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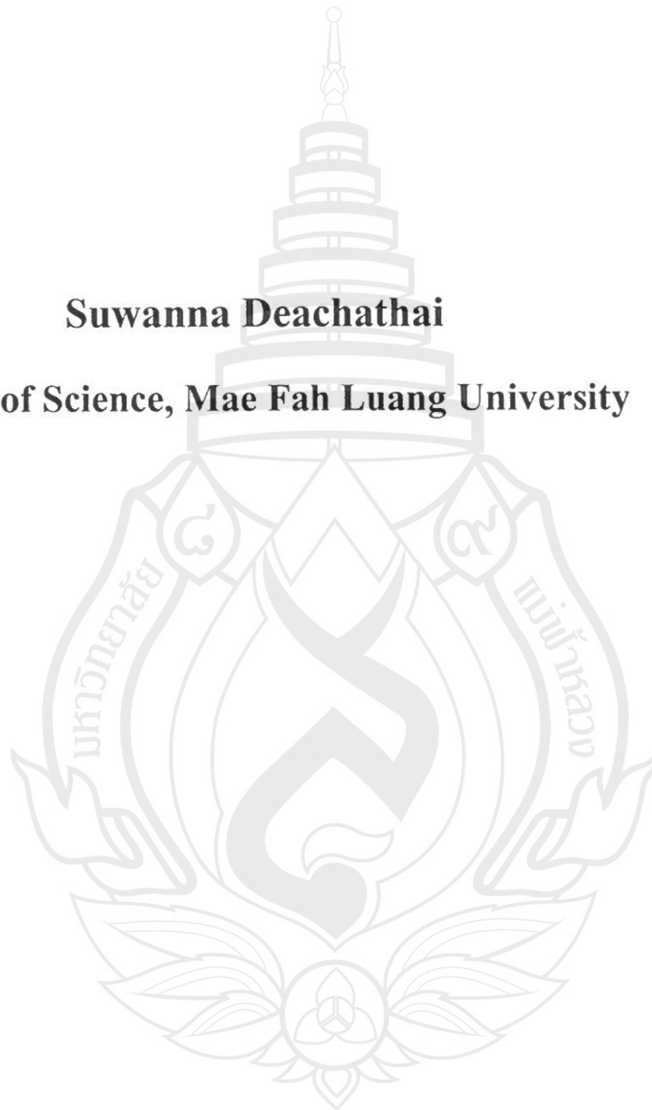
Chemical Constituents from the Roots of *Mucuna macrocarpa* Wallich



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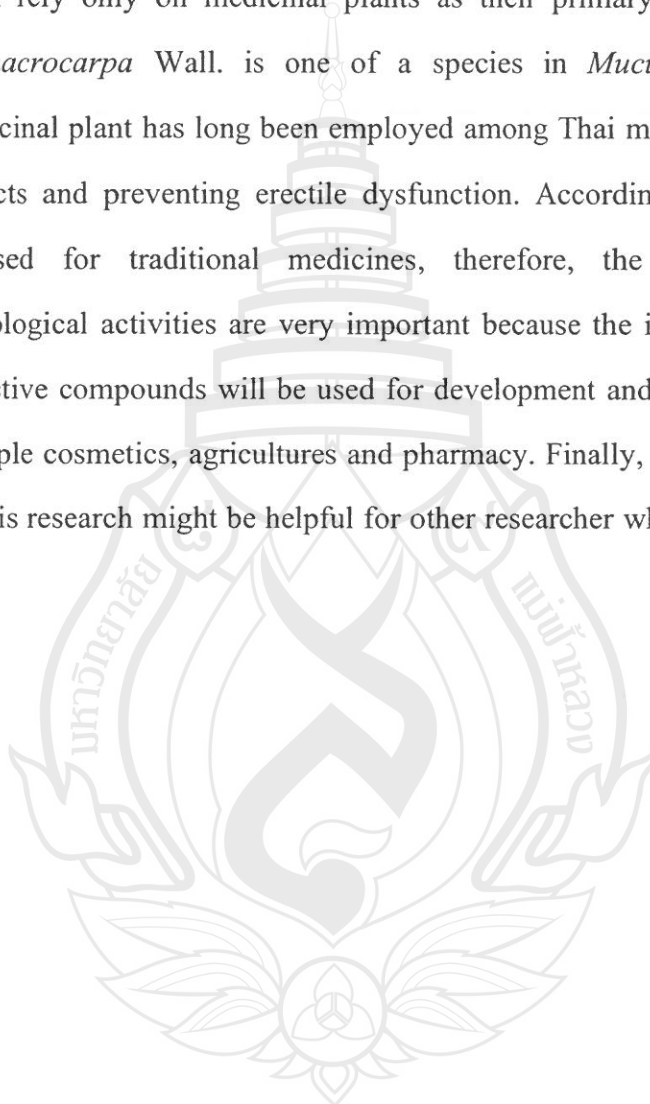
from Mae Fah Luang University

2010

PREFACE

Plants have been used worldwide in traditional medicines for the treatment of diseases. It is estimated that even today approximately two-thirds to three-quarters of the world's population rely only on medicinal plants as their primary source of medicines. *Mucuna macrocarpa* Wall. 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. According to many plants have been used for traditional medicines, therefore, the study of phytochemistry and biological activities are very important because the information from the study of bioactive compounds will be used for development and apply into related fields, for example cosmetics, agricultures and pharmacy. Finally, I hope that the information from this research might be helpful for other researcher who needs to use these information.

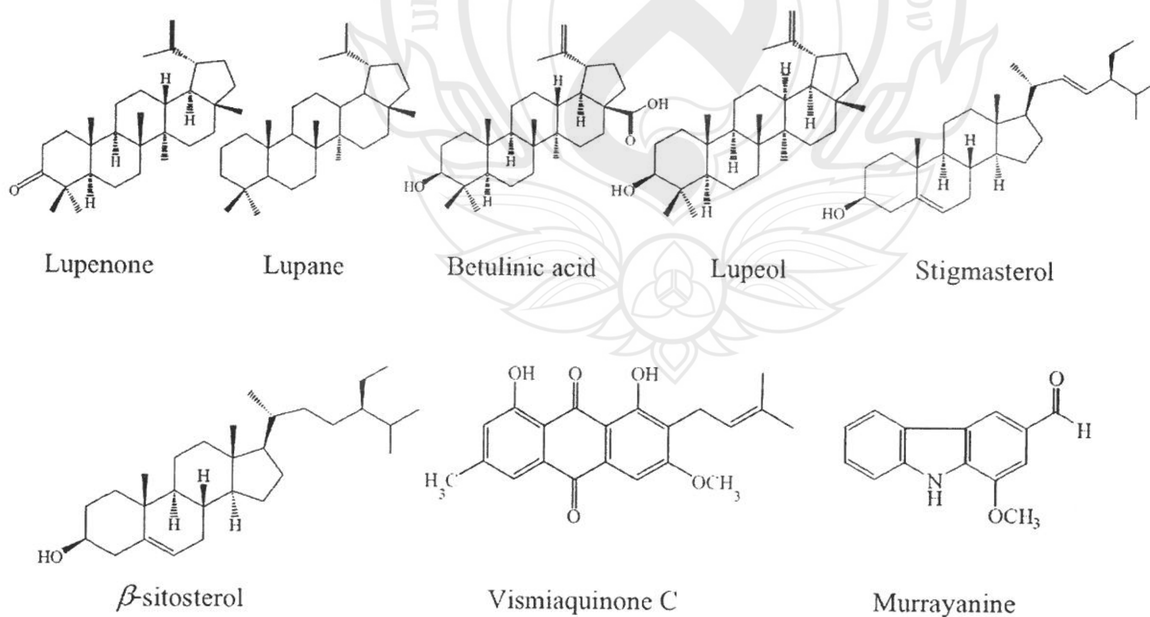


ABSTRACT

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

The crude methanolic extract was found to show strong antioxidation activity (IC_{50} 0.25 μg/mL) better than BHT (IC_{50} 0.41 μg/mL). The crude acetone extract showed good antioxidation activity with IC_{50} 1.19 μg/mL whereas the crude dichloromethane extract showed weakly activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay. Pure compounds showed weak antioxidative activity.

Compounds lupenone and vismiaquinone C exhibited the antibacterial activity against *Bacillus cereus*, *Pseudomonas fluorescens* and *Salmonella typhimurium* with MIC values of 32-64 μg/mL.



ACKNOWLEDGEMENT

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Suwanna Deachathai

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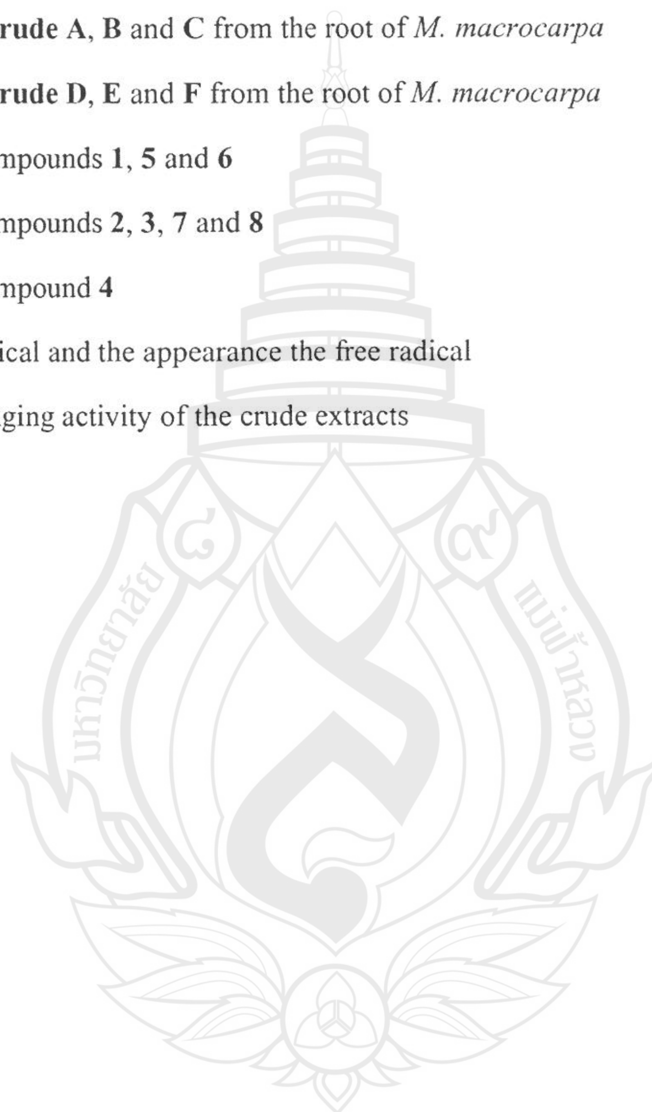


CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

Synthesis of many important drugs makes use of natural product starting materials. Researches are conducted in order to find major constituents with biological activity to be used as drugs or in synthesis of analog or derivatives. Pure compounds extracted from many plants and many parts of the plants are explored and tested for biological activities. However, elucidation of chemical constituents from natural products and biological activity testing are only the initial step in the process of study to find new compounds and acquire basic knowledge of biological activities. The important process is the application of the knowledge in pharmacology and medicine.

1.2 Objectives

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

1.3 Scope of study

Extraction and isolation of secondary metabolite from the roots of *M. macrocarpa*, Characterization of all isolates by spectroscopic methods (UV, IR and NMR) and evaluation of antioxidative and antimicrobial activities of crude extracts and pure compounds.

1.4 Benefit

M. macrocarpa which is one of the medicinal plants, was investigated for the chemical constituents and biological activities. Therefore, some active compounds might be applied into the cosmetic, pharmacy or agriculture. This work might be published in international journals.

1.5 Abbreviations and Symbols

s = *singlet*

d = *doublet*

t = *triplet*

m = *multiplet*

br s = *broad singlet*

dd = *doublet of doublet*

kg = kilogram

g = gram

mg = milligram

µg = microgram

mM = millimolar

µM = micromolar

mL = milliliter

µL = microliter

cm^{-1} = reciprocal centimeter (wave number)

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

CHAPTER 2

LITERATURE REVIEWS

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 later 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 (Cherdshewasart *et al.,* 2004b).

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 woodland of tropical areas. General characteristics of Black Kwao Krua including:

Leave: Semi-leathery, with rushedbrown backs, terminal leaflet in long elliptical shape, cuspidate, (Chi-Wen Kuo *et al.,* 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 *et al.,* 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.

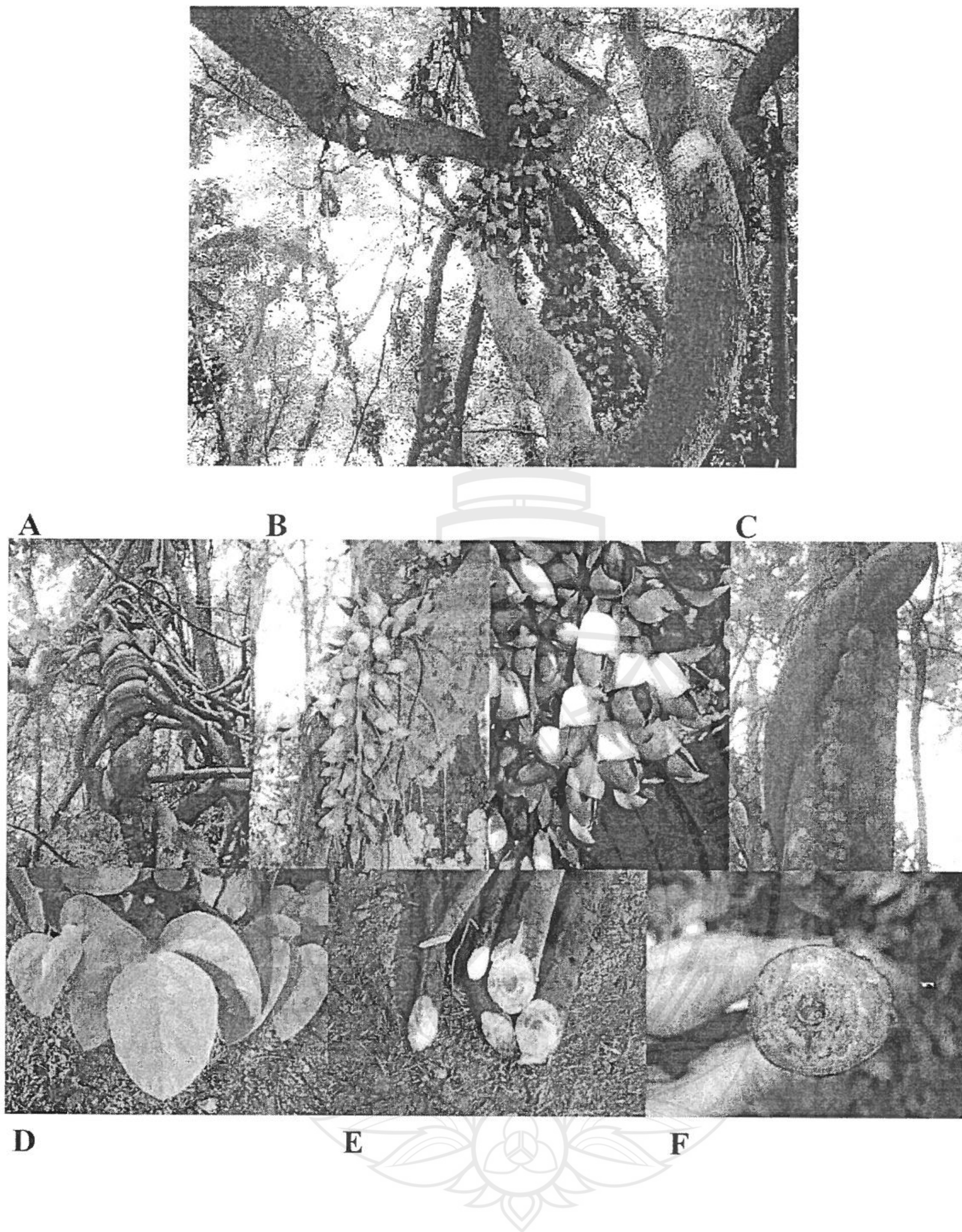


Figure 1 *Mucuna macrocarpa* Wall.

(**A**: climber stem, **B**: mature flowers, **C**: green fruit, **D**: trifoliate leaves,

E: stem-like tuber, **F**: fluid is exuded from the stem)

2.2 Chemical constituents isolated 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 1** shows the chemical constituents isolated from *Mucuna* genus.

Table 1 Compounds isolated from the *Mucuna* genus

Scientific name (investigated part)	Compound	Structure	References
M. acuminata			
(seed)	L-dopa	1	Lubis *et al.*, 1981
M. aterrima			
(seed)	L-dopa	1	Dexenbuchler *et al.*, 1971; Amarasekera *et al.*, 1980
	3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	2	Amarasekera *et al.*, 1980
	3-Carboxy-1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	3	
(leaves and stem)	Tetracosanolic acid triacontyl ester	-	Nogueira *et al.*, 1996
	Triacontan-1-olalkane	-	
M. birdwoodiana			
(stalk)	3-*O*-(6-*O*-Methyl-β-D-glucuronopyranosyl)asiatic acid	4	Ding *et al.*, 1991

Table 1 (continued)

Scientific name (investigated part)	Compound	Structure	References
M. birdwoodiana (stalk)	3-*O*-(6-*O*-Methyl-β-D-glucuronopyranosyl)-28-*O*-β-D-glucopyranoside	-	Ding *et al*., 1991
	3-*O*-[α-L-Arabinopyranosyl (1→2)]-6-*O*-methyl-β-D-glucuronopyranoside	-	
	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*., 1987a
	N-(*trans*-Feruloyl)tyramine	7	
(seed)	L-dopa	1	Cai *et al.*, 1990
(stem)	2,6-Dimethoxy phenol	8	Goda *et al*., 1987
	Syringic acid	9	
	Valillic acid	10	
M. capitata (seed)	Proteid	-	Katiyar *et al*., 1981
M. cochinchinensis (fruit)	L-dopa	1	Su *et al*., 1992

Table 1 (continued)

Scientific name (investigated part)	Compound	Structure	References
M. currannii (seed)	Hydrocyanic acid	11	Laurena *et al.,* 1994
M. deeringiana (leaves)	Stizolobic acid	12	Ellis *et al.*, 1976
(seed)	3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	2	Amarasekera *et al.*, 1980
	3-Carboxy-1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	3	
M .diabolica (seed)	L-dopa	1	Lubis *et al.*, 1981
M. flagellipes (seed)	Haemagglutinin	-	Mbadiwe *et al.*, 1978
M. gigantea (seed)	L-dopa	1	Lubis *et al.*, 1981
M. holtionii (seed)	L-dopa	1	Dexenbuchler *et al.*, 1971
M. imbricata (seed oil)	Linoleic acid	13	Badami *et al.*, 1975
	Oleic acid	14	
	Palmitic acid	15	
	Stearic acid	16	

Table 1 (continued)

Scientific name (investigated part)	Compound	Structure	References
M. macrocarpa			
(root)	L-dopa	1	Chen *et al*., 1993
(part not specified)	Friedelin	17	Hu *et al*., 1994
	Lupenone	18	
	2,3-Dihydroxypropyl ester hexacosanoic acid	19	
	2,3-Dihydroxypropyl ester pentacosnoic acid	20	
M. macrophylia			
(seed)	L-dopa	1	Lubis *et al*., 1981
	Tannins	-	Debelmas *et al*., 1973
	Lecithin	21	Panikkar *et al*., 1987
	Alkaloid P-R	22-24	Rakshit *et al*., 1956
	Alkaloid S	-	
	Alkaloid X	25	
	Prurienidine	-	
	threo-12,13-Dihydroxy-*cis*-9-octadec enoic acid	-	Hasan *et al*., 1980
	cis-12,13-Epoxy-*trans*-9-octadec enoic acid	-	
	threo-12,13-Dihydroxy-*trans*-9-octadec enoic acid	-	

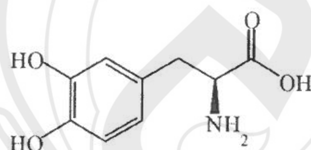
Table 1 (continued)

Scientific name (investigated part)	Compound	Structure	References
(seed oil)	Vernolic acid	26	Ahmad *et al.*, 1978
(fruit)	Indole alkaloid	27	Smith *et al.*, 1977
(leaves)	Bufotenine	28	Ghosal *et al.*, 1971
	Choline	29	
	N,N-Dimethyl tryptamine	30	
(leaves and stem)	*N,N*-Dimethyl methoxy tryptamine	33	Smith *et al.*, 1977
(suspention culture)	Dopamine	34	Wichers *et al.*, 1993
M. sempervirens			
(seed)	Mucuna lectin msl	-	Zhou *et al.*, 1996
	D-pinitol	35	Plouvire, 1962
(leaves)	6,8-Di-*C*-α-L-arabinosylapigenin	36	Ishikura *et al.*, 1988
	8-*C*-α-L-arabinosyl luteoin	37	
	Isoorientin	38	Ishikura *et al.*, 1988
M. sloanei			
(seed)	L-dopa	1	Rai *et al.*, 1977
M. species	L-dopa	1	Amarasekera *et al.*, 1980
(seed)	3-Carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	2	
	3-Carboxy-1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	3	

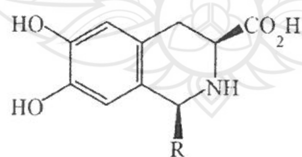
Table 1 (continued)

Scientific name (investigated part)	Compound	Structure	References
M. urens (seed)	L-dopa	1	Dexenbuchler *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 *et al.*, 1981

2.3 Structure of compounds from *Mucuna macrocarpa*



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

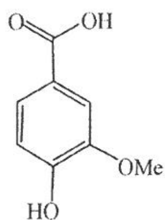
3-*O*-(6-*O*-Methyl-β-D-glucuronopyranosyl) asiatic acid

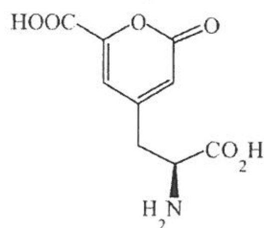
R_1	R_2	R_3	
CH_3	CH_3	-	**5:** Mucunagenin A
H	CH_3	CH_3	**6:** Mucunagenin B

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

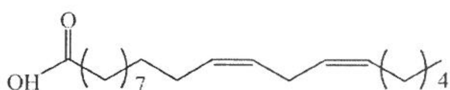
R = H **8:** 2,6-Dimethoxy phenol

R = CO_2H **9:** Syringic acid

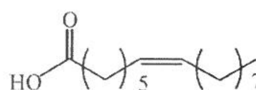




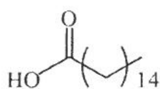
10: Valillic acid **11:** Hydrocyanic acid **12:** Stizolobic acid



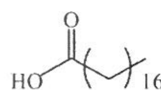
13: Linoleic acid



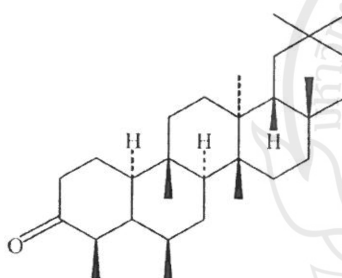
14: Oleic acid



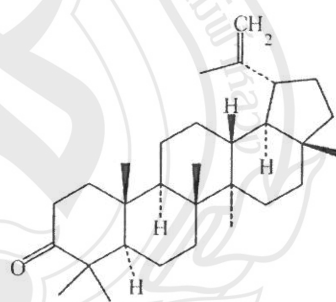
15: Plamitic acid



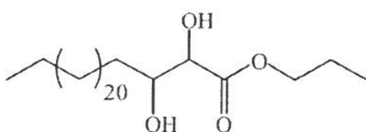
16: Stearic acid



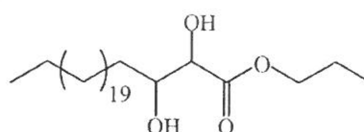
17: Friedelin



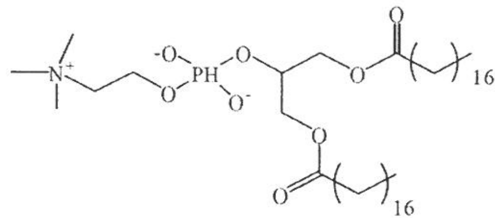
18: Lupenone



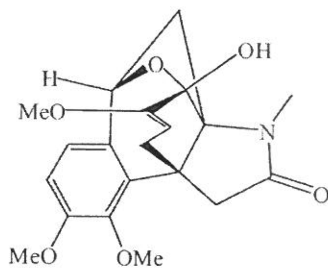
19: 2,3-Dihydroxypropyl ester heaxacosnoic acid



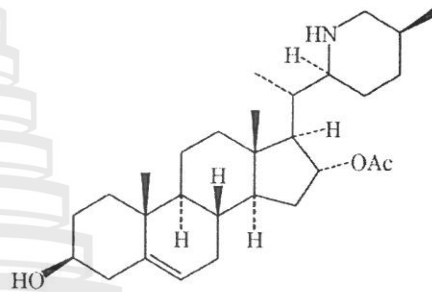
20: 2,3-Dihydroxypropyl ester pentacosnoic acid



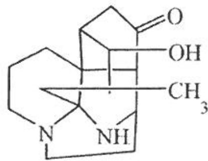
21: Lecithin



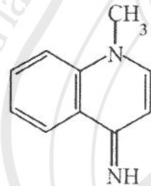
22: Alkaloid P



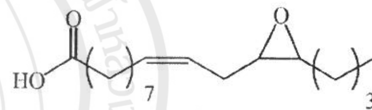
23: Alkaloid Q



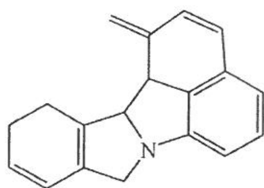
24: Alkaloid R



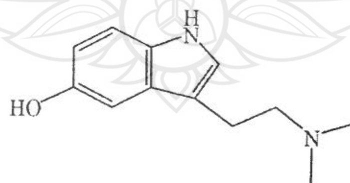
25: Alkaloid X



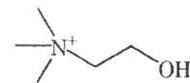
26: Vernolic acid



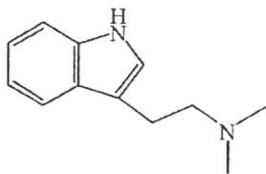
27: Indole alkaloid



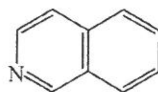
28: Bufotenine



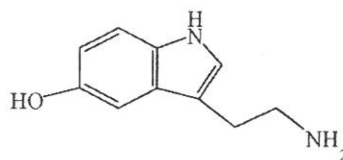
29: Choline



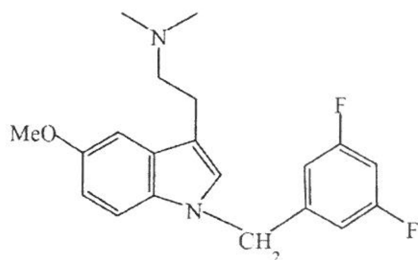
30: *N,N*-Dimethyl tryptamine



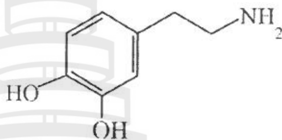
31: Isoquinoline



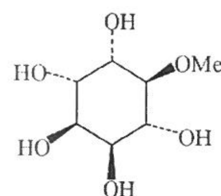
32: 5-Hydroxy tryptamine



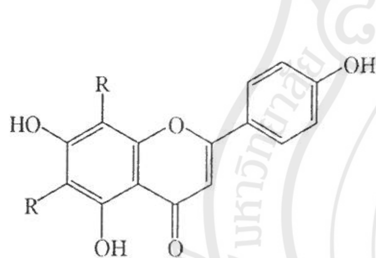
33: *N,N*-Dimethyl methoxy tryptamine



34: Dopamine

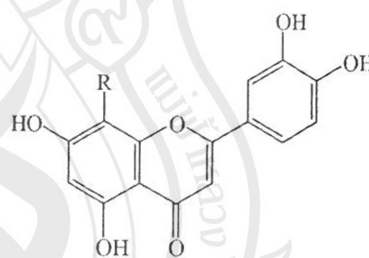


35: D-pinitol



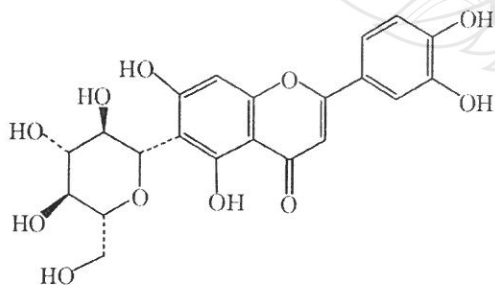
36: R=α-L-arabinosyl

6,8-Di-*C*-α-L-arabinosyl apigenin

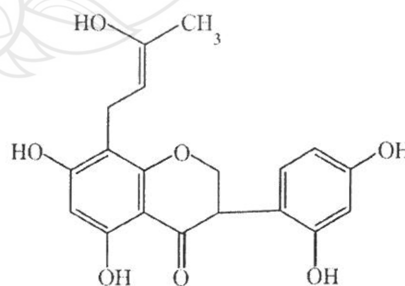


37: R= α-L-arabinosyl

8-*C*-α-L-arabinosyl luteoin



38: Isoorientin



39: Kievitone

2.4 Biological activities of *Mucuna* genus

M. macrocarpa has been tested antimicrobial 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 microbial growth (Saisavoey, 2006). *Mucuna macrocarpa* has been used by the local Thai people as folk medicine, purposes of tonic effects and preventing erectile dysfunction (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 (Cherdshewasart *et al.*, 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 (Cherdshewasart *et al.*, 2004b). Ethanolic extracts from Black Kwao Krua had no effect on intracavernous pressure and blood pressure in the adult male rats (Smitasiri *et al.*, 2004).

The natural antioxidants have attracted attention because some synthetic antioxidants have been found to be carcinogenic and harmful to lungs and liver (Yamasaki *et al.*, 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, 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, 1999). In addition, aqueous extracts of Black Kwao Krua have been reported to show strong antioxidation activity (Sang-Arun *et al.*, 2001).

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.

CHAPTER 3

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 ε in method solution. ^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded on Brüker AVANCE 400 and/or 300 MHz or Varian UNITY INOVA 500 MHz NMR. Spectra were recorded in $CDCl_3$ or CD_3OD solution and were recorded as δ value in ppm down field from TMS (internal standard δ0.00). 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, gentamycin and streptomycin) were used for control maker of antimicrobial 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* ATCC25932 and methicillin-resistant strain MRSA SK1) were supported 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, to give, after evaporation, 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 2**.

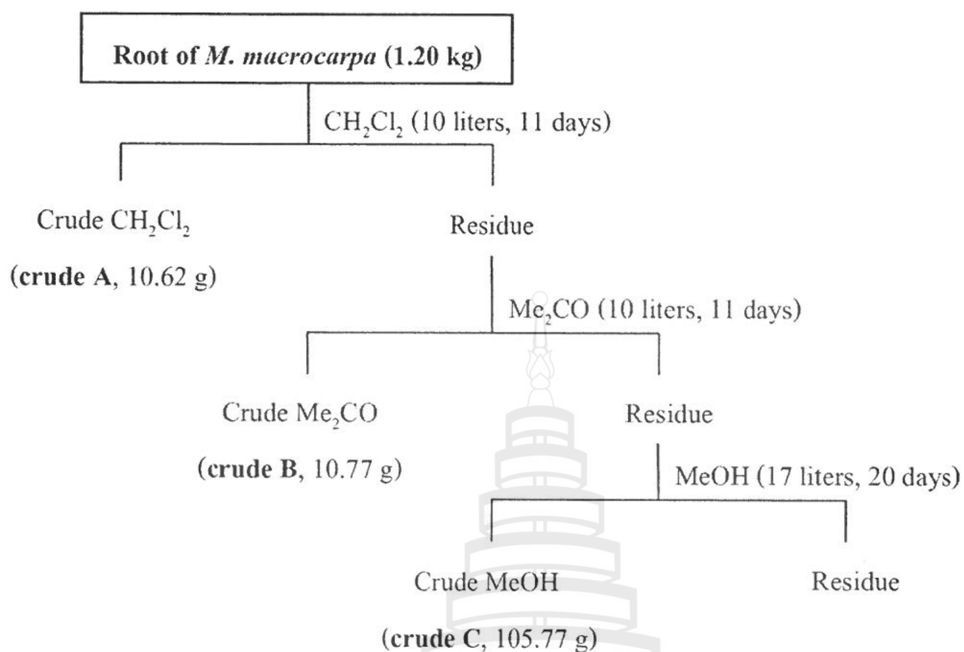


Figure 2 Extraction of **crude A, B** and **C** from the root of *M. macrocarpa*

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**.

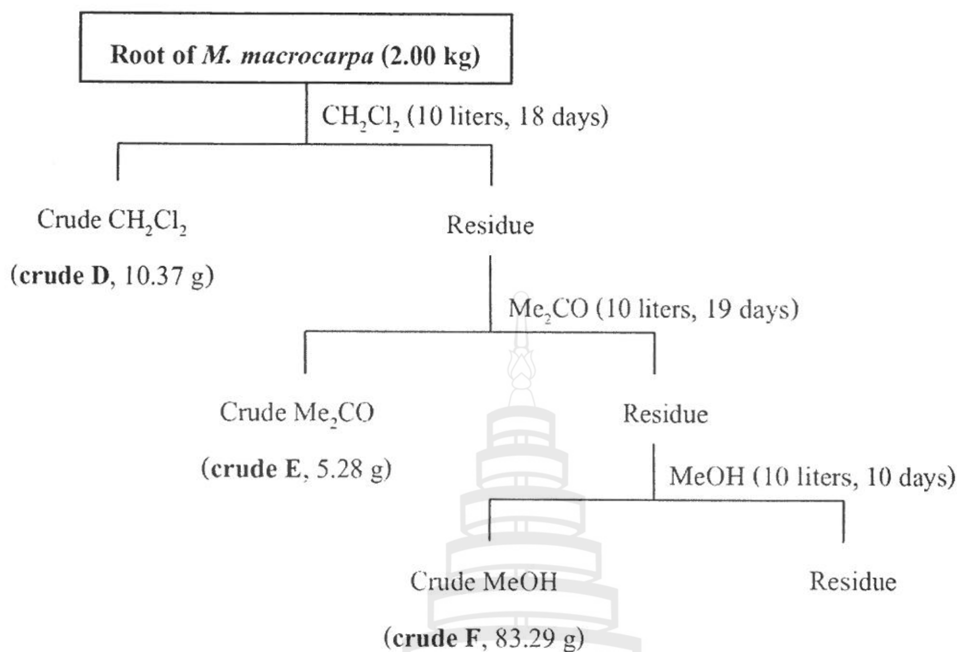


Figure 3 Extraction of **crude D, E** and **F** from the root of *M. macrocarpa*

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 2**. The selected fractions were further purified to give compounds **1**, **5** and **6** (**Figure 4**).

Table 2 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
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

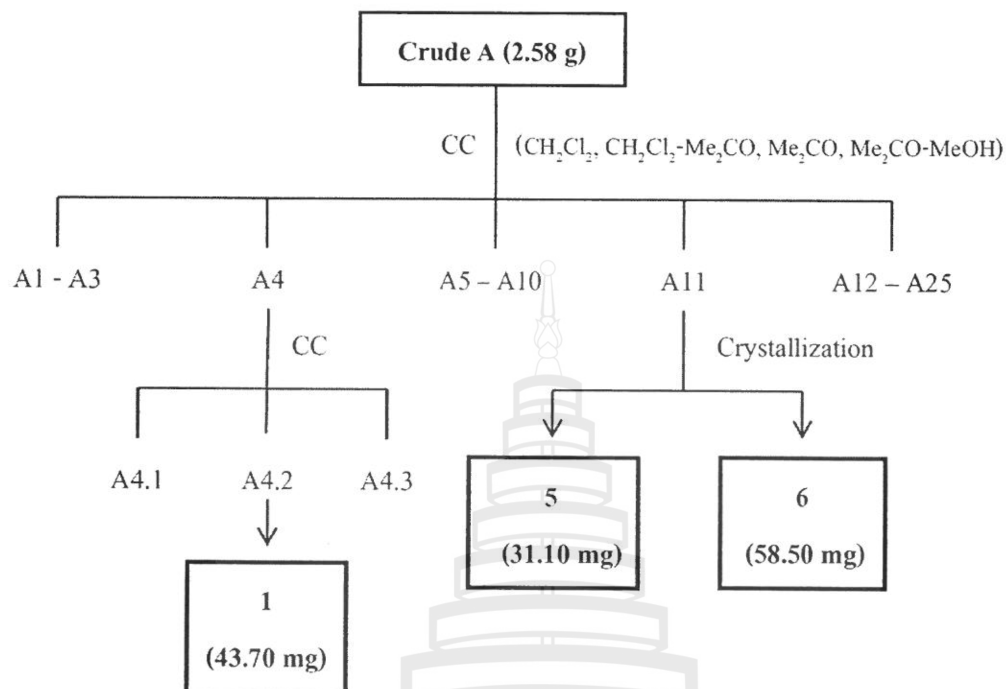


Figure 4 Isolation of compounds **1**, **5** and **6**

Isolation of compound 1

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 compound **1** as purple viscous liquid (40.80 and 43.70 mg, respectively).

Isolation of compounds 5 and 6

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 **5** (31.10 mg). The filtrate was further recrystallized from CH_2Cl_2-Me_2CO (8:2). A white solid **6** was collected (58.50 mg).

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**). The selected fractions were further purified to give 4 compounds as shown in **Figure 5**.

Table 3 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

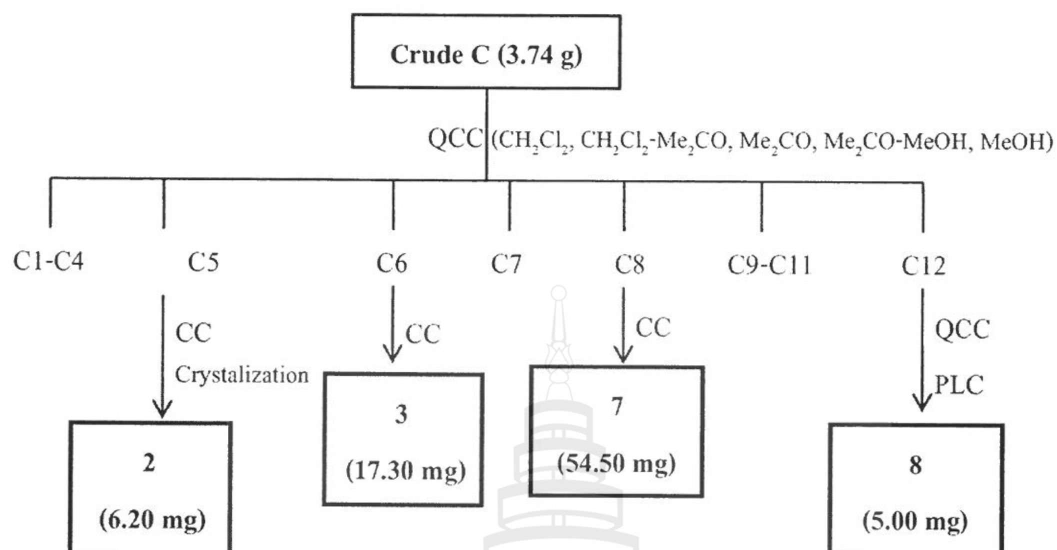


Figure 5 Isolation of compounds **2**, **3**, 7 and **8**

Isolation of compound 2

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 (12.6 mg) which contained one major component was recrystallized in the mixture of hexane-CH_2Cl_2 (8:2) to give **2** (6.20 mg) as a white solid.

Isolation of compound 3

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). Compound **3** was obtained from the fraction C6.13 as a white solid (17.30 mg).

Isolation of compound 7

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 **7** as an orange viscous liquid (54.50 mg).

Isolation of compound 8

Fraction C12 was purified by QCC using silica gel and eluted with gradiently CH_2Cl_2-Me_2CO, Me_2CO, CH_2Cl_2-MeOH and MeOH to give 17 fractions (C12.1-C12.17). Fraction C12.4 (11.0 mg) which contained one major component was further purified by PLC using 60% CH_2Cl_2-hexane (2 elutions) as an eluent to give **8** (5.00 mg) as a yellow solid.

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 4**. Compound **4** was obtained from the fractions D9 and D10 (**Figure 6**).

Isolation of compound 4

Fractions D9 and D10 were recrystallized in the mixture of hexane-CH_2Cl_2 (9:1) to give a white solid of **4** (31.30 mg).

Table 4 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

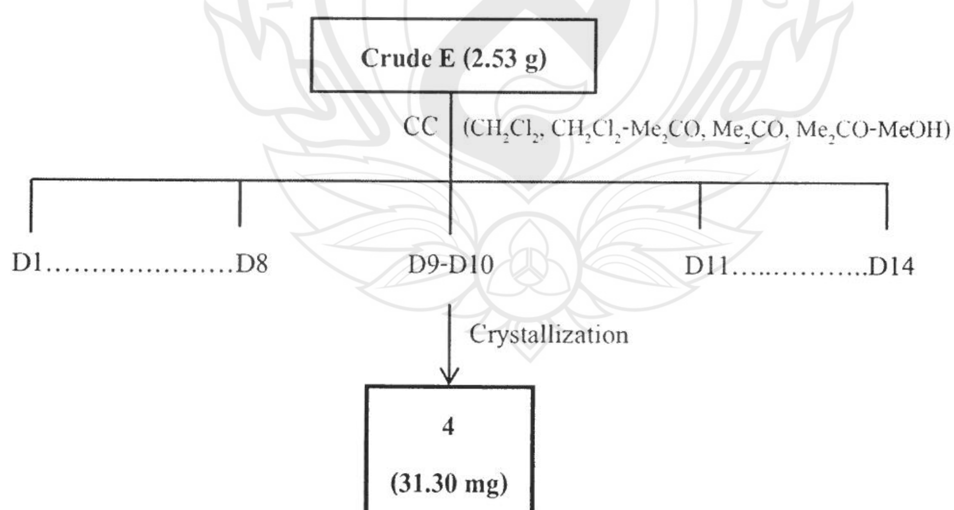


Figure 6 Isolation of compound **4**

3.5 DPPH radical scavenging assay

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).

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

3.5.1 Screening on the free radical scavenging activity of crude extracts and pure compounds

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. The screening on the free radical scavenging activity of pure compounds was performed like crude extracts except the final concentration was made at 50 μM.

3.5.2 Evaluation of IC_{50} of the crude extracts

Crude B, **C** and **F** extracts 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 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 results were expressed as % inhibition. The concentration that needed to decrease % inhibition of DPPH solution to 50% inhibition concentration (IC_{50}) was obtained by linear regression analysis of dose-response curve.

3.6 Antimicrobial activity assays

The paper disc diffusion method (Lorian, 1996) was used to screen the antimicrobial activity. Minimum inhibition concentrations (MICs) were determined by broth microdilution method (CLSI M7-A4, 2002) for bacterial.

3.6.1 Paper disc diffusion method

The paper disc diffusion method (Lorian, 1996) was used to screen the antimicrobial activity of the crude extracts. Three-five colonies of microbial culture are transferred to nutrient broth and incubated for 3 hrs at 35 ^{O}C, 150 rpm shaking incubator. The turbidity of microbial 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 culture into agar plate with sterile cotton swab. Place filter paper, containing of the microorganism, on the agar plates,

then drop 10 μL of crude extracts on the filter paper. Plates kept in the incubator at 35 °C for 18 h. This performs in duplicate for each extracts. The clear zone on the plate express in antimicrobial activity. CH_2Cl_2, Me_2CO and MeOH are used for testing markers for crude extracts of *M. macrocarpa* received from these solvents perform extraction. Antibiotic paper disc is used for control marker of antimicrobial activity.

3.6.2 Broth microdilution method

3.6.2.1 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 concentrations were 200 μg/mL. The inoculated plate were incubated at 35 °C for 16-18 h. Then drop 0.18% resazurin 10 μL in microtiter plate and incubated in 35 °C for 2-3 h. The blue color showed sample can inhibit microbial growth and pink color shown sample can not inhibit microbial growth. The test was performed in triplicates for each sample. Vancomycin, gentamycin and Streptomycin were used as a positive control drug.

3.6.2.2 Determination of minimum inhibition concentration

Minimum inhibition concentrations (MICs) were determined by the Broth microdilution method (CLSI M7-A4, 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 sample in broth ranged from 1280–2.5 μg/mL. Add 50 μL of inoculum suspensions in each well (final concentration $1x10^4$ CFU/well). The inoculated plate were incubated at 35 °C for 16-18 h. Drop 0.18% resazurin 10 μL in microtiter plate and incubated in 35 °C for 2-3 h. The blue color showed sample can inhibit microbial growth and pink color shown sample can not inhibit microbial growth. MICs were

recorded by reading the lowest concentration that inhibited visible growth. The test was performed in triplicates. Vancomycin, gentamycin and streptomycin were used as a positive control drug. Growth controls were performed on agar containing DMSO.

CHAPTER 4

RESULTS AND DISCUSSION

The roots of *M. macrocarpa* were collected from Chiang Rai province on March, 2002 and December, 2006. The roots of *M. macrocarpa* were extracted with dichloromethane, acetone and methanol, successively. Isolation and purification of dichloromethane and methanolic extracts (March, 2002) gave three compounds (**1**, **5** and **6**) and four compounds (**2**, **3**, **7** and **8**), respectively. One compound (**4**) was obtained from acetone extract of the material on December, 2006. Their structures were determined using spectroscopic data evidence, especially 1D and 2D NMR spectral data.

4.1 Spectroscopic data of pure compounds

Compound 1

Melting point: 169-171 $^{\circ}C$

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) ($CDCl_3$) (δ ppm) : 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

Compound 2

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)

Compound 3

Melting point : 282-288 °C

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

^{1}H NMR (300 MHz) ($CDCl_3$) (δ ppm) : 4.77 (1H, *br qd*, *J*=2.5, 1.5 Hz, H-29), 4.73 (1H, *br d*, *J*=2.5 Hz, H-29), 3.18 (1H, *dd*, *J*=11.0, 5.0 Hz, H-3), 3.04 (1H, *dt*, *J*=11.0, 5.0 Hz, 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 Hz, H-5)

Compound 4

Melting point : 213-215 °C

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

^{1}H NMR (300 MHz) ($CDCl_3$) (δppm) : 4.68 (1H, *d*, *J*=2.4 Hz, H-29), 4.56 (1H, *m*, H-29), 3.39 (1H, *dd*, *J*=5.7, 1.5 Hz, H-3), 2.39 (*ddd*, *J*=5.7, 5.7, 5.4 Hz, 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), 0.78 (3H, *s*, H-24), 0.82 (3H, *s*, H-25), 0.78 (3H, *s*, H-28)

Compound 5

Melting point : 156-157 °C

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

^{1}H NMR (300 MHz) ($CDCl_3$) (δ ppm) : 0.70 (3H, *s*, H-18), 0.74 (3H, *d*, H-26), 0.75 (3H, *t*, H-29), 0.77 (3H, *d*, H-27), 0.94 (1H, *m*, H-9), 1.00 (1H, *m*, H-14, H-15), 1.05 (3H, *s*, H-19), 1.08 (*m*, H-1), 1.15 (*m*, H-12, H-28), 1.20 (1H, *q*, 9.9, H-17), 1.22 (3H, *d*, H-21), 1.30 (*m*, H-16), 1.45 (1H, *m*, H-8), 1.46 (1H, *m*, H-24), 1.50 (*m*, H-2, H-7, H-11, H-15), 1.52 (*m*, H-28), 1.59 (1H, *m*, 6.4, H-25), 1.77 (*m*, H-16), 1.80 (*m*, H-1), 1.82 (*m*, H-2), 1.94 (*m*, H-7, H-12), 2.00 (1H, *m*, H-20), 2.19 (*m*, H-4), 2.29 (*m*, H-4), 3.46 (1H, *m*, H-3), 4.94 (1H, *dd*, 8.6, H-23), 5.10 (1H, *dd*, 15.2, H-22), 5.28 (1H, *m*, H-6)

^{13}C NMR (75 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, 31.9, 31.9, 31.9, 29.1, 29.1, 28.9, 28.3, 25.4, 24.4, 21.2, 21.1, 19.4, 19.0, 12.2, 12.1

Compound 6

Melting point : 139-142 ^{o}C

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

^{1}H NMR (300 MHz) ($CDCl_3$) (δ ppm) : 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 Hz, 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)

Compound 7

^{1}H NMR (400 MHz) ($CDCl_3$) (δ ppm) : 12.43 (1H, *s*, 1-OH), 12.15 (1H, *s*, 8-OH), 7.62 (1H, *d*, *J*=1.2, H-4), 7.40 (1H, *s*, H-5), 7.07 (1H, *d*, *J*=1.2, H-2), 5.20 (1H, *t*, *J*=7.0, H-2′), 4.02 (3H, *s*, 3-OCH_3), 3.43 (2H, *d*, *J*=7.0, H-1′), 1.80 (3H, *s*, H-5′), 1.68 (3H, *s*, H-4′)

^{13}C NMR (100 MHz) ($CDCl_3$) (δ ppm) : 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, 22.71, 21.15, 17.88

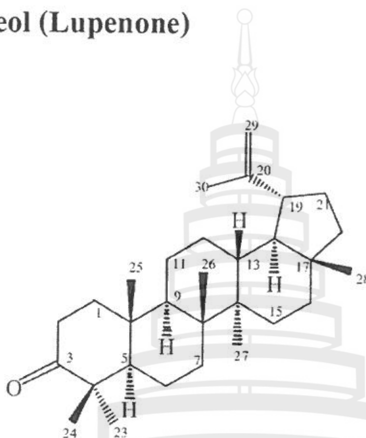
Compound 8

^{1}H NMR (400 MHz) ($CDCl_3$) (δ ppm) : 10.05 (1H, *s*, 2-CHO), 8.64 (1H, *br*, NH), 8.20 (1H, *d*, *J* = 4.00 Hz, H-4), 8.12 (1H, *d*, *J* = 8.00 Hz, H-5), 7.50 (1H, *dd*, *J* = 8.00, 4.00 Hz, H-8), 7.51 (1H, *t*, *J* = 8.00, H-7), 7.46 (1H, *d*, *J* = 4.00 Hz, H-2), 7.32 (1H, *t*, *J* = 8.00 Hz, H-6), 4.07 (3H, *s*, 1-OCH_3)

^{13}C NMR (100 MHz) ($CDCl_3$) (δ ppm) : 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

4.2 Structural determination

Compound 1: 3-oxo-Lupeol (Lupenone)



Compound 1 was isolated as a white solid, m.p. 169-171 ^{o}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 and 2.40, 1.09 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 5)**. 3-*oxo*-Lupeol was assigned for **1**. It was known as lupenone (Boonsri, 2004). Lupenone has been reported to possess antimicrobial, antiviral, anticancer and antiinflammatory activity (Prachayasittikul *et al.,* 2009).

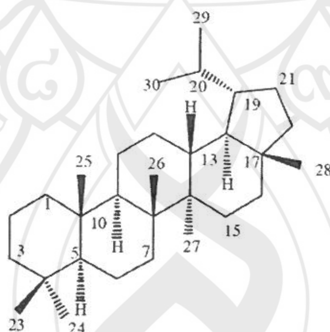
Table 5 NMR spectral data of compound **1**

Position	Compound **1**		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	-	105.9
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*)	15.8	0.93 (*s*)	15.8

Table 5 (continued)

Position	Compound **1**		Lupenone	
	δ_H (*multiplicity*, J_{Hz})	δ_C	δ_H (*multiplicity*, J_{Hz})	δ_C
26	1.07 (3H, *s*)	16.0	1.07 (*s*)	16.0
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) H_b : 4.58 (1H, *m*)	109.0	4.68 (*d*, 2.1) 4.57 (*m*)	109.4
30	1.72 (*m*)	19.4	1.70 (*s*)	19.3

Compound 2: Lupane

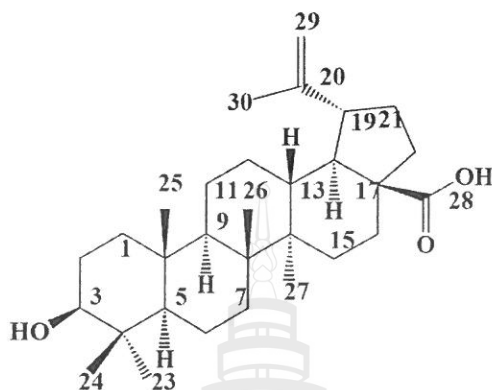


Compound 2 was is isolated as a white solid, m.p. 235-243 $^{\circ}$C. The IR spectrum showed the absorption band of C-H stretching (2928, 2870 cm^{-1}). The ^{1}H NMR spectrum (**Table 6**) indicated that it was a triterpene derivative of lupenone (**1**). The ^{1}H NMR spectrum showed the characteristic signals similar to lupenone except two multiplet signals of methylene protone H-3 at δ 1.40 and 2.40 and the siglet 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 indentical with lupane.

Table 6 NMR spectral data of compound **2**

Position	δ_H (*multiplicity*, J_{Hz})	
	Compound **2**	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*)

Compound 3: Betulinic acid



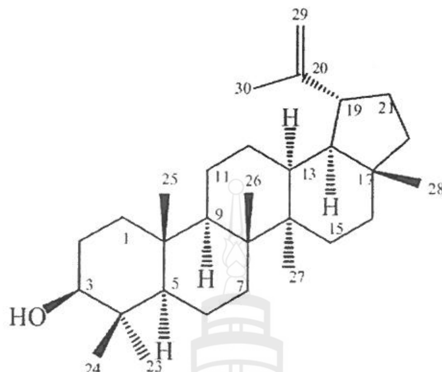
Compound **3** was isolated as a white solid; m.p. 282-288 ^{O}C. The IR spectrum showed the absorption band of O=H stretching at 3470 cm^{-1}. The ^{1}H NMR ($CDCl_3$, 300 MHz) spectral data (**Table 7**) showed the resonances of an oxymethine proton (δ 3.18, *dd,* 11.0 and 5.0 Hz, H-3), five methyl groups (δ 0.97, 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 **3** and betulinic acid (Boonsri, 2004) indicated that **3** had same structure as betulinic acid.

Betulinic acid has been reported to exhibit anti-HIV-1, antibacterial, antifungal, antiplasmodial and anti-inflammatory activities (Yogeeswari *et al.*, 2005). Betulinic acid has also been reported to inhibit growth of cancer cells, without affecting normal cells (Einzhammer *et al.*, 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 7 NMR spectral data of compound **3**

Position	δ_H (*multiplicity*, J_{Hz})	
	Compound **3**	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, *brd*, 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	4.73 (1H, *br d,* 2.5), 4.77 (1H, *br qd*, 2.5, 1.5)	4.73 (1H, *br d,* 2.1,), 4.59 (1H, *br qd*, 2.1, 1.2)
30	1.69 (3H, *s*)	1.69 (3H, *s*)

Compound 4: Lup-20(29)-en-3*β*-ol (Lupeol)



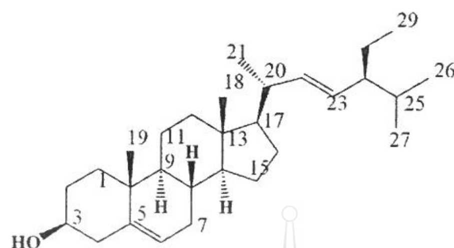
Compound **4** was obtained as a white solid, m.p. 213-215 ^{o}C. The IR spectrum exhibited the absorption band of O-H stretching at 3313 cm $^{-1}$. The ^{1}H NMR spectrum indicated that it was a triterpene derivative of **3**. The ^{1}H NMR spectrum (**Table 8**) showed the characteristic signal of a terminal olifinic methylene protons at δ 4.68 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 (δ 3.39, *dd*, *J* = 5.7 and 1.5 Hz, H-3) and seven methyl groups (δ 0.96 (H-23), 0.78 (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-3*β*-ol which was known as lupeol (Imam *et al.*, 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 (Patočka 2003). Moreover, lupeol also exhibit antiinflammatory and antiarthritic activities (Agarwal *et al.*, 2003).

Table 8 NMR spectral data of compound **4**

Position	δ_H (*multiplicity*, J_{Hz})	
	Compound **4**	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	0.78 (3H, *s*)	0.79 (3H, *s*)
25	0.82 (3H, *s*)	0.85 (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*)

Compound 5: 5,22-stigmastadien-3β-ol (Stigmasterol)

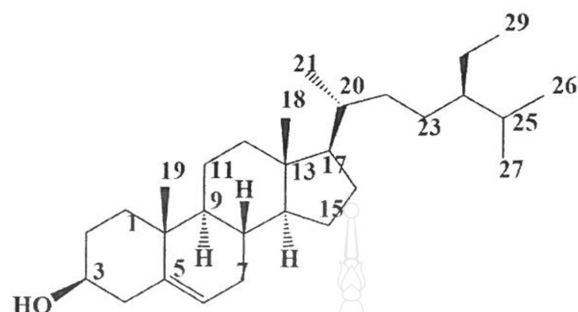


Compound **5** was obtained as a white solid, m.p. 156-157 $^{\circ}$C. In IR spectrum, the absorption band of O-H stretching (3426 cm^{-1}) and C-H stretching (2936 and 2867 cm^{-1}) were shown. The ^{1}H NMR spectrum (**Table 9**) contained an oxymethine proton signal at δ3.56-3.48, three olefinic protons at δ5.36-5.33 (*m*), 5.16 (*dd*) and 5.02 (*dd*) and six methyl groups at δ 1.02, 1.05, 0.86, 0.82, 0.80 and 0.69. The ^{1}H NMR data, optical rotation value and melting point were corresponded to the previous reported data (Forgo and Köver, 2004). Thus, compound **5** was assigned to be stigmasterol.

Table 9 NMR spectral data of compound **5**

Position	δ_H (*multiplicity*, J_{Hz})	
	Compound 5	Stigmasterol
3	3.56-3.48 (1H, *m*)	3.51 (*m*)
6	5.36-5.33 (1H, *m*)	5.34 (*m*)
18	0.69	0.70 (*s*)
19	1.02	10.1 (*s*)
21	1.05	1.03 (*d*, 6.2)
22	5.16 (1H, *dd*, 15.0, 8.0)	5.17 (*dd*, 15.2, 8.6)
23	5.02 (1H, *dd*, 15.0, 8.0)	5.04 (*dd*, 15.2, 8.6)
26	0.86	0.85 (*d*, 6.4)
27	0.80	0.80 (*d*, 6.4)
29	0.82	0.81 (*t*, 7.3)

Compound 6: Stigmast-5-en-3β-ol (β-Sitosterol)



Compound **6** was a white solid, m.p. 139-142 $^{\circ}$C. The IR spectrum showed the sbsorption band of O-H stretching (3426 cm^{-1}) and C-H stretching (2936, 2851 cm^{-1}). The ^{1}H NMR spectral data (**Table 10**) 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 **6** was proposed to be stigmast-5-en-3β-ol. It was known as β-sitosterol (Nguyen *et al.*, 2004).

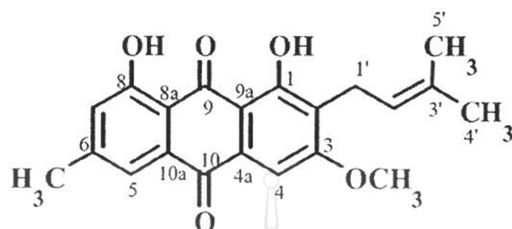
Table 10 NMR spectral data of compound **6**

Position	δ_H (*multiplicity*, J_{Hz})	
	Compound **6**	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*)

Table 10 (continued)

Position	δ_H (*multiplicity*, J_{Hz})	
	Compound **6**	β-Sitosterol
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)

Compound 7: Vismiaquinone C



Compound **7** was isolated as an orange viscous liquid. The ^{1}H NMR spectral data (**Table 11**) showed two sharp *singlet* signals of two chelated hydroxyl groups at δ 12.43 (1H, 1-OH) and 12.15 (1H, 8-OH). Two *singlet* 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 assignments were supported by ^{3}J correlations of H-7 to C-5, C-8a and 6-CH_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 2.45 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 of 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 11**) suggested that compound **7** contained three methyl carbons, a methylene carbon, four methine carbons, ten quaternary carbons, a methoxy carbon and two carbonyl carbons. The proposed structure and 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 *et al.*, 1997).

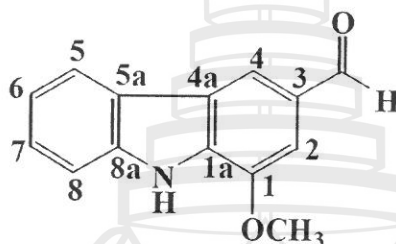
Table 11 NMR spectral data of compound **7**

Position	Compound 7				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- CH_3	7.58 (*d*, 1.6)	121.0
6	-	148.29	C	-	-	133.6
7	7.07 (1H, *d*, 1.2)	124.42	CH	C-5, C-8, C-8a, 6- CH_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*, 7.0)	22.71	CH_2	C-3, C-2, C-1, C-2′, C-3′	3.40 (*d*, 7.0)	22.1
2′	5.20 (1H, *t*, 7.0)	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*)	-	-	C-2, C-9a	12.12 (*s*)	-
8-OH	12.15 (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 11 (continued)

Position	Compound 7				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

Compound 8: 3-formyl-1-methoxycarbazole (Murrayanine)



Compound **8** was isolated as a yellow solid. The ^{1}H NMR spectral data (**Table12**) exhibited a pair of *meta*-coupled protons at δ 7.46 (*d*, *J* = 4.0 Hz) and 8.20 (*d*, *J* = 4.0 Hz) for H-2 and H-4, respectively. The coupling of the aromatic protons indicate that the ring A is *ortho*-substituted with two *doublets* at δ 8.12 and 7.50 attributed to H-5 and H-8, respectively. Another two pairs of *triplets* 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 compound 8 as 3-formyl-1-methoxycarbazole which was known as murrayanine (Abu Bakar *et al.*, 2007).

Table 12 NMR spectral data of compound **8**

Position	Compound **8**				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*, 4.00)	103.54	CH	C-1, C-1a, C-4, CHO	7.47 (1H, *s*)	103.5
3	-	130.19	C	-	-	130.16
4	8.20 (1H, *d*, 4.00)	120.42	CH	C-1a, C-2, C-4a, CHO	8.20 (1H, *s*)	120.41
4a	-	123.64	C	-	-	123.64
5	8.12 (1H, *d*, 8.00)	120.71	CH	C-5a, C-7, C-8a	8.13 (1H, *d*,7.32)	120.69
5a	-	123.68	C	-	-	123.64
6	7.32 (1H, *t*, 8.00)	120.72	CH	C-5a, C-7	7.34 (1H, *t*, 8.24)	120.69
7	7.51(1H, *t*, 8.00)	111.51	CH	C-5, C-6	7.53(1H, *t*, 8.24)	111.47
8	7.50 (1H, *dd*, 8.00, 4.00)	126.65	CH	C-6, C-8a	7.50 (1H, *d*, 6.44)	126.6
8a	-	139.43	C	-	-	134.1
1-OMe	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*)	-

4.3 Evaluation of antioxidative 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 $5x10^{-4}$ 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). Coexistance 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 7**.

Figure 7 DPPH free radical and the appearance the free radical

4.3.1 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 results were expressed as % inhibition (**Table 12**, **Figure 7**). The activity was exhibited by the acetone as well as by the methanolic extracts.

Table 13 The average absorption and % inhibition of the crude extracts (at final concentration 100 μg/mL)

sample	Average absorbances (517 nm)					%inhibition (at 30 min)
	0 min	15 min	30 min	45 min	60 min	
Control (0.05 mM DPPH)	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

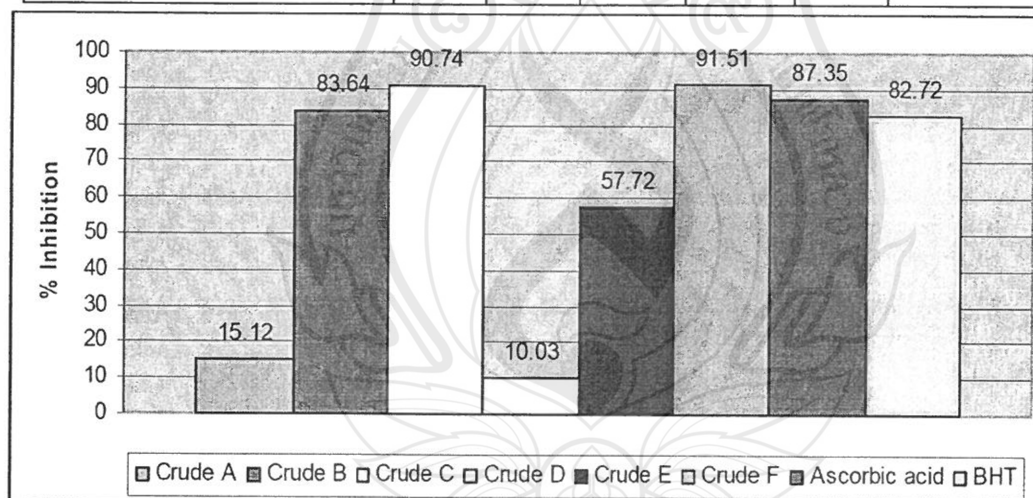


Figure 7 Radical scavenging activity of the crude extracts

The assessment of the antioxidative 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 average absorption and % inhibition

of **Crude B**, **C**, **E**, **F** and standard antioxidants at various concentrations were shown in **Table 14**. The oxidation effect was evaluated as the concentration required to scavenge 50% DPPH free radical. Their IC_{50} were exhibited at 1.19, 0.25, 4.37, and 0.21 mg/mL, respectively whereas IC_{50} of ascorbic acid and BHT were shown at 0.19 and 0.41 mg/mL (**Table 15**).

Table 14 The average absorption and % inhibition of **Crude B**, **C**, **E**, **F** and standard antioxidants at various concentrations

Sample Conc.	**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	-	-	-	-	0.330	46.60	-	-	-	-	-	-
3.0	0.119	80.74	0.062	89.97	0.376	39.16	0.059	90.54	0.027	95.63	0.097	84.30
2.0	0.197	68.12	0.059	90.45	0.416	32.67	0.082	86.73	0.027	95.63	0.123	80.10
1.0	0.360	41.75	0.068	89.00	0.530	14.24	0.247	60.03	0.033	94.66	0.182	70.55
0.5	0.498	19.42	0.114	81.55	-	-	0.453	26.70	0.028	95.47	0.233	62.30
0.25	0.561	9.22	0.370	40.13	-	-	0.529	14.40	0.037	94.01	0.388	37.22
0.125	0.605	2.10	0.446	27.83	-	-	0.576	6.80	0.128	79.29	0.478	22.65
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 15 IC_{50} values of crude extracts and standard antioxidants

Sample	IC_{50} (mg/mL, 30 min)
Crude B	1.19
Crude C	0.25
Crude E	4.37
Crude F	0.21
Ascorbic acid	0.19
BHT	0.41

4.3.2 Free radical scavenging activity of pure compounds

To determine the free radical scavenging activity of pure compounds. The sample tested at the final concentration of 50 μM. The absorption of the solutions were measured at 517 nm 30 min (**Table 16**). Ascorbic acid and BHT were used as reference compounds. The activity was expressed in the % inhibition (**Table 17**). The pure compounds showed to weak activity.

Table 16 The average absorption and % inhibition of pure compounds

sample	Average absorbance (517 nm)					%inhibition
	0 min	15 min	30 min	45 min	60 min	(at 30 min)
Control (0.05 mM DPPH)	0.634	0.637	0.639	0.641	0.644	-
DPPH + compound **1**	0.637	0.633	0.627	0.627	0.627	1.88
DPPH + compound **2**	0.634	0.638	0.636	0.632	0.633	0.47
DPPH + compound **4**	0.636	0.639	0.636	0.634	0.633	0.47
DPPH + compound **5**	0.626	0.638	0.636	0.637	0.638	0.47
DPPH + compound **6**	0.626	0.632	0.629	0.627	0.629	1.56
DPPH + compound **7**	0.645	0.637	0.637	0.636	0.636	0.31
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

Table 17 % Inhibition of tested compounds and standard antioxidants (50 μM)

Sample	% inhibition ± S.D. (50 μM, 30 min)
Control (0.05 mM DPPH)	-
Compound **1**	1.83 ± 0.95
Compound **2**	0.52 ± 0.42
Compound **4**	0.52 ± 1.18
Compound **5**	0.42 ± 0.27
Compound **6**	1.51 ± 0.97
Compound **7**	0.26 ± 0.72
Ascorbic acid	95.93 ± 1.01
BHT	51.17 ± 4.69

4.4 Evaluation of antimicrobial activity

4.4.1 Antimicrobial activity of crude extracts

Dried roots of *M. macrocarpa* were extracted with CH_2Cl_2, Me_2CO and MeOH to give **crude A** to **F**. Each extract was screen for antimicrobial activity on *Staphylococcus aureus* (SA), methicillin-resistant strain MRSA SK1, *Bacillus cereus* (BC), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Pseudomonas fluorescens* (PF), and *Salmonellae typhimurium* (ST) using paper disc diffusion method. The results were presented in **Table 18**. It was found that the extracts **C** and **F** showed inhibition zone whereas the other extracts showed no activity. Thus, crude **C** and **F** were then selected for further study MIC value by broth microdilution method. The results were shown in **Table 19**.

Table 18 Inhibition zone of antibacterial activity screening of crude extracts

Sample	Diameter of clear 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	–	–	–	–
Gentamycin	–	–	–	19.01	18.74	16.23	21.20

– no inhibition zone

Table 19 MIC value of crude extracts **C** and **F**

Sample	Antimicrobial 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	-
Gentamycin	-	-	-	0.500

4.4.2 Antibacterial activity of pure compounds

Some of the pure compounds obtained from each extract were evaluated for their antibacterial activity againt, *Staphylococcus aureus* (SA), methicillin-resistant strain MRSA SK1, *Bacillus cereus* (BC), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Pseudomonas fluorescens* (PF), and *Salmonellae typhimurium* (ST) by broth microdilution method. The compounds **1** and **7** exhibited the antimicrobial activity ageinst *Bacillus cereus* (BC), *Pseudomonas fluorescens* (PF) and *Salmonellae typhimurium* (ST) with MIC values of 32-64 μg/mL (**Table19**), however it was less active than vancomycin and entamycin, the standard antibiotic. The other compounds were showed no activity.

Table20 MIC value of pure compounds

Sample	Antimicrobial activity (MIC, µg/mL)						
	Gram Positive			Gram Negative			
	SA	MRSA SK1	BC	EC	PA	PF	ST
Compound **1**	>200	>200	64	>200	>200	64	32
Compound **2**	>200	>200	>200	>200	>200	>200	>200
Compound **4**	>200	>200	>200	>200	>200	>200	>200
Compound **5**	>200	>200	>200	>200	>200	>200	>200
Compound **6**	>200	>200	>200	>200	>200	>200	>200
Compound **7**	>200	200	32	>200	>200	64	64
Vancomycin	0.5	0.5	0.5	-	-	-	-
Gentamycin	-	-	-	0.5	0.5	0.5	0.5

CHAPTER 5

CONCLUSION

Investigation of the chemical constituents of dichloromethane, acetone and methanolic extracts from the root of *M. macrocarpa* let to the isolation of compounds: lupenone (**1**), lupane (**2**), betulinic acid (**3**), lupeol (**4**), stigmasterol (**5**), β-sitosterol (**6**), vismiaquinone C (**7**) and murrayanine (**8**).

The crude extracts and some pure compounds were examined for their antioxidation properties by DPPH free radical scavenging assay and antibacterial activities. Crude methanolic extracts (**crude F** and **crude C**) were showed strong antioxidation activity with IC_{50} 0.21 and 0.25 mg/mL, respectively than that of BHT (IC_{50} 0.41 mg/mL). Crude acetone extracts (**crude B** and **crude E**) were showed antioxidation activity with IC_{50} 1.19 and 4.37 mg/mL, respectively. Whereas the pure compounds showed weak activity.

The antimicrobial activity found compounds **1** and **7** inhibited the growth of *Bacillus cereus*, *Pseudomonas fluorescens* and *Salmonella typhimurium* with MIC values of 32-64 µg/mL.

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Education background

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2006	Ph.D.	Organic Chemistry	Prince of Songkla University
2001	M.Sc.	Organic Chemistry	Prince of Songkla University
1999	B.Sc.	Chemistry	Prince of Songkla University

Educational Attainment

1995-1999 Bachelor of Science in Chemistry. Prince of Songkla University.

1999-2001 Master of Science in Organic Chemistry. Prince of Songkla University.
Research Supervisor: Asst. Prof. Dr. Wilawan Mahabusarakam.
Thesis Title: Chemical Constituents from *Derris scandens* and Antioxidation Properties.

2002-2005 Ph.D. Student in Organic Chemistry. Prince of Songkla University.
Research Supervisor: Asst. Prof. Dr. Wilawan Mahabusarakam.
Thesis Title: Chemical Constituents from the flowers, fruits and seeds of *Garcinia dulcis* and Antioxidation Properties.

2003 Visiting Ph.D. student at State Key Laboratory of Phytochemistry & Plant Resources in West China, Kunming Institute of Botany, Chinese

Academy of Sciences, Heilongtan, Kunming 650204, China (31 October 2003 –21 November 2003).

Research Supervisor: Prof. Dr. Chong-Ren YANG & Assoc. Prof. Dr. Ying-Jun Zhang.

Research Title: Isolation of High Polarity Compounds from *Garcinia dulcis*.

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Radicals, Oxidative Stress and Their Evaluation Methods, 15-16 December, 2008. **(Poster presentation)**

2. Trinop Promgool, Manita Treesub and **Suwanna Deachathai**, Chemical constituents from the roots of *Garcinia cowa* Roxb., antimicrobial and antioxidation properties., 34th Congress on Science and Technology of Thailand (STT 34), 31 October – 2 November, 2008. **(Oral presentation)**
3. Achjanee Somsri and **Suwanna Deachathai**, Chemical Constituents from the Root of *Mucuna macrocarpa* Wall., International Conference on Mathematics and Natural Sciences 2008, 28-30 October, 2008. **(Oral presentation)**
4. Rolly Yulianthi and **Suwanna Deachathai**, Chemical constituents from dried leaves of *Camellia sinensis* var. *assamica* in Chiang Rai, Thailand, International Conference on Mathematics and Natural Sciences 2008, 28-30 October, 2008. **(Oral presentation)**
5. **Suwanna Deachathai,** Chemical constituents from the roots of *Garcinia cowa* Roxb., International Conference on Mathematics and Natural Sciences 2008, 28-30 October, 2008. **(Oral presentation)**
6. Achjanee Somsri and **Suwanna Deachathai** (2007) Chemical Constituents from the Root of *Mucuna macrocarpa* Wall., Antimicrobial and Antioxidation Properties. TSB 19th Annual Meeting, Bangkok, Thailand. (Poster Presentation)
7. Achjanee Somsri and **Suwanna Deachathai** (2007) Chemical Constituents from the Root of *Mucuna macrocarpa* Wall., Antimicrobial and Antioxidation Properties. STT 33, Walailak University, Nakorn Sri Thummarat, Thailand. **(Poster Presentation)**
8. P. Thongbai, P. Damrongkul Eungwanichayapant, **S. Dechathai,** S. Popluechai, S.

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