CHEMICAL CONSTITUENTS FROM THE ROOT OF *MUCUNA MACROCARPA* WALL., ANTIBACTERIAL AND ANTIOXIDATION PROPERTIES

ACHJANEE SOMSRI

MASTER OF SCIENCE

PROGRAM IN BIOTECHNOLOGY

MAE FAH LUANG UNIVERSITY

2010

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.....CHAIRPERSON

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Achjanee Somsri

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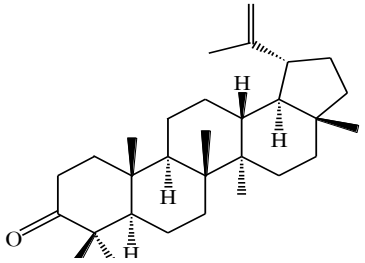
Author	Achjanee Somsri
Degree	Master of Science (Biotechnology)
Supervisory Committee	Dr. Suwanna Deachathai Assoc. Prof. Yuthana Smitasiri Dr. Panom Winyayong

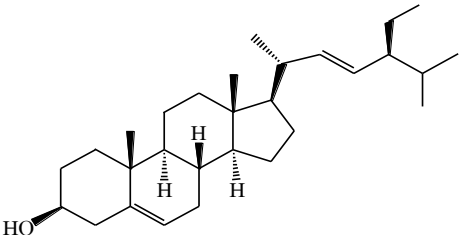
ABSTRACT

Chromatographic separation of the roots of *Mucuna macrocarpa* led to the isolation of nine compounds. They were four triterpenoids: lupenone, lupane, betulinic acid and lupeol, two steroids: stigmasterol and β-sitosterol, one anthraquinone: vismiaquinone C, one alkaloid: murrayanine and one benzaldehyde: 4-methoxybenzaldehyde. Their structures were elucidated on the basis of spectroscopic techniques.

Bioactivity assays showed that the compounds lupenone and vismiaquinone C exhibited antibacterial activities against *Bacillus cereus*, *Pseudomonas fluorescens* and *Salmonella typhimurium* with the minimum inhibition concentrations (MICs) values of 32-64 µg/mL. The crude methanolic extracts (on 2002 and 2006) showed strong antioxidation activities (IC_{50} 0.31 and 0.88 mg/mL, respectively). The crude acetone extracts (on 2002 and 2006) showed good antioxidation activities with the IC_{50} of 1.32 and 3.30 mg/mL, respectively. The crude dichloromethane extracts showed weak activity in 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay. All of the compounds showed weak antioxidative activities when tested with DPPH radical and less than 10% for radical scavenging activity.



Lupenone



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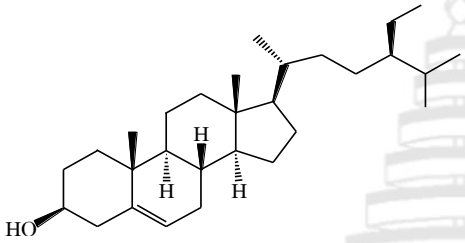
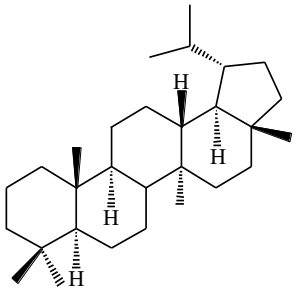


β-Sitosterol



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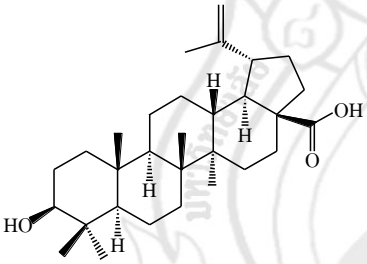
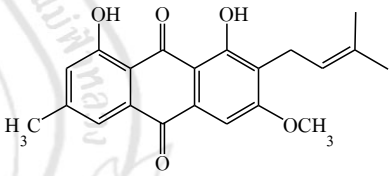
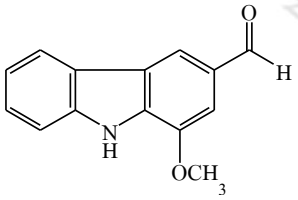


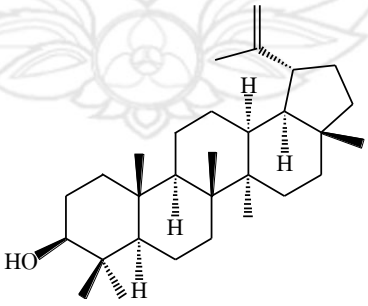
Betulinic acid



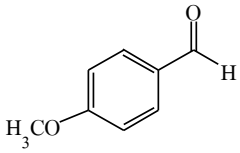
Vismiaquinone C



Murrayanine



Lupeol



4-Methoxybenzaldehyde

Keywords: *Mucuna macrocarpa* / antibacterial / antioxidation

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ABBREVIATION AND SYMBOLS

s	=	*singlet*
d	=	*doublet*
t	=	*triplet*
q	=	*quartet*
m	=	*multiplet*
br s	=	*broad singlet*
br d	=	*broad doublet*
br qd	=	*broad quartet of doublets*
dd	=	*doublet of doublet*
ddd	=	*doublet of doublet of doublet*
dt	=	*doublet of triplet*
kg	=	kilogram
g	=	gram
mg	=	milligram
µg	=	microgram
mM	=	millimolar
µM	=	micromolar
mL	=	milliter
µL	=	microliter
cm^{-1}	=	reciprocal centimeter (wave number)
h	=	hour
min	=	minute
%	=	percentage
cm	=	centimeter
mm	=	minlimeter

ABBREVIATION AND SYMBOLS (continued)

nm	=	nanometer
m.p.	=	melting point
δ	=	chemical shift relative to TMS
J	=	coupling constant
ε	=	molar extinction coefficient
°C	=	degree celcius
MHz	=	Megahertz
ppm	=	part per million
c	=	concentration
λ_{max}	=	maximum wavelengh
IR	=	Infrared
UV	=	Ultraviolet-Visible
^{1}H NMR	=	Proton Nuclear Magnetic Resonance
^{13}C NMR	=	Carbon Nuclear Magnetic Resonance
CC	=	Column Chromatography
PLC	=	Preparative Thin-layer Chromatography
TLC	=	Thin-layer Chromatography
TMS	=	tetramethylsilane
DMSO	=	dimethylsulfoxide
$CDCl_3$	=	deuterochloroform
CD_3OD	=	tetradeuteromethanol
MICs	=	Minimum Inhibition Concentrations
MHA	=	Mueller Hinton Agar
MHB	=	Mueller Hinton Broth
NSS	=	Normal Saline Solution

ABBREVIATION AND SYMBOLS (continued)

CFU	=	Colony Forming Unit
rpm	=	Revolutions per minute
A	=	absorbance
IC_{50}	=	50% Inhibition Concentration
BHT	=	butylated hydroxytoluene
DPPH	=	1,1-diphenyl-2-picrylhydrazyl radical

CHAPTER 1

INTRODUCTION

1.1 *Mucuna macrocarpa*

Thailand is in a tropical area and has sunlight all year round. For this reason, the varieties of plants are found including those with medicinal properties. The plants are sources of natural medicines which are neglected for a long time since the modern science occupied the livelihood of Thai people. Medicinal properties of each plant depend on its chemical constituents.

Mucuna genus is in the family of Fabaceae and distributed in the north of Thailand. According to the information from SciFinder Scholar database, twenty-two species have been found in Thailand.

Mucuna genus can be found in the tropics and subtropics of both hemispheres. These have pods and tropical climbing vines, those flowers and seed pods hang from long stems that stretch all the way to the forests canopy. Some of the pods look like the pea pods. Most of the flowers of the *Mucuna* are like the flower of a pea and range color. The seeds are round or disk shaped, they are varies tough, and are varied in color from brown, white, black and green. The genus *Mucuna* ADANSON comprises about 160 species distributed over the area of tropics and subtropics (Chen, 1991). *M. macrocarpa* in the northern of Thailand can be found in Doi Tung, Chiang Rai province; Doi Chiang Dao and Doi Suthep, Chiang Mai province; besides, the external distribution are Burma, China, India and Japan. *Mucuna macrocarpa* Wall., known as Black Kwao Krua, is one of a species in *Mucuna* genus. Traditionally, this medicinal plant has long been employed among Thai males for the purposes of tonic effects and preventing erectile dysfunction (Wichai Cherdshewasart, Warakorn Cheewasopit & Porntipa Picha, 2004b).

1.2 Objectives

The objectives of this work were to investigate the chemical constituents from the roots of *M. macrocarpa* and examine their antibacterial and antioxidation activities.

CHAPTER 2

LITERATURE REVIEWS

2.1 General characteristics of *M. macrocarpa*

The genus *Mucuna* ADANSON comprises about 160 species distributed over the area of tropics and subtropics (Chen, 1991), 33 accepted species of climbing vines and shrubs of the family Fabaceae, found worldwide in the woodlands of tropical areas.

Leave: Semi-leathery, with rushedbrown backs, terminal leaflet in long elliptical shape, cuspidate, (Chi, Nien, Chao, Hsin & Chung, 2004) 8-18 cm long and 4-10 cm broad, petiole long, hairy as branchlets**.**

Inflorescences: paniculate, but look like raceme through abbreviation of lateral branches, 15-30 cm long, subsessile, brown-velvety, lateral branches reduced to tuber-like organ, many, fasciculate, Bracts eaducous, Pedicels 1-2 cm long, hairy as rachis. Bracteoles eaducous (Yoichi & Hiroyushi, 1981).

Flowers: 5.5-7 cm long, dark purple but with standard of greenish gray and keel-petal of purplish. Calyx obliquely campanulate, brown-velvety on both sides and with sparsely long stinging brown hairs (0.5-1 mm long) outside, 4 lobed; upper lobe broadly triangular, obtuse to rounded at apex, 4-5 mm long, lower one 7-9 mm long, as long as or shorter than tube (Yoichi et al., 1981).

Pods: woody, green in living state, compressed, linear, 20-50 cm long, 3-5 cm wide, 4-12 seeds septate, angled but not winged along both sides near margin, loosely constricted between seeds, ferrugineous-tomentose.

Seeds: dark brown, broadly elliptic, 2.2-2.5 cm in longer dimension compressed.

Figure 2.1 *Mucuna macrocarpa* Wall.

Note. A: climber stem, B: fluid is exuded from the stem, C: mature flowers, D: green fruit, E: trifoliate leaves, F: stem-like tuber, G: ripe fruit

2.2 Chemical constituents from *Mucuna* genus

According to NAPRALERT database Science direct and Chemical Abstracts, several types of compounds have been reported to be present in *Mucuna* genus such as triterpenes, alkaloids and flavonoids. Table 2.1 shows the chemical constituents isolated from *Mucuna* genus.

Table 2.1 Compounds isolated from the *Mucuna* genus

Scientific name (investigated part)	Compound	Structure	References
M. acuminata			
(seed)	L-dopa	1	Lubis & Sastrapradha, 1981
M. aterrima			
(seed)	L-dopa	1	Amarasekera & Jansz, 1980
	3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	2	
	3-Carboxy-1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	3	
	L-dopa	1	Daxenbichler, Van Etten, Hallinan, Earle & Barclay, 1971
(leaves and stem)	Tetracosanolic acid triacontyl ester	-	Nogueira, de Oliveira, & Ferraz, 1996
	Triacontan-1-olalkane	-	
M. birdwoodiana			
(stalk)	3-*O*-(6-*O*-Methyl-β-D-glucuronopyranosyl) asiatic acid	4	Ding, Kinjo, Yang & Nohara, 1991
	3-*O*-(6-*O*-Methyl-β-D-glucuronopyranosyl)-28-*O*-β-D-glucopyranoside	-	

Table 2.1 (continued)

Scientific name (investigated part)	Compound	Structure	References
M. birdwoodiana	3-*O*-[α-L-Arabinopyranosyl(1→2)]-6-*O*-methyl-β-D-glucuronopyranoside	-	Ding et al., 1991
(stalk)	3-O-[α-L-Arabinopyranosyl(1→2)]-6-*O*-methyl-β-D-glucuronopyranosyl maslinic acid	-	
	Mucunagenin a	5	
	Mucunagein b	6	
	Benzenoid	-	Goda et al., 1987
	N-(*trans*-Feruloyl)tyramine	7	
(seed)	L-dopa	1	Cai & Zhu, 1990
(stem)	2,6-Dimethoxy phenol	8	Goda et al., 1987
	Syringic acid	9	
	Valillic acid	10	
M. capitata			
(seed)	Proteid	-	Katiyar & Niranjan, 1981
M. cochinchinensis			
(fruit)	L-dopa	1	Su, Tang, Xu & Zeang, 1992
M. currannii			
(seed)	Hydrocyanic acid	11	Laurena, Revilleza & Mendoza, 1994
M. deeringiana			
(leaves)	Stizolobic acid	12	Ellis, 1976
(seed)	3-Carboxy-6,7-dihydroxy-1,2,3,4-Tetrahydroisoquinoline	2	Amarasekera et al., 1980

Table 2.1 (continued)

Scientific name (investigated part)	Compound	Structure	References
M. deeringiana	3-Carboxy-1-methyl-6,7-dihydroxy-	3	Amarasekera et al.,
(seed)	1,2,3,4-tetrahydroisoquinoline		1980
(suspension culture)	L-dopa	1	Remmen & Ellis, 1980
M .diabolica			
(seed)	L-dopa	1	Lubis et al., 1981
M. flagellipes			
(seed)	Haemagglutinin	-	Mbadiwe & Agogbua, 1978
M. gigantea			
(seed)	L-dopa	1	Lubis et al., 1981
M. holtionii			
(seed)	L-dopa	1	Daxenbuchler et al., 1971
M. imbricata			
(seed oil)	Linoleic acid	13	Badami & Patil, 1975
	Oleic acid	14	
	Palmitic acid	15	
	Stearic acid	16	
M. macrocarpa			
(root)	L-dopa	1	Chen, Zhen, Xu, Pan & Tang, 1993
(part not specified)	Friedelin	17	Hu, Luo & Cai, 1994
	Lupenone	18	
	2,3-Dihydroxypropyl ester hexacosanoic acid	19	

Table 2.1 (continued)

Scientific name (investigated part)	Compound	Structure	References
M. macrocarpa (part not specified)	2,3-Dihydroxypropyl ester pentacosnoic acid	20	Hu, Luo & Cai, 1994
M. macrophylia (seed)	L-dopa	1	Lubis et al., 1981
M. nivea (seed)	L-dopa	1	Amarasekera et al., 1980
	3-Carboxy-6,7-dihydroxy-1,2,3,4-etrahydroisoquinoline	2	
	Hydrocyanic acid	11	Jansz & Pieris, 1978
M. pruriens (seed)	L-dopa	1	Hussain & Manyam, 1997
	Tannins	-	Debelmas, Dobremez, Michel & Benarroche, 1973
	Lecithin	21	Panikkar, Majella & Pillai, 1987
	Alkaloid P	22	Rakshit & Majumdar, 1956
	Alkaloid Q	23	
	Alkaloid R	24	
	Alkaloid S	-	
	Alkaloid X	25	
	Prurienidine	-	
	threo-12,13-Dihydroxy-*cis*-9-octadec enoic acid	-	Hasan, Sherwani, Ahmad, I., Ahmad, F. & Osman, 1980

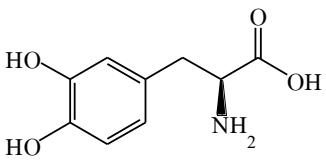
Table 2.1 (continued)

Scientific name (investigated part)	Compound	Structure	References
M. pruriens	*cis*-12,13-Epoxy-*trans*-9-octadec enoic acid	-	Hasan et al., 1980
(seed)	*threo*-12,13-Dihydroxy-*trans*-9-octadec enoic acid	-	
(seed oil)	Vernolic acid	26	Ahmad, 1965
(fruit)	Indole alkaloid	27	Smith, 1977
(leaves)	Bufotenine	28	Ghosal, Singh &
	Choline	29	Bhattacharya, 1971
	N,*N*-Dimethyl tryptamine	30	
	Isoquinoline	31	Ghosal et al., 1971
(pod trichomes)	5-Hydroxy tryptamine	32	Ghosal et al., 1971
(leaves and stem)	*N*,*N*-Dimethyl methoxy tryptamine	33	Smith, 1977
(suspention culture)	Dopamine	34	Wichers, Visser, Huizing & Pras, 1993
M. sempervirens			
(seed)	Mucuna lectin msl	-	Zhou, Zeng, Bao, & Boa, 1996
	D-pinitol	35	Plouvire, 1962
(leaves)	6,8-Di-*C*-α-L-arabinosyl apigenin	36	Ishikura & Yushitama,
	8-*C*-α-L-arabinosyl luteoin	37	1988
	Isoorientin	38	Ishikura et al., 1988
M. sloanei			
(seed)	L-dopa	1	Rai & Saidu, 1977
M. species	L-dopa	1	Amarasekera et al., 1980
(seed)	3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	2	

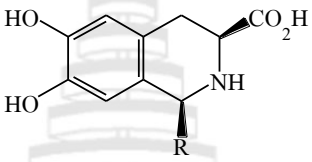
Table 2.1 (continued)

Scientific name (investigated part)	Compound	Structure	References
M. species (seed)	3-Carboxy-1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	3	Amarasekera et al., 1980
M. urens (seed)	L-dopa	1	Daxenbuchler et al., 1971
M. utilis (seed)	L-dopa	1	Amarasekera et al., 1980
	3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	2	
	Kievitone	39	Narayanaswamy & Mahadevan, 1981

Structure of compounds from *Mucuna macrocapa*

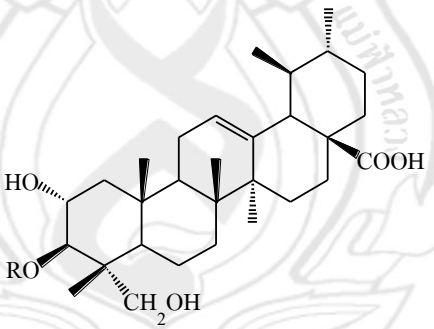


1: L-dopa



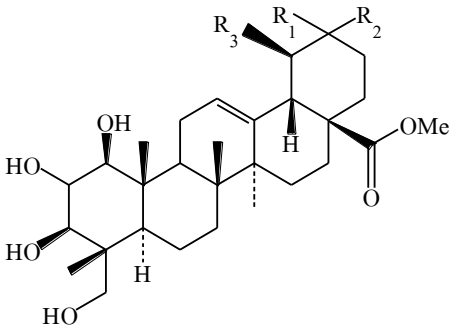
2: R= H 3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline

3: R= CH_3 3-Carboxy-1-methyl-6,7-dihydroxy-1,2,3,4- tetrahydroisoquinoline

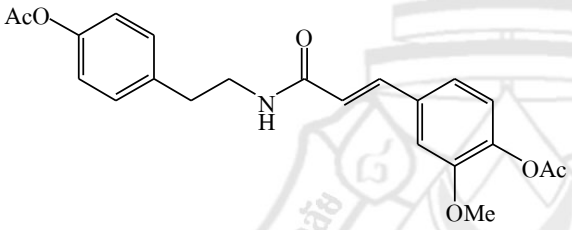


4: R = 6-*O*-Methyl-β-D-glucuronopyranosyl

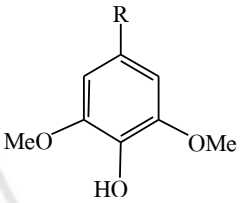
3-*O*-(6-*O*-Methyl-β-D-glucuronopyranosyl) asiatic acid



R_1	R_2	R_3	
CH_3	CH_3	H	**5:** Mucunagenin A
H	CH_3	CH_3	**6:** Mucunagenin B

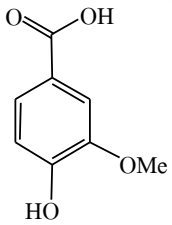


7: *N*-(*trans*-Feruloyl) tyramine



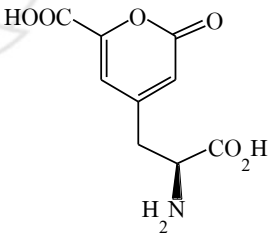
R = H **8:** 2,6-Dimethoxy phenol

R = CO_2H **9:** Syringic acid

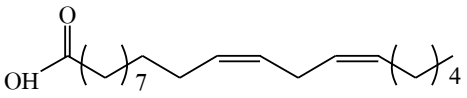


10: Valillic acid

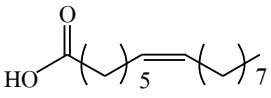
11: Hydrocyanic acid



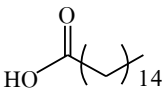
12: Stizolobic acid



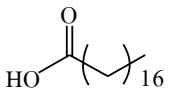
13: Linoleic acid



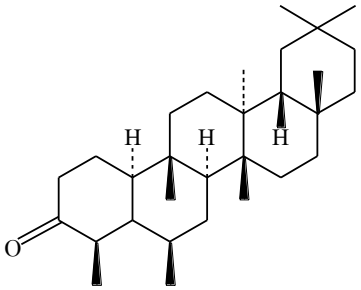
14: Oleic acid



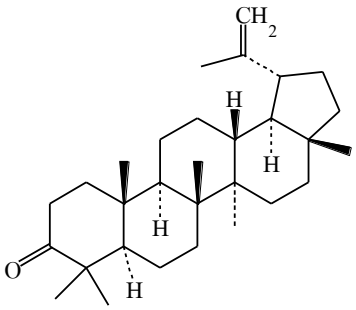
15: Plamitic acid



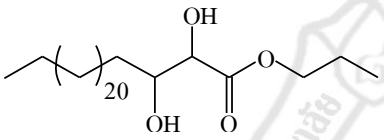
16: Stearic acid



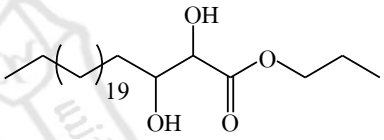
17: Friedelin



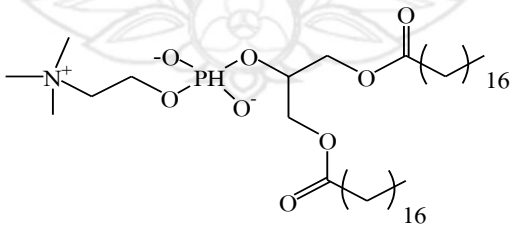
18: Lupenone



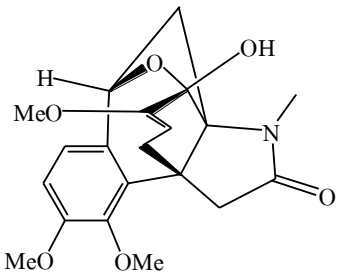
19: 2,3-Dihydroxypropyl ester hexacosnoic acid



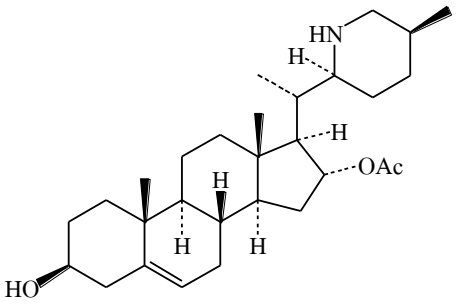
20: 2,3-Dihydroxypropyl ester pentacosnoic acid



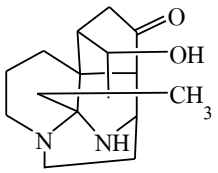
21: Lecithin



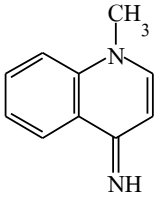
22: Alkaloid P



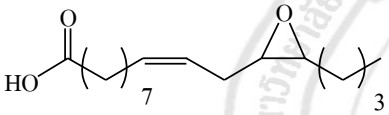
23: Alkaloid Q



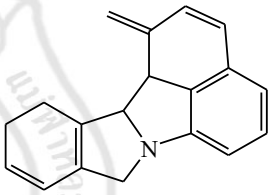
24: Alkaloid R



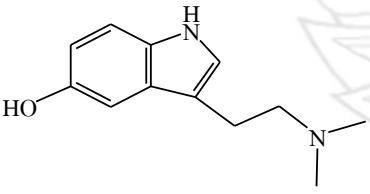
25: Alkaloid X



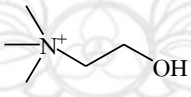
26: Vernolic acid



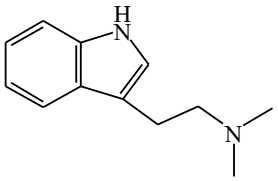
27: Indole alkaloid



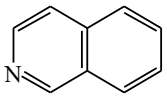
28: Bufotenine



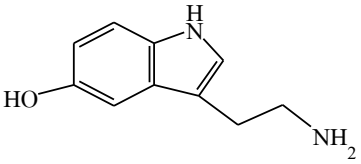
29: Choline



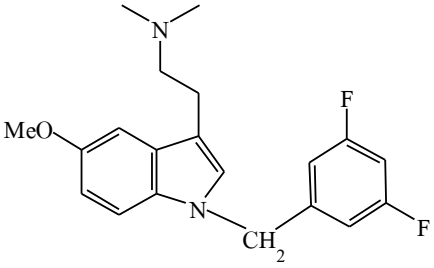
30: *N*,*N*-Dimethyl tryptamine



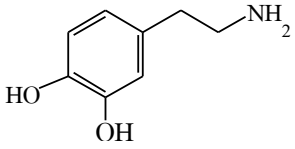
31: Isoquinoline



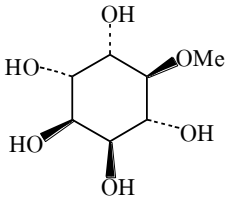
32: 5-Hydroxy tryptamine



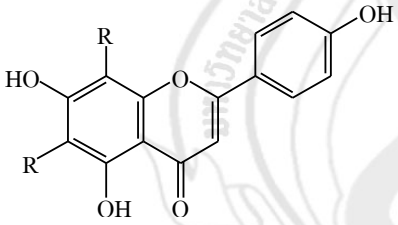
33: *N,N*-Dimethyl methoxy tryptamine



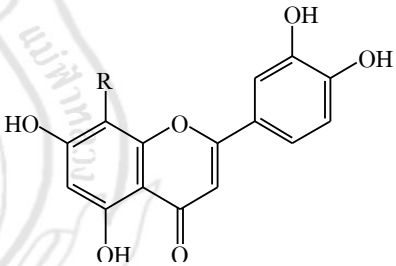
34: Dopamine



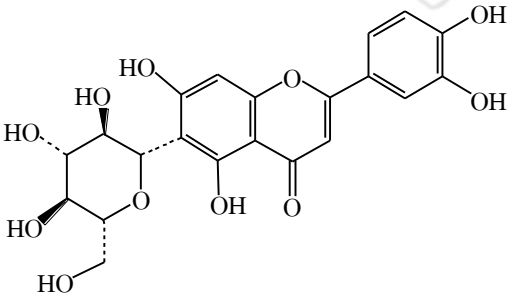
35: D-pinitol



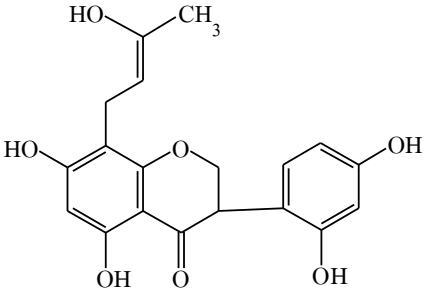
36: R=α-L-arabinosyl 6,8-Di-*C*-α-L-arabinosyl apigenin



37: R= α-L-arabinosyl 8-*C*-α-L-arabinosyl luteoin



38: Isoorientin



39: Kievitone

2.3 Antibacterial activity

M. macrocarpa has been tested antibacterial activity of crude extracts (hexane, ethyl acetate and methanol) using the disc diffusion method was selected to serve this purpose based on the protocol of Arias. The result shows crude extracts can not inhibit bacterial growth (Tanatorn Saisavory, 2006).

2.4 Antioxidation activity

The natural antioxidants have attracted attention because some synthetic antioxidants have been found to be carcinogenic and harmful to lungs and liver (Yamasak, Hashimato, Kokusenya, Miyamayo & Sato, 1994). Reactive oxygen species such as hydroxyl ($OH^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and the superoxide anion ($O_2^{\bullet -}$) are constantly produced as a result of metabolic reactions in living systems (Wang et al., 1999). A growing body of evidence indicates that various pathological conditions, including cardiovascular disease, arthritis, various cancers and Alzheimer's disease, are associated, at least in part, with the damaging effects of uncontrolled free radical production (Wang et al.,1999). In addition, aqueous extracts of Black Kwao Krua have been reported to show strong antioxidation activity (Janya Sang-Arun, Yuthana Smitasiri, Supak Poungbangpho & Maitree Suttajit, 2001).

2.5 Other biological activity

Mucuna macrocarpa has been used by the local Thai people as folk medicine, purposes of tonic effects and preventing erectile dysfunction (Wichai Cherdshewasart et al., 2004b). Anti-proliferation effects of Black Kwao Krua on the growth of HeLa cells showed the strongest effect. The 50% growth inhibition (ED_{50}) was determined to be 393.85 μg/mL for the Black Kwao Krua and out of range for the rest (Wichai Cherdshewasart, Warakorn Cheewasopit & Porntipa Picha 2004a). Black Kwao Krua ethanol extract led to no proliferation and a strong anti-proliferation effect on the growth of MCF-7 cell at the medium and high concentrations. The plant extract were

no characteristic as phytoestrogens and did not exhibit any estrogenic effect (Wichai Cherdshewasart et al., 2004b). Ethanolic extracts from Black Kwao Krua had no effect on intracavernous pressure and blood pressure in the adult male rats (ยุทธนา สมิตะสิริ, จิณณวัตร มานะเสถียร, สิทธิศักดิ์ ปิ่นมงคลสกุล และสมศรี วงศ์เรือน, 2547). In addition the biological activities and ethnomedical applications of *Mucuna* genus were shown in Table 2.2 and 2.3. Although a number of biological properties from *M. macrocarpa* have been recognized, no study on the antioxidant potential and antimicrobial activity from chemical constituents has been described.

Table 2.2 Biological activities of *Mucana* genus

Scientific name (investigated part)	**Biological activity**	**References**
M. argyrophylla		
(seed)	Hemagglutinin activity	Giral, Sotelo, Lucas & De
	Trypsin inhibition	La Vega, 1978
	Trypsin inhibition	Laurena et al., 1994
M. aterrima		
(leave and stem)	Nematocidal activity	Nogueira et al., 1996
M. capitata		
(seed)	Toxic effect	Niranjan & Katiyar, 1981
M. currannii		
(seed)	Trypsin inhibition	Laurena et al., 1994
M. ferruginea		
(stem)	Antibacterial activity	Chen, Lin & Namba, 1989
M. flagellipes		
(fruit and seed)	Autonomic effects	Sandberg & Cronlund, 1977

Table 2.2 (continued)

Scientific name (investigated part)	**Biological activity**	**References**
M. imbricate		
(part not specified)	Analgesic activity	Dhawan, Patnaik, Rastogi,
	Antibacterial activity	Singh & Tandon, 1977
	Anticonvulsant activity	
	Antifungal activity	
	Antiinflammatory activity	
	Antispasmodic activity	
	Antitumor activity	
	Antiyeast activity	
	Barbiturate potentiation	
	Diuretic activity	
	Hypoglycemic activity	
	Hypolipemic activity	
	Hypothermic activity	
	Semen coagulation	
	Spermicidal effect	
	Toxicity assessment	
M. macrocarpa		
(stem)	Analgesic activity	Bhakuni et al., 1988
	Anticonvulsant activity	
	Antiprotozoan activity	
	Antiviral activity	
	Diuratic activity	
	Hypothermic activity	
	Spasmolytic activity	
	Toxicity assessment	

Table 2.2 (continued)

Scientific name (investigated part)	Biological activity	References
M. monosperma		
(root)	Abortifacient effect	Kamboj, 1988
M. pruriens		
(entire plant)	Fertility promotion effect	Madaan, 1985
M. pruriens		
(entire plant)	Insecticide activity	Sievers, Archer, Moore & McGovran, 1949
	Bening prostatic hyperplasia improvement	Mukherjee, Ghosh & De, 1984
	Genitourinary effect	Rathore & Saraswat, 1986
	Antibacterial activity	Chen, Lin & Namba, 1987
(fruit)	Antispasmodic activity	Dhar M. L., Dhar, M. M.,
	Cytotoxic activity	Dhawan, Mehrotra & Ray,
	Hypoglycemic activity	1968
	Toxicity assessment	
	Analgesic activity	Jauk, Galati, Kirjavainen,
	Antiinflammatory activity	Forestieri & Trovato, 1993
	Antipyretic activity	
(leaves)	Antihypercholesteroleolemic activity	Jauk, Galati, Forestieri,
	Antihyperlipemic activity	Kirjavainen & Trovato, 1989
	Analgesic activity	Jauk et al., 1993
	Antiinflammatory activity	
	Antipyretic activity	
	Anticoagulant activity	Houghton & Skari, 1994
(leave, root and seed)	Antiinflammatory activity	Dabral & Sharma, 1983

Table 2.2 (continued)

Scientific name (investigated part)	Biological activity	References
M. pruriens		
(part not specified)	Anabolic activity Androgenic effect	Jayatilak, P. G., Pardanani, D. S., Dattatreya, M. B. & Sheth, A. R., 1976
(root)	Antispasmodic activity Cytotoxic activity Hypoglycemic activity Toxicity assessment	Dhar et al., 1968
(part not specified)	Aphrodisiac activity Histamine release stimulation Melatonin level increase Serotonin releasing effect	Bhargava & Singh, 1978 Upadhya, Shukla, Agrawal & Dubey, 1988
	Antiradiation effect	Ohta, Sakurai, Inoue, Takahashi & Shinoda, 1987
(seed)	Hypoglycemic activity	Dhawan et al., 1977
	Spasmogenic activity	Uguru, Aguiyi & Gesa, 1997
	Nematocidal activity	Kiuchi, Hioki, Nakamura, Miyashita & Tsuday, 1989
	Penis erectile stimulant	Sankaran, 1984
	Nematocidal activity	Ali, Mikage, Kiuchi, Tsuda & Kondo, 1991
	Hemagglutinin activity Trypsin inhibition	Udedibie & Carlini, 1998

Table 2.2 (continued)

Scientific name (investigated part)	Biological activity	References
M. pruriens		
(seed)	Bronchodilator activity	Carbajal, Casaco, Arruzazabala, Gonzalez & Fuentes, 1991
	Antigalactagogue effect	Vaidya, R. A., Aloorkar, S. D., Sheth, A. R., & Pandya, S. K, 1978
	Antiparkinson activity	Hussain et al., 1997; Nath et al., 1981; Vaidya, A. B., et al 1978; Manyam, 1990
	Aphrodisiac activity FSH release inhibition FSH release stimulation effect FSH synthesis stimulation Gonadotropin release stimulation Gonadotropin synthesis stimulation LH- release inhibition LH- release stimulation LH- synthesis stimulation	Jayatilak, Sheth & Mugatwala, 1976 Jayatilak et al., 1976
	Aphrodisiac activity	Rao & Parakh, 1978
	Cholinesterase inhibition	Nath et al., 1981
	Embryotoxic effect Teratogenic activity	Nath, Sethi, Singh & Jain, 1992
	Hypocholesterolemic activity Hypoglycemic activity	Pant, Uddin, Bhardwaj & Tewari, 1968

Table 2.2 (continued)

Scientific name (investigated part)	Biological activity	References
M. pruriens		
(seed)	Prolactin inhibition	Vaidya, A. B., et al 1978
	Sexual behavior modification	Mitra, Muralidhar & Rao,
	Spermatogenic effect	1996
	Testosterone level increase	Mitra, Muralidhar & Rao,
		1996
	Sexual behavior modification	Amin, Khan, Rahman &
		Khan, 1996
	Spermatogenic effect	Solepure, Joshi, Deshkar,
		Muzumdar & Shirole, 1976
	Spermatogenic effect	Pardanani et al., 1976
	Taenicide activity	Feroz, Khare & Srivastava,
		1982
	Plant growth inhibitor	Fuiji, Shibuya & Yasuda,
		1992
	Skeletal muscle stimulant activity	Aguiti, Uguru, Johnson,
	Smooth muscle stimulant activity	Obi & Adoga, 1997
	Antihyperglycemic activity	Akhtar, 1992
	Hypoglycemic activity	
	Plant growth inhibitor	Fujii, Shibuya & Yasuda,
		1991
	Prostaglandin synthetase inhibition	Goda, Shibuya &
		Sankawa, 1987
M. sloanei		
(fruit, root and leaves)	Molluscicidal activity	Medina & Woodbury,
		1979

Table 2.2 (continued)

Scientific name (investigated part)	Biological activity	References
M. species		
(fresh stem bark)	CNS effect	Cox, 1989
	Spasmolytic activity	
M. urens		
(seed)	Antibacterial activity	Caceres et al., 1995
M. utilis		
(seed)	Analgesic activity	Abraham et al., 1986
	Antiviral activity	
	Diuretic activity	
	Spasmogenic activity	
	Toxicity assessment	

Table 2.3 Ethnomedical applications of *Mucuna* genus

Scientific name (investigated part)	Ethnomedical application	References
M. bracteata		
(entire plant)	Homostatic	Jamir, 1990
M. colletii		
(seed)	Cancer	Sasithorn Wasuwat, 1967
M. coriacea		
(part not specified)	Treat urinary schistosomiasis	Ndamba, Nyazyma,
(root)	Styptic for extended menstruation	Makaza, Anderson &
		Kaondera, 1994
		Haerdi, 1964
M. currannii		
(seed)	Food	Laurena et al., 1994
M. flagellipes		
(intire plant)	Administered as an emmenagogue	Bouquet & Debray, 1974
M. gigantean		
(root)	Treat schistosomiasis	Hostettmann, 1984
(seed)	Aphrodisiac	Burkill, 1966
M. macocarpa		
(entire plant)	Medicinally	Sebastian & Bhandari,
(stem)		1984
	Treat diabetes mellitus	Lin, 1992
M. monosperma		
(leaves)	Check external hemorrhages	Alam, 1992
M. poggei		
(seed)	Food	Johns, Mhoro & Sanaya,
		1996

Table 2.3 (continued)

Scientific name (investigated part)	Ethnomedical application	References
M. pruriens		
(fruit)	Intestinal parasites	Weniger, Rousier, Daguilh, Henrys & Anton, 1986
	Geven interally to children in case of Stomach worms	Joshi, Patel & Mehta, 1980
	Anthelmintic	Sebastian et al., 1984
	Irritant	
(entire plant)	Emmenagogue	Bouquet et al., 1974
(leaves)	Uterine stimulant	Ghosal et al., 1971
	Drysentery	
	Aphaodisiac	
	Nerve tinic	
	Diuretic	
	Corpion stings	Nagaraja & Rao, 1990
	Treat snakebite	Houghton et al., 1994
(leaves and stem)	Applied to cut	Anderson, 1986
	Applied to burn	
	Burn	
(part not specified)	Abortifacient	Casey, 1960
	Antifertility agent	
	Cancer	Jain & Tarafder, 1970
	Improve sexual function	Nisteswar & Murthy, 1989
	Improve male function	
	Medicinally	Duke, 1975
	Worms	Oake & Morris, 1958

Table 2.3 (continued)

Scientific name (investigated part)	Ethnomedical application	References
M. pruriens		
(part not specified)	Herb	Upadhya et al., 1988
(plant juice)	Emmenagogue	Viera, 1959
(root)	Emmenagogue	Saha, Savini & Kasinathan, 1961
	Aphaodisiac	Bhattarai, 1992
	Gout	Jain, 1989
	Rheumatism	
	Kidney stone	Pushpangadan & Atal, 1984
	Blood purifier	
	Diuretic	
	Delirium ayuredic and unani medicine	Kapoor & Kapoo, 1980
	Relieve dysmenorrhea	Hemadri, & Sasibhushana Rao, 1983
(pod)	Anthelmintic	Bhandary, Chandrashekhar & Kaveriappa, 1995
(seed)	Vermifuge	Vitalyos, 1979
	Aphaodisiac	Viera, 1959 Burkill, 1966; Bhandary et al., 1995; Suwan, 1970; Mitra et al., 1996; Amico, 1977
	Diarrhea	Girach, Aminuddin, Siddioui & Khan, 1994

Table 2.3 (continued)

Scientific name (investigated part)	Ethnomedical application	References
M. pruriens		
(seed)	Antivenin	Selvanayahgam, Gnanavendan, Balakrishnan & Rao, 1994
	Scorpion stings and snakebite	Nagaraju et al., 1990
	Intestinal worms	Ayensu, 1978
	Cure night dreams and impotency	Jain, 1963
	Promote fertilitiy	
	Aphrodisiac to increase seminal fluid and Manly vigour	Das, 1955
	Emmenagogue	
	Aphrodisiac in ayurveda and unani medicine	Kapoor et al., 1980
	Nervine	
	Nerve tonic	Nath et al., 1992
	Abortion	Kumar & Prabhakar, 1987
	Pulmonary tuberculosis Treat male impotence and sterility	Rao et al., 1978 ; Pardanani et al., 1976
	Sexual debility	Dixit & Pandey, 1984
	Persistent coughs	Akhtar, 1992
	Diabetes	Pushpangadan et al., 1984
	Aphrodisiac	Udedibie et al., 1998
M. rostrata		
(seed)	Against hemorrhoids	Duke, 1994
	Antidote	
	Diuretic	

Table 2.3 (continued)

Scientific name (investigated part)	Ethnomedical application	Reference
M. species		
(part not specified)	Abortive	Debelmas, 1975
(stem)	Relieve stomach pains	Holdsworth & Balun, 1992
M. urens		
(part not specified)	Treat gonorrhea	Duke, 1994
	Treat migraine	
	Vermifuge	
M. urens		
(sap)	Bites and sting	Coee & Anderson, 1996
	Digestive	Caceres et al., 1995
(seed)	Gonorrhea	

CHAPTER 3

RESEARCH METHODOLOGY

3.1 General methods

Melting points were recorded in °C and were measured on BÜCHI model B-540 visual Melting Point Apparatus. Infrared spectra were recorded by using Perkin-Elmer FTSFT-IR/Spectrum GX spectrometer. Major bands (λ_{max}) were recorded in wave number (cm^{-1}). Ultraviolet (UV) absorption spectra were recorded using UV-Lamp and cabinet spectrometer (Vilber Lourmat/ France). Principal bands (λ_{max}) were recorded as wavelengths (nm) and log $\mathcal{E}$ in method solution. ^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded on Brüker AVANCE 300 MHz and 400 MHz Bruker FTNMR Ultra Shield. Spectra were recorded in $CDCl_3$ or CD_3OD solution and were recorded as δvalue in ppm down field from TMS (internal standard δ 0.00). Optical rotation $[\alpha]_D$ values were determined with a JASCO-P-1020 polarimeter. Solvents of extraction and chromatography were distilled at their boiling point ranges prior to use. Solvents for crystallization were analytical grade reagent. Pre-coated TLC aluminum sheets of silica gel 60 GF_{254} (20x20 cm, layer thickness 0.2 mm) were use for analytical purposes and the compounds were visualized under ultraviolet light and/or vanillin sulphuric acid reagent. Plates of silica gel GF_{245}, 20x20 cm, thinkness 1.00 mm, activated at 110 °C for 3 h were utilized in the case of preparative TLC. Quick column chromatography was performed on silica gel 60 GF_{254} (Merck). Column chromatography was performed by using silica gel (Merck) type 100 (70-230 mesh ASTM) and 60 (0.063-6.200 mesh ASTM). The analytical grade of absolute ethanol, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Fluka), ascorbic acid (Fluka) and butylated hydroxytoluene (BHT, Fluka) was used for antioxidation activity testing and absorbance were measured by spectrophotometer (Thermo/Genesys 20). The nutrient agar (CRITERION dehydrated culture media) and dimethyl sulfoxide (DMSO) were used for antibacterial activity

testing against 7 strains of microorganism (*Bacillus cereus*, *Escherichia coli*, *Pseudomonas fluorescens, Pseudomonas aeruginosa, Salmonellae typhimurium*, *Staphylococcus aureus* and methicillin-resistant strain MRSA SK1). Antibiotic paper disc and drug (vancomycin, gentamicin and streptomycin) were used for control maker of antibacterial activity.

3.2 Plant material and microorganism culture materials

The root of *Mucuna macrocarpa* was collected in the first time on March, 2002 and the second time on December, 2006 from Doitung, Chiang Rai province, in the Northern part of Thailand.

Three microorganism cultures (*Bacillus cereus* TISTR 678, *Pseudomonas fluorescens* TISTR 358*, Salmonellae typhimurium* TISTR 292) were collected from TISTR in Mae Fah Luang University. And four microorganisms (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853*, Staphylococcus aureus* ATCC 25932 and methicillin-resistant strain MRSA SK1) were suported by Department of Microbiology, Faculty of Science, Prince of Songkla University.

3.3 Extraction and Isolation

3.3.1 Extraction of *M. macrocarpa* (March, 2002)

The root of *M. macrocarpa* (1.20 kg) was chopped and immersed in dichloromethane (11 days), acetone (11 days) and methanol (20 days), respectively, after evaporation, to give the dichloromethane extract (**crude A**, 10.62 g), acetone extract (**crude B**, 10.87 g) and methanolic extract (**crude C**, 105.77 g). The process of extraction was shown in Figure 3.1.

3.3.2 Extraction of *M. macrocarpa* (December, 2006)

The root of *M. macrocarpa* (2.00 kg) was chopped and immersed in dichloromethane (18 days), acetone (19 days) and methanol (20 days), respectively, to give, after evaporation, the

dichloromethane extract (**crude D**, 10.37 g), acetone extract (**crude E**, 5.28 g) and methanolic extract (**crude F**, 83.29 g). The process of extraction was shown in Figure 3.2.

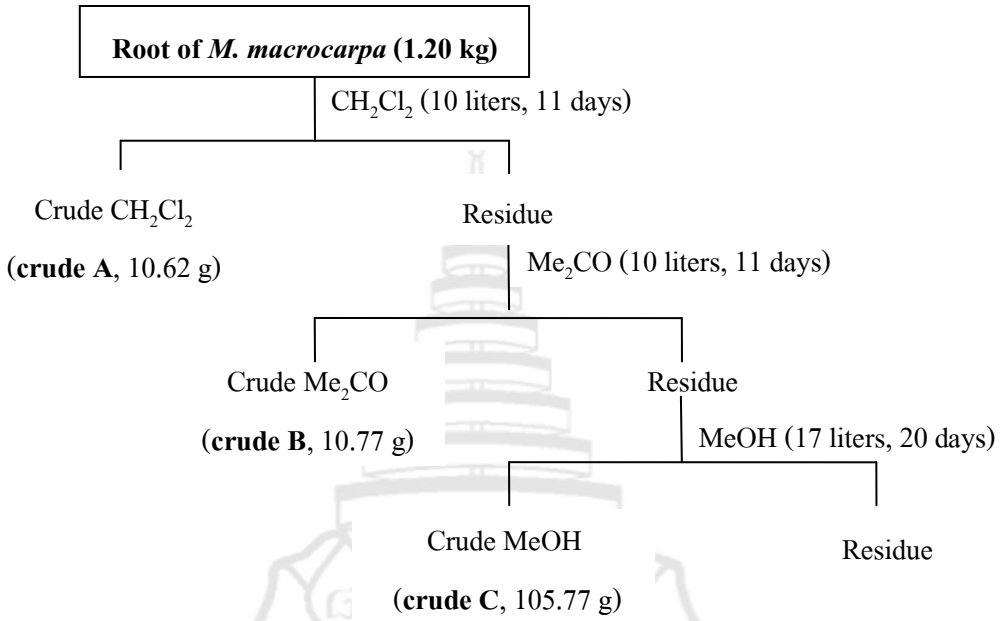


Figure 3.1 Extraction of **crude A**, **B** and **C** from the root of *M. macrocarpa*

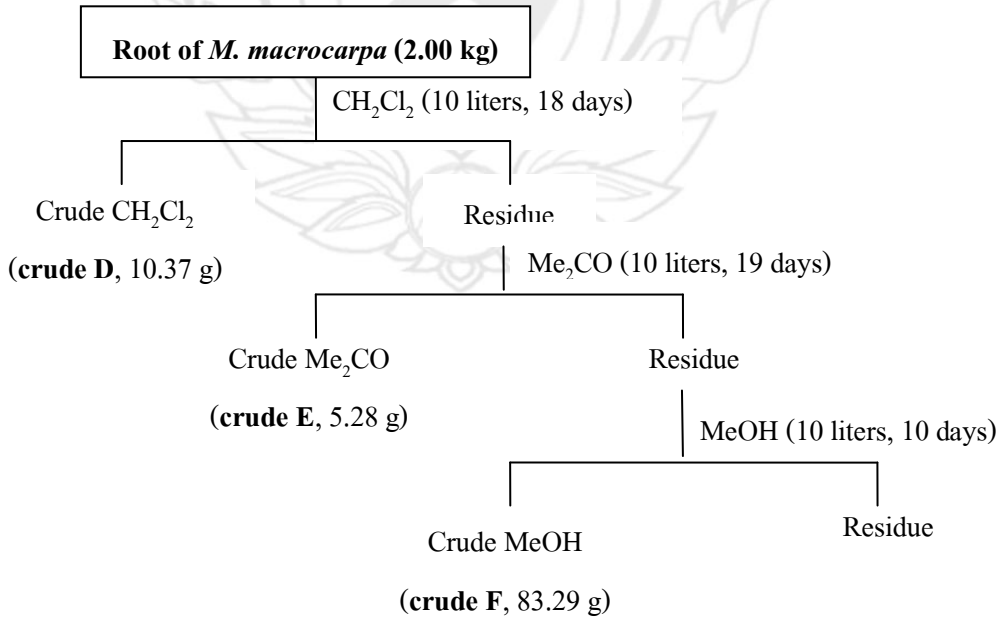


Figure 3.2 Extraction of **crude D**, **E** and **F** from the root of *M. macrocarpa*

3.4 Purification

3.4.1 Purification of crude A

Crude A (2.58 g) was subjected to CC using silica gel as a stationary phase and gradiently eluted with CH_2Cl_2, CH_2Cl_2-Me_2CO, Me_2CO, Me_2CO-MeOH and MeOH. On the basis of TLC characteristic, the similar TLC chromatograms were combined to afford 25 fractions (A1-A25) as shown in Table 3.1. The selected fractions were further purified by CC and crytallization. Three pure compounds were obtained. (Figure 3.3)

Table 3.1 Physical characteristic and weight of fractions obtained from **crude A**

Fraction	Weight (g)	Physical characteristic
A1	0.0959	Yellow viscous liquid
A2	0.0815	Yellow viscous liquid
A3	0.0593	Yellow viscous liquid mixed with white solid
A4	0.0992	Purple viscous liquid
A5	0.1730	Deep orange viscous liquid
A6	0.0982	Brown viscous liquid
A7	0.0564	Blue viscous liquid
A8	0.0535	Deep blue viscous liquid
A9	0.0321	Red–orange viscous liquid
A10	0.0970	Purple viscous liquid mixed with white solid
A11	0.1540	White and colorless solid
A12	0.0474	Pink viscous liquid mixed with white solid
A13	0.0875	Pink viscous liquid mixed with white solid
A14	0.4472	Pale green viscous liquid mixed with white solid
A15	0.0134	Green viscous liquid mixed with white solid
A16	0.0897	Green viscous liquid
A17	0.0065	Brown viscous liquid

Table 3.1 (continued)

Fraction	Weight (g)	Physical characteristic
A18	0.0831	Yellow viscous liquid
A19	0.0620	Yellow viscous liquid mixed white solid
A20	0.0946	White solid
A21	0.0342	Yellow viscous liquid
A22	0.0084	Yellow viscous liquid
A23	0.0342	Yellow viscous liquid mixed with white solid
A24	0.0453	Yellow viscous liquid mixed with white solid
A25	0.0674	Yellow viscous liquid

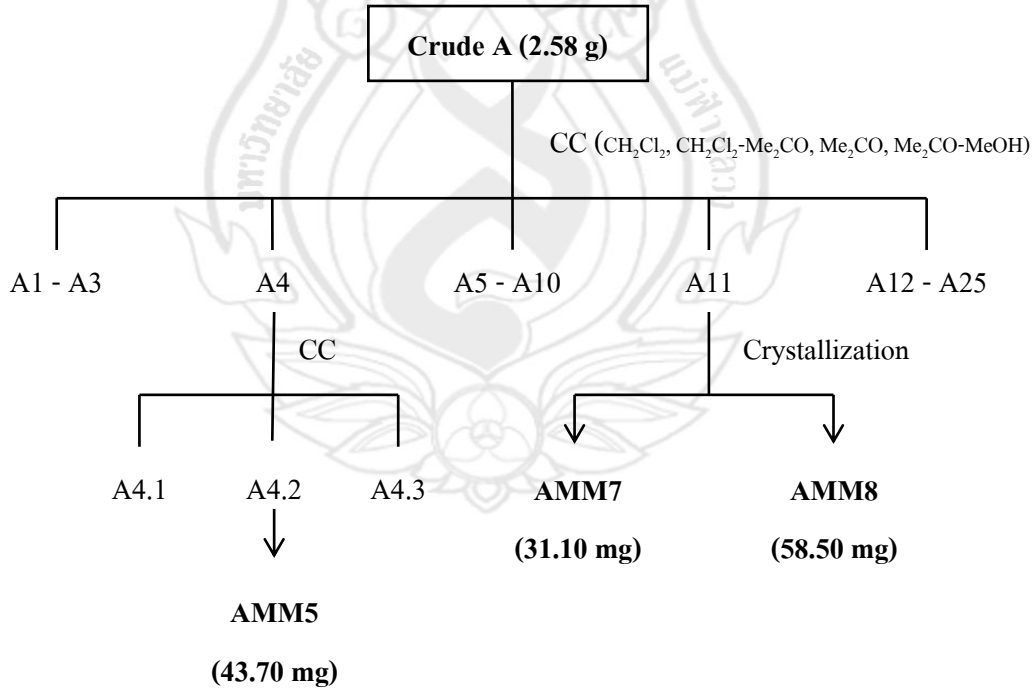


Figure 3.3 Isolation of compounds **AMM5**, **AMM7** and **AMM8**

Isolation of AMM5

Fraction A4 was further purified by CC using silica gel and eluted with 50% hexane-CH_2Cl_2 to give 3 fractions. Fractions A4.2 and A4.3 were obtained **AMM5** as purple viscous liquid (40.80 and 43.70 mg, respectively).

Melting point : 169-171 °C

IR (neat) ν (cm^{-1}) : 1705 (C=O stretching), 2942 (C-H stretching)

^{1}H NMR (300 MHz) ($CDCl_3$) (δ ppm) : 4.70 (1H, *d*, *J*=2.4 Hz, H_a-29), 4.58 (1H, *m*, H_b-29), 2.40 (*m,* H-2), 2.35 (*m*, H-19), 1.90 (*m,* H-21, H-22), 1.72 (*m*, H-30), 1.71 (*m*, H-12, H-15), 1.66 (*m*, H-13), 1.48 (*m,* H-16), 1.43 (*m,* H-6, H-7, H-13), 1.42 (*m*, H-1), 1.36 (*m*. H-9), 1.36 (*m*, H-18), 1.35 (*m*, H-16), 1.32 (*m*, H-5), 1.21 (*m,* H-1), 1.09 (*m*, H-2), 1.08 (3H, *s*, H-23), 1.07 (3H, *s*, H-26), 1.00 (3H, *s*, H-24), 0.95 (3H, *s*, H-27), 0.94 (3H, *s*, H-25), 0.79 (3H, *s*, H-28)

^{13}C NMR (75 MHz) ($CDCl_3$) (δppm) : 218.3, 151.0, 109.0, 54.9, 49.8, 48.2, 48.0, 47.4, 43.0, 42.9, 40.8, 40.0, 39.6, 38.2, 36.9, 35.5, 33.6, 34.2, 29.9, 27.4, 26.7, 25.2, 21.5, 21.1, 19.7, 19.4, 18.0, 16.0, 15.8, 14.5

Isolation of AMM7 and AMM8

Fraction A11 was dissolved in CH_2Cl_2 the white solid formed was collected and further crystallized from 50% hexane-CH_2Cl_2 to give a white solid of **AMM7** (31.10 mg). The filtrate was further recrystallized from CH_2Cl_2-Me_2CO (8:2). A white solid **AMM8** was collected (58.50 mg).

AMM7

Melting point : 156-157 °C

IR (neat) ν (cm^{-1}) : 3426 (O-H stretching), 2936, 2867 (C-H stretching)

^{1}H NMR (400 MHz) ($CDCl_3$) (δ ppm) : 5.28 (*m*, H-6), 5.08 (H-22), 4.94 (H-23), 3.46 (*m*, H-3), 2.19 (*m*, H-4), 2.17 (*m*, H-20), 1.94 (H-7),1.83 (*m*, H-2) 1.81(*m*, H-1), 1.59 (H-24), 1.58 (*m*, H-11), 1.50 (*m*, H-2, H-7, H-15), 1.46 (H-23), 1.44 (*m*, H-8), 1.43 (*m*, H-28), 1.19 (*m*, H-12, H-28), 1.13 (*m*, H-16), 1.02 (*m*, H-1, H-14, H-15, H-17, H-19, H-21), 0.89 (*m*, H-9), 0.74 (H-26, H-27, H-29), 0.62 (H-18)

^{13}C NMR (100 MHz) ($CDCl_3$) (δppm) : 140.8, 138.3, 129.3, 121.4, 71.8, 56.9, 56.1, 51.3, 51.1, 42.3, 40.5, 39.8, 39.7, 37.3, 36.5, 31.9, 31.9, 31.9, 28.9, 28.3, 25.4, 25.4, 24.4, 21.2, 21.1, 19.4, 19.0, 12.2, 12.1

AMM8

Melting point : 139-142 °C

IR (neat) ν (cm^{-1}) : 3426 (O-H stretching), 2936, 2851 (C-H stretching)

^{1}H NMR (300 MHz) ($CDCl_3$) (δppm) : 5.36 (1H, *m*, H-6), 3.53 (1H, *m*, H-3), 2.31 (1H, *m*, H-4), 2.24 (1H, *m*, H-4), 2.03 (*m*, H-12), 1.98 (2H, *m*, H-7), 1.96 (*m*, H-2), 1.86 (*m*, H-16), 1.84 (*m*, H-1), 1.83 (*m*, H-16), 1.66 (*m*, H-25), 1.59 (*m*, H-2), 1.57 (*m*, H-11), 1.50 (*m*, H-8), 1.29 (*m*, H-22), 1.28 (*m*, H-12, H-20), 1.25 (*br s*, H-28), 1.16 (2H, *m*, H-23), 1.15 (*m*, H-15, H-17), 1.08 (*m*, H-1, H-15), 1.02 (*m*, H-11, H-14), 1.01 (3H, *s*, H-19), 1.00 (*s*, H-22), 0.93 (*m*, H-9, H-24), 0.92 (3H, *d*, *J*=6.5, H-21), 0.85 (3H, *t*, *J*=8.0, H-29) 0.84 (3H, *d*, *J*=6.5, H-26), 0.81 (3H, *d*, *J*=6.5, H-27), 0.63 (3H, *s*, H-18)

3.4.2 Purification of crude C

The **crude C** (3.74 g) was subjected to QCC using silica gel as stationary phase and eluted with CH_2Cl_2, CH_2Cl_2-Me_2CO, Me_2CO, Me_2CO-MeOH and MeOH. Fractions with the similar TLC chromatograms were combined to afford 12 fractions (C1-C12) (Table 3.2). The selected fractions were further purified to give 5 compounds as showed in Figure 3.4.

Table 3.2 Physical characteristic and weight of fractions obtained from **crude C**

Fraction	Weight (g)	Physical characteristic
C1	0.0543	Yellow viscous liquid
C2	0.0086	Yellow viscous liquid mixed with white solid
C3	0.1593	Yellow viscous liquid mixed with white solid
C4	0.0714	Yellow viscous liquid mixed with white solid
C5	2.8526	Brown viscous liquid mixed with white solid
C6	0.8080	Deep yellow viscous liquid mixed with white solid
C7	0.1959	Deep yellow viscous liquid mixed with white solid
C8	2.6579	Deep yellow viscous liquid
C9	0.1298	Brown viscous liquid
C10	0.4321	Brown viscous liquid
C11	0.1671	Brown viscous liquid
C12	10.3414	Brown viscous liquid

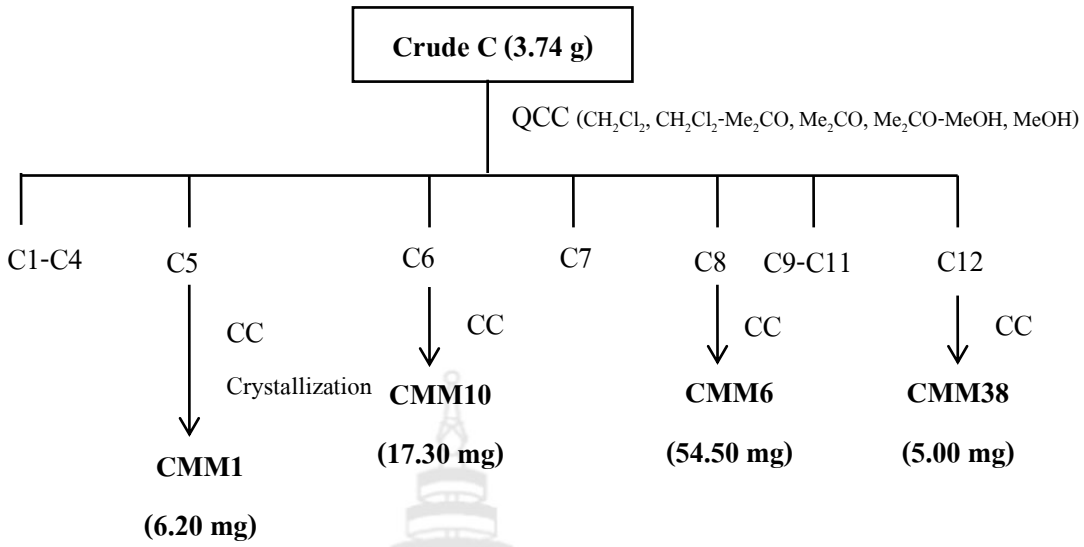


Figure 3.4 Isolation of compounds **CMM1**, **CMM10**, **CMM6** and **CMM3**

Isolation of CMM1

Fraction C5 was purified by CC using silica gel and eluted with gradiently hexane-CH_2Cl_2 to give 9 fractions (C5.1-C5.9). Fraction C5.8 (12.60 mg) which contained one major component was recrystallized in the mixture of hexane-CH_2Cl_2 (8:2) to give **CMM1** (6.20 mg) as a white solid.

Melting point : 235-243 °C

IR (neat) ν (cm^{-1}) : 2928, 2870 (C-H stretching)

^{1}H NMR (300 MHz) ($CDCl_3$) (δ ppm) : 2.40 (*m*, H-2, H-3, H-19), 2.30 (*m*, H-2), 1.80 (*m*, H-21, H-22), 1.42 (*m*, H-16), 1.40 (*m*, H-3, H-11, H-20), 1.62 (*m*, H-12, H-13, H-15), 1.62 (*s*, H-30),1.35 (*m*, H-1), 1.20 (*m*, H-1, H-5, H-6, H-7, H-9, H-11, H-16, H-18), 1.02 (*s*, H-26), 1.00 (*s*, H-23, H-29), 0.95 (*s*, H-24), 0.90 (*s*, H-25), 0.89 (*s*, H-27), 0.71 (*s*, H-28)

Isolation of CMM10

Fraction C6 was purified on CC and eluted with CH_2Cl_2 and CH_2Cl_2-Me_2CO to give 17 fractions (C6.1-C6.17). **CMM10** was obtained from the fraction C6.13 as a white solid (17.30 mg).

Melting point : 282-288 °C

IR (neat) ν (cm^{-1}) : 3470 (O-H stretching), 2943, 2870 (C-H stretching)

^{1}H NMR (400 MHz) ($CDCl_3$) (δppm) : 4.77 (1H, *br qd*, *J*=2.5, 1.5, H-29), 4.73 (1H, *br d, J*=2.5, H-29), 3.18 (1H, *dd, J*=11.0, 5.0, H-3), 3.04 (1H, *dt*, *J*=11.0, 5.0, H-19), 2.28 (1H, *m*, H-13), 2.25 (1H, *m*, H-16), 1.98 (1H, *m*, H-22), 1.94 (1H, *m*, H-21), 1.71 (1H, *m*, H-12), 1.69 (3H, *s*, H-30), 1.65 (1H, *m*, H-1), 1.59 (2H, *m*, H-2), 1.57 (1H, *m*, H-18), 1.55 (1H, *m*, H-6), 1.52 (1H, *m*, H-15), 1.50 (1H, *m*, H-16), 1.41 (1H, *m*, H-21), 1.40 (1H, *m*, H-11), 1.38 (1H, *m*, H-22), 1.37 (1H, *m*, H-6), 1.37 (2H, *m*, H-7), 1.27 (1H, *m*, H-9), 1.23 (1H, *m*, H-11), 1.16 (1H, *m*, H-15), 1.15 (1H, *m*, H-12), 0.97 (3H, *s*, H-27), 0.96 (3H, *s*, H-23), 0.94 (3H, *s*, H-26), 0.88 (1H, *m*, H-1), 0.81 (3H, *s*, H-25), 0.75 (3H, *s*, H-24), 0.68 (1H, *d*, *J*=10.0, H-5)

Isolation of CMM6

Fraction C8 was rechromatographed on CC and eluted with hexane-CH_2Cl_2, CH_2Cl_2, CH_2Cl_2-Me_2CO, and Me_2CO to give 28 fractions (C8.1-C8.28). Fraction C8.12 was purified by CC and eluted with hexane, hexane-CH_2Cl_2, CH_2Cl_2 to give 11 fractions (C8.12.1-C8.12.11). Fraction C8.12.5 contained one major component were further purified by CC using 50% hexane-CH_2Cl_2 as an eluent to afford pure **CMM6** as an orange viscous liquid (54.50 mg).

^{1}H NMR (400 MHz) ($CDCl_3$) (δppm) : 12.43 (1H, *s*, 1-OH), 12.15 (1H, *s*, 8-OH), 7.62 (1H, *s*, H-5), 7.40 (1H, *s*, H-4), 7.07 (1H, *s*, H-7), 5.20 (1H, *t*, H-2'), 4.02 (3H, *s*, 3-OCH_3), 3.43 (2H, *d*, H-1'), 2.45 (3H, *s*, 6-CH_3) 1.80 (3H, *s*, H-5'), 1.69 (3H, *s*, H-4')

^{13}C NMR (100 MHz) ($CDCl_3$) (δppm) : 191.26, 182.20, 163.55, 162.43, 161.71, 148.29, 133.16, 133.01, 132.79, 124.42, 124.19, 121.13, 120.57, 113.70, 110.69, 103.34, 56.27, 25.83, 22.15, 22.15, 17.88

DEPT 135° ($CDCl_3$) (δ ppm) : CH_3 : 56.27, 25.83, 22.15, 17.88; CH_2 : 22.15; CH : 124.42, 121.13, 120.57, 103.34

DEPT 90° ($CDCl_3$) (δppm) : CH : 124.42, 121.13, 120.57, 103.34

Isolation of CMM38

Fraction C12 was purified by QCC using silica gel and eluted with gradiently CH_2Cl_2-Me_2CO, Me_2CO, Me_2CO-MeOH and MeOH to afford 17 fractions (C12.1-C12.17). Fraction C12.4 (11.00 mg) was further purified by PLC using 60% CH_2Cl_2–hexane (2 elutions) as an elutent to give **CMM38** (5.00 mg) as a yellow solid.

^{1}H NMR (400 MHz) ($CDCl_3$) (δppm) : 10.05 (1H, *s*, 2-CHO), 8.64 (1H, *br s*, N-H), 8.20 (1H, *d*, *J*=0.8, H-4), 8.12 (1H, *d*, *J*=8.0, H-5), 7.51 (1H, *t*, *J*=8.0, H-7), 7.50 (1H, *dd*, *J*=1.2, 5.6 , H-8), 7.46 (1H, *d*, *J*=0.8, H-2), 7.32 (1H, *t*, *J*=6.8, H-6), 4.07 (3H, *s*, 1-OCH_3)

^{13}C NMR (100 MHz) ($CDCl_3$) (δppm) : 191.90, 146.10, 139.43, 134.09, 130.19, 126.65, 123.68, 123.64, 120.72, 120.71, 120.42, 111.51, 103.54, 55.82

DEPT 135° ($CDCl_3$) (δppm) : CH_3 : 55.82; CH : 191.90, 126.65, 120.72, 120.71, 120.42, 111.51, 103.54

3.4.3 Purification of crude E

Crude E (2.53 g) was fractionated by CC eluted with CH_2Cl_2, CH_2Cl_2-Me_2CO, Me_2CO and Me_2CO-MeOH in polarity gradient manner. The eluents containing similar components were combined into 14 fractions (D1-D14) as shown in Table 3.3. The fractions D9 and D10 were further purified by crytallization. **DMM1** were obtained. (Figure 3.5)

Table 3.3 Physical characteristic and weight of fractions obtained from **crude E**

Fraction	Weight (g)	Physical characteristic
D1	0.2201	Yellow viscous liquid
D2	0.1165	Yellow viscous liquid
D3	0.0054	Deep brown viscous liquid
D4	0.1689	Deep green viscous liquid mixed with white solid
D5	0.0074	Green viscous liquid mixed with white solid
D6	0.0582	Green viscous liquid mixed with white solid
D7	0.0102	Green viscous liquid mixed with white solid
D8	0.0061	Yellow viscous liquid mixed with white solid
D9	0.1903	Yellow viscous liquid mixed with white solid
D10	0.1384	Yellow viscous liquid mixed with white solid
D11	0.0713	Yellow viscous liquid mixed with white solid
D12	0.1432	Yellow viscous liquid mixed with white solid
D13	0.0934	Yellow viscous liquid mixed with white solid
D14	0.0539	Yellow viscous liquid mixed with white solid

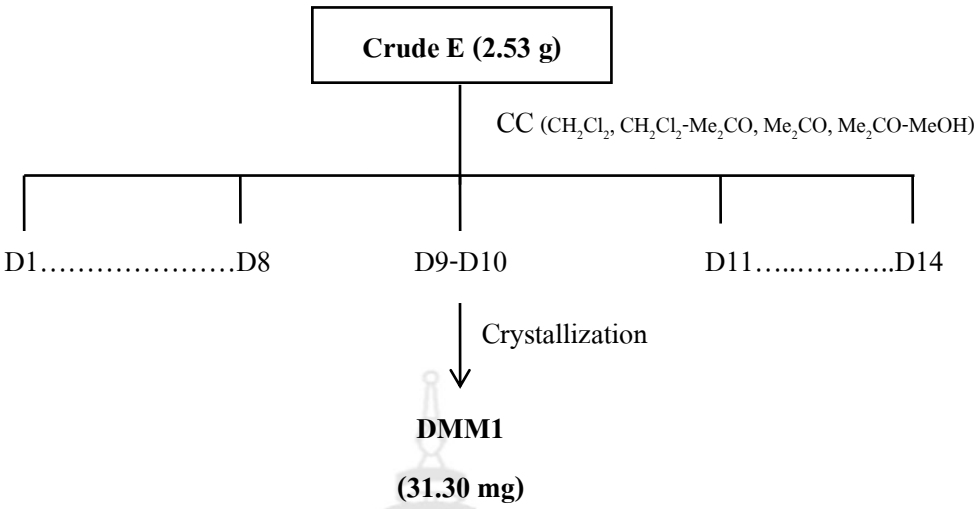


Figure 3.5 Isolation of compound **DMM1**

Isolation of DMM1

Fractions D9 and D10 were recrystallized in the mixture of hexane-CH_2Cl_2 (9:1) to give a white solid of **DMM1** (31.30 mg).

Melting point : 213-215 °C

IR (neat) ν (cm^{-1}) : 3313 (O-H stretching)

^{1}H NMR (300 MHz) ($CDCl_3$) (δ ppm) : 4.68 (1H, *d*, *J*=2.4, H-29), 4.56 (1H, *m*, H-29), 3.39 (1H, *dd, J*=5.7, 1.5, H-3), 2.39 (*ddd*, *J*=5.7, 5.7, 5.4, H-19), 1.95 (1H, *m*, H-21), 1.70 (*m*, H-2, H-12), 1.68 (3H, *s*, H-30), 1.65 (*m*, H-13), 1.52 (*m*, H-15), 1.50 (2H, *m*, H-1), 1.45 (*m*, H-6, H-11), 1.40 (*m*, H-5, H-16, H-22), 1.35 (*m*, H-18), 1.20 (*m*, H-7, H-9, H-22), 1.03 (3H, *s*, H-26), 0.96 (3H, *s*, H-23), 0.93 (3H, *s*, H-27), 0.84 (3H, *s*, H-24), 0.82 (3H, *s*, H-25), 0.78 (3H, *s*, H-28)

3.4.4 Purification of crude F

Crude F (30.99 g) was fractionated by QCC eluted with CH_2Cl_2-Me_2CO, Me_2CO and Me_2CO-MeOH. Fractions with the similar TLC chromatograms were combined to afford 10 fractions (F1-F10) (Table 3.4). The selected fractions were further purified to give compound **FMM2** as showed in Figure 3.6.

Table 3.4 Physical characteristic and weight of fractions obtained from **crude F**

Fraction	Weight (g)	Physical characteristic
F1	0.1213	Brown viscous liquid
F2	0.2128	Brown viscous liquid mixed with white solid
F3	0.2122	Brown viscous liquid
F4	0.3549	Deep brown viscous liquid
F5	0.2775	Deep brown viscous liquid
F6	0.4234	Deep brown viscous liquid
F7	1.7847	Deep brown viscous liquid
F8	7.3251	Deep brown viscous liquid
F9	6.1240	Deep brown viscous liquid
F10	2.7351	Deep brown viscous liquid

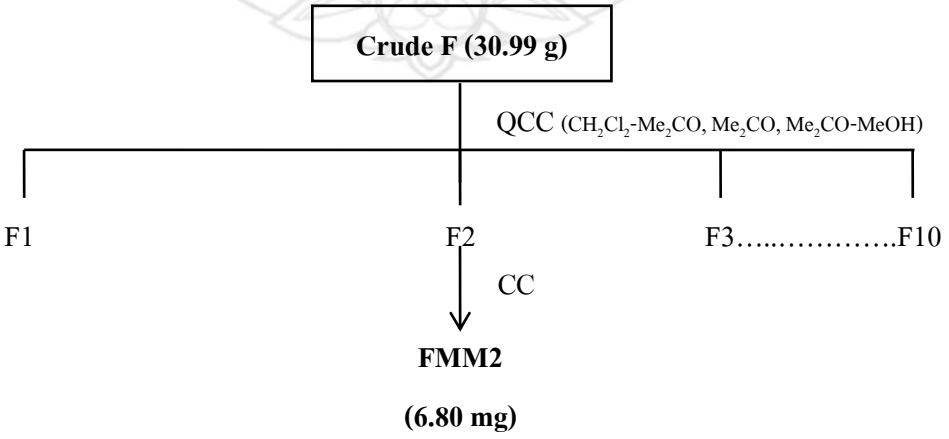


Figure 3.6 Isolation of compound **FMM2**

Isolation of FMM2

Fraction F2 was purified by CC using silica gel and eluted with gradiently CH_2Cl_2-Me_2CO, Me_2CO, Me_2CO-MeOH and MeOH to afford 15 fractions (F2.1-F2.15). Fraction F2.12 (22.3 mg) was further purified by CC using 5% Me_2CO - CH_2Cl_2 to afford 2 fractions. Fraction F2.12.1 (12.4 mg) was further purified by CC using CH_2Cl_2 to give **FMM2** (6.8 mg) as a yellow viscous liquid.

^{1}H NMR (400 MHz) ($CDCl_3$) (δppm) : 9.60 (1H, *s*, H-1), 7.25 (2H, *d*, *J* =3.2, H-2, H-6), 6.53 (2H, *d, J*=3.2, H-3, H-5), 4.73 (3H, *s*, 4-OCH_3)

3.5 Antibacterial activity assays

The paper disc diffusion method (Lorian, 1996) was used to screen crude for the antibacterial activity. Broth microdilution method (CLSI, 2002) was used to screen pure compounds and determined minimum inhibition concentrations (MICs).

3.5.1 Paper disc diffusion method

The paper disc diffusion method (Lorian, 1996) was used to screen the antibacterial of the crude extracts. Three-five colonies of bacterial culture are transferred to nutrient broth and incubated for 3 h. at 35 °C, 150 rpm shaking incubator. The turbidity of bacterial suspension was adjusted with 0.85% NaCl (normal saline solution, NSS) compared to 0.5 McFarland standard. The cell culture is determined using total plate count. Spread the cuture into agar plate with sterile cotton swab. Sterile filter paper was placed on the agar plates, containing of the microorganism, then droped 10 µL of crude extracts on the filter paper. Plates kept in the incubator at 35 °C for 18 h. This was performed in triplicate for each crude extracts. The diameter of clear zone shown on plates was then measured and expressed in millimeters as its antimicrobial activity. Growth controls were performed on agar containing DMSO. Antibiotic paper disc is used for control marker of antibacterial activity.

3.5.2 Broth microdilution method

3.5.2.1 Screening of pure compounds

Test samples were dissolved in dimethyl sulfoxide (DMSO) and mixed with melted Mueller Hinton Broth (MHB) in microtiter plates. Add 50 µL of inoculum suspensions in each well. Final concentration of the test samples were 200 µg/mL. The inoculated plate were incubated at 35 ºC for 16-18 h. Drop 0.18% resazurin 10 µL in microtiter plate and incubated in 35 ºC for 2-3 h. The blue color showed sample can inhibit bacterial growth and pink color shown sample can not inhibit bacterial growth. This was performed in triplicate for each sample.

3.5.2.2 Determination of minimum inhibition concentration (MICs)

Minimum inhibition concentrations (MICs) were determined by the Broth microdilution method (CLSI, 2002) for bacterial. Test samples were dissolved in dimethyl sulfoxide (DMSO). Serial 2-fold dilutions of the test samples were mixed with melted Mueller

Hinton Broth (MHB) in microtiter plates. Final concentration of the test crude sample in broth ranged from 1280–2.5 µg/mL and test pure compound in broth ranged from 128–0.25 µg/mL . Add 50 µL of inoculum suspensions in each well (final concentration $1x10^4$ CFU/well). The inoculated plate were incubated at 35 ºC for 16-18 h. Drop 0.18% resazurin 10 µL in microtiter plate and incubated in 35 ºC for 2-3 h. The blue color showed sample can inhibit bacterial growth and pink color shown sample can not inhibit bacterial growth. MICs were recorded by reading the lowest concentration that inhibited visible growth. The test was performed in triplicates. Vancomycin and gentamicin were used as a positive control drug.

3.6 DPPH radical scavenging assays

The potential antioxidant activities of the crude extract and pure compounds isolated from the roots of *Mucuna macrocarpa* were assessed on the basis of scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The DPPH assay is one of the methods used for evaluation of antioxidative activity. The following assay procedure was modified from those described in previous report (Subhadhirasakul and Khumfang, 2000). The test solution in absolute ethanol (50 µL) was mixed with 0.05 mM DPPH solution in ethanol (3 mL). The absorbance (A) was then measured at 517 nm on spectrophotometer. BHT and ascorbic acid were used as a positive control. The measurements were performed at least in triplicate. The result expressed as percentage inhibition. The concentration of the sample at 50% inhibition (IC_{50}) was obtained by linear regression analysis of dose-response curve, which was plotted between % inhibition and concentration (Subhadhirasakul and Khumfang, 2000).

$$\% \text{ inhibition} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

3.6.1 Screening on the free radical scavenging activity of crude extracts

The crude material was dissolved in absolute ethanol to prepare the solution with concentration of 6.1 mg/mL. The solution of each sample (50 µL) was mixed with 0.05 mM DPPH ethanolic solution (3 mL) in a cuvette to give the solution with the final concentration of

100 μg/mL. The trapping effect was assessed by measuring the absorbance change of the solution at 517 nm against 0.05 mM DPPH ethanolic solution after 15, 30, 45 and 60 min. Ascorbic acid and BHT were used as a positive control. The measurements were performed at least in triplicate. The degree of loss of color implied the activity.

3.6.2 Evaluation of 50% inhibition concentration (IC_{50}) of the crude extracts

Crude **B**, **C**, **E** and **F** showed the strong activity, they were then selected for further study. The solution of DPPH (0.05 mM, 3 mL) was mixed with the sample at concentration of 4.0, 3.0, 2.0, 1.0, 0.5, 0.25, and 0.125 mg/mL. The absorbances were measured at 517 nm for 30 minute. The concentration that needed to decrease % inhibition of DPPH solution to 50% inhibition (IC_{50}) was obtained by linear regression analysis of dose-response curve.

3.6.3 Screening on the free radical scavenging activity of pure compounds

The pure compound was dissolved in absolute ethanol to prepare the solution with concentration of 50 μM. The solution of each sample (50 μL) was mixed with 0.05 mM DPPH ethanolic solution (3 mL) in a cuvette to give the solution with the final concentration of 10 μM.

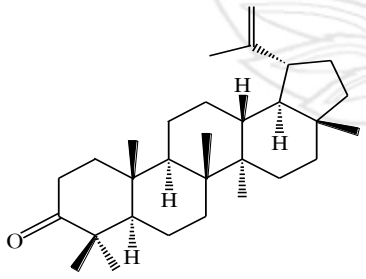
CHAPTER 4

RESULTS AND DISCUSSION

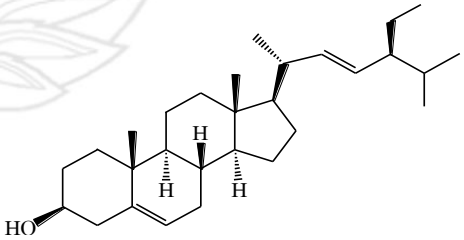
4.1 Structural elucidation of compounds isolate from the root of *M. macrocarpa*

The roots of *M. macrocarpa* were collected from Chiang Rai province on March, 2002 and December, 2006. The root of *M. macrocarpa* were extracted with dichloromethane, acetone and methanol, successively. Isolation and purification of dichloromethane and methanolic extracts (2002) gave three compounds (**AMM5**, **AMM7** and **AMM8**) and four compounds (**CMM1**, **CMM10**, **CMM6** and **CMM38**), respectively. Compound **DMM1** was obtained from the acetone extract and compound **FMM2** was obtained from the methanolic extract from the roots of *M. macrocarpa* which was collected in 2006.

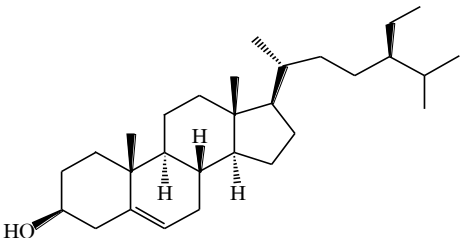
Their structures were determined using spectroscopic data evidence, especially 1D and 2D NMR spectral data.



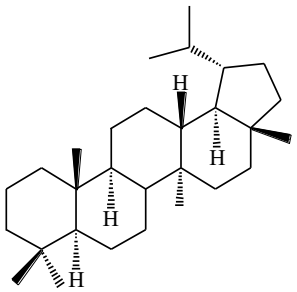
Lupenone (**AMM5**)



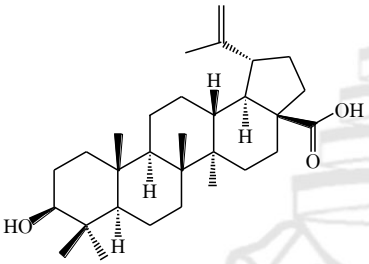
Stigmasterol (**AMM7**)



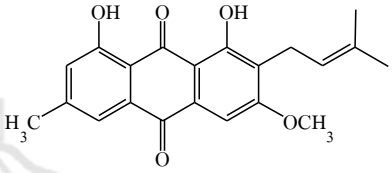
β-sitosterol (**AMM8**)



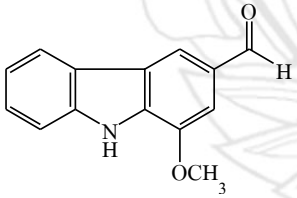
Lupane (**CMM1**)



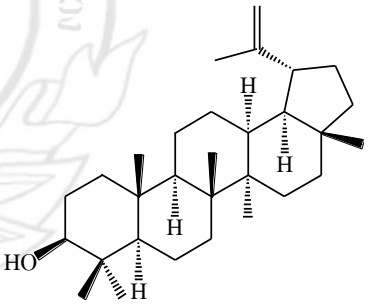
Betulinic acid **(CMM10)**



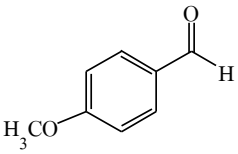
Vismiaquinone C **(CMM6)**



Murrayanine (**CMM38**)

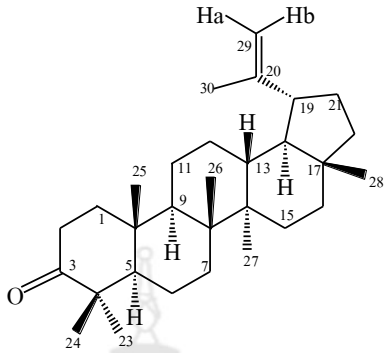


Lupeol **(DMM1)**



4-Methoxybenzaldehyde **(FMM2)**

AMM5: 3-oxo-Lupeol (Lupenone)



AMM5 was isolated as a white solid, m.p. 169-171 °C. The IR spectrum showed the absorption band of C=O stretching at 1705 cm^{-1}. The ^{1}H NMR spectrum exhibited the resonances of an isoprenyl side chain at δ 1.72 (H-30), 4.70 (*d*, *J* = 2.4 Hz, H_a-29) and 4.58 (*m*, H_b-29). Four *multiplet* signals at δ 1.42, 1.21, 1.09 and 2.40 were in agree with the α,β-unsaturated ketone. The six methyl groups H-23, H-24, H-25, H-26, H-27 and H-28 resonated at δ 1.08, 1.00, 0.94, 1.07, 0.95 and 0.79, respectively. The ^{13}C NMR spectra displayed 30 peaks and confirmed the above data through the resonances displayed at δ 19.4, 109.0, 151.0, as well as at δ 33.6, 39.6 and 218.3 for the isopropenyl and α,β-unsaturated carbonyl groups, respectively. The absence of the methine proton signal at C-3 indicated that the carbonyl groups located at C-3. The resulting structure was confirmed by ^{13}C NMR spectral data (Table 4.1). 3-*oxo*-Lupeol was assigned for **AMM5**. It was known as lupenone (Sompong Boonsri, 2004).

Lupenone has been reported to possess antimicrobial, antiviral, anticancer and anti-inflammatory activities (Supaluk Prachayasittikul et al., 2009).

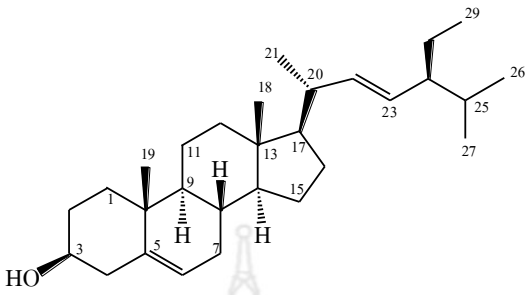
Table 4.1 NMR spectral data of **AMM5**

Position	AMM5		Lupenone	
	δ_H (*multiplicity*, J_{Hz})	δ_C	δ_H (*multiplicity*, J_{Hz})	δ_C
1	1.21 (*m*), 1.42 (*m*)	39.6	1.20 (*m*), 1.40 (*m*)	39.6
2	1.09 (*m*), 2.40 (*m*)	34.2	1.89 (*m*), 2.43 (*m*)	34.1
3	-	218.3	-	218.2
4	-	47.4	-	47.3
5	1.32 (*m*)	54.9	1.30 (*m*)	54.9
6	1.43 (*m*)	19.7	1.45 (*m*)	19.7
7	1.43 (*m*)	33.6	1.45 (*m*)	33.6
8	-	40.8	-	40.8
9	1.36 (*m*)	49.8	1.38 (*m*)	49.8
10	-	36.9	-	36.9
11	1.43 (*m*)	21.5	1.28 (*m*), 1.45 (*m*)	21.5
12	1.71 (*m*)	25.2	1.70 (*m*)	25.2
13	1.66 (*m*)	38.2	1.68 (*m*)	38.2
14	-	43.0	-	42.9
15	1.71 (*m*)	27.4	1.70 (*m*)	47.4
16	1.35 (*m*), 1.48 (*m*)	35.5	1.36 (*m*), 1.48 (*m*)	35.5
17	-	42.9	-	43.0
18	1.36 (*m*)	48.2	1.38 (*m*)	48.2
19	2.35 (*m*)	48.0	2.43 (*m*)	48.0
20	-	151.0	-	150.8
21	1.90 (*m*)	29.9	1.90 (*m*)	29.8
22	1.90 (*m*)	40.0	1.90 (*m*)	40.0
23	1.08 (3H, *s*)	26.7	1.07 (*s*)	26.6
24	1.00 (3H, *s*)	21.1	1.02 (*s*)	21.0
25	0.94 (3H, *s*)	16.0	0.93 (*s*)	16.0
26	1.07 (3H, *s*)	15.8	1.07 (*s*)	15.8

Table 4.1 (continued)

Position	AMM5		Lupenone	
	δ_H (*multiplicity*, J_{Hz})	δ_C	δ_H (*multiplicity*, J_{Hz})	δ_C
27	0.95 (3H, *s*)	14.5	0.95 (*s*)	14.5
28	0.79 (3H, *s*)	18.0	0.79 (*s*)	18.0
29	H_a : 4.70 (1H, *d*, 2.4)	109.0	4.68 (*d*, 2.1)	109.4
	H_b : 4.58 (1H, *m*)		4.57 (*m*)	
30	1.72 (*m*)	19.4	1.70 (*s*)	19.3

AMM7: 5,22-Stigmastadien-3β-ol (Stigmasterol)



AMM7 were obtained as a white solid, m.p. 156-157 °C. In IR spectrum, the absorption band of O-H stretching (3426 cm^{-1}) and C-H stretching (2936 and 2867 cm^{-1}) were shown. The ^{1}H NMR spectrum (Table 4.2) contained an oxymethine proton signal at δ 3.46, three olefinic protons at δ 5.28, 5.08 and 4.94 and six methyl groups at δ 1.20, 1.20, 0.74, 0.74, 0.74 and 0.70. The ^{1}H NMR data, optical rotation value and melting point were corresponded to the previous reported data (Forgo & Kövér, 2003). Therefore, **AMM7** was assigned to be 5,22-stigmastadien-3β-ol or stigmasterol.

Stigmasterol does seem to be play a role in reducing inflammation, which may because it is a precursor to chemical compounds which can limit inflammatory processes. Sterols like stigmasterol have also been recommended for their cholesterol lowering abilities, although more study is needed to determine which compounds perform this function, and how they work in the body. Stigmasterol was active in reducing the oedema by a similar proportion. Stigmasterol produced a 41% inhibition at doses of 0.5 mg/ear, in the acute inflammation model. (García, Sáenz, Gómez & Fernández, 1999).

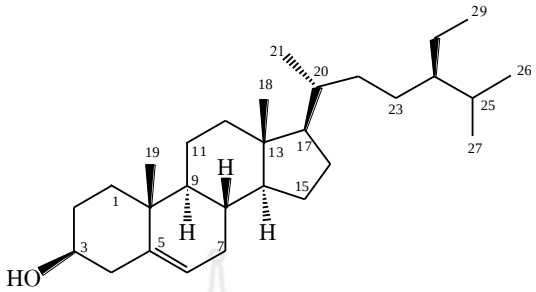
Table 4.2 NMR spectral data of **AMM7**

Position	AMM7		Stigmasterol	
	δ_H (*multiplicity*, J_{Hz})	δ_C	δ_H (*multiplicity*, J_{Hz})	δ_C
1	1.81 (1H, *m*), 1.02 (1H, *m*)	37.3	1.84 (1H, *m*, 14.5, 3.6), 1.08 (1H, *m*, 14.9, 4.7)	37.6
2	1.83 (1H, *m*), 1.50 (1H, *m*)	28.9	1.83 (1H, *m*, 15.4, 4.6), 1.51 (1H, *m*, 11.2)	31.9
3	3.46 (1H, *m*)	71.8	3.51 (1H, *m*, 4.8, 11.2)	70.2
4	2.19 (2H, *m*)	42.3	2.30 (1H, *m*, 13.2, 2.1), 2.23 (1H, *m,* 1.9, 2.6)	42.5
5	-	140.8	-	140.8
6	5.28 (1H, *d*, 4.8)	121.4	5.34 (1H, *m*, 5.3, 1.9)	121.8
7	1.94 (1H, *m*), 1.50 (1H, *m*)	31.9	1.97 (1H, *m*, 14.5, 2.1), 1.50 (1H, *m*, 14.3)	32.1
8	1.44 (1H, *m*)	31.9	1.46 (1H, *m*, 11.3, 11.2)	32.2
9	0.89 (1H, *m*)	50.1	0.94 (1H, *m*, 11.4, 4.9)	50.5
10	-	36.5	-	36.5
11	1.58 (1H, *m*)	24.4	1.50 (2H, *m*, 13.2, 3.4)	21.2
12	1.19 (2H, *m*)	39.8	2.00 (1H, *m*, 13.2, 3.2), 1.18 (1H, *m*, 3.1)	40.0
13	-	39.7	-	42.2
14	1.02 (1H, *m*)	56.9	1.01 (1H, *m*, 10.6, 8.3)	57.1
15	1.50 (1H, *m*), 1.02 (1H, *m*)	24.3	1.56 (1H, *m*, 11.1), 1.06 (1H, *m*)	24.5
16	1.13 (2H, *m*)	28.3	1.72 (1H, *m*, 10.8, 8.7), 1.28 (1H, *m*, 10.7)	28.9
17	1.02 (1H, *m*)	56.1	1.15 (1H, *q*, 9.9)	56.3
18	0.62 (3H, *s*)	12.2	0.70 (3H, *s*)	12.2
19	1.02 (3H, *s*)	21.1	1.01 (3H, *s*)	19.5

Table 4.2 (continued)

Position	AMM7		Stigmasterol	
	δ_H (*multiplicity*, J_{Hz})	δ_C	δ_H (*multiplicity*, J_{Hz})	δ_C
20	2.17 (1H, *m*)	40.5	2.06 (1H, *m*, 6.2, 8.6)	40.4
21	1.02 (3H, *br s*)	21.2	1.03 (3H, *d*)	21.4
22	5.08 (1H, *m*)	138.3	5.17 (1H, *dd*, 15.2)	138.3
23	4.94 (1H, *m*)	129.3	5.04 (1H, *dd*, 8.6)	129.7
24	1.46 (1H, *m*)	51.3	1.54 (1H, *m*)	51.5
25	1.59 (1H, *m*)	31.9	1.55(1H, *m*. 6.4)	32.2
26	0.74 (3H, *s*)	19.4	0.85 (3H, *d*)	21.2
27	0.74 (3H, *br s*)	19.0	0.80 (3H, *d*)	19.2
28	1.43 (1H, *m*), 1.19 (1H, *m*)	25.4	1.43 (1H, *m*, 13.4, 7.3, 3.6), 1.18 (1H, *m*, 7.3, 5.7)	25.4
29	0.74 (3H, *br s*)	12.1	0.81 (3H, *t*)	12.2

AMM8: Stigmast-5-en-3β-ol (β-Sitosterol)



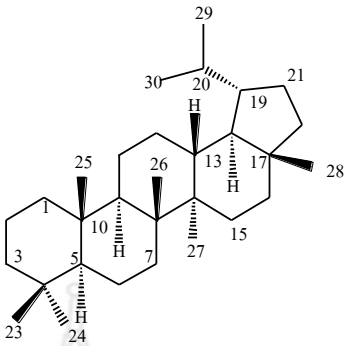
AMM8 was isolated as a white solid, m.p. 139-142 °C. The IR spectrum showed the absorption band of O-H stretching (3426 cm^{-1}) and C-H stretching (2936, 2851 cm^{-1}). The ^{1}H NMR spectral data (Table 4.3) revealed the presence of an olefinic proton at δ 5.36 (1H, *m*, H-6) and an oxymethine proton at δ 3.53 (1H, *m*, H-3). The signals of six methyl groups were shown at δ 0.63 (*s*, H-18), 0.81 (*d*, *J* = 6.5 Hz, H-27), 0.84 (*d*, *J* = 6.5 Hz, H-26), 0.85 (*t*, *J* = 8.0 Hz, H-29), 0.92 (*d*, *J* = 6.5 Hz, H-21) and 1.01 (*s*, H-19). Accordingly the structure of **AMM8** was proposed to be stigmast-5-en-3β-ol. It was known as β-sitosterol (Nguyen et al., 2004).

Sitosterol is a plant sterol and like its synthetic analogues hydrocortisone and corticosterone it has powerful skin properties including the reduction in skin erythema (skin redness), the reduction of pruritis (skin itching) and the reduction in inflammation (Axterer, Müller, Dweck, GmbH & Weckdata, 2010). The mixture of stigmasterol and β-sitosterol exhibited antimicrobial action against *Saccharomyces cerevisiae* ATCC 2610 (Supaluk Prachayasittikul et al., 2009).

Table 4.3 NMR spectral data of **AMM8**

Position	δ_H (*multiplicity*, J_{Hz})	
	AMM8	**β-Sitosterol**
1	1.08 (*m*), 1.84 (*m*)	1.06 (*m*), 1.85 (*m*)
2	1.59 (*m*), 1.96 (*m*)	1.61(*m*), 1.95 (*m*)
3	3.53 (1H, *m*)	3.54 (1H, *m*)
4	2.24 (1H, *m*), 2.31 (1H, *m*)	2.27 (1H, *m*), 2.36 (1H, *m*)
6	5.36 (1H, *m*)	5.38 (1H, *m*)
7	1.98 (2H, *m*)	1.98 (2H, *m*)
8	1.50 (*m*)	1.52 (*m*)
9	0.93 (*m*)	0.93 (*m*)
11	1.02 (*m*), 1.57 (*m*)	1.02 (*m*), 1.56 (*m*)
12	1.28 (*m*), 2.03 (*m*)	1.18 (*m*), 2.02 (*m*)
14	1.02 (*m*)	1.01 (*m*)
15	1.08 (*m*), 1.15 (*m*)	1.08 (*m*), 1.12 (*m*)
16	1.83 (*m*), 1.86 (*m*)	1.83 (*m*), 1.86 (*m*)
17	1.15 (*m*)	1.12 (*m*)
18	0.63 (3H, *s*)	0.68 (3H, *s*)
19	1.01 (3H, *s*)	1.00 (3H, *s*)
20	1.28 (*m*)	1.36 (*m*)
21	0.92 (3H, *d*, 6.5)	0.92 (3H, *d*, 6.4)
22	1.00 (*s*), 1.29 (*m*)	1.00 (*s*), 1.34 (*m*)
23	1.16 (2H, *m*)	1.18 (2H, *m*)
24	0.93 (*m*)	0.95 (*m*)
25	1.66 (*m*)	1.66 (*m*)
26	0.84 (3H, *d*, 6.5)	0.82 (3H, *d*, 6.8)
27	0.81 (3H, *d*, 6.5)	0.84 (3H, *d*, 6.8)
28	1.25 (*br s*)	1.26 (*br s*)
29	0.85 (3H, *t*, 8.0)	0.84 (3H, *t*, 7.6)

CMM1: Lupane

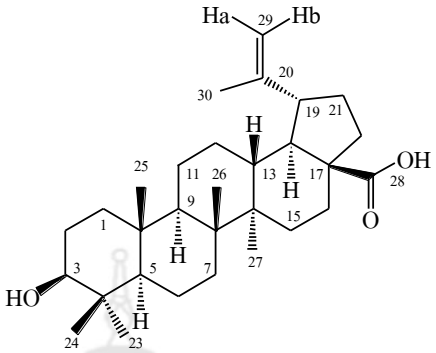


CMM1 was isolated as a white solid, m.p. 235-243 °C. The IR spectrum showed the absorption band of C-H stretching (2928, 2870 cm^{-1}). The ^{1}H NMR spectrum (Table 4.4) indicated that it was a triterpene derivative of lupenone (**AMM5**). The ^{1}H NMR spectrum showed the characteristic signals similar to lupenone except two *multiplet* signals of methylene protone H-3 at δ 1.40 and 2.40 and the *singlet* signal of methyl proton H-29 at δ 1.00. The ^{1}H NMR spectral data as well as melting point and IR spectrum were identical with lupane. Lupane triterpene and derivatives were produced highly effective against influenza A and herpes simplex type 1 viruses *in vitro* (Baltina et al., 2003).

Table 4.4 NMR spectral data of **CMM1**

Position	δ_H (*multiplicity*, J_{Hz})	
	CMM1	**Lupenone**
1	1.20 (*m*), 1.35 (*m*)	1.20 (*m*), 1.40 (*m*)
2	2.40 (*m*), 2.30 (*m*)	1.89 (*m*), 2.43 (*m*)
3	1.40 (*m*), 2.40 (*m*)	-
5	1.20 (*m*)	1.30 (*m*)
6	1.20 (*m*)	1.45 (*m*)
7	1.20 (*m*)	1.45 (*m*)
9	1.20 (*m*)	1.38 (*m*)
11	1.20 (*m*), 1.40 (*m*)	1.28 (*m*), 1.45 (*m*)
12	1.62 (*m*)	1.70 (*m*)
13	1.62 (*m*)	1.68 (*m*)
15	1.62 (*m*)	1.70 (*m*)
16	1.20 (*m*), 1.42 (*m*)	1.36 (*m*), 1.48 (*m*)
18	1.20 (*m*)	1.38 (*m*)
19	2.40 (*m*)	2.43 (*m*)
20	1.40 (*m*)	-
21	1.80 (*m*)	1.90 (*m*)
22	1.80 (*m*)	1.90 (*m*)
23	1.00 (*s*)	1.07 (*s*)
24	0.95 (*s*)	1.02 (*s*)
25	0.90 (*s*)	0.93 (*s*)
26	1.02 (*s*)	1.07 (*s*)
27	0.89 (*s*)	0.95 (*s*)
28	0.71 (*s*)	0.79 (*s*)
29	1.00 (*s*)	4.68 (*d*, 2.1), 4.57 (*m*)
30	1.62 (*s*)	1.70 (*s*)

CMM10: Betulinic acid



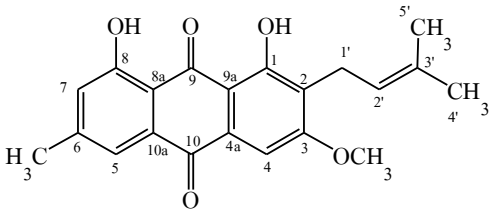
Compound **CMM10** was isolated as a white solid; m.p. 282-288 °C. The IR spectrum showed the absorption band of O-H stretching at 3470 cm^{-1}. The ^{1}H NMR ($CDCl_3$, 400 MHz) spectral data (Table 4.5) showed the resonances of an oxymethine proton (δ 3.18, *dd,* 11.0 and 5.0 Hz, H-3), five methyl groups (δ0.97, H-27; 0.96, H-23; 0.94, H-26; 0.81, H-25 and 0.75, H-24) and isopropenyl side chain (δ 4.73, *br d, J*= 2.5 Hz, H_a-29; 4.77, *br qd, J* = 2.5 and 1.5 Hz, H_b-29; 1.69, *s*, H-30). Comparison of its NMR data with those of **CMM10** and betulinic acid (Sompong Boonsri, 2004) (Table 4.5) indicated that **CMM10** had same structure as betulinic acid.

Betulinic acid has been reported to exhibit anti-HIV-1, antibacterial, antifungal, antiplasmodial and anti-inflammatory activities (Yogeeswari & Sriram, 2005). Betulinic acid has also been reported to inhibit growth of cancer cells, without affecting normal cells (Einzhammer & Xu, 2004). Betulinic acid showed selective cytotoxicity against human melanoma, neuroectodermal and malignant brain tumor cell line, induced apoptosis in human neurablastoma cell line (Baltina et al., 2003).

Table 4.5 NMR spectral data of **CMM10**

Position	δ_H (*multiplicity*, J_{Hz})	
	CMM10	**Betulinic acid**
1	1.65 (1H, *m*), 0.88 (1H, *m*)	1.68 (1H, *m*), 0.95 (1H, *m*)
2	1.59 (2H, *m*)	1.60 (2H, *m*)
3	3.18 (1H, *dd,* 11.0, 5.0)	3.18 (1H, *dd,* 10.2, 6.0)
5	0.68 (1H, *d*, 10.0)	0.7 (1H, *br d*, 10.5)
6	1.55 (1H, *m*), 1.37 (1H, *m*)	1.55 (1H, *m*), 1.40 (1H, *m*)
7	1.37 (2H, *m*)	1.40 (2H, *m*)
9	1.27 (1H, *m*)	1.28 (1H, *m*)
11	1.40 (1H, *m*), 1.23 (1H, *m*)	1.47 (1H, *m*), 1.24 (1H, *m*)
12	1.15 (1H, *m*), 1.71 (1H, *m*)	1.72 (2H, *m*)
13	2.28 (1H, *m*)	2.28 (1H, *m*)
15	1.52 (1H, *m*), 1.16 (1H, *m*)	1.54 (1H, *m*), 1.19 (1H, *m*)
16	1.50 (1H, *m*), 2.25 (1H, *m*)	2.21 (2H, *m*)
18	1.57 (1H, *m*)	1.60 (1H, *m*)
19	3.04 (1H, *dt*, 11.0, 5.0)	3.04 (1H, *dt*, 11.1, 4.5)
21	1.94 (1H, *m*), 1.41 (1H, *m*)	1.98 (2H, *m*)
22	1.98 (1H, *m*), 1.38 (1H, *m*)	1.99 (1H, *m*), 1.43 (1H, *m*)
23	0.96 (3H, *s*)	0.97 (3H, *s*)
24	0.75 (3H, *s*)	0.77 (3H, *s*)
25	0.81 (3H, *s*)	0.83 (3H, *s*)
26	0.94 (3H, *s*)	0.94 (3H, *s*)
27	0.97 (3H, *s*)	0.99 (3H, *s*)
29	H_a : 4.73 (1H, *br d,* 2.5),	4.73 (1H, *br d,* 2.1,),
	H_b : 4.77 (1H*, br qd*, 2.5, 1.5)	4.59 (1H*, br qd*, 2.1, 1.2)
30	1.69 (3H, *s*)	1.69 (3H, *s*)

CMM6: 1,8-Dihydroxy-3-methoxy-6-methyl-2-(3-methyl-2-butenyl)anthraquinone (Vismiaquinone C)



CMM6 was isolated as an orange viscous liquid. The ^{1}H NMR spectral data (Table 4.6) showed two sharp *singlet* signals of two chelated hydroxyl groups at δ 12.43 (1H, 1-OH) and 12.15 (1H, 8-OH). Two *doublet* signals in aromatic region, δ 7.07 (1H) and 7.62 (1H) appearing as meta-coupling were proposed for the signals of H-7 and H-5, respectively. These assignment were supported by ^{3}J correlations of H-7 to C-5, C-8a and 6-$\underline{C}H_3$; H-5 to C-7, C-8a and C-10 on HMBC experiment. A *singlet* signal of the aromatic proton H-4 was at δ 7.40 according to the correlation to C-2, C-3, C-4a, C-9a and C-10 from the HMBC experiment. The spectrum further showed the typical signals of a prenyl side chain which appeared at δ 1.69 (3H, *s*, H-4′), 1.80 (3H, *s*, H-5′), 3.43 (2H, *d*, H-1′) and 5.20 (1H, *t*, H-2′). This prenyl unit was assigned to be at C-2 and was supported by the correlation to H-1′ to C-1, C-2 and C-3. A signal of methoxy group appearing as a *singlet* at δ 4.02 was indicated to be at C-3 by the ^{3}J correlation of methoxy protons to C-3. The ^{13}C NMR spectral data (Table 4.6) suggested that **CMM6** contained three methyl carbons, a methylene carbon, four methine carbons, ten quaternary carbons, a methoxy carbon and two carbonyl carbons. The proposed structure, the spectral data were found to be corresponded to 1,8-dihydroxy-3-methoxy-6-methyl-2-(3-methyl-2-butenyl)anthraquinone or vismiaquinone C (Nagem & Oliverira, 1997).

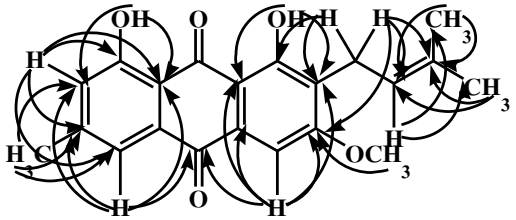


Figure 4.1 HMBC correlations of **CMM6**

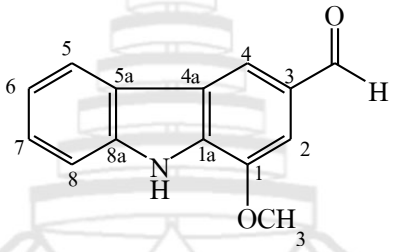
Table 4.6 NMR spectral data of **CMM6**

Position	CMM6				Vismiaquinone C	
	δ_H (*multiplicity*, J_{Hz})	δ_C	DEPT	HMBC	δ_H (*multiplicity*, J_{Hz})	δ_C
1	-	161.71	C	-	-	162.8
2	-	124.19	C	-	-	124.6
3	-	163.55	C	-	-	162.1
4	7.40 (1H, *s*)	103.34	CH	C-3, C-2, C-4a, C-10, C-9a	7.34 (*s*)	103.7
4a	-	133.01	C	-	-	133.4
5	7.62 (1H, *d*, 1.2)	121.13	CH	C-7, C-8a, C-10, 6- $\underline{C}H_3$	7.58 (*d,* 1.6)	121.0
6	-	148.29	C	-	-	133.6
7	7.07 (1H, *d*, 0.8)	124.42	CH	C-5, C-8, C-8a, 6- $\underline{C}H_3$	7.04 (*d*, 1.6)	124.8
8	-	162.43	C	-	-	163.9
8a	-	113.70	C	-	-	114.1
9	-	191.26	C	-	-	191.7
9a	-	110.69	C	-	-	111.1
10	-	182.20	C	-	-	182.7
10a	-	132.79	C	-	-	133.6
1′	3.43 (2H, *d*)	22.15	CH_2	C-3, C-2, C-1, C-2′, C-3′	3.40 (*d*, 7.0)	22.1
2′	5.20 (1H, *t*)	120.57	CH	C-4′, C-5′	5.16 (*t*, 7.0)	121.5
3′	-	133.16	C	-	-	126.3
4′	1.69 (3H, *s*)	25.83	CH_3	C-2′, C-3′	1.66 (*s*)	22.6
5′	1.80 (3H, *s*)	17.88	CH_3	C-2′, C-3′	1.78 (*s*)	22.5
1-OH	12.43 (1H, *s*) 12.15	-	-	C-2, C-9a	12.12 (*s*)	-
8-OH	(1H, *s*)	-	-	C-7, C-8a	12.39 (*s*)	-
3-OCH_3	4.02 (3H, *s*)	56.27	CH_3	C-3	4.01(*s*)	56.3

Table 4.6 (continued)

Position	CMM6				Vismiaquinone C	
	δ_H (*multiplicity*, J_{Hz})	δ_C	DEPT	HMBC	δ_H (*multiplicity*, J_{Hz})	δ_C
6-CH_3	2.45 (3H, *s*)	22.15	CH_3	C-7, C-6, C-5	2.42 (*s*)	20.5

CMM38: 3-Formyl-1-methoxycarbazole (Murrayanine)



CMM38 was isolated as a yellow solid. The ^{1}H NMR spectral data (Table 4.7) exhibited a pair of *meta*-coupled protons at δ 7.46 (*d*, *J*= 0.8 Hz) and 8.20 (*d*, *J* = 0.8 Hz) for H-2 and H-4, respectively. The coupling of the aromatic protons indicate that the ring A is *ortho*-substitued with two *doublets* at δ8.12 and 7.50 attributed to H-5 and H-8, respectively. Another two pair of *triplet* at δ 7.32 and 7.51 were due to H-6 and H-7, respectively. The ^{1}H NMR also showed signals for one methoxyl group (1-OCH_3) was present at δ 4.07 which was supported by the HMBC correlation of 1-OCH_3 to C-1. The presence of a formyl group on C-3 was proved by the existence of a formyl signal at δ 10.05 in the ^{1}H NMR spectrum and a carbonyl signal at δ 191.90 in the ^{13}C NMR spectrum. Therefore, the above spectral data indicated the structure of **CMM38** as 3-formyl-1-methoxycarbazole which was known as murrayanine (Abu Bakar et al., 2007). The biological of carbazole alkaloids inhibiting the cell cycle and inducing cell death of cancer cells have been studied in detail by Cui, Cai, Yan and Yao (2002). It has been reported by previous that carbazole alkaloids possess various biological activities such as anti-tumor, anti-oxidative, anti-mutagenic and anti-inflammatory activities (Mackeen et al., 1997).

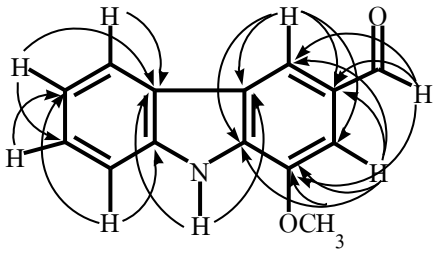
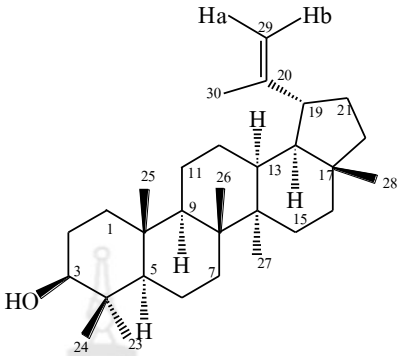


Figure 4.2 HMBC correlations of **CMM38**

Table 4.7 NMR spectral data of **CMM38**

Position	CMM38				Murrayanine	
	δ_H (*multiplicity*, J_{Hz})	δ_C	DEPT	HMBC	δ_H (*multiplicity*, J_{Hz})	δ_C
1	-	146.10	C	-	-	146.1
1a	-	134.09	C	-	-	139.4
2	7.46 (1H, *d*, 0.8)	103.54	CH	3-CHO, C-1, C-4, C-1a	7.47 (1H, *s*)	103.5
3	-	130.19	C	-	-	130.2
4	8.20 (1H, *d*, 0.8)	120.42	CH	3-CHO, C-1a, C-2, C-4a	8.20 (1H, *s*)	120.4
4a	-	123.64	C	-	-	123.6
5	8.12 (1H, *d*, 8.0)	120.71	CH	C-5a	8.13 (1H, *d*,7.32)	120.7
5a	-	123.68	C	-	-	123.6
6	7.32 (1H, *t*, 6.8)	120.72	CH	C-5a, C-7	7.34 (1H, *t*, 8.24)	120.7
7	7.51 (1H, *t*, 8.0)	111.51	CH	C-6	7.53 (1H, *t*, 8.24)	111.5
8	7.50 (1H, *dd*, 1.2, 6.8)	126.65	CH	C-6, C-8a	7.50 (1H, *d*, 6.44)	126.6
8a	-	139.43	C	-	-	134.1
1-OCH_3	4.07 (3H, *s*)	55.82	CH_3	C-1	4.08 (3H, *s*)	55.8
3-CHO	10.05 (1H, *s*)	191.90	CH	C-1, C-2, C-4	10.06 (1H, *s*)	191.9
NH	8.64 (1H, *br s*)	-		C-4a, C-5a	8.61 (1H, *br s*)	-

DMM1: Lup-20(29)-en-3β-ol (Lupeol)



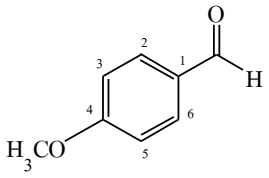
DMM1 was obtained as a white solid, m.p. 213-215 °C. The IR spectrum exhibited the absorption band of O-H stretching at 3313 cm^{-1}. The ^{1}H NMR spectrum indicated that it was a triterpene derivative of **AMM5**. The ^{1}H NMR spectrum (Table 4.8) showed the characteristic signal of a terminal olifinic methylene protons at δ4.68 and 4.56 (1H each, *d*, *J* = 2.4 Hz) for H_a-29 and H_b-29, respectively. The ^{1}H NMR spectrum showed the resonance of an oxymethine proton (δ3.39, *dd*, *J* = 5.7 and 1.5 Hz, H-3) and seven methyl groups (δ0.96 (H-23), 0.84 (H-24), 0.82 (H-25), 1.03 (H-26), 0.93 (H-27), 0.78 (H-28) and 1.68 (H-30)) were observed. ^{1}H and ^{13}C NMR spectral data as well as melting point were identical with lup-20(29)-en-3β-ol which was known as lupeol (Imam, Azhar, Hasan, Ali & Ahmed, 2007)

Lupeol was reported to possess beneficial effects as a therapeutic and preventive agent for a range of disorders and decreasing carcinogenesis and ameliorating inflammation. Lupeol has been shown to exhibit various pharmacological activities under *in vitro* conditions (Saleem, 2009), strong anti-mutagenic activity under *in vitro* and *in vivo* systems (Lira et al., 2008) and no toxicity in animal studies (Patocˇka, 2003). Moreover, lupeol also exhibit anti-inflammatory and antiarthritic activities (Agarwal & Rangari, 2003). Lupeol was inhibited to bacterial which is harmonize with the result of Supaluk Prachayasittikul et al. (2010)

Table 4.8 NMR spectral data of **DMM1**

Position	δ_H (*multiplicity*, J_{Hz})	
	DMM1	**Lupeol**
1	1.50 (2H, *m*)	0.68 (2H, *d*)
2	1.70 (*m*)	1.61 (1H, *d*), 1.54 (1H, *q*)
3	3.39 (1H, *dd,* 5.7*,* 1.5)	3.18 (1H, *dd*)
5	1.40 (*m*)	0.69 (1H, *d*)
6	1.45 (*m*)	1.54 (1H, *d*), 1.39 (1H, *q*)
7	1.20 (*m*)	1.41 (2H, *m*)
9	1.20 (*m*)	1.28 (1H, *d*)
11	1.45 (*m*)	1.42 (1H, *d*), 1.29 (1H, *q*)
12	1.70 (*m*)	1.07 (1H, *q*), 1.68 (2H, *d*)
13	1.65 (*m*)	1.67 (1H, *t*)
15	1.52 (*m*)	1.71 (1H, *t*), 1.01 (1H, *d*)
16	1.40 (*m*)	1.49 (1H, *d*), 1.38 (1H, *t*)
18	1.35 (*m*)	1.37 (1H, *t*), 0.91 (1H, *t*)
19	2.39 (*ddd*, 5.7, 5.7, 5.4)	2.39 (1H, *m*)
21	1.95 (1H, *m*)	1.93 (1H, *m*)
22	1.40 (1H, *m*), 1.20 (*m*)	1.42 (1H, *m*), 1.20 (1H, *m*)
23	0.96 (3H, *s*)	0.98 (3H, *s*)
24	0.84 (3H, *s*)	0.79 (3H, *s*)
25	0.82 (3H, *s*)	0.27 (3H, *s*)
26	1.03 (3H, *s*)	1.04 (3H, *s*)
27	0.93 (3H, *s*)	0.97 (3H, *s*)
28	0.78 (3H, *s*)	0.84 (3H, *s*)
29	4.68 (1H, *d*, 2.4), 4.56 (1H, *m*)	4.69 (1H, *m*), 4.56 (1H, *m*)
30	1.68 (3H, *s*)	1.69 (3H, *s*)

FMM2: 4-Methoxybenzaldehyde



FMM2 was isolated as a white solid. The ^{1}H NMR spectrum (Table 4.9) showed the characteristic signal of formyl group at δ 9.60 (1H, *s*). The AA′BB′ resonances of aromatic protons H-2, H-6 and H-3, H-5 were displayed at δ 7.25 (*d*, *J*=3.2 Hz) and δ 6.53 (*d*, *J*=3.2 Hz), respectively. A *singlet* signal at δ 4.73 was assigned for 4-OCH_3. The assignment suggested that **FMM2** was 4-methoxybenzaldehyde.

Table 4.9 NMR spectral data of **FMM2**

Position	δ_H (***multiplicity***, J_{Hz})
1	9.60 (1H, *s*)
2, 6	7.25 (2H, *d*, 3.2)
3, 5	6.53 (2H, *d,* 3.2)
4-OCH_3	4.73 (3H, *s*)

4.2 Evaluation of antibacterial activity

4.2.1 Antibacterial activity of crude extracts

Dried roots of *M. macrocarpa* were extracted with CH_2Cl_2, Me_2CO and MeOH to give crudes **A** to **F**. Each extract was tested for antibacterial activity on *Staphylococcus aureus* (SA), *Staphylococcus aureus* methicillin-resistant strain (MRSA SK1), *Bacillus cereus* (BC), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Pseudomonas fluorescens* (PF), and *Salmonellae typhimurium* (ST). The results of antibacterial activity test were presented in Table 4.10. Crude **A**, **B**, **D** and **E** did not show inhibition zone. Only the methanolic extract exhibited antibacterial activity, the crude **C** was able to inhibit the growth of *S. aureus* and *S.aureus* MRSA SK1 and crude **F** was able to inhibit the growth of *B. cereus* and *S. typhimurium*. Table 4.11 shows MICs values of crude **C** exhibited *S. aureus* and *S. aureus* MRSA SK1 at 1280 μg/mL. Crude **F** exhibited *B. cereus* and *S. typhimurium* at 640 and 1280 μg/mL, respectively. Thus, crude **F** exhibited a stronger antibacterial activity against Gram positive bacteria than it did Gram negative bacteria. The reason for different sensitivity of crude **F** against Gram positive and Gram negative bacteria could be ascribed to the morphological differences between these microorganisms, Gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes. The Gram positive give more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer & Gerhardt, 1971). Vacomycin is bactericidal against Gram positive bacterial; binds to the peptide side chain of *N*-acetylmuramic acid. By doing so, it blocks synthesis of peptodoglycan, resulting in weakening of the cell wall and ultimately, cell lysis. Streptomycin and gentamicin were bactericidal against aerobic and facultative bacteria; binds to the 30S ribosome subunit, blocking the initiation of translation and coursing the misreading of mRNA (Nester, Anderson, Roberts, Pearsall & Naster, 1994).

Table 4.10 Antibacterial activity of crude extracts determined by paper disc diffusion method

Sample	Diameter of inhibition zone (mm)						
	Gram Positive			Gram Negative			
	SA	MRSA SK1	BC	EC	PA	PF	ST
Control	-	-	-	-	-	-	-
Crude **A**	-	-	-	-	-	-	-
Crude **B**	-	-	-	-	-	-	-
Crude **C**	6.90	10.04	-	-	-	-	-
Crude **D**	-	-	-	-	-	-	-
Crude **E**	-	-	-	-	-	-	-
Crude **F**	-	-	7.35	-	-	-	7.80
Vancomycin	17.63	17.75	16.03	-	-	-	-
Gentamicin	-	-	-	19.01	18.74	-	-
Streptomycin	-	-	-	-	-	16.23	21.20

Notes. - = no inhibition zone

Table 4.11 MIC values of crudes **C** and **F** determined by broth microdilution method

Sample	Antibacterial activity (MIC, μg/mL)			
	SA	MRSA SK1	BC	ST
Crude **C**	1280	1280	NT	NT
Crude **F**	NT	NT	640	1280
Vancomycin	0.500	1.000	0.500	-
Gentamicin	-	-	-	0.500

4.2.2 Antibacterial activity of pure compounds

Some of the pure compounds obtained from each extract were evaluated for their antibacterial activity against, *Staphylococcus aureus* (SA), *Staphylococcus aureus* methicillin-resistant strain (MRSA SK1), *Bacillus cereus* (BC), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Pseudomonas fluorescens* (PF), and *Salmonellae typhimurium* (ST). The compounds **AMM5** (Lupenone) and **CMM6** (Vismiaquinone C) exhibited the antibacterial activity ageinst *B. cereus*, *Ps. fluorescens* and *S. typhimurium* with MIC values of 32-64 μg/mL (Table 4.12). **CMM6** results were similar to these of the studied of Kuete et al. (2007). Structure of **CMM6** (Vismiaquinone C) have a phenol-based chemical that destroys bacteria by disrupting cell walls and membranes. The antimicrobial mechanisms were explained by Cowan and Talaro (1999). The properties of membrane disruption could be explained for antimicrobial potency of our crude extracts and chemical compounds. However, **CMM6** was less active than vancomycin and gentamicin, the standard antibiotic agents. Compounds **AMM7**, **AMM8**, **CMM1** and **DMM1** did not show the antibacterial activity. The other compounds were not tested for the antibacterial activity because the quantities of these pure compounds were not enough to test the activity.

Table 4.12 Antibacterial activity of pure compounds determined by broth microdilution method

Sample	Antibacterial activity (MIC, μg/mL)						
	Gram Positive			Gram Negative			
	SA	MRSA SK1	BC	EC	PA	PF	ST
AMM5	>200	>200	64	>200	>200	64	32
AMM7	>200	>200	>200	>200	>200	>200	>200
AMM8	>200	>200	>200	>200	>200	>200	>200
CMM1	>200	>200	>200	>200	>200	>200	>200
CMM6	>200	200	32	>200	>200	64	64
DMM1	>200	>200	>200	>200	>200	>200	>200
Vancomycin	0.5	0.5	0.5	-	-	-	-
Gentamicin	-	-	-	0.5	0.5	0.5	0.5

4.3 Evaluation of antioxidation activity

Evaluation of antioxidative effects has been carried out by various methods. The DPPH assay is one of the methods used for antioxidant testing on free radical terminator because its odd electron can be used as a convenient tool for the antioxidant assay. The DPPH free radical is dark violet solid, its solubility is not great, alcoholic solution having concentrations of approximately 5 x10^{-4} are nevertheless densely colored. Its solution shows a strong absorption band at λ 517 nm (in ethanol), when DPPH radical accepts an electron or hydrogen radical, a more stable compound will be formed and consequently its characteristic absorption at 517 nm vanishes (deep violet turns colorless). The capacity of the substances to donate electrons can be estimated from the degree of loss color (Blois, 1958). Coexistence of an antioxidant compound (AH) and free radical ($DPPH^{\bullet}$) leads to the disappearance of DPPH free radical and the appearance the free radical ($A^{\bullet}$) as shown in Figure 4.3.

Figure 4.3 DPPH free radical and the appearance of the free radical

4.3.1 Screening on the free radical scavenging activity of crude extracts

To determine the scavenging activity, the crude extracts of *M. macrocarpa* roots were tested for scavenging activity at the final concentration of 100 μg/mL. The activity was monitored by following the decrease of absorbance of the solution at 517 nm for 30 min. The result (Table 4.13) indicated that the crude extracts were able to the DPPH radical scavenging.

Table 4.13 The average absorbtion and % inhibition of the crude extracts (100 µg/mL)

sample	Average absorbances (517 nm)					% inhibition
	0 min	15 min	30 min	45 min	60 min	(at 30 min)
Control	0.649	0.649	0.648	0.648	0.648	-
DPPH + crude **A**	0.624	0.603	0.550	0.529	0.523	15.12
DPPH + crude **B**	0.636	0.185	0.106	0.094	0.087	83.64
DPPH + crude **C**	0.523	0.072	0.060	0.057	0.056	90.74
DPPH + crude **D**	0.643	0.572	0.583	0.572	0.563	10.03
DPPH + crude **E**	0.637	0.442	0.274	0.227	0.211	57.72
DPPH + crude **F**	0.589	0.159	0.055	0.053	0.052	91.51
DPPH + Ascorbic acid	0.423	0.026	0.082	0.024	0.021	87.35
DPPH + BHT	0.591	0.269	0.112	0.100	0.096	82.72

Control = 0.05 mM DPPH

Crude **F** is most able to scavenge the DPPH radical followed by crude **C**, **B**, **E**, **A** and **D**, respectively. Percentage of inhibition of crude **B**, **C**, **E** and **F** were more than 50% at 30 min (Figure 4.4). Crude **A** and **D** were presented percentage of inhibition less than 50% at 30 min.

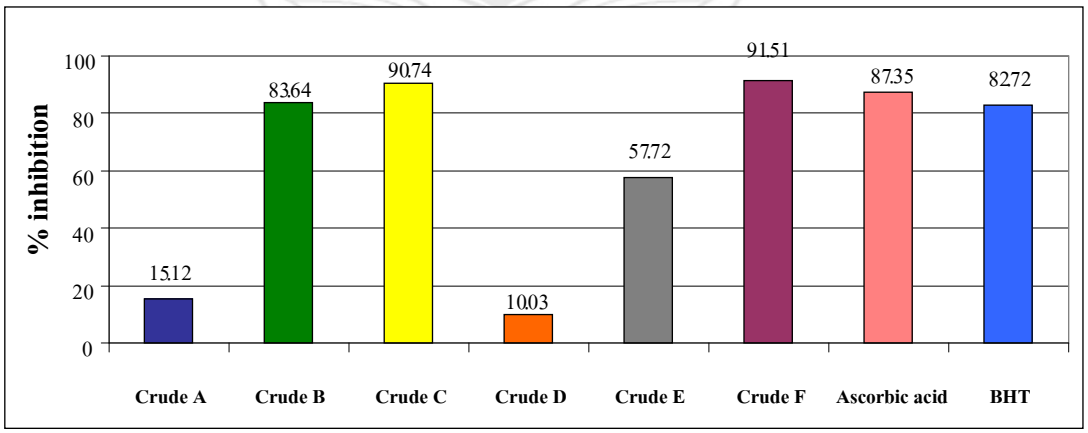


Figure 4.4 Radical scavenging activity of the crude extracts (final concentration at 100 µg/mL)

4.3.2 Evaluation of 50% inhibition concentration (IC_{50}) of the crude extracts

The results were expressed as % inhibition as shown in Table 4.14. The average absorption and % inhibition of the crude **B**, **C**, **E** and **F** at various concentrations were at 4.0, 3.0, 2.0, 1.0, 0.5, 0.25, and 0.125 mg/mL.

Table 4.14 The average absorption and % inhibition of the sample solutions and standard antioxidants at various concentrations

concentration	Crude B		Crude C		Crude E		Crude F		Ascorbic acid		BHT	
(mg/mL)	A	% I	A	% I	A	% I	A	% I	A	% I	A	% I
4.0	-	-	-	-	0.304	50.81	-	-	0.027	95.63	0.116	81.23
3.0	0.119	80.74	0.062	89.97	0.330	46.60	0.048	92.23	0.027	95.63	0.104	83.17
2.0	0.197	68.12	0.059	90.45	0.376	39.16	0.062	89.97	0.033	94.66	0.166	73.14
1.0	0.360	41.75	0.068	89.00	0.416	32.67	0.252	59.22	0.028	95.47	0.275	55.50
0.5	0.498	19.42	0.114	81.55	0.530	14.24	0.464	24.92	0.037	94.01	0.301	51.29
0.25	0.561	9.22	0.370	40.13	-	-	0.545	11.81	0.128	79.29	0.420	32.04
0.125	0.605	2.10	0.446	27.83	-	-	0.585	5.34	0.540	2.62	0.492	20.39
Control	0.618	-	0.618	-	0.618	-	0.618	-	0.618	-	0.618	-

Control = 0.05 mM DPPH

A = Average absorbances of samples at 517 nm for 30 min

% I = % inhibition of samples at 517 nm for 30 min

Table 4.15 IC_{50} values of tested crude extracts and standard antioxidants

Sample	IC_{50} (mg/mL, 30 min)
Crude **B**	1.32
Crude **C**	0.31
Crude **E**	3.30
Crude **F**	0.88
Ascorbic acid	0.19
BHT	0.49

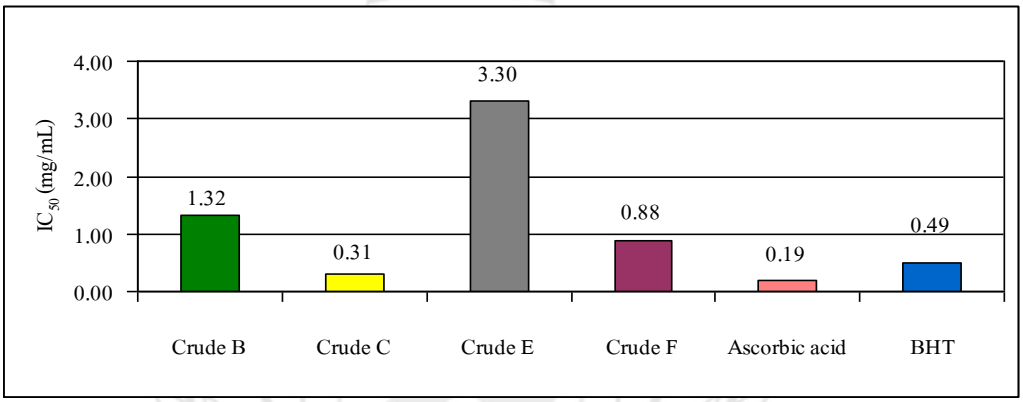


Figure 4.5 Crude **B**, **C**, **E** and **F** were evaluated for IC_{50}

The assessment of the antioxidation activity of the crude material was extended. In comparable to the standard antioxidant (BHT and ascorbic acid) and crude **B**, **C**, **E** and **F** were evaluated for IC_{50}. The oxidation effect was evaluated as the concentration required to scavenge 50% DPPH free radical. Their IC_{50} were exhibited at 1.32, 0.31, 3.30 and 0.88 mg/mL, respectively. (Table 4.15 and Figure 4.5). Crude **C** showed strong antioxidation activity, which was better than BHT (IC_{50} 0.49 mg/mL). The crude **B**, **E** and **F** were founded to showed antioxidation activities with IC_{50} 1.32, 3.30 and 0.88 mg/mL, according to the methanolic extract give the highest antioxidation activity of the extracts. In contrast dichloromethane extract gave the least potent antioxidant activity (Buran Pansawan, Supak Poungbangpho & Maitree Suttajit, 2006).

4.3.3 Screening on the free radical scavenging activity of pure compounds

The testing was performed as in **4.3.1** except the final concentration was made at 50 μM. The results were shown in Table 4.16.

Table 4.16 The average absorption and % inhibition of pure compounds (50 μM)

sample	Average absorbances (517 nm)					% inhibition
	0 min	15 min	30 min	45 min	60 min	(at 30 min)
Control	0.634	0.637	0.639	0.641	0.644	-
DPPH + **AMM5**	0.637	0.633	0.627	0.627	0.627	1.88
DPPH + **AMM7**	0.626	0.638	0.636	0.637	0.638	0.47
DPPH + **AMM8**	0.626	0.632	0.629	0.627	0.629	1.56
DPPH + **CMM1**	0.634	0.638	0.636	0.632	0.633	0.47
DPPH + **CMM6**	0.645	0.637	0.637	0.636	0.636	0.31
DPPH + **DMM1**	0.636	0.639	0.636	0.634	0.633	0.47
DPPH + Ascorbic acid	0.624	0.169	0.026	0.023	0.023	95.93
DPPH + BHT	0.630	0.500	0.312	0.236	0.202	51.17

Control = 0.05 mM DPPH

To determine the antioxidation activity of the pure compounds. The samples tested at the final concentration of 50 μM, the absorption of the solution were measured at 517 nm (30 min). Ascorbic acid and BHT were used as reference compounds. The other compounds were did not test the antioxidation activity, because quantity of these pure compounds were not enough to test activity. The activity was expressed in the 95.93 and 51.17 % inhibition, respectively. (Table 4.17).

Table 4.17 % inhibition of tested compounds and standard antioxidants (50 μM)

Sample	% inhibition (50 μM, 30 min)
AMM5	1.88
AMM7	0.47
AMM8	1.56
CMM1	0.47
CMM6	0.31
DMM1	0.47
Ascorbic acid	95.93
BHT	51.17

These results of pure compounds shown low % inhibition, indicate low ability to donate electron the DPPH radical scavenging. The triterpenes (**AMM5**, **CMM1** and **DMM1**) and steroids (**AMM7** and **AMM8**) were isolated from the root of *M. macrocarpa* shows low level of hydrogen radical donating. Vismiaquinone C (**CMM6**) is probable to be antioxidant compound, because its structure consist of phenol, able to donated hydrogen radical. When DPPH radical accepts electron or hydrogen radical, then DPPH is turn colorless. However, in this study **CMM6** showed low % inhibition might cause by the absorption of **CMM6** itself with orange color that absorb the light at 564 nm which close to the absorption of DPPH (purple color) at 517 nm. The other methods to evaluate the antioxidant capacities then should be used to confirm the antioxidant activity of **CMM6** such as Trolox equivalent antioxidant capacity (TEAC), Total phenolic content (TPC) and Total antioxidant capacity (TAC) (Janya Sang-Arun et al., 2001).

CHAPTER 5

CONCLUSIONS

Investigation of the chemical constituents of dichloromethane, acetone and methanolic extracts from the root of *M. macrocarpa* on March, 2002 and December, 2006 led to the isolation of nine compounds: lupenone (**AMM5**), stigmasterol (**AMM7**), β-sitosterol (**AMM8**), lupane (**CMM1**), vismiaquinone C (**CMM6**), betulinic acid (**CMM10**), murrayanine (**CMM38**), lupeol (**DMM1**) and 4-methoxybenzaldehyde (**FMM2**).

Bioactivity assays showed that the compounds lupenone and vismiaquinone C exhibited antibacterial activities against *Bacillus cereus*, *Pseudomonas fluorescen*s and *Salmonella typhimurium* with the minimum inhibition concentrations (MICs) values of 32-64 μg/mL.

The crude extracts and some pure compounds were examined for their antioxidation properties by DPPH free radical scavenging assay. Crude acetone (2002), methanolic (2002), acetone (2006) and methanolic (2006) extracts were showed strong antioxidation activity with IC_{50} 1.32, 0.31, 3.30 and 0.88 mg/mL. Whereas the pure compounds showed weak activity.

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APPENDIXES

APPENDIX A

Preparations

Preparation of microbial

Bacterial

Method 1

1. Streak culture from stock culture into MHA plate
2. Incubated in 35 °C 18-24 h.
3. to give the culture on plate, select 3-5 single colonies to sterile MHB
4. incubated in 35 °C and 150 rpm. 3 h.
5. compare turbidity with 0.5 McF standard for bacteria and adjust with 0.85% NSS
6. the culture already to use

Method 2

1. Streak culture from stock culture into MHA plate
2. Incubated in 35 °C 18-24 h.
3. Corrected 3-5 single colonies to 1 mL of 0.85% NSS in sterile tube.
4. compare turbidity with 0.5 McFarland standard, 2 McFarland standard for yeast and adjust with 0.85% NSS
5. the culture already to use

Preparations of Media

Mueller-Hinton agar (MHA), Difco

1 liter of media including	Beef extract powder	2.0	g
	Acid digest of casein	17.5	g
	Soluble starch	1.5	g
	Agar	17.5	g

Mueller-Hinton agar (MHB), Difco

1 liter of media including	Beef extract powder	2.0	g
	Acid digest of casein	17.5	g
	Soluble starch	1.5	g

Nutrient agar (NA), Difco

1 liter of media including	Beef extract	3.0	g
	Peptone	5.0	g
	Agar	15.0	g

Nutrient broth (NB), Difco

1 liter of media including	Beef extract	3.0	g
	Peptone	5.0	g

Sabouraud dextrose agar (SDA), Difco

1 liter of media including	Peptic digest of animal tissue	5.0	g
	Peptic digest of animal tissue	5.0	g
	Dextrose	40.0	g
	Agar	15.0	g

Sabouraud dextrose broth (SDB), Difco

1 liter of media including	Enzymatic digest of casein	10.0	g
	Dextrose	20.0	g

Preparation of standard drug

Prepared stock solution of vancomycin, gentamicin (concentration 16 mg/mL)

- Weight 0.016 g of drug in sterile water 1 mL
- Kept in -20 °C

Turbidity standards (McFarland)

McFarland 0.5 turbidity standards are available from various manufacturers. Alternately, the 0.5 McFarland may be prepared by adding 0.5 mL of a 1.175% (w/v) barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) solution to 99.5 mL of 1% (v/v) sulfuric acid. The turbidity standard is then aliquoted into test tubes identical to those used to prepare the inoculum suspension. Seal the McFarland standard tubes with wax, Parafilm, or some other means to prevent evaporation. McFarland standards may be stored for up to 6 months in the dark at room temperature (22 to 25°C). Discard after 6 months or sooner if any volume is lost. Before each use, shake well, mixing the fine white precipitate of barium sulfate in the tube. The accuracy of the density of a prepared McFarland standard should be checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standard, the absorbance at a wavelength of 625 nm should be 0.08 to 0.1. Alternately, the accuracy of the McFarland standard may be verified by adjusting a suspension of a control strain (e.g., *E. coli* ATCC 25922) to the same turbidity, preparing serial 10-fold dilutions, and then performing plate counts. The adjusted suspension should give a count of 108 colony forming units/mL.

0.85% Normal saline

NaCl	8.5 g
Distilled water	1 liter

Dissolve NaCl in water, heating if necessary. May be sterilized by autoclaving or membrane filtration. Store at ambient temperature for up to 6 months with caps tightened to prevent evaporation.

APPENDIX B

IR and NMR Spectrum

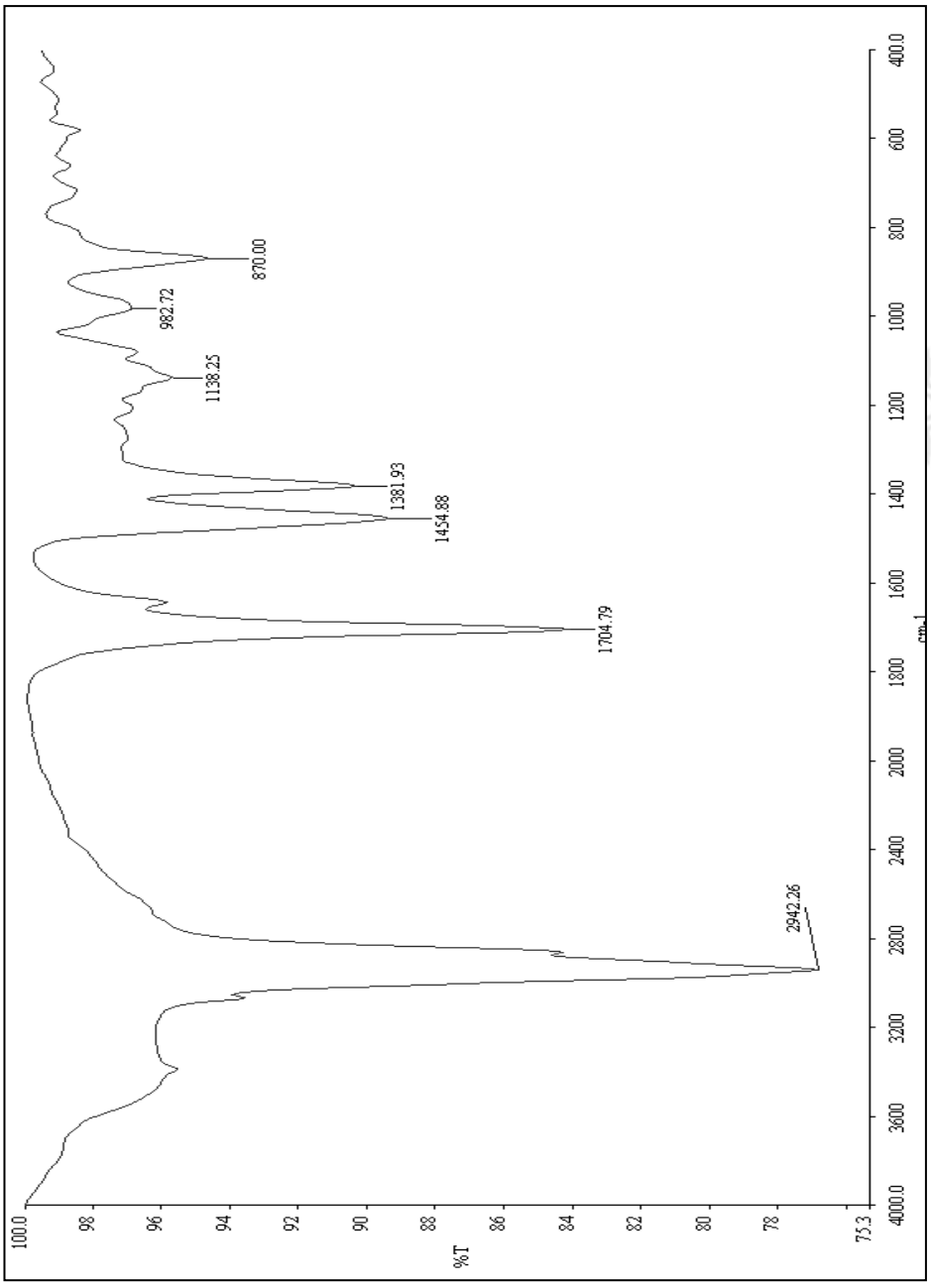

Figure B1 IR (KBr) spectrum of **AMM5**

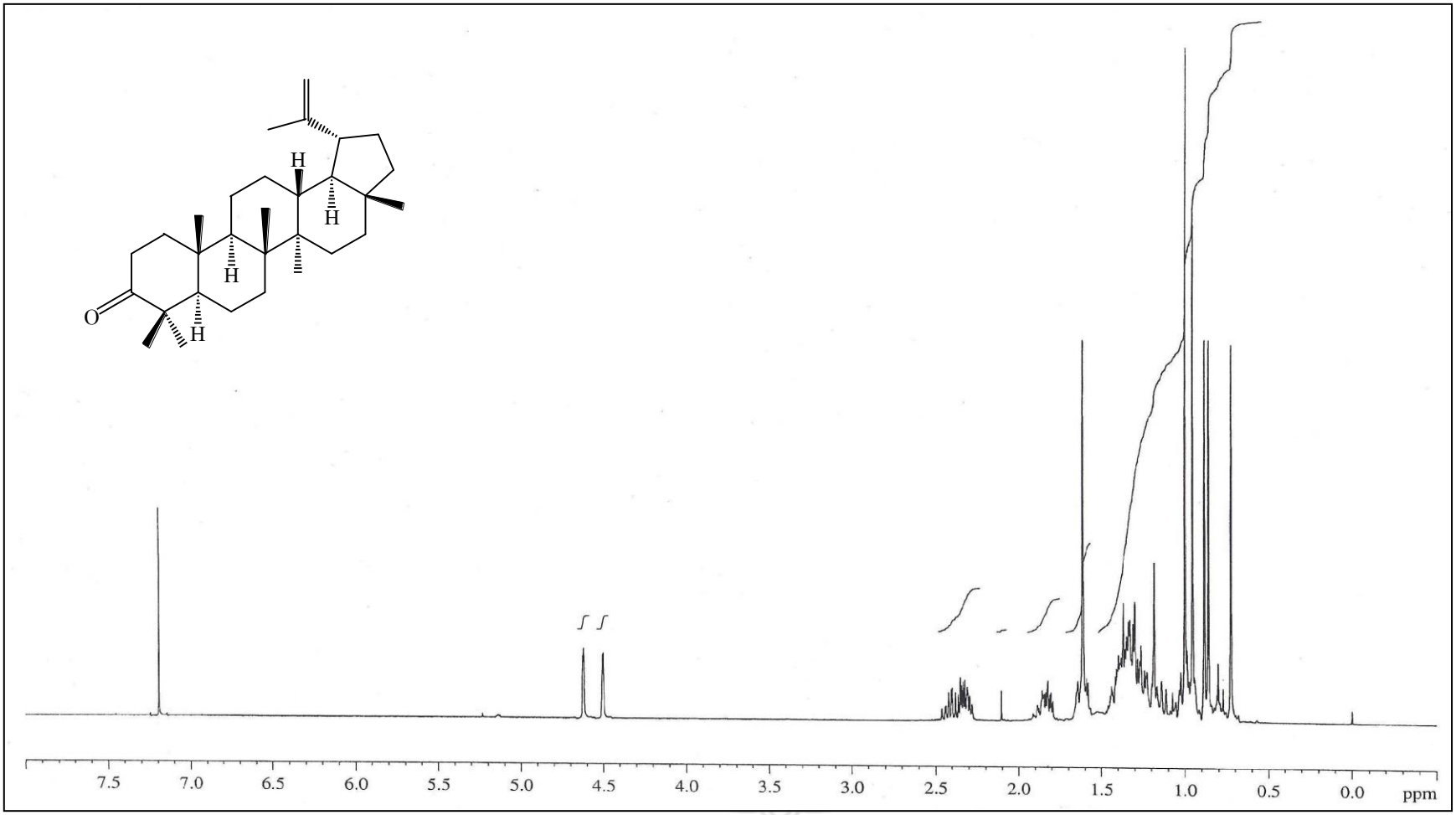

Figure B2 ^{1}H NMR (300 MHz) ($CDCl_3$) spectrum of **AMM5**

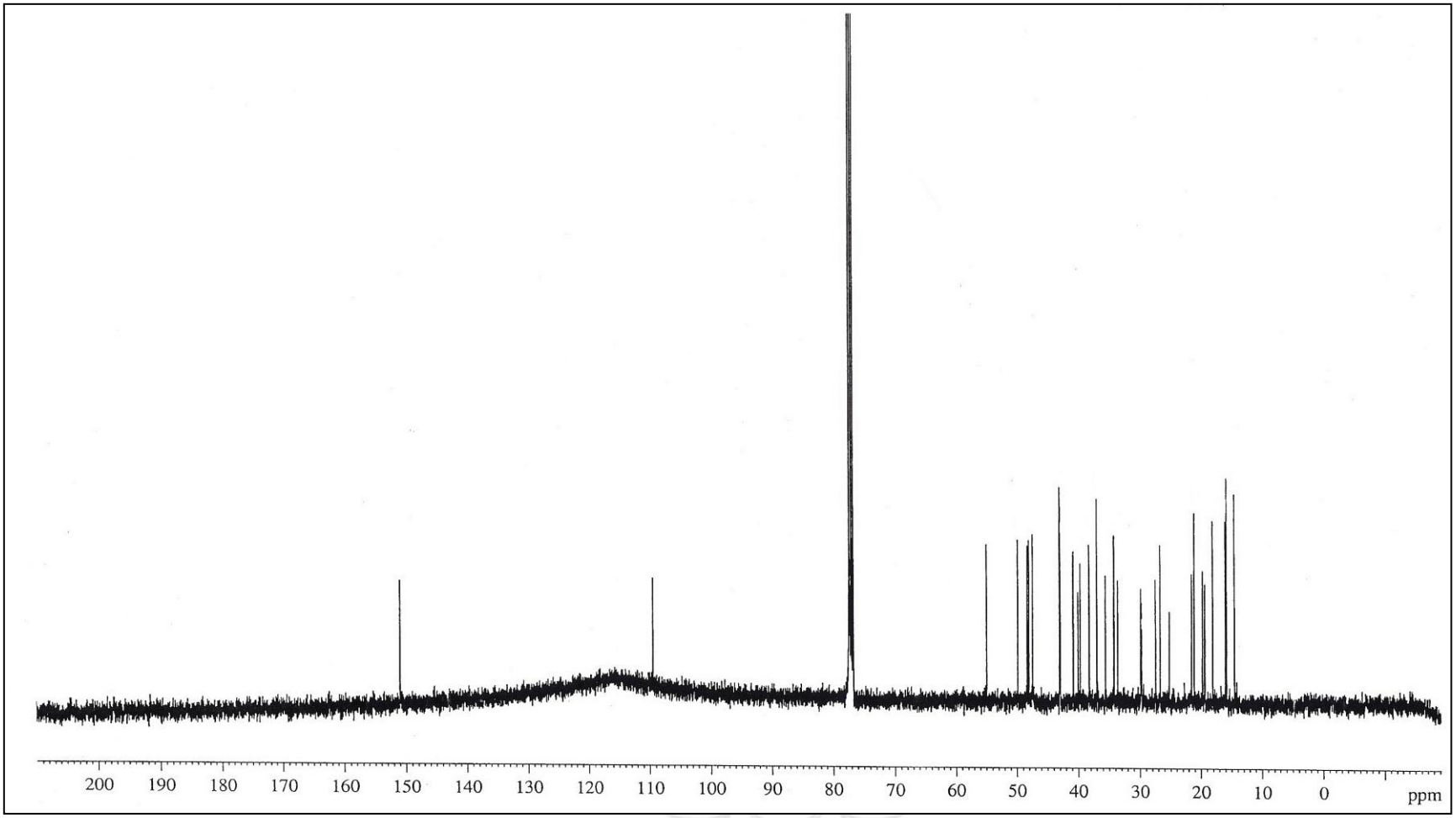

Figure B3 ^{13}C NMR (75 MHz) ($CDCl_3$) spectrum of **AMM5**

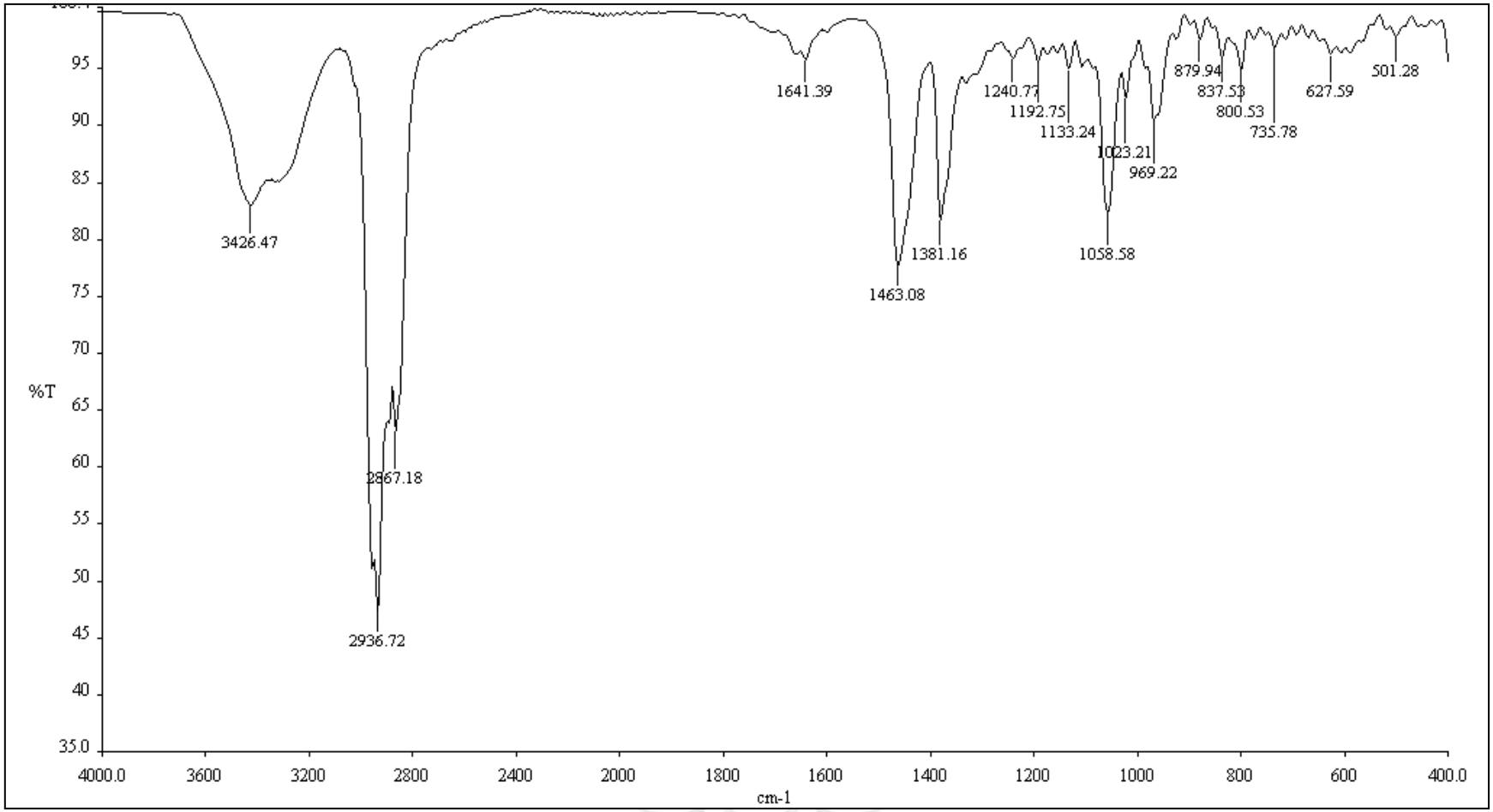

Figure B4 IR (KBr) spectrum of **AMM7**

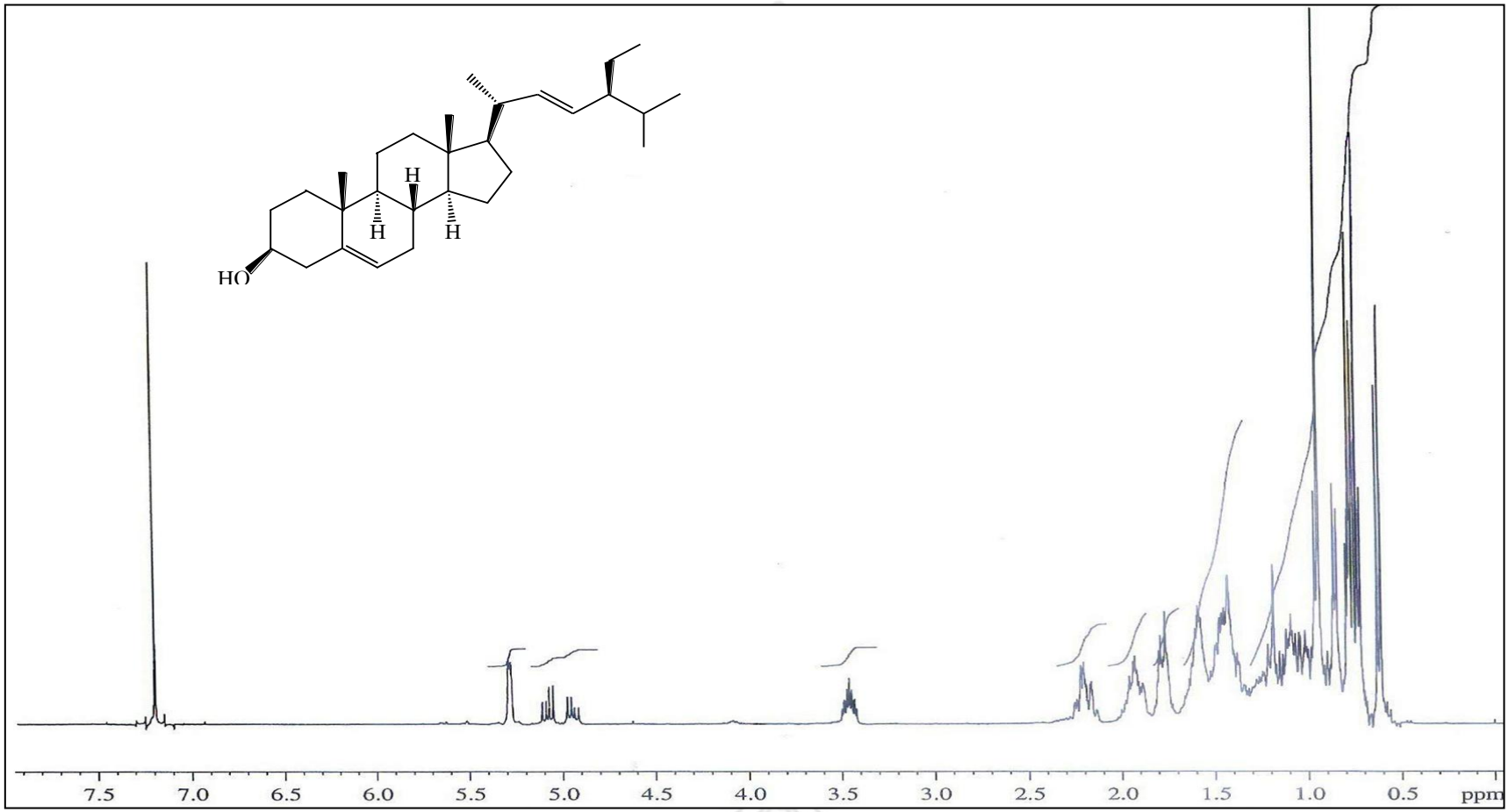

Figure B5 ^{1}H NMR (300 MHz) ($CDCl_3$) spectrum of **AMM7**

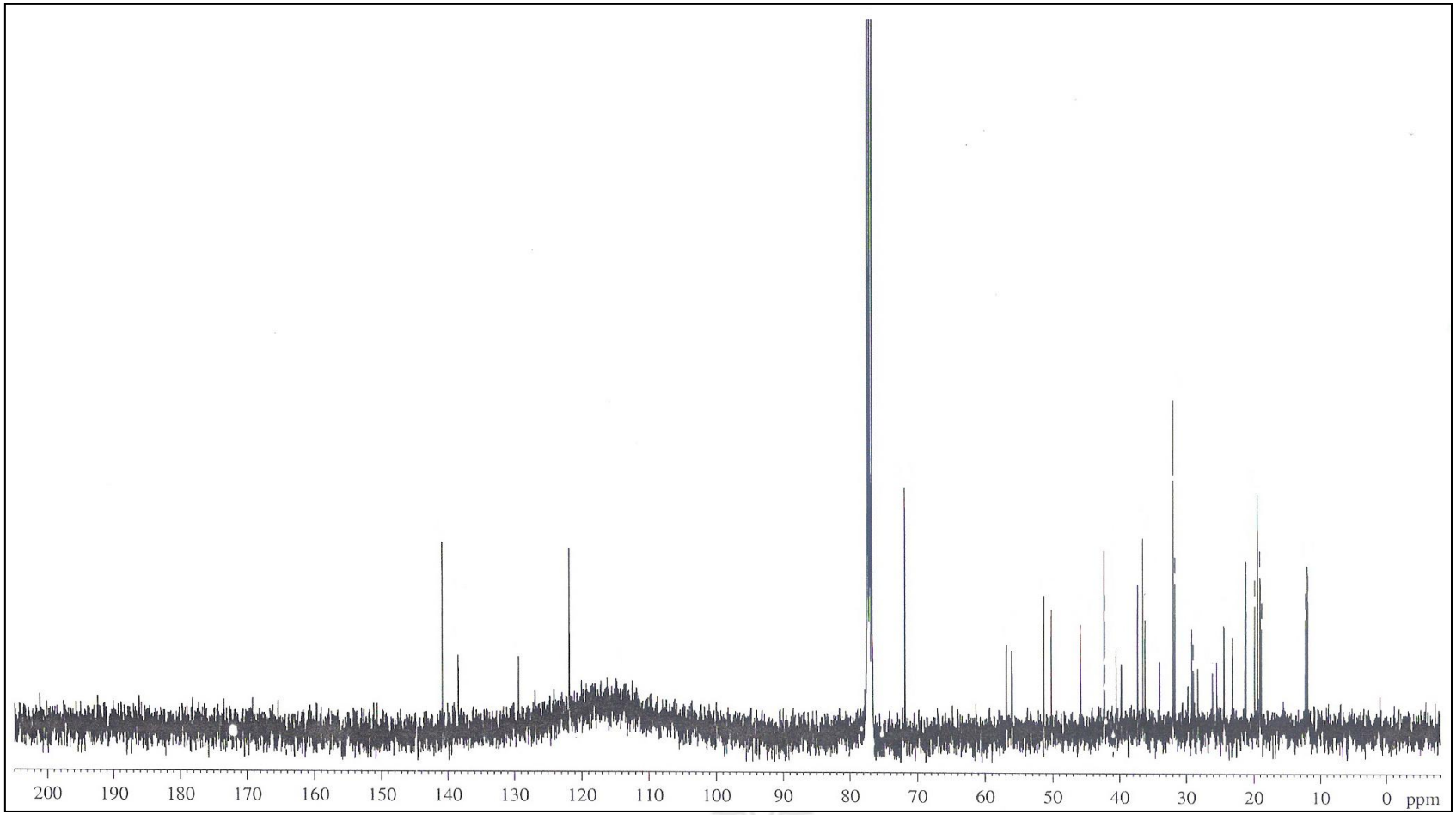

Figure B6 ^{13}C NMR (75 MHz) ($CDCl_3$) spectrum of **AMM7**

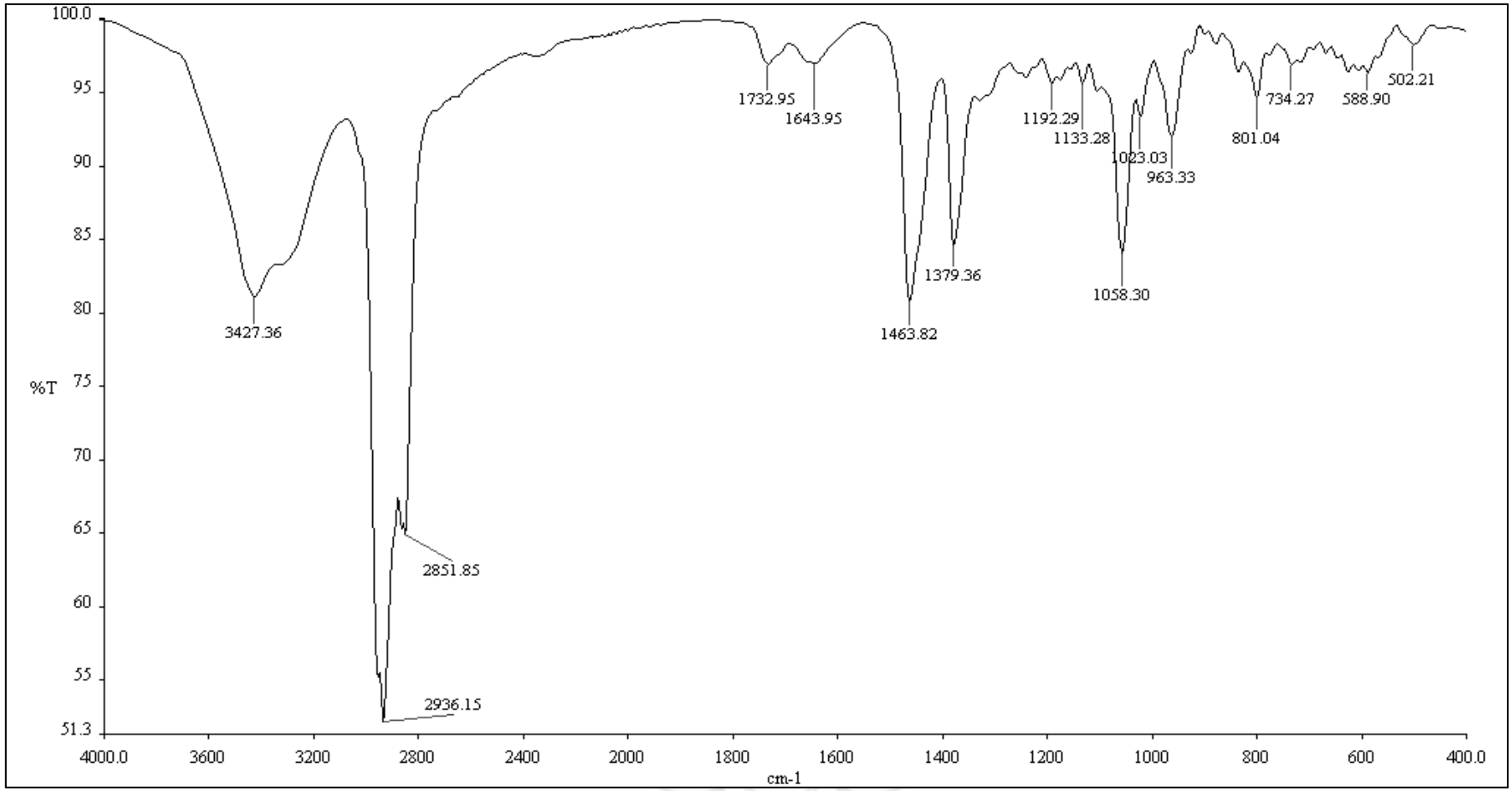

Figure B7 IR (KBr) spectrum of **AMM8**

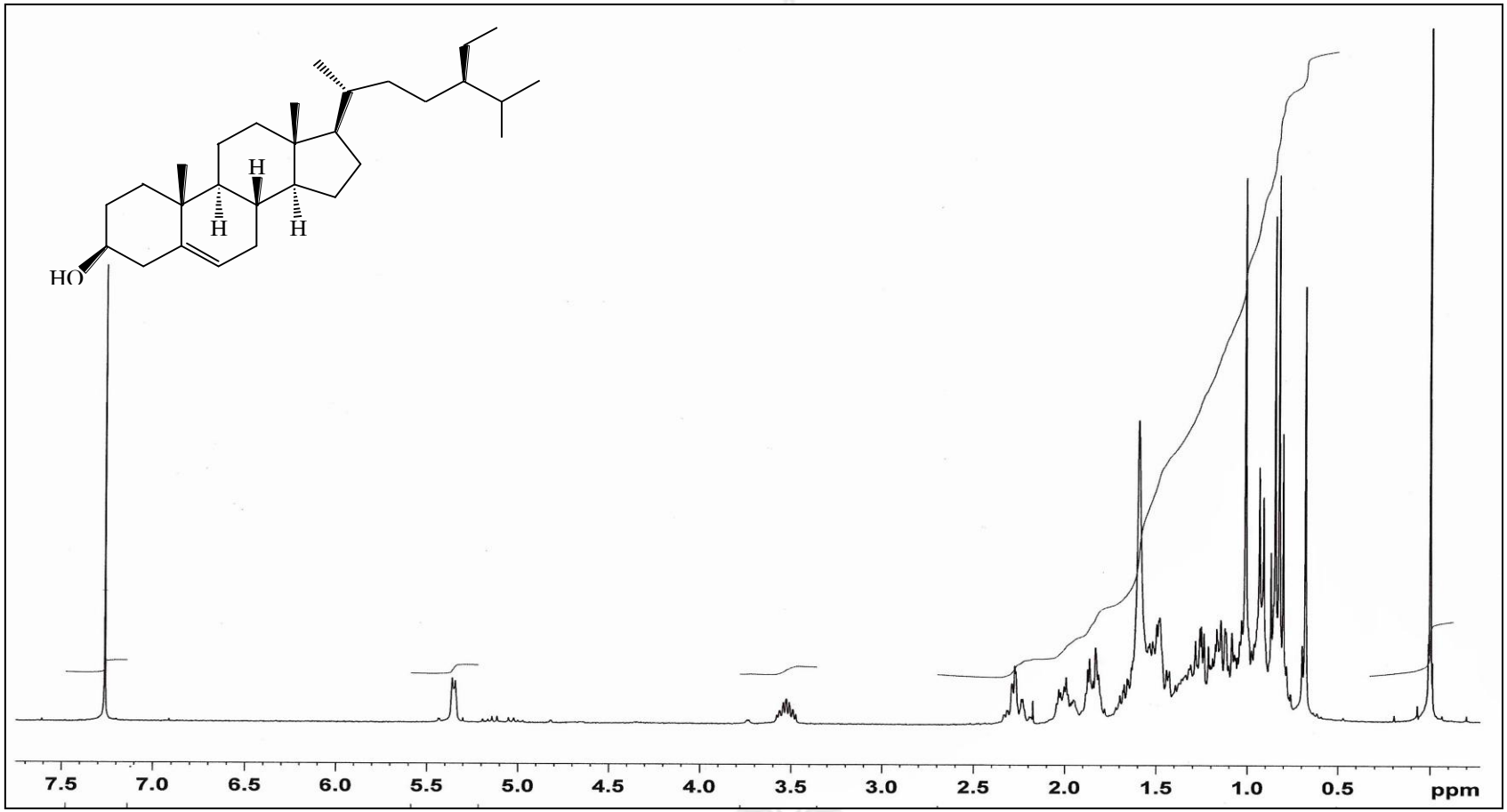

Figure B8 ^{1}H NMR (300 MHz) ($CDCl_3$) spectrum of **AMM8**

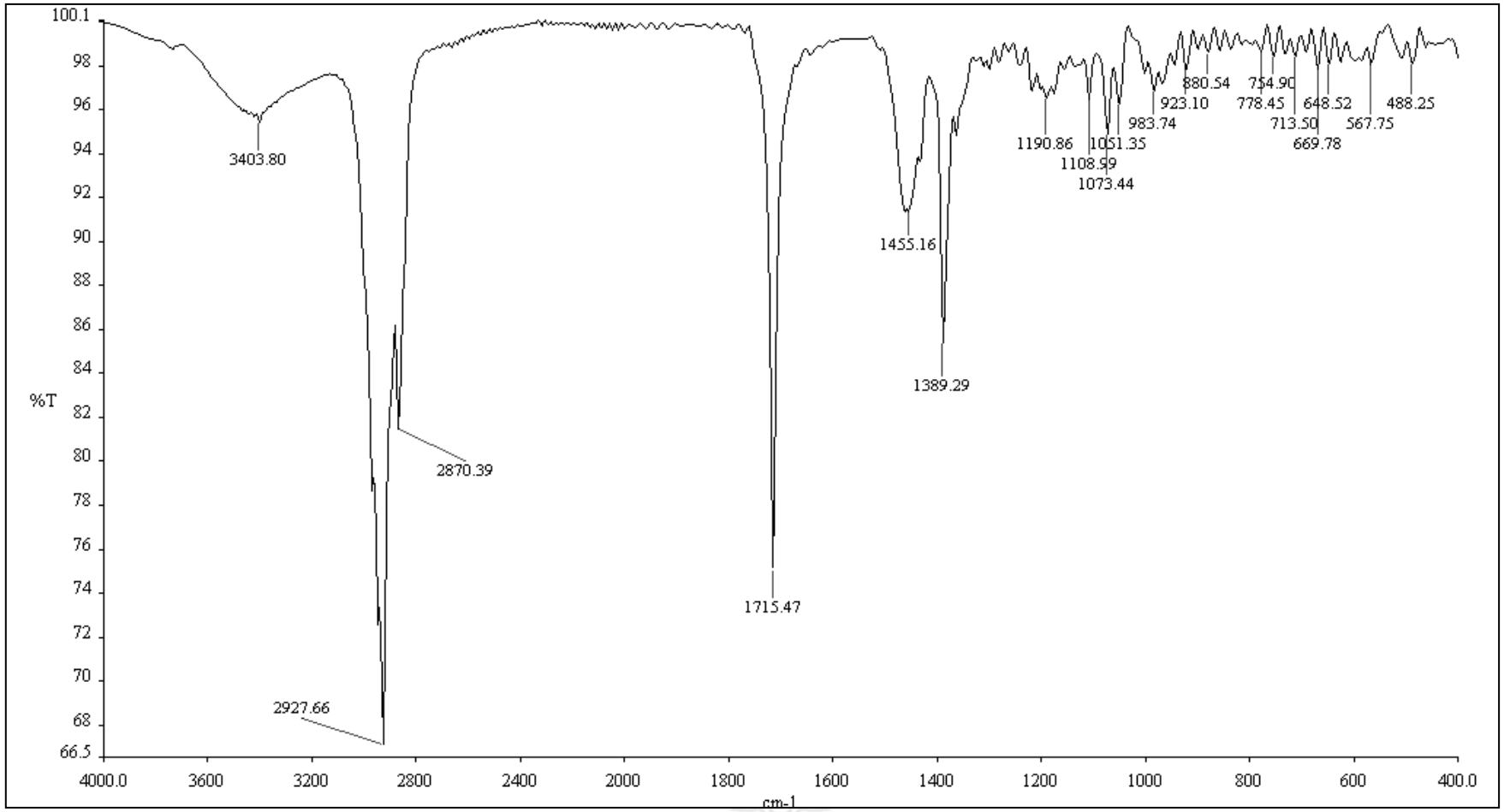

Figure B9 IR (KBr) spectrum of **CMM1**

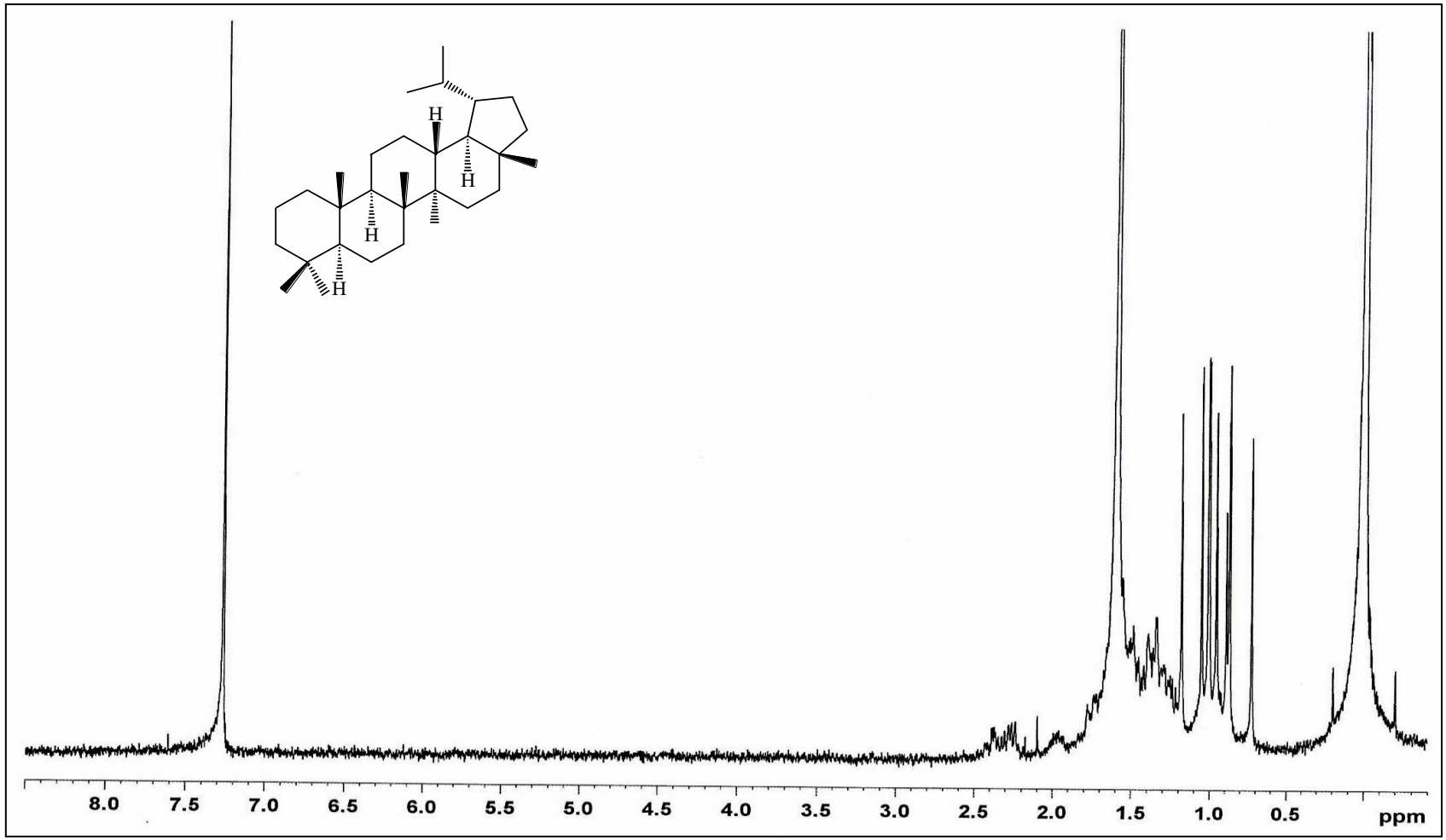

Figure B10 ^{1}H NMR (300 MHz) ($CDCl_3$) spectrum of **CMM1**

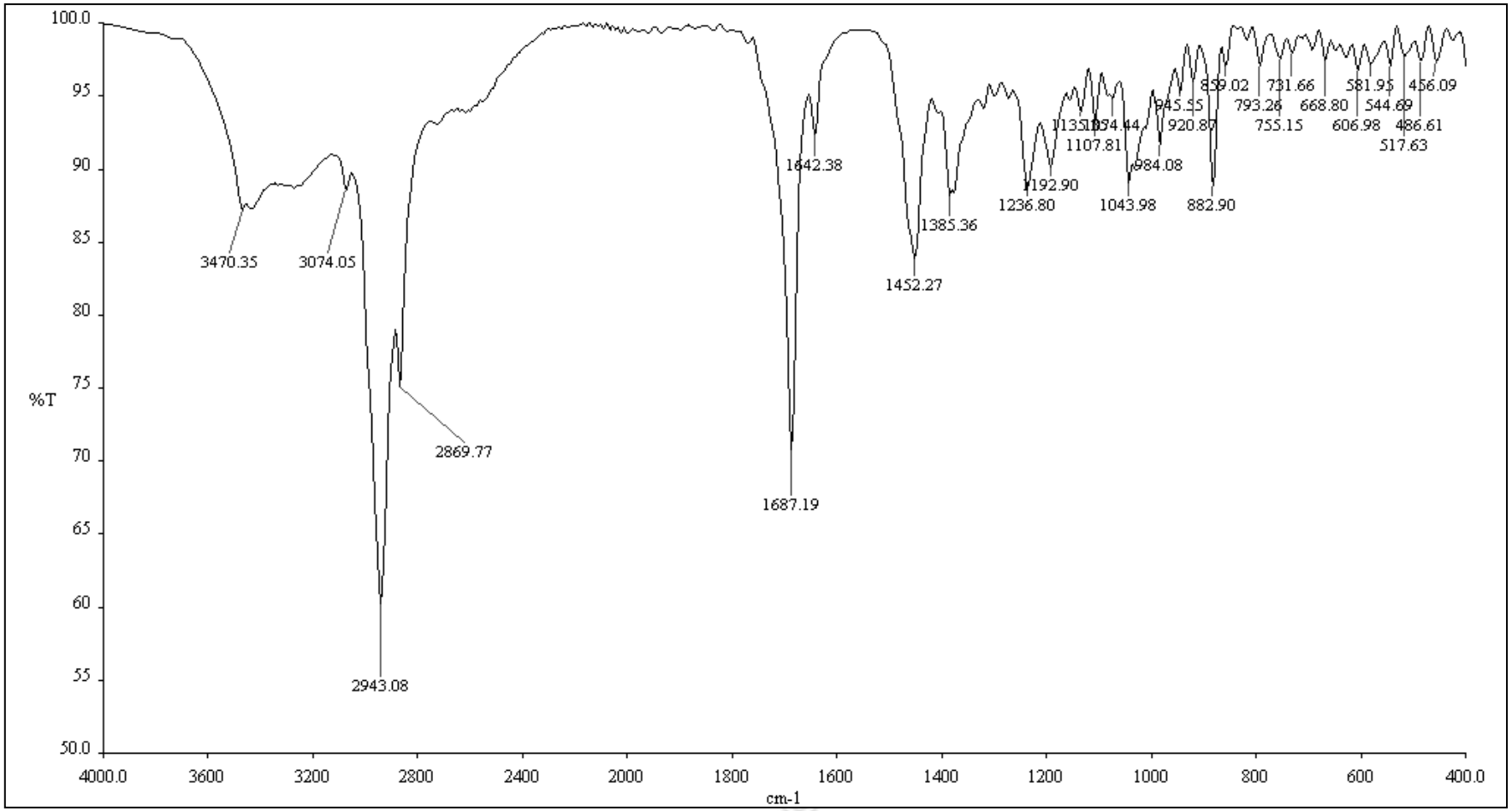

Figure B11 IR (KBr) spectrum of **CMM10**

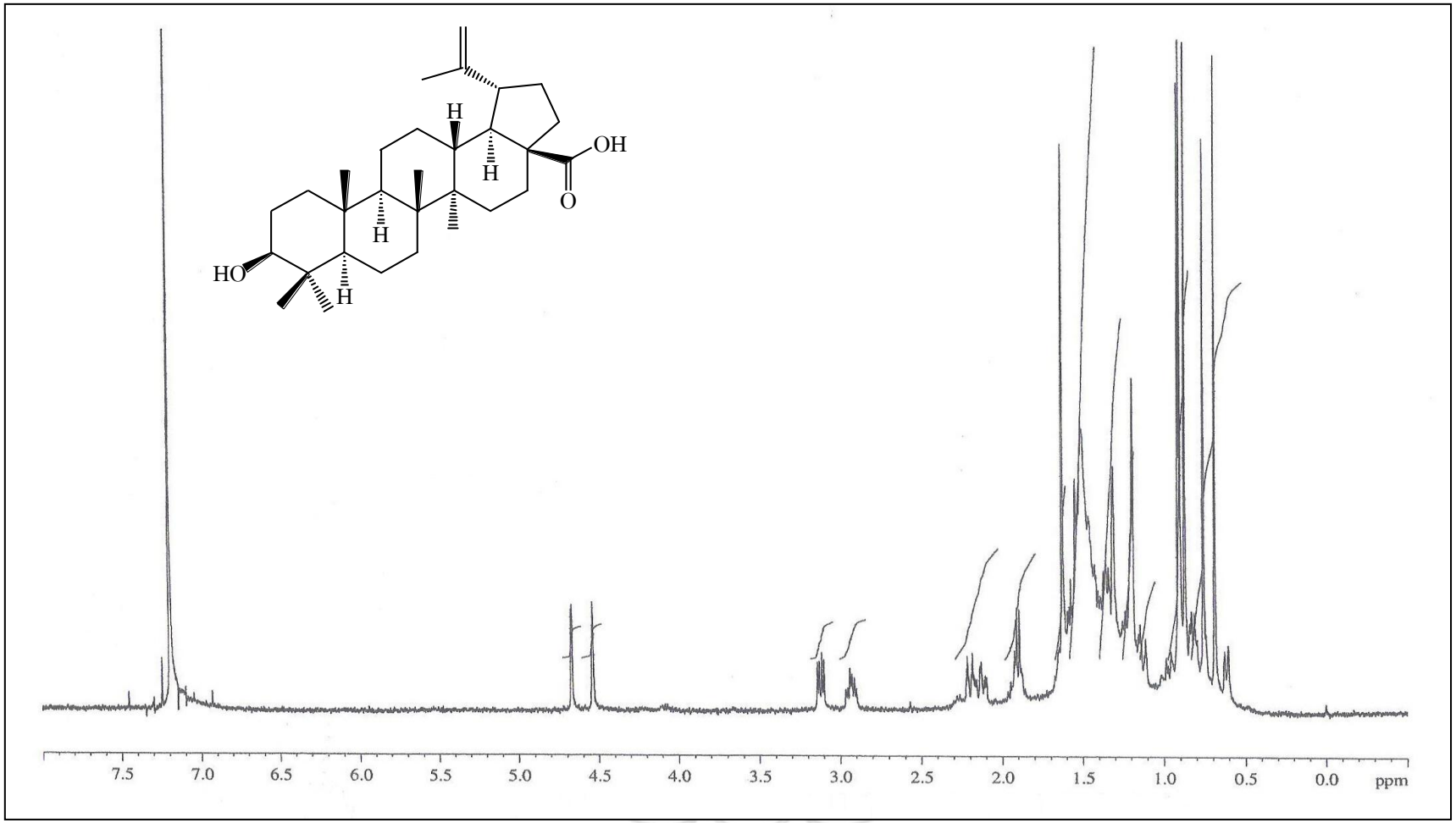

Figure B12 ^{1}H NMR (400 MHz) ($CDCl_3$) spectrum of **CMM10**

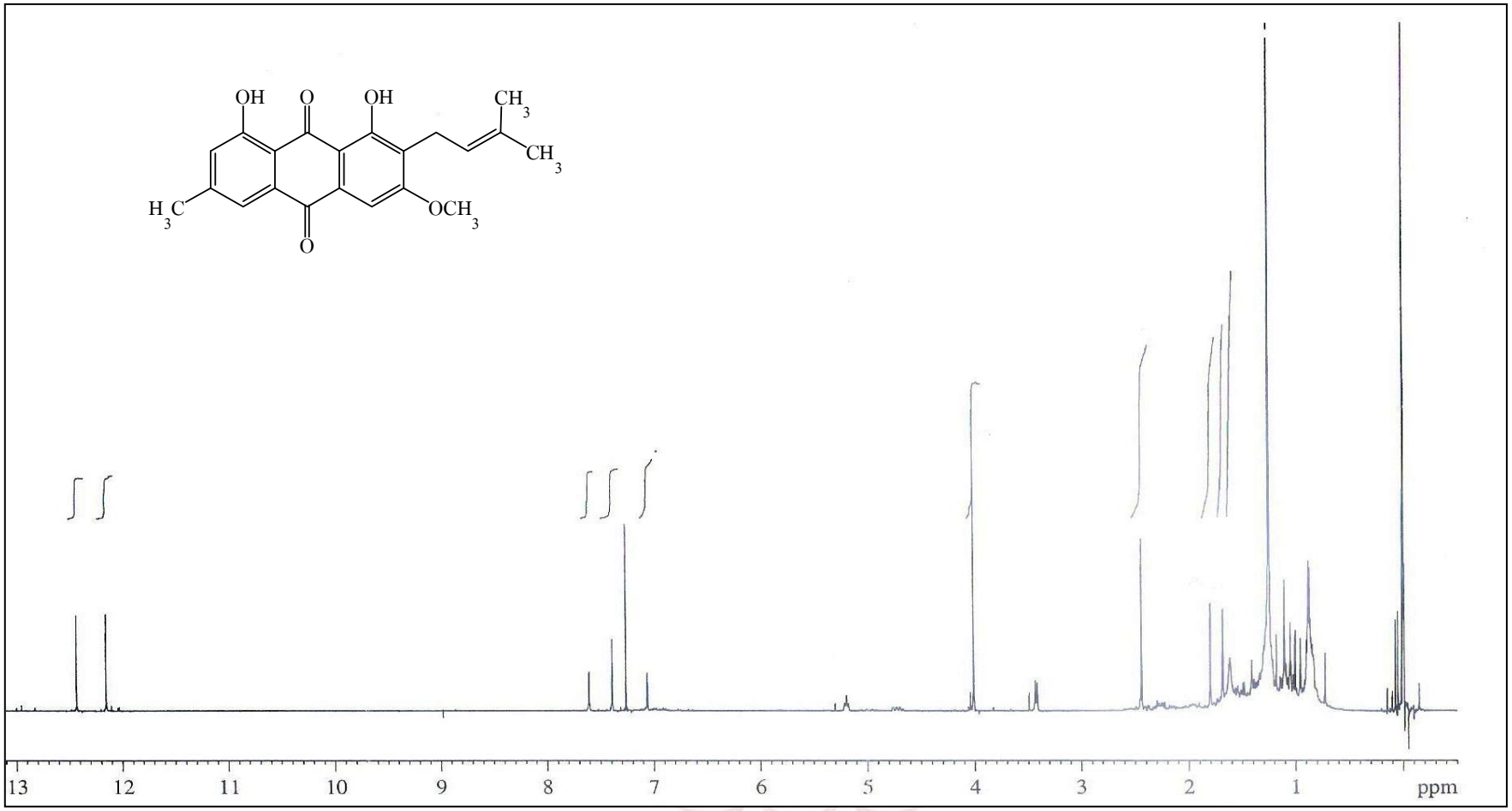

Figure B13 ^{1}H NMR (400 MHz) ($CDCl_3$) spectrum of **CMM6**

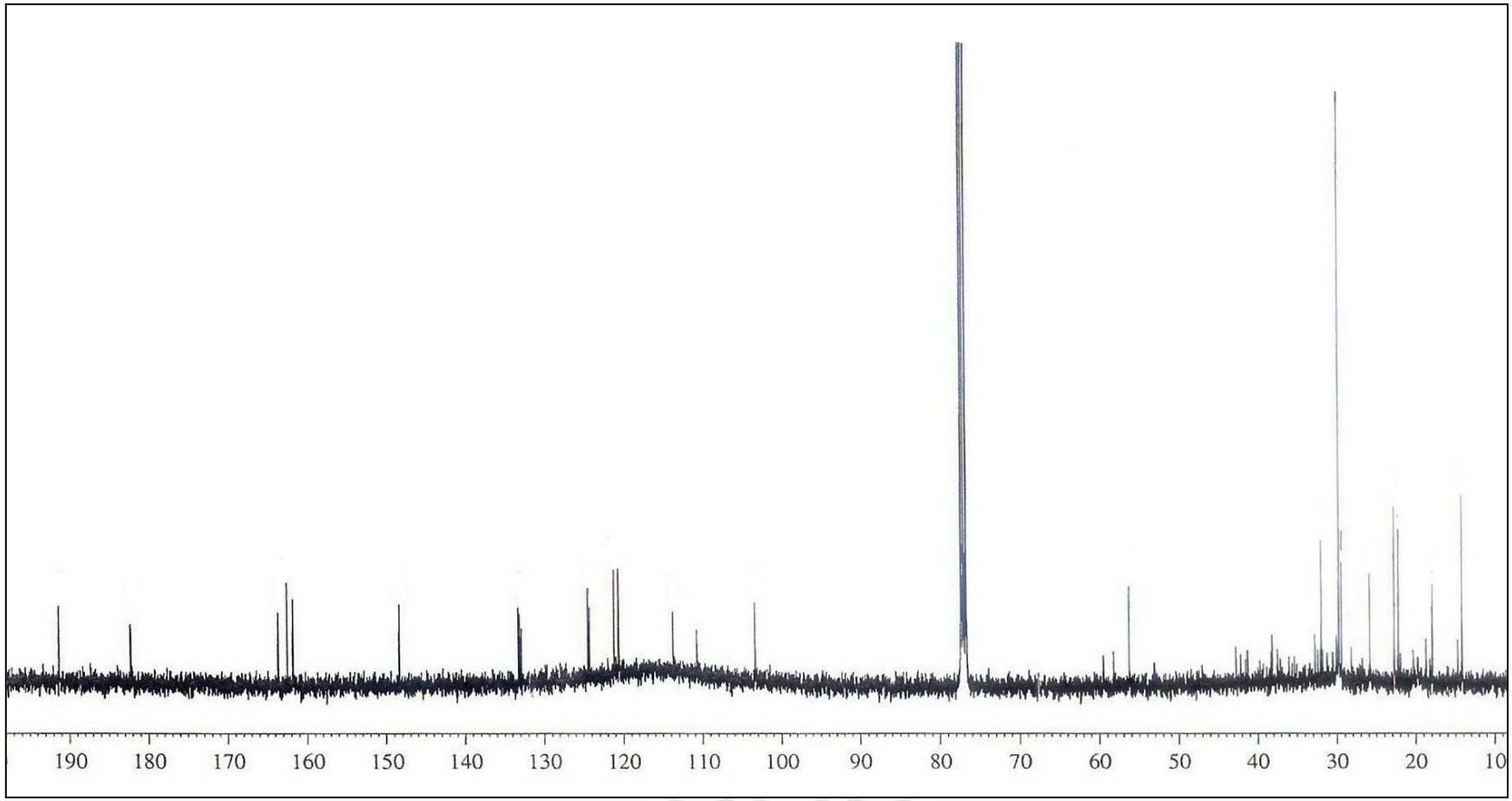

Figure B14 ^{13}C NMR (100 MHz) ($CDCl_3$) spectrum of **CMM6**

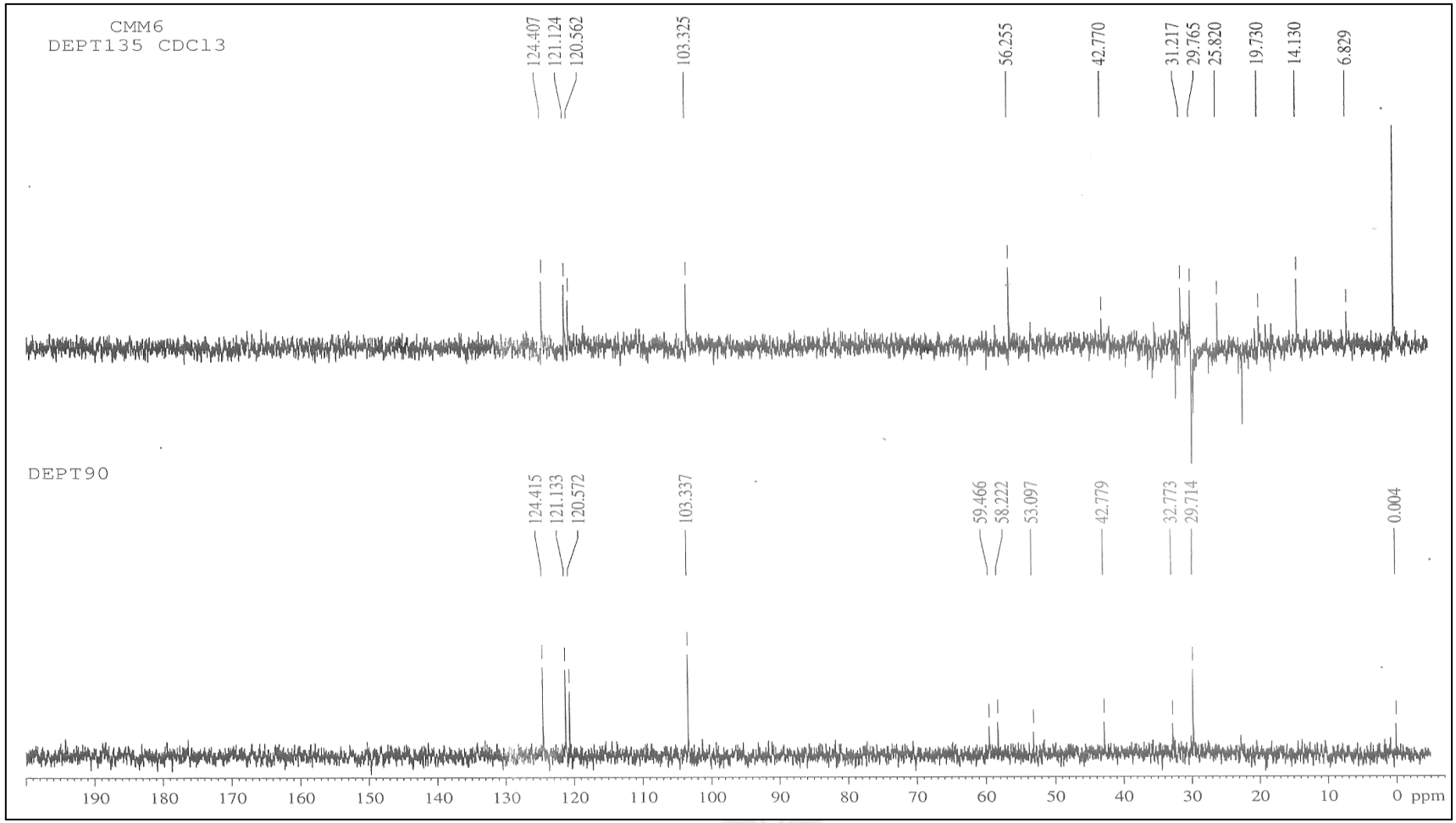

Figure B15 DEPT 135° and DEPT 90° ($CDCl_3$) spectrum of **CMM6**

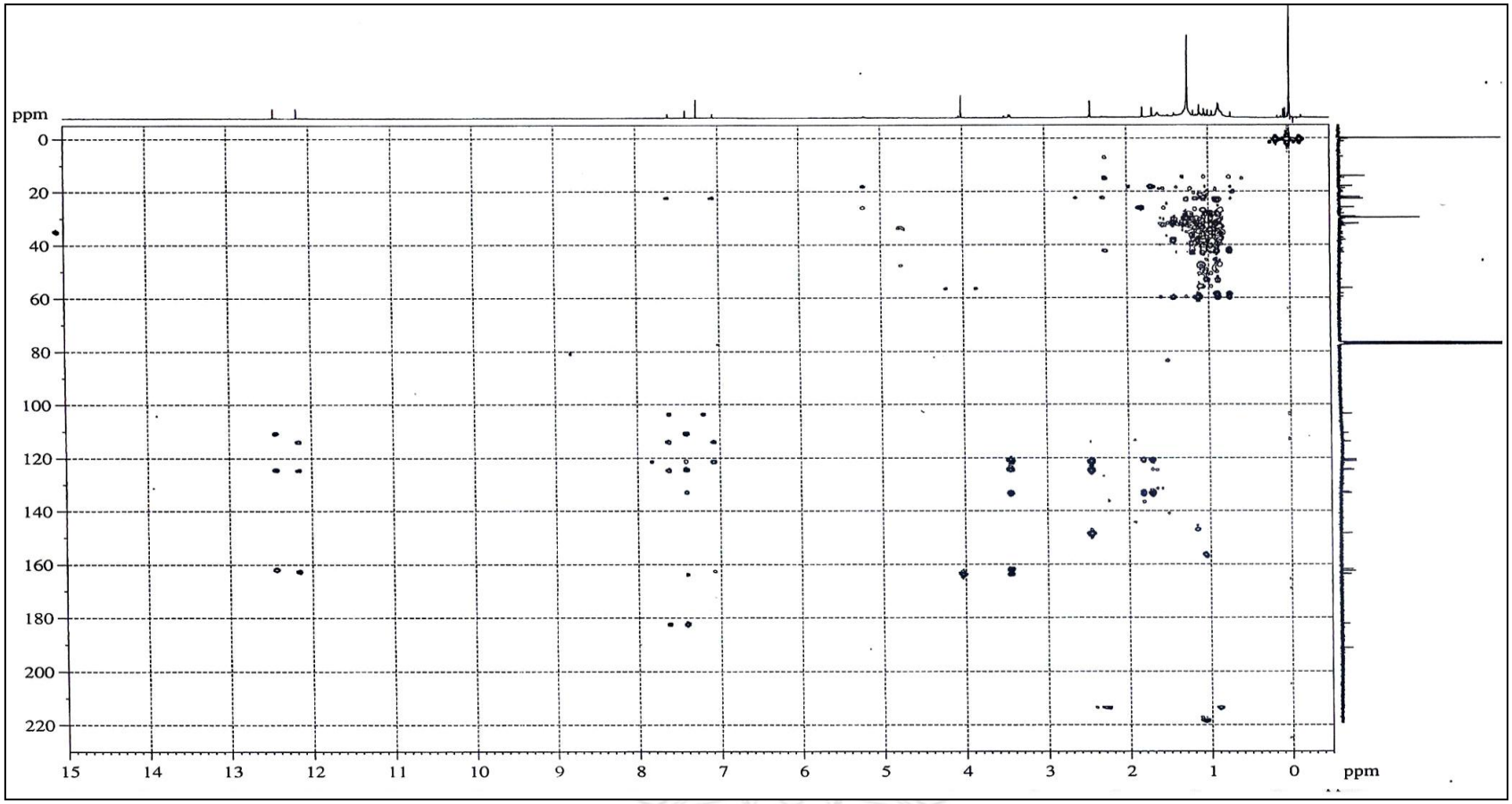

Figure B16 HMBC correlations of **CMM6**

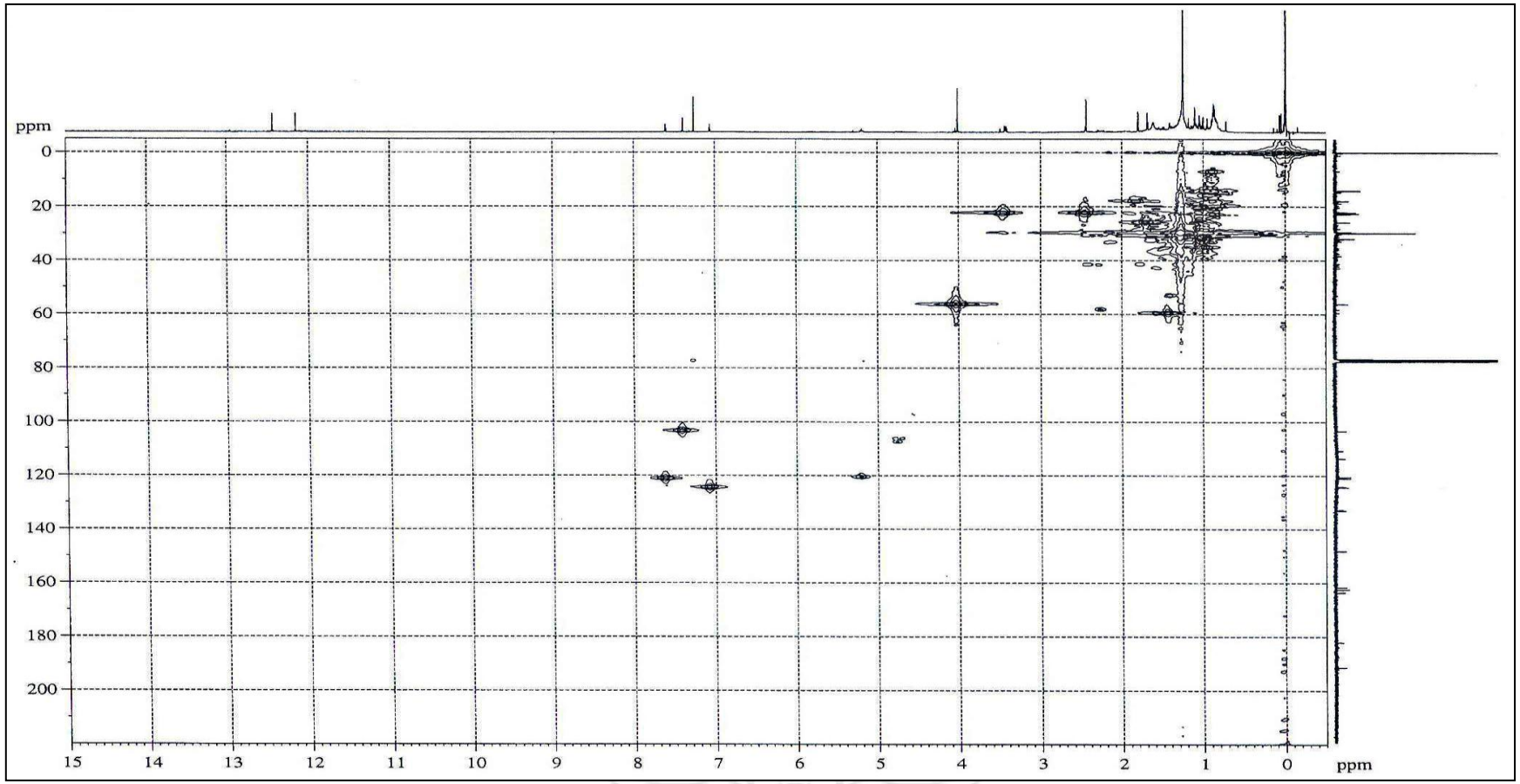

Figure B17 HMQC correlations of **CMM6**

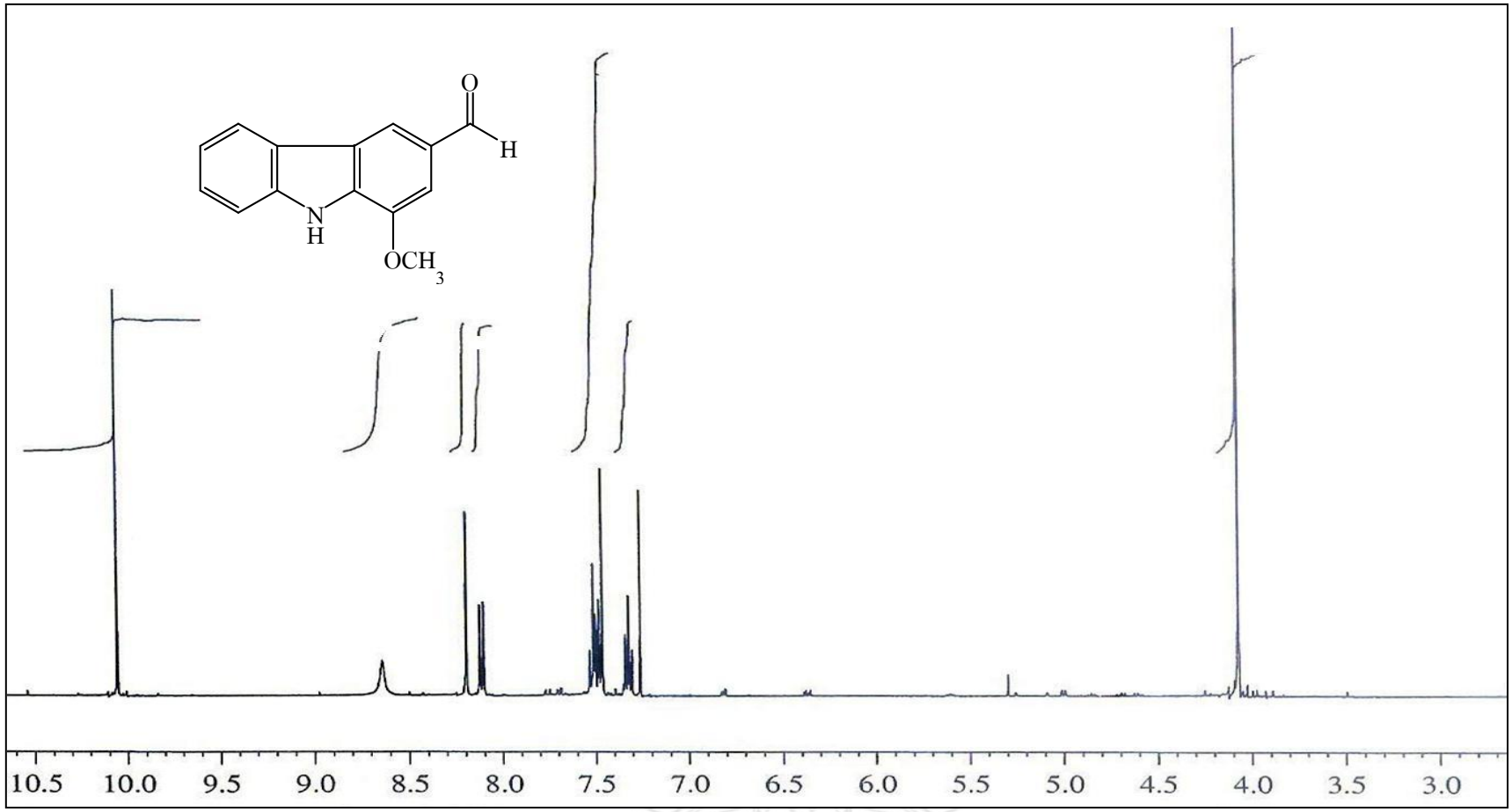

Figure B18 ^{1}H NMR (400 MHz) ($CDCl_3$) spectrum of **CMM38**

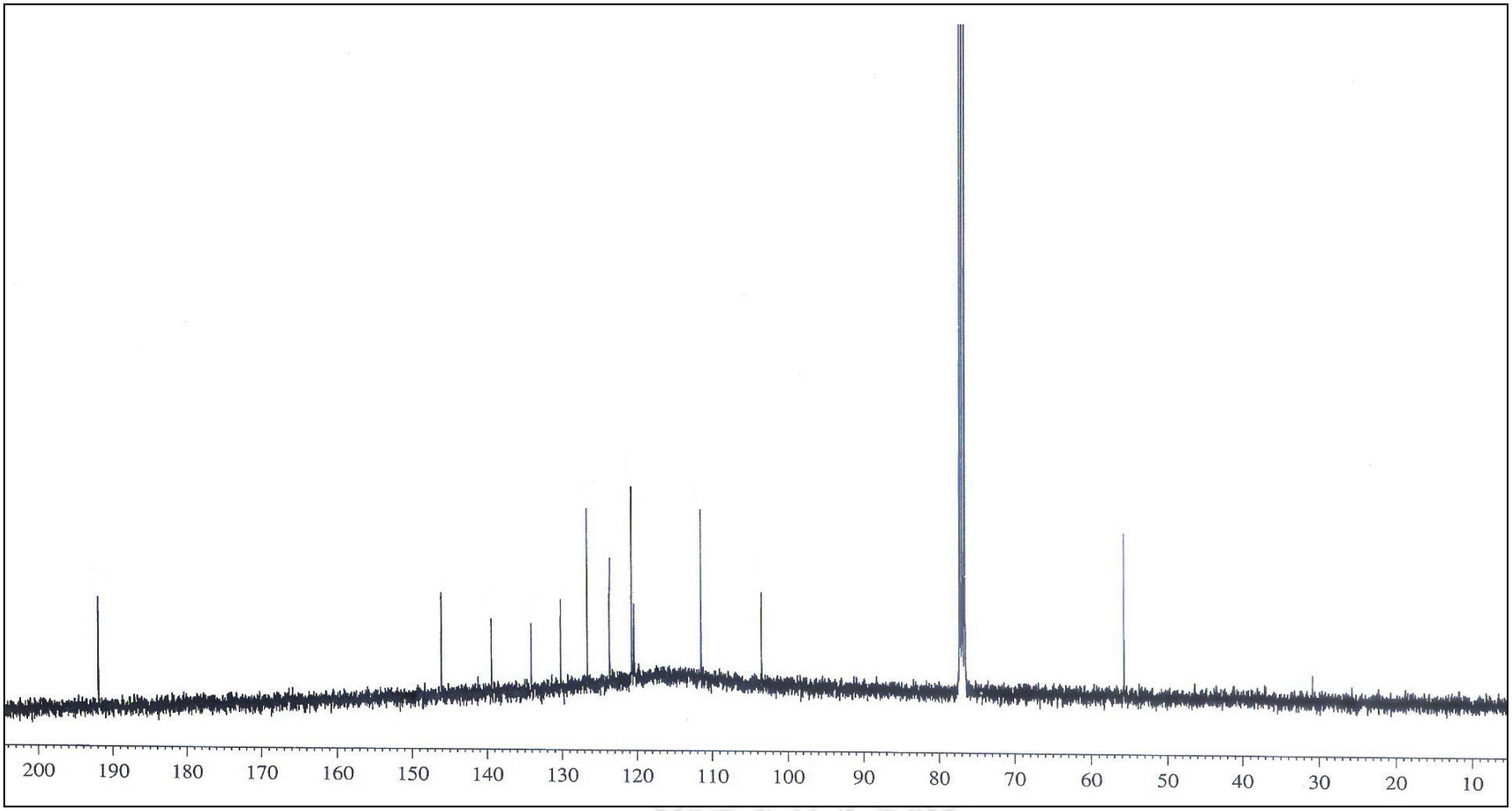

Figure B19 ^{13}C NMR (100 MHz) ($CDCl_3$) spectrum of **CMM38**

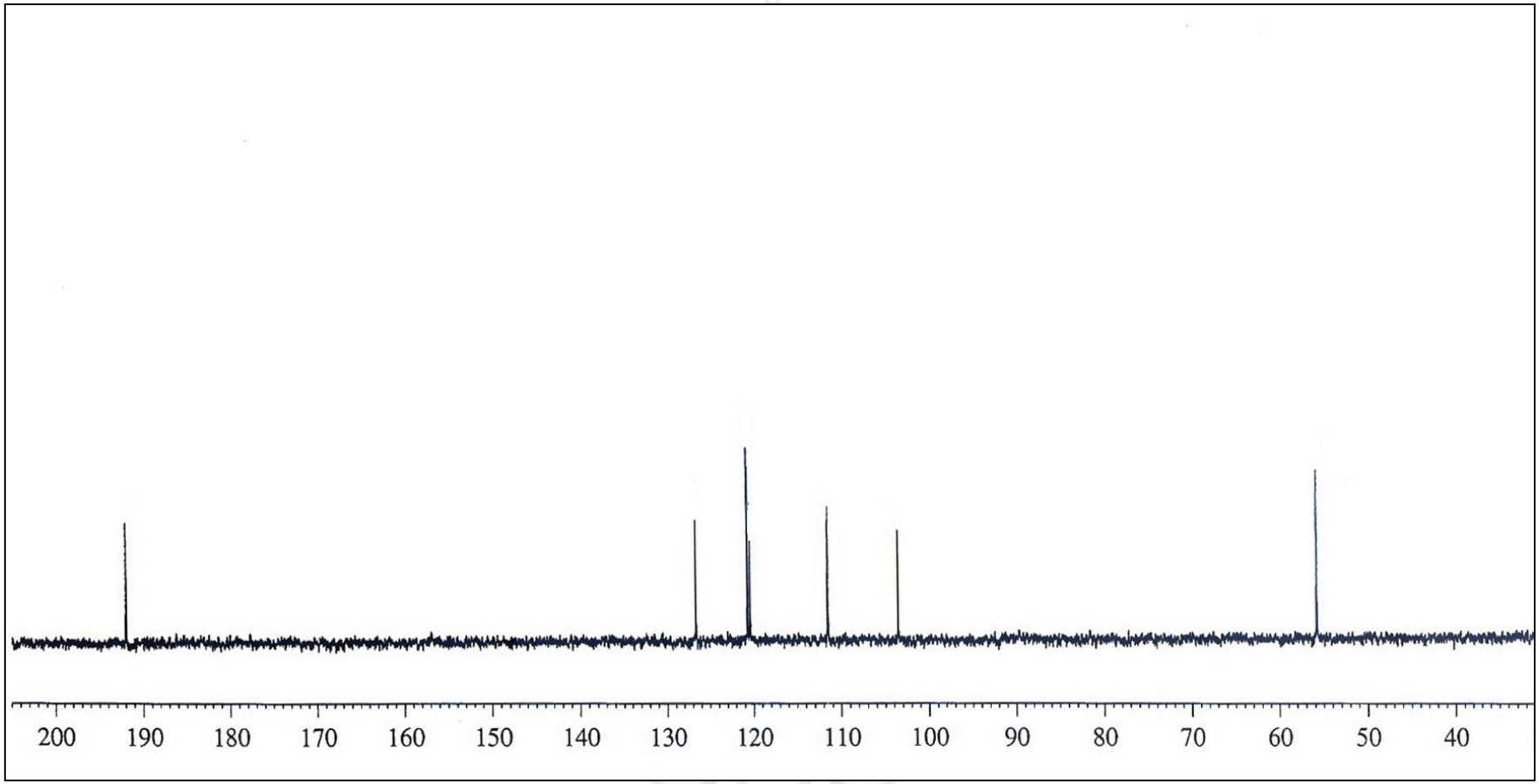

Figure B20 DEPT 135° ($CDCl_3$) spectrum of **CMM38**

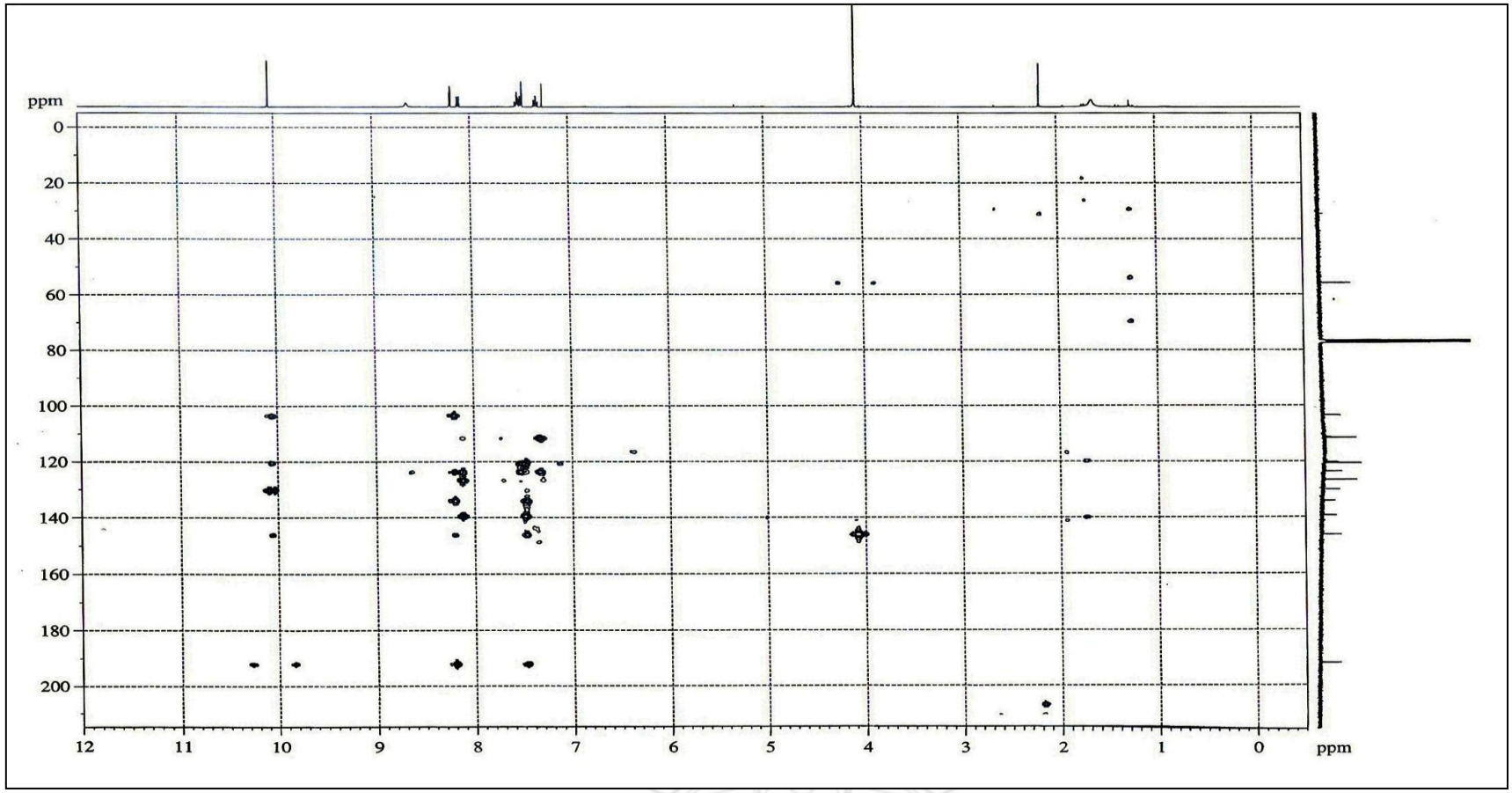

Figure B21 HMBC correlations of **CMM38**

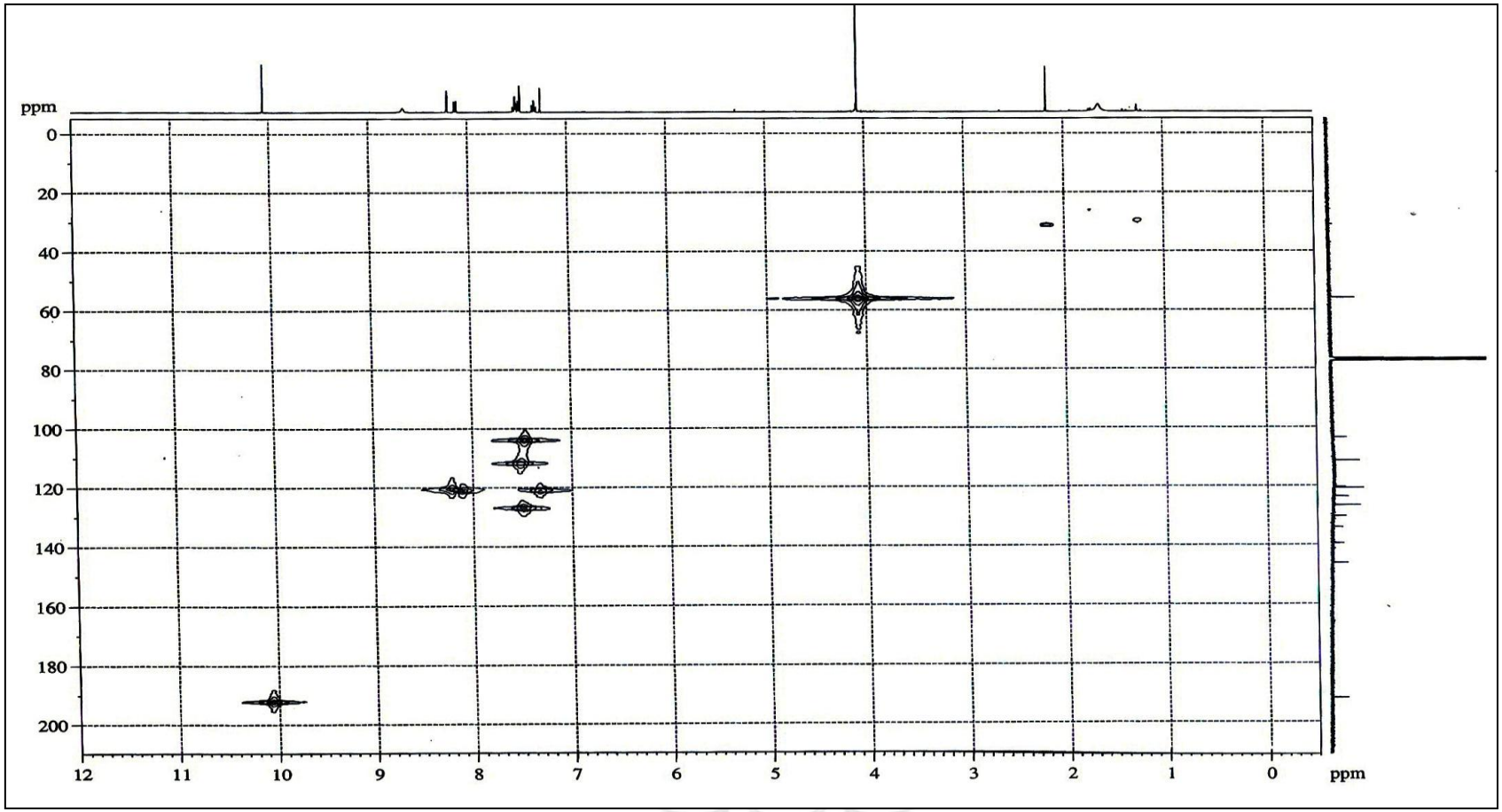

Figure B22 HMQC correlations of **CMM38**

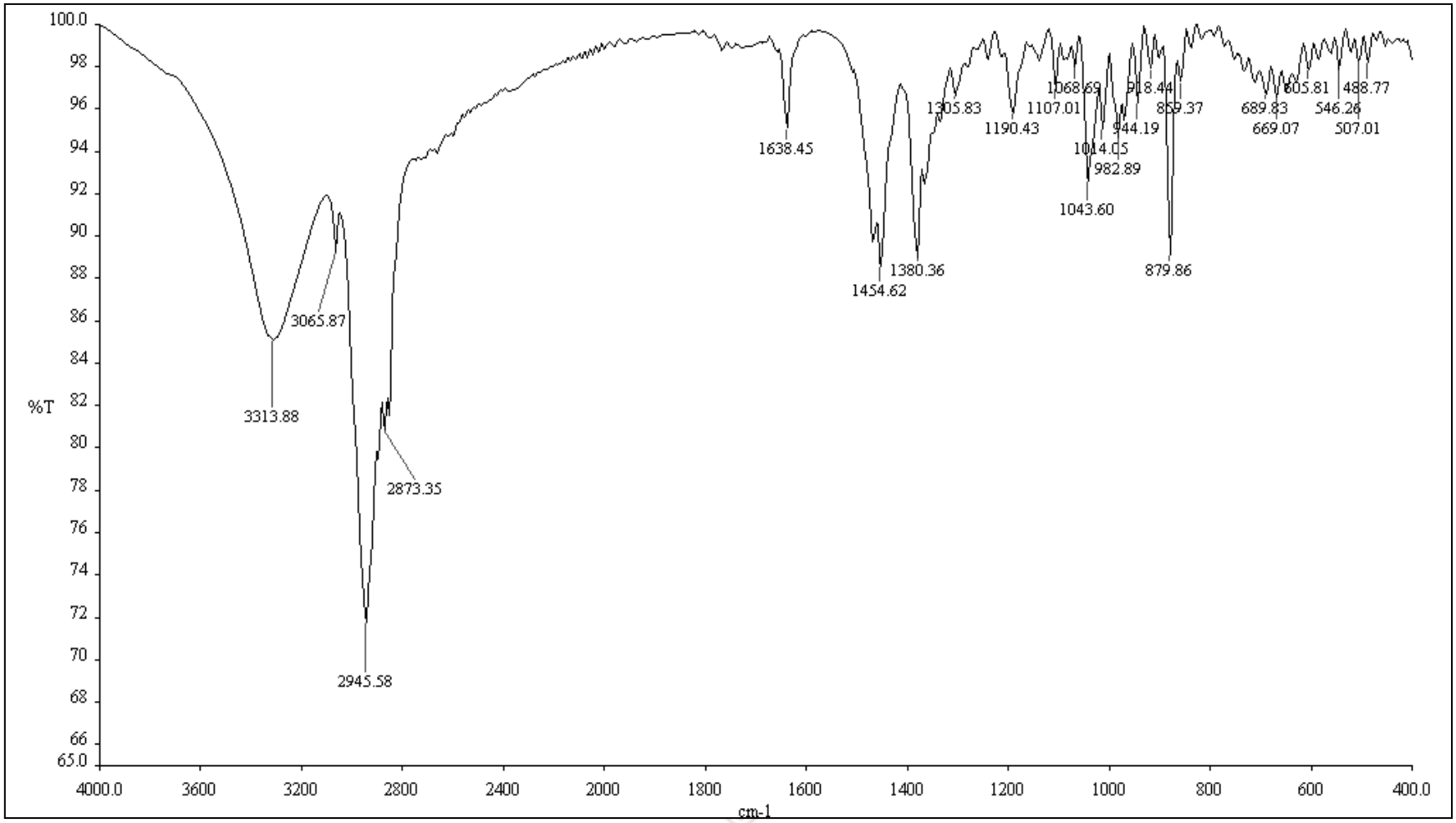

Figure B23 IR (KBr) spectrum of **DMM1**

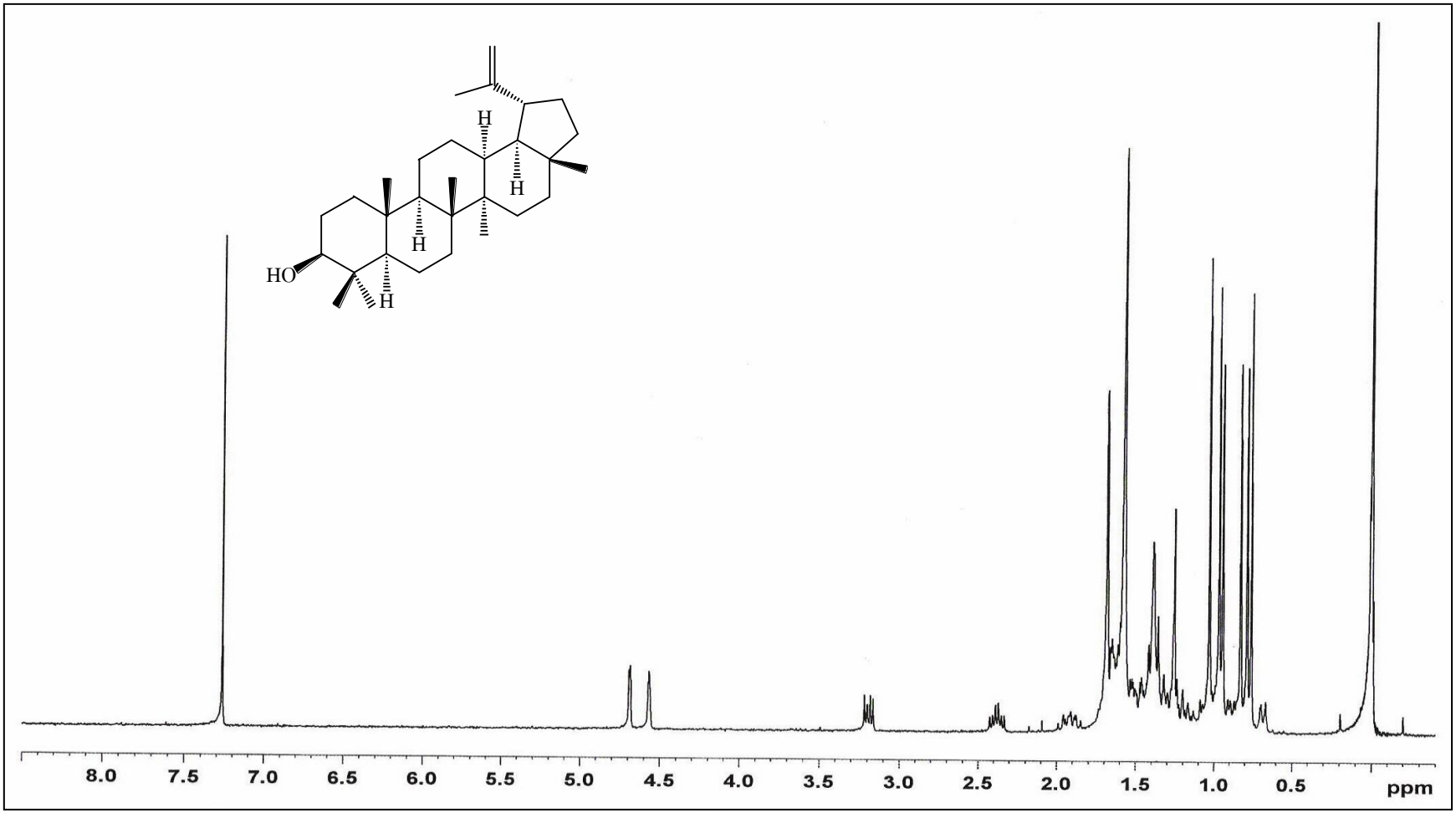

Figure B24 ^{1}H NMR (300 MHz) ($CDCl_3$) spectrum of **DMM1**

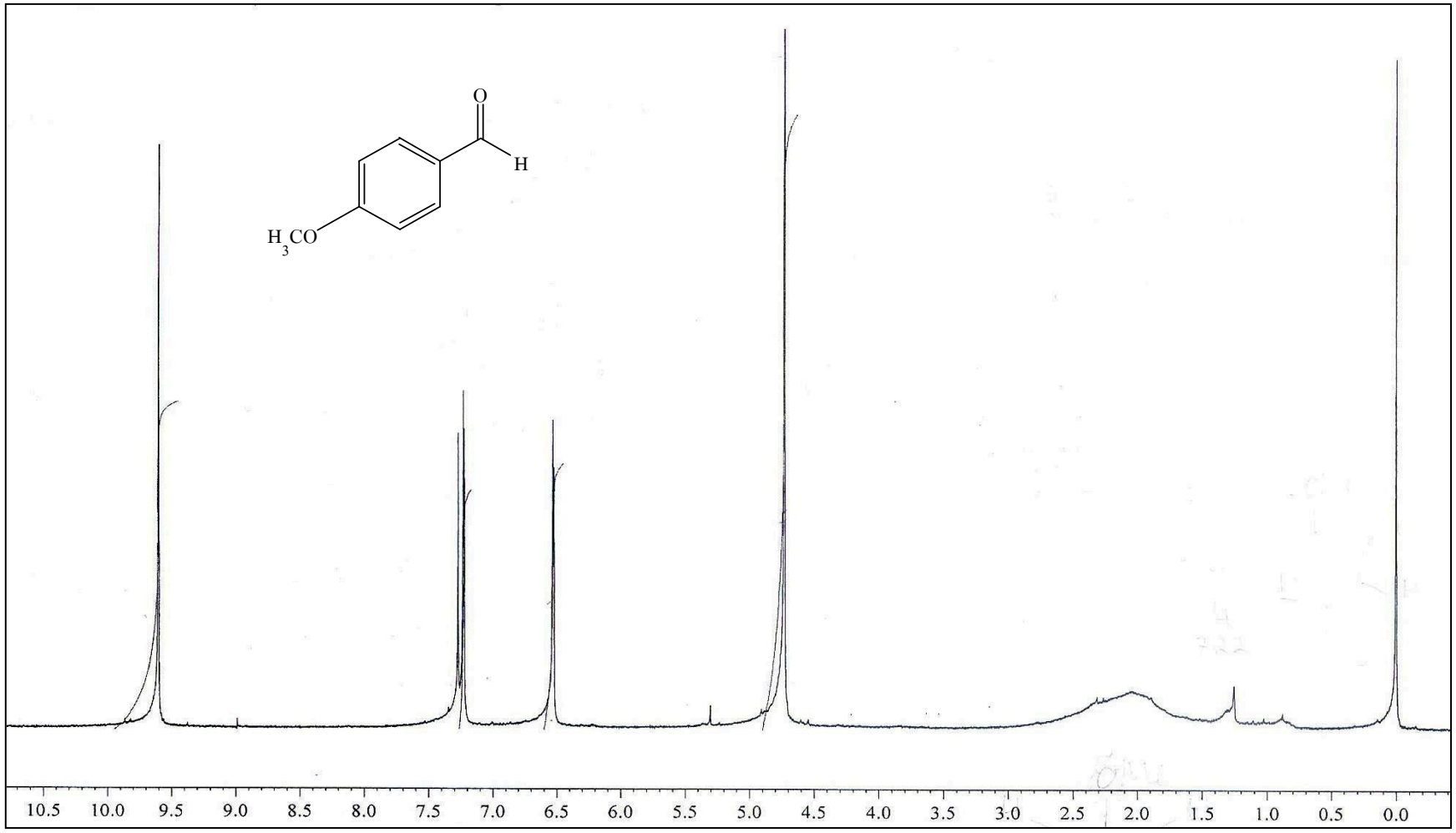

Figure B25 ^{1}H NMR (400 MHz) ($CDCl_3$) spectrum of **FMM2**

CURRICULUM VITAE

CURRICULUM VITAE

NAME Ms. Achjanee Somsri

DATE OF BIRTH 3 August 1982

ADDRESS 59/7 Pasang, Dokkhamtai, Phayao 56120

EDUCATIONAL BACKGROUND

2004 Bachelor of Science
Major in Food Technology
Mae Fah Luang University

WORK EXPERIENCE

2005 Production supervisor
Cal Intertrade Co., Ltd.