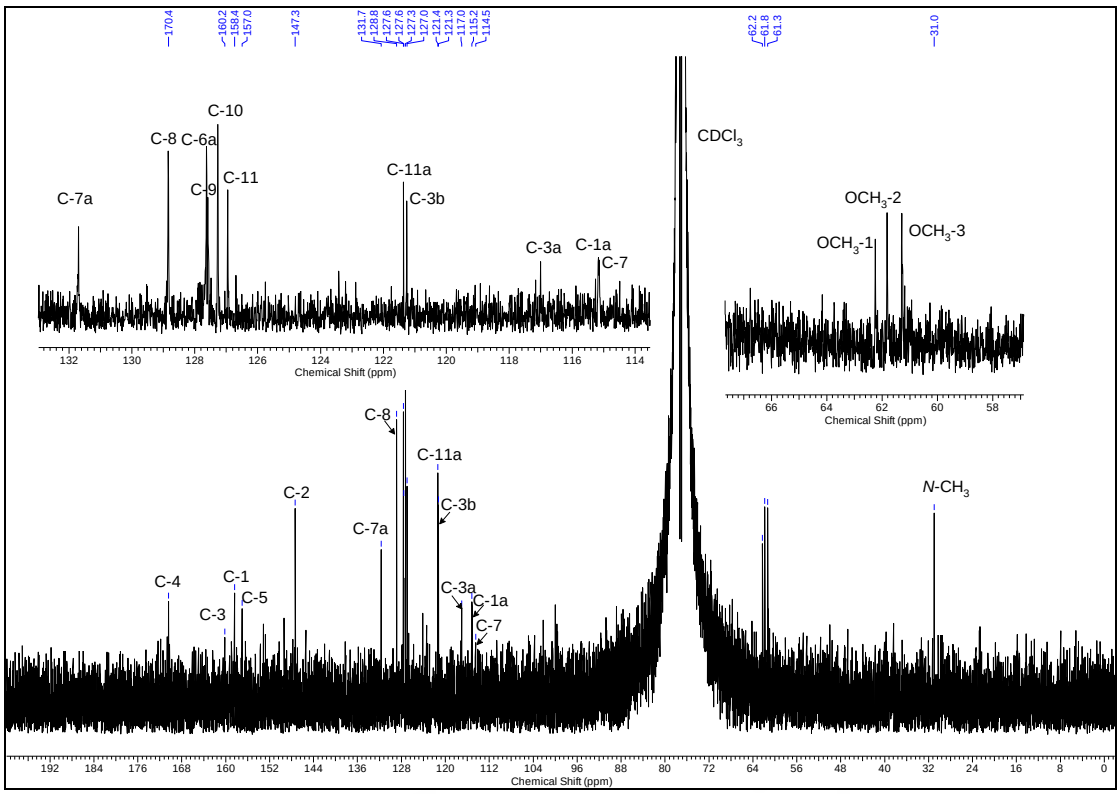


Figure 4.42. ^{13}C NMR spectrum of **ACB7**

The resonance of carbon for **ACB7** was compared with compound ouregidione (**88**) that have similar basic skeleton. This compound was previously isolated from the mixture of twigs and leaves of *Pseuduvaria trimera* (Sesang et al., 2014). Moreover, the assignment of **ACB7** structure was also confirmed by comparison with literature values reported for *N*-methylouregidione (**86**), which was isolated from *P. macrophylla* (Mahmood et al., 1986) and *P. setosa* (Wirasathien et al., 2006) as summarized in Table 4.9. This compound also known as 3-methoxycepharadione-B or 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine.

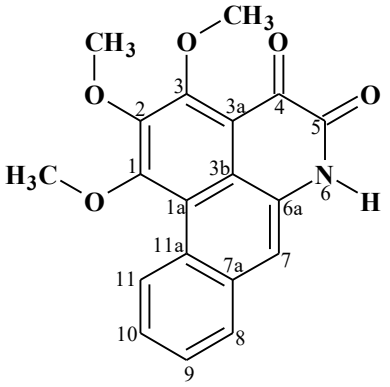


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Table 4.9

*NMR spectral data ($CDCl_3$, 500 MHz) of **ACB7** and ^1H NMR ($CDCl_3$, 300 MHz) of N-methylouregidione (**86**) and ^{13}C NMR of Ouregidione (**88**)*

Position	ACB7	*N*-methylouregidione (86)	ACB7	Ouregidione (88)
	δ^1H (ppm)	*δ^1H (300 MHz)	$\delta^{13}C$ (ppm)	**$\delta^{13}C$ (ppm)
1			158.4	158.66
1a			115.2	116.26
2			147.3	147.54
3			160.2	160.44
3a			117.0	117.62
3b			121.3	120.31
4			170.4	175.41
5			157.0	157.63
6a			127.63	128.38
7	7.63 (1H, *s*)	7.60 (1H, *s*)	114.5	116.0
7a			131.7	131.77
8	7.92 (1H, *m*)	7.88 (1H, *m*)	128.8	128.48
9	7.66	7.63	127.59	127.57
10	(2H, *m*)	(2H, *m*)	127.3	127.44
11	9.48 (1H, *m*)	9.45 (1H, *m*)	127.0	127.24
11a			121.4	121.23
N-CH_3	3.87 (3H, *s*)	3.84 (3H, *s*)	31.0	-
OCH_3-1	4.09 (3H, *s*)	4.07 (3H, *s*)	62.2	62.08
OCH_3-2	4.13 (3H, *s*)	4.11 (3H, *s*)	61.8	61.74
OCH_3-3	4.18 (3H, *s*)	4.16 (3H, *s*)	61.3	61.17

Note: *Measured at 300 (^1H) in $CDCl_3$, δ TMS = 0 (Wirasathien et al., 2006)
**Measured at 125 (^{13}C) in $CDCl_3$ (Sesang et al., 2014)

4.4 Antibacterial analysis

According to Balouiri et al., (2016), there are various antimicrobial susceptibility testing methods that are available such as agar disc diffusion, agar well diffusion, agar overlay, cross streak, TLC-bioautography, broth dilution and agar dilution. However, the most well known and commonly used are disc diffusion, well diffusion, broth dilution and agar dilution method. In this study, antibacterial activity test was completed using agar disc diffusion and broth dilution methods. These methods were most preferred in previous reported studies by Bagul & Sivakumar (2016) and Ataee et al. (2012) because it is simple, labour-efficient, low cost and reliable.

4.4.1 Determination of antibacterial activity by disc diffusion assay

Diameter zone of inhibition for different concentrations (200, 100, 75, 50 and 25 mg/mL) of hexane, dichloromethane and methanol extracts from the barks of *A. cylindrica* against two tested microorganisms, *S. aureus* and *P. aeruginosa* are presented in Table 4.10. Zones of inhibition were measured from the circumference of the disks to the circumference of the inhibition zones (Ncube et al., 2008).

Table 4.10

Inhibition zones of hexane, dichloromethane and methanol extracts against microbial growth of S. aureus and P. aeruginosa

Extracts	Concentration (mg/mL)	Diameter of inhibition zone (mm)*	
		Gram positive	Gram negative
		S. aureus	***P. aeruginosa***
Hexane	200	-	-
	100	-	-
	75	-	-
	50	-	-
	25	-	-
	0	-	-
Dichloromethane	200	9.33 ± 0.58	11.00 ± 0.00
	100	9.00 ± 1.00	10.67 ± 0.58
	75	8.33 ± 0.58	10.00 ± 0.00
	50	7.67 ± 0.58	9.00 ± 0.00
	25	7.33 ± 0.58	8.67 ± 0.58
	0	-	-
Methanol	200	-	-
	100	-	-
	75	-	-
	50	-	-
	25	-	-
	0	-	-

Note: Samples was dissolved in methanol. 0 mg/ml concentration means that there was no sample, only methanol and act as negative control; - = no zone of inhibition;
* Values given in mean ± standard deviation (n = 3)

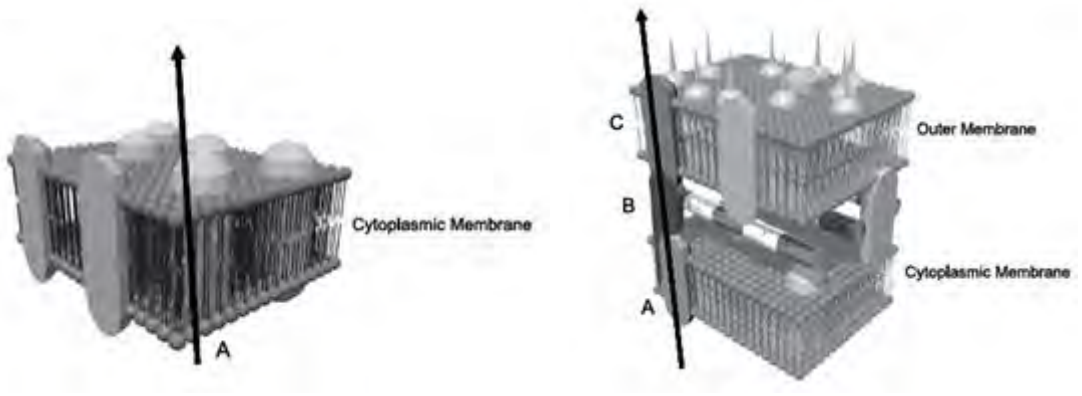
The results of the present study revealed that among the three extracts tested; only dichloromethane extract showed antibacterial activity against microbial growth of Gram positive bacteria, *S. aureus* and Gram negative bacteria, *P. aeruginosa*. On the contrary, hexane and methanol extracts from the barks of *A. cylindrica* were inactive against the tested microorganisms. According to Dharajiya et al. (2012), Chitravadivu et al. (2009) and Dogruoz et al. (2008), the polarity of solvent used in the plant extraction process may affect the antibacterial activity. This is because

different solvent polarity can extract different group of chemical compounds. In the present study, the results showed that the antibacterial compounds from the barks of *A. cylindrica* were better extracted with dichloromethane than hexane and methanol. Dichloromethane had been reported to extract compounds of intermediate polarity such as alkaloids and flavonoids (Seidel, 2006). Alkaloids and flavonoids had been reported to have broad spectrum of pharmacology effect including antibacterial property (Ncube et al., 2008). Therefore, the antibacterial activity demonstrated by the dichloromethane extract may due to the high concentration of alkaloids or flavonoids in the extract, while the inactive results of hexane and methanol extracts indicated the insolubility of antibacterial compounds from this plant material in these solvent.

In addition, inhibition zones exhibited by the dichloromethane extract of *A. cylindrica* barks were in dose-dependent manner. Besides that, this extract was found to be more active against *P. aeruginosa* with inhibition zone of 11.00 ± 0.00 mm at a concentration of 200 mg/mL as compared to *S. aureus*. The standard deviations of the mean were zero because all triplicate performed same diameter of inhibition zone.

Gram negative bacteria are naturally less permeable against most of antibacterial agents because the outer lipopolysaccharide wall layer forms permeability barrier compared with Gram positive bacteria which do not have outer membrane layer (Richmond, 1978). Therefore, low permeability of Gram negative bacteria limiting the entry of antibacterial agents into the bacterial cell and prevent the access of antibacterial agent (Kumar & Varela, 2013). Furthermore, the intrinsic resistance of all bacteria to antibacterial agent was also contributed by the efflux pumps (Blair et al., 2015).

Efflux pumps are transport proteins of the bacterial membrane that actively expel toxic substance, including antibiotic from within the cell into the external environment (Webber & Piddock, 2003). There are five families of efflux proteins, including the resistance nodulation division (RND) family, the major facilitator superfamily (MFS), the adenosine triphosphate binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (Schindler & Kaatz, 2016; Fernández & Hancock, 2012; Piddock, 2006). Four families of the efflux pumps, MFS, ABC, SMR and MATE are widely distributed in both Gram positive and gram negative bacteria, while RND is only found in Gram negative bacteria which function as tripartite complex system. The tripartite complex system composed of transporter efflux protein in the cytoplasmic membrane, an accessory protein in the periplasmic space and an outer membrane protein (Blanco et al., 2016; Sun et al, 2014). In addition, broad substrate specificity of RND pumps may contribute to multi drug resistance phenotype. Figure 4.43 shows the efflux proteins of Gram positive and Gram negative bacteria.



(a) (b)

Figure 4.43. Diagram of the cytoplasmic membrane of Gram positive bacteria (a) and the cytoplasmic membrane and the outer membrane of Gram negative bacteria (b). Cytoplasmic membrane protein (A); periplasmatic space protein (B); and outer membrane protein (C). The arrows indicate the drug efflux (adapted from Moreira et al., 2004).

Seven compounds isolated from hexane extract, alkaloid and neutral fractions of the dichloromethane extract of *A. cylindrica* barks were evaluated for their antibacterial activity against *S. aureus* and *P. aeruginosa*. The tested isolated chemical compounds were stigmasterol (**81**), kinabaline (**82**), muniranine (**83**), *O*-methylmoschatoline (**8**), lysicamine (**84**), atherospermidine (**85**) and *N*-methylouregidione (**86**). These compounds were screened for antibacterial activities at only one concentration (1 mg/mL) due to the limited quantity of the compounds available. Positive control used in this assay was ampicillin, while methanol used as negative control.

Ampicillin is broad-spectrum penicillin that effective against both Gram negative and positive bacteria. It is an antibiotic with β-lactam ring and amino group, which attribute its ability in inhibiting the construction of the cell wall of bacteria and

leads to cell lysis (Sharma et al., 2013). Ampicillin inhibits the synthesis of bacterial cell wall by inactivating transpeptidase, an enzyme that crosslink the peptidoglycan chain to form rigid cell wall. However, the resistance bacteria may produce an extracellular *β*-lactamase which inactivates antibiotics through the *β*-lactam ring hydrolysis (Chudobova et al., 2014). Alkaloids, the dominant group of compounds isolated from *Alphonsea* species also bind to the active site of enzyme and inhibit its activity for cell development (Cushnie et al., 2014). The results of antibacterial activity exhibited by the isolated compounds from *A. cylindrica* barks and ampicillin (1 mg/mL) against two tested microorganisms are summarized in Table 4.11.

Table 4.11

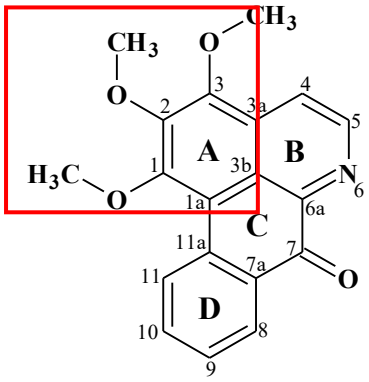
Diameter of inhibition zone of compounds from A. cylindrica barks and ampicillin against S. aureus and P. aeruginosa

Isolated compounds	**Diameter of inhibition zone (mm)***	
	Gram positive	**Gram negative**
	S. aureus	***P. aeruginosa***
Stigmasterol (**81**)	-	-
Kinabaline (**82**)	-	-
Muniranine (**83**)	-	-
O- methylmoschatoline (**8**)	8.33 ± 0.58	9.67 ± 0.58
Lysicamine (**84**)	11.33 ± 0.58	13.33 ± 1.53
Atherospermidine (**85**)	-	-
N- methylouregidione (**86**)	-	-
Methanol (negative control)	-	-
Ampicillin (positive control)	11.00 ± 1.00	9.67 ± 0.58

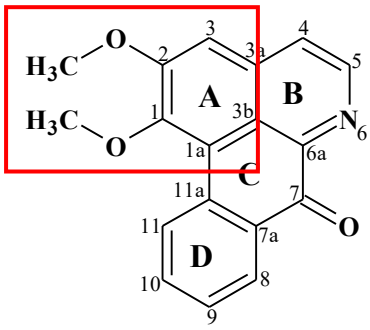
Note: *Values given in mean ± standard deviation (n = 3); - = no inhibition zone

From Table 4.11, it was observed those two oxoaporphine alkaloids, compound **8** and compound **84** demonstrated antibacterial activities against *S. aureus* and *P. aeruginosa* with diameter zone of inhibition ranging from 8.33 ± 0.58 to 13.33 ± 1.53 mm. These results implied that antibacterial property of the active compound was related to compound with 7-oxoaporphine alkaloid skeleton.

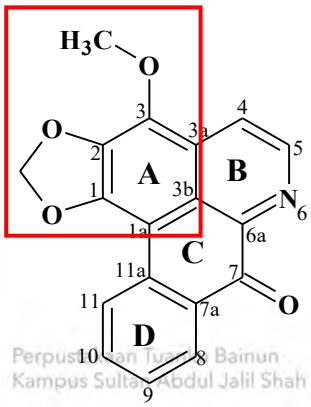
Previous study by Tavares et al. (2014) had reported that different substitution pattern in the alkaloid compounds of same basic skeleton may influencing their antimicrobial activity. Compound **8** and **84** are similar in skeleton structure differing only in the substitution at position C-1, C-2 and C-3 of ring **A**. Compound **8**, with methoxyl group at carbon C-1, C-2 and C-3 of ring **A** and compound **84** with methoxyl group at carbons C-1 and C-2 were found to be active against three microorganisms tested. However, compound **85**, that features the methylenedioxy group at position C-1, C-2 and methoxyl group at C-3 were inactive against all the microorganisms tested. Circles in Figure 4.44 showed the different substitution in ring **A** of compound **8**, **84** and **85**. In addition, these results were in agreement with previous studies by Tan et al. (2015), Yusof et al. (2015) and Omar et al. (2013), who reported that *O*-methylmoschatoline (**8**) and lysicamine (**84**) exhibited antibacterial activity, while atherospermidine (**85**) was inactive. Therefore, these results suggested that the substitution at carbons C-1, C-2 and C-3 of ring **A** in oxoaporphine alkaloid were important for antibacterial activity.



8



84



85

Figure 4.44. Structure of compounds **8**, **84** and **85**

4.4.2 Minimum inhibition concentration (MIC)

Due to the positive results in the disc diffusion assay, dichloromethane extract and active compounds, *O*-methylmoschatoline (**8**) and lysicamine (**84**) were further tested to determine their MIC values.

The determination of MIC through the spectrophotometric method was defined as the concentration at which there was a sharp decline in the absorbance value (Devienne & Raddi, 2002). Results of MICs obtained from spectrophotometric measurement for dichloromethane extract, compound **8**, compound **84** and ampicillin against *S. aureus* and *P. aeruginosa* are showed in Figure 4.45 - 4.48.

The plot of bacterial optical density (OD) at absorbance wavelength 620 nm (OD_{620}) *versus* concentration of dichloromethane extracts (µg/mL) for two tested microorganisms showed in Figure 4.45. From the graph, the MIC values of dichloromethane extract were found to be 1300 and 800 µg/mL against *S. aureus* and *P. aeruginosa* respectively. The MIC value was determined as the graph start to give OD_{620} zero value and means that there was no growth of bacteria.

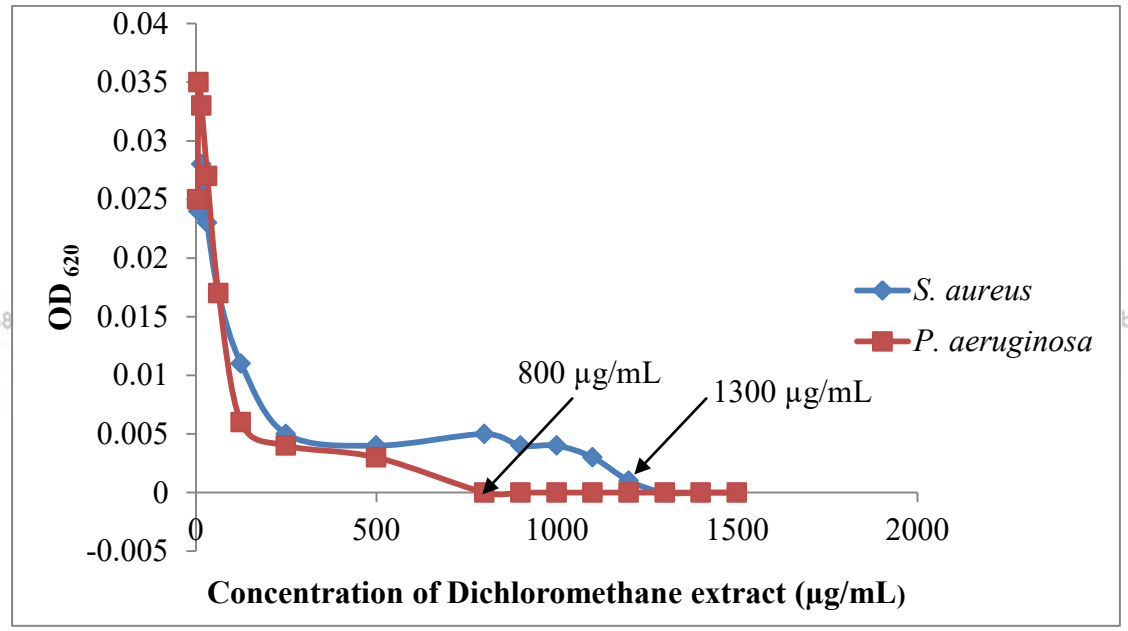


Figure 4.45. Minimum inhibition concentration of dichloromethane extract from *A. cylindrica* bark against *S. aureus* and *P. aeruginosa*

From Figure 4.46, the MIC value for compound **8** against *P. aeruginosa* was 500 µg/mL while 250 µg/mL against *S. aureus.* The absorbance values for compound **8** against *P. eruginosa* were not achieved the OD_{620} zero value, but the line of the graph becomes more horizontal from 500 µg/mL, meaning that the growth of bacteria continued to decline.

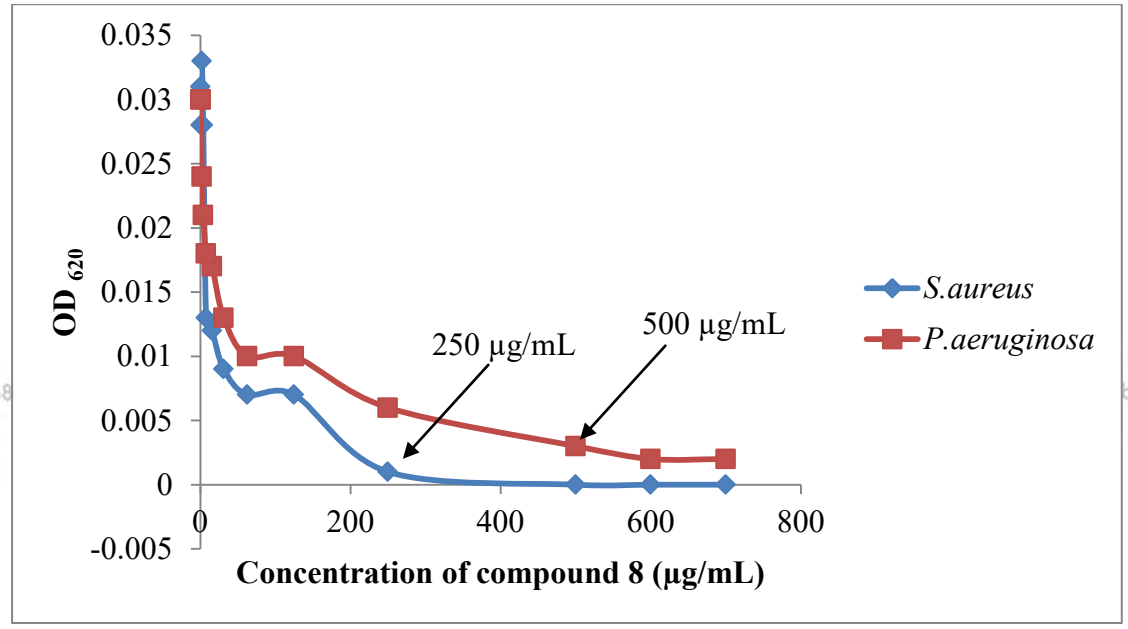


Figure 4.46. Minimum inhibition concentration of compound **8** from *A. cylindrica* bark against *S. aureus and P. aeruginosa*

MIC value for compound **84** against *S. aureus* and *P. aeruginosa* is summarized in Figure 4.47. From the graph, MIC value for compound **84** against *S. aureus* gave the value, 125 µg/mL, while MIC value against *P. aeruginosa* was 250 µg/mL since the graph start to decline at this point.

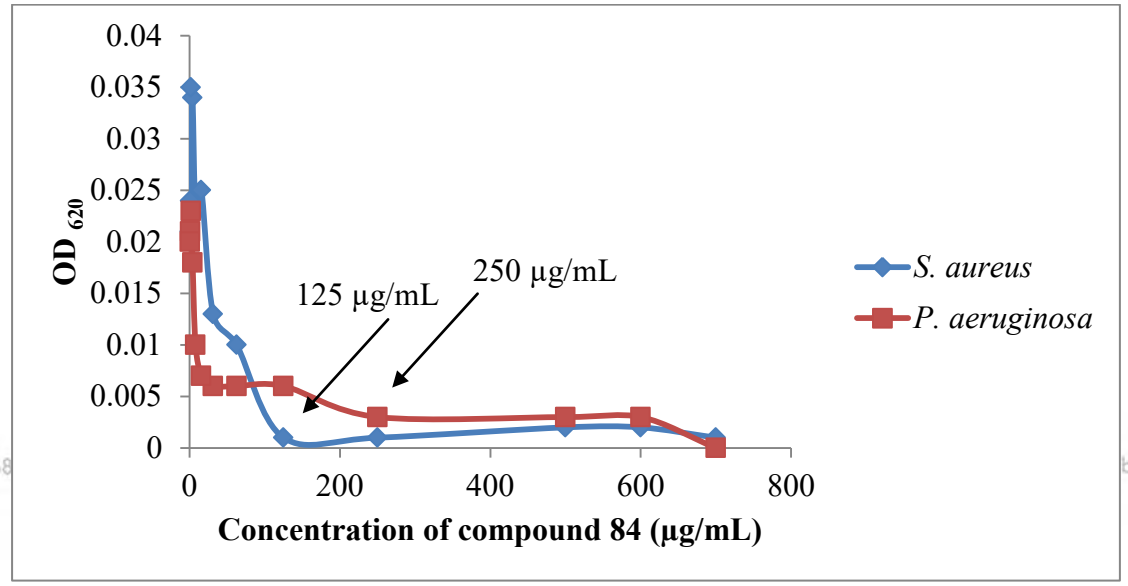


Figure 4.47. Minimum inhibition concentration of compound **84** from *A. cylindrica* bark against *S. aureus* and *P. aeruginosa*

Figure 4.48 displays the MIC value of ampicillin against *S. aureus* and *P. aeruginosa* at 300 µg/mL with zero value of bacteria OD_{620}. Therefore, there was no growth of bacteria at 300 µg/mL.

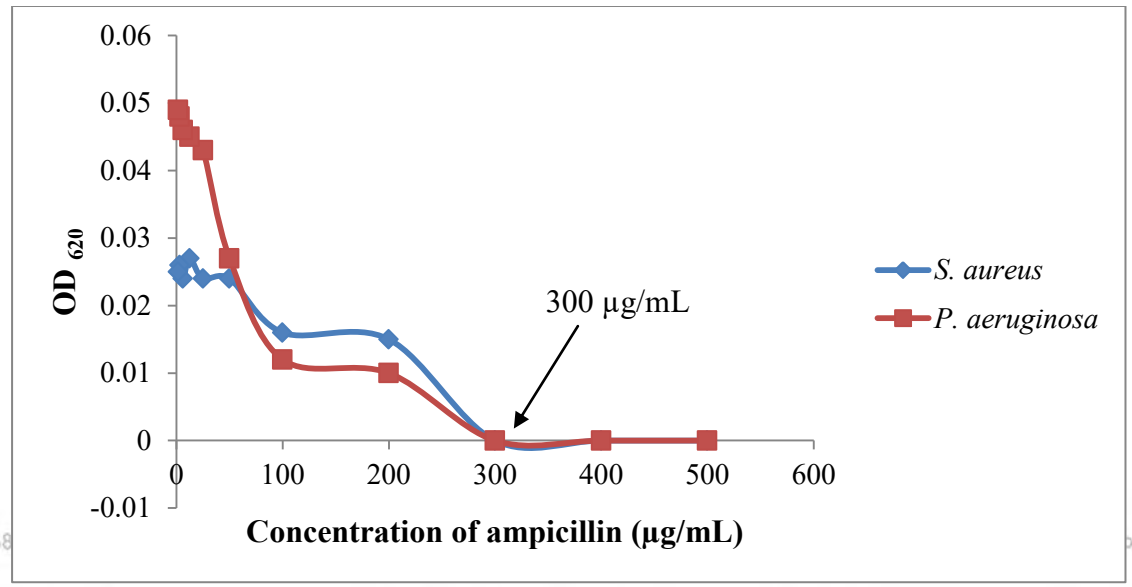


Figure 4.48. Minimum inhibition concentration of ampicillin against *S. aureus* and *P. aeruginosa*

From the above result, it was found that the MIC value of lysicamine against *S. aureus*, 125 µg/ml is smaller than MIC value of ampicillin, 300 µg/mL. Therefore, these finding suggested that lysicamine is more potent than ampicillin as antibacterial agent towards *S. aureus*.

CHAPTER 5

CONCLUSIONS

Phytochemical studies on the barks of *A. cylindrica* King. had led to the isolation of seven compounds; one terpenoid, two azafluorenone alkaloids, three oxoaporphine alkaloids and one 4,5-dioxoaporphine alkaloid. The isolated compounds were identified as stigmasterol (**81**), kinabaline (**82**), muniranine (**83**), *O*-methylmoschatoline (**8**), lysicamine (**84**), atherospermidine (**85**) and *N*-methylouregidione (**86**). All the known isolated compounds were isolated for the first time from *Alphonsea* sp. and muniranine (**83**) or 6-hydroxy-5,7,8-trimethoxyonychine was successfully isolated as a new derivative of azafluorenone alkaloid.

The antibacterial activity demonstrated by the dichloromethane extract and compounds from alkaloid fraction of dichloromethane extract, compound **8** and compound **84** against *S. aureus* and *P. aeruginosa* supported the traditional application of this species in the treatment of bacterial-related diseases (Batugal et al., 2004) while the antibacterial activity of compound **82**, **83** and **86** were investigated for the first time. However, these compounds were found to be inactive against the tested microorganisms, *S. aureus* and *P. aeruginosa*. Besides that, the antibacterial activity results of compound **8**, **84** and **85** also suggested that the substitution pattern of oxoaporphine alkaloid, at position carbon C-1, C-2 and C-3 of ring **A** are important for antibacterial activity. Moreover, compound **84** was found to be a potent antibacterial agent as the MIC value (125 µg/mL) showed by this compound was smaller than the MIC value of ampicillin, 300 µg/mL.

Recommendations for future study

From this study, we learn that *A. cylindrica* bark contain varieties of chemical compounds especially alkaloid compound. According to Amel et al. (2012), different plant part may contain different chemical compounds or different concentration of bioactive compound. Therefore, it was suggested to study the other part of this plant such as leaves or fruits which may contain different group and unique structure of chemical compounds with potential medicinal properties.

As the mass of the isolated chemical compounds were not enough for other studies, it was also recommended to the researcher in future study to synthesize the

chemical compounds in order to increase the amount of the compounds. Since this is the first phytochemical and biological study for *A. cylindrica*, it was also suggested to screen the extracts and compounds from *A. cylindrica* for other bioactivities such as antioxidant, antimicrobial, anti-inflammation, and anticancer. Besides that, the active compounds also have to be subjected to extensive scientific experiment like pharmacological, toxicological and clinical test before developed as new drug (Chitravadivu et al., 2009). In addition, the bioactive compound also can be further studied for structure-activity relationship in order to understand the mechanism of bioactivities.

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APPENDICES

Publication/articles in proceedings/ presented at conferences/ seminar

1. **Abdul Talip, M**., Syed Abdul Azziz, S. S., Chee Fah, W., Awang, K., Naz, H., Bakri, Y. M., Ahmad, M. S. & Litaudon, M. 2017. New azafluorenone derivative and antibacterial activities of *Alphonsea cylindrica* barks. Natural Product Science. (Accepted).

2. Syed Abdul Azziz, S. S., **Abdul Talip, M.**, Chee Fah, W., Alimon, H., Abu Bakar, N., Wan Mahamod, W. R., Yahaya, R., Ahmad, M. S. and Juahir, Y. 2015. Determination of Malaysian Herbs and Spices as Biopreservative Agents in Food Products. American Journal of Plant Science, 6: 718-724 (Published).

3. Saripah Salbiah Syed Abdul Azziz, **Munirah Abdul Talip**, Wong Chee Fah, Norlaili Abu Bakar, Wan Rusmawati Wan Mahamod, Rozita Yahaya, Mohamad Syahrizal Ahmad, Yusnita Juahir, Khalijah Awang, Humera Naz, Nor Hadiani Ismail (24 – 25th March 2015). Studies on alkaloid from barks of *Alphonsea cylindrica* King. International Conference on Natural Products 2015, (Johor Bahru).

4. **Munirah Abdul Talip**, Saripah Salbiah Syed Abdul Azziz, Wong Chee Fah, Khalijah Awang, Humera Naz (10 – 11th October 2015). Phytochemical investigation from the barks of *Alphonsea cylindrica* King. The 3rd International Postgraduate Conference on Science and Mathematics 2015 (IPCSM'15), (Tanjong Malim, Perak).

5. **Munirah Abdul Talip**, Saripah Salbiah Syed Abdul Azziz, Wong Chee Fah, Mohamad Syahrizal Ahmad, Yuhanis Mhd Bakri, Humera Naz & Khalijah Awang (19th November 2016). Antibacterial properties of extracts and compounds from *Alphonsea cylindrica* bark against pathogenic bacteria. The 4th International Postgraduate Conference on Science and Mathematics 2016 (IPCSM'16), (Tanjong Malim, Perak).