องค์ประกอบทางเคมีของแว่นแก้ว *Hydrocotyle umbellata* Linn. และผลต่อการเติบโตของไมยราบยักษ์ *Mimosa pigra* Linn.

นางสาววาสนา พรรคเจริญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมี ภาควิชาเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2545

ISBN 974-17-9869-5

CHEMICAL CONSTITUENTS FROM *Hydrocotyle umbellata* LINN. AND EFFECT ON GROWTH OF *Mimosa pigra* LINN.

Miss Wasana Pakjarurn

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

Department of Chemistry
Faculty of Science
Chulalongkorn University

Academic Year 2002
ISBN 974-17-9869-5

Thesis Title	CHEMICAL CONSTITUENTS FROM *Hydrocotyle umbellata* Linn. AND EFFECT ON GROWTH OF *Mimosa pigra* Linn.
By	Miss Wasana Pakjarurn
Field of Study	Chemistry
Thesis Advisor	Assistant Professor Warinthorn Chavasiri, Ph.D.
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Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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(Namthip Sitachitta, Ph.D.)

วาสนา พรรคเจริญ : องค์ประกอบทางเคมีของแว่นแก้วและผลต่อการเติบโตของไมยราบยักษ์ (CHEMICAL CONSTITUENTS FROM *Hydrocotyle umbellata* Linn. AND EFFECT ON GROWTH OF *Mimosa pigra* Linn.) อ. ที่ปรึกษา: ผศ. ดร.วรินทร ชวศิริ, อ. ที่ปรึกษาร่วม : ดร. ศิริพร ซึงสนธิพร, 128 หน้า. ISBN 974-17-9869-5.

ผลการทดสอบฤทธิ์ทางชีวภาพเบื้องต้น ชี้ว่า สิ่งสกัดเฮกเซนและคลอโรฟอร์มของแว่นแก้วมีฤทธิ์ทางชีวภาพ สามารถแยกสารได้ 9 ชนิด และพิสูจน์โครงสร้างโดยอาศัยสมบัติทางกายภาพ, ปฏิกิริยาเคมี และหลักฐานทางสเปกโทรสโกปี คือ ของผสมของน้ำมันหอมระเหย (chamigrene, α-bergamotene, α-santalol, germacrene D, β-caryophyllene และ β-pinene), ของผสมของแอลกอฮอล์โซ่ตรง, ของผสมของเอสเทอร์โซ่ตรง, ของผสมของ stigmasterol และ stigmasta-7, 25-dien-3-D-glucopyranoside, β-D-glucopyranosyl tetratriacontan-2,4-dienoate และ methyl oleanolate 3-*O*-(β-D-glucopyranoside)$_3$ สารสองชนิดหลัง พบว่า เป็นสารใหม่ นอกจากนี้ ได้นำแว่นแก้วสดมากลั่นไอน้ำได้น้ำมันหอมระเหย ซึ่งวิเคราะห์ได้ว่าประกอบด้วย longicyclene, α-bergamotene, β-caryophyllene, 1H-benzocycloheptene-2, 4α, 5, 6, 7, 8, 9, 9α-octahydro-3, 5, 5-trimethylene-(*4αS-cis*) และ α-santalol เป็นองค์ประกอบ งานวิจัยนี้ เป็นการศึกษาองค์ประกอบทางเคมีของพืชชนิดนี้เป็นครั้งแรก การศึกษาฤทธิ์ในการยับยั้งการเติบโตของต้นกล้าไมยราบยักษ์ *Mimosa pigra* Linn. พบว่า methyl oleanolate 3-*O*-(β-D-glucopyranoside)$_3$ แสดงฤทธิ์ยับยั้งการเติบโตส่วนรากได้ดีที่สุด 70%, β-D-glucopyranosyl tetratriacontan-2,4-dienoate 63% และ น้ำมันหอมระเหยจากต้นสด 46% ที่ความเข้มข้น 1000 ส่วนในล้านส่วน นอกจากนี้ ได้ศึกษาฤทธิ์ในการยับยั้งการเติบโตของต้นกล้าหญ้าข้าวนก *Echinochloa crus-galli* Beauv., หญ้าขจรจบดอกเล็ก *Pennisetum pedicellatum* Schult., ผักกาดหอม *Lactuca sativa* Linn., ผักเบี้ยหิน *Trianthema portulacastrum* Linn. และ ก้นจ้ำขาว *Bidens pilosa* Linn. พบว่า methyl oleanolate glycoside สามารถยับยั้งการเติบโตส่วนรากของพืชทุกชนิดได้ดีที่สุด

ภาควิชา.....เคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา.....เคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2545..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4372408723 : MAJOR CHEMISTRY

KEY WORD: *Hydrocotyle umbellata* Linn. / *Mimosa pigra* Linn. / ALLELOPATHY /

WASANA PAKJARURN : CHEMICAL CONSITUENTS OF *Hydrocotyle umbellata* Linn. AND EFFECT ON GROWTH OF *Mimosa pigra* Linn. THESIS ADVISOR : ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., THESIS CO-ADVISOR : SIRIPORN ZUNGSONTIPORN, Ph. D.,128 pp. ISBN 974-17-9869-5.

The preliminary bioassay results indicated that the hexane and chloroform extracts of *Hydrocotyle umbellata* Linn. were biologically active. Nine substances were isolated and elucidated their structural formulas by means of physical properties, chemical reactions and spectroscopic evidences as a mixture of essential oils (chamigrene, α-bergamotene, α-santalol, germacrene D, β-caryophyllene and β-pinene), a mixture of long chain carboxylic acids, a mixture of long chain aliphatic alcohols, a mixture of long chain aliphatic esters, a mixture of stigmasterol and stigmasta-7,25-dien-3-ol, stigmasteryl-3-D-glucopyranoside, β-D-glucopyranosyl tetratriacontan-2,4-dienoate and methyl oleanolate 3-*O*-(β-D-glucopyranoside)$_3$. The last two compounds were disclosed to be new naturally occurring compounds. Besides, the hydrodistillation of the fresh whole plants was conducted to gain the essential oil. Longicyclene, α-bergamotene, β-caryophyllene, 1H-benzoheptene,2,4α,5,6,7,8,9,9α-octahydro 3,5,5-trimethyl-9-methylene-(*4αS-cis*) and α-santalol were analyzed as its composition. This is the first report of the chemical constituents from this plant. Plant growth inhibitory study against seedling of *Mimosa pigra* Linn., disclosed methyl oleanolate glycoside showed the highest root inhibitory activity 70% at 1000 ppm, followed by β-D-glucopyranosyl tetratriacontan-2,4-dienoate and essential oil from fresh plants, which gave 63% and 46% inhibitory activities, respectively. Furthermore, seedling growth inhibitory effect against selected plants: *Echinochloa crus-galli* Beauv., *Pennisetum pedicellatum* Schult., *Lactuca sativa* Linn., *Trianthema portulacastrum* Linn. and *Bidens pilosa* Linn. were also investigated. Methyl oleanolate 3-*O*-(β-D-glucopyranoside)$_3$ exhibited the highest inhibitory effect activity against root growth on these studied plants.

Department.....Chemistry……Student's signature.....

Field of study…….Chemistry.....…..Advisor's signature.....

Academic year…….....2002…..……..Co-advisor's signature.....

Acknowledgement

On this thesis's success, the author wishes to express her extremely grateful acknowledgement to her advisor Assistant Professor Dr. Warinthorn Chavasiri and thesis co-advisor Dr. Siriporn Zungsotiporn for their helpful guidance, understanding, valuable suggestions, supervision and continuous encouragements throughout the course of this research. The author also would like to express her appreciation to Professor Dr. Padet Sidisunthorn, Professor Dr. Udom Kokpol, Dr. Namthip Sitachitta for their comments, corrections and assistance as thesis examiners. Moreover, thanks are extended to the Graduate School and the Department of Chemical, Faculty of Science, Chulalongkorn University for the financial support. Pursuing the master program at Chulalongkorn University would have been impossible without this financial support. The author acknowledged to Natural Products Research Unit, Faculty of Science, Chulalongkorn University for the support of chemical and laboratory facilities. Thanks are also extended to the Weed Science Sub-division, Botany and Weed Science Division, Department of Agriculture, Thailand for plant growth study experiment.

Deep gratitude is acknowledged to her parents, her elder brother, her aunts for their inexhaustible love, encouragement and support. Thanks to her friends for friendship and helps throughout the entire course of study. Unforgettably, the author would like especially to thank Mr. Theerasak Pirotsathaporn for tuition fees support during the whole studying.

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List of Abbreviations

AHP	=	Aniline hydrogen phthalate
br	=	Broad
n-BuOH	=	*normal*-Butanol
°C	=	Degree Celsius
$CDCl_3$	=	Deuterated chloroform
CIGAR	=	Constant time Inverse-detected Gradient Accordion Rescaled long-range heteronuclear multiple bond corelation
$CHCl_3$	=	Chloroform
cm^{-1}	=	Unit of wave number
^{13}C NMR	=	Carbon-13 Nuclear Magnetic Resonance
d	=	Doublet (NMR)
dd	=	Doublet of doublet (NMR)
dec	=	Decompose
DMSO-d_6	=	Deuterated dimethylsulfoxide
EtOAc	=	Ethyl acetate
EtOH	=	Ethanol
g	=	Gram (s)
GC/MS	=	Gas Chromatograph / Mass Spectrometry
HMQC	=	Heteronuclear Single Quantum Coherence
^1H NMR	=	Proton-1 Nuclear Magnetic Resonance
H_2O	=	Water
Hz	=	Hertz (NMR)
IR	=	Infrared
J	=	Coupling constant
Kg	=	Kilogram (s)
m	=	Multiplet (NMR)
m	=	Medium (IR)

List of Abbreviations (continued)

MeOH	=	Methanol
mg	=	Milligram (s)
min	=	Minute
mL	=	Milliliter (s)
mm	=	Millimeter (s)
m.p.	=	Melting point
MS	=	Mass Spectrometry
MW	=	Molecular weight
m/z	=	Mass to charge ratio
NIST	=	National Institute of Standards and Technology
No.	=	Number
ppm	=	Part per million
ppt	=	Precipitate
R_f	=	Retarding factor in chromatography
s	=	Singlet (NMR)
s	=	Strong (IR)
t	=	Triplet (NMR)
TLC	=	Thin Layer Chromatography
UV	=	Ultraviolet
v/v	=	Volume by volume
w	=	Weak (IR)
w/w	=	Weight by weight
δ	=	Unit of chemical shift

CHAPTER I

INTRODUCTION

Plants have been not only the most important source of food but also essential source of chemical substances used for the direct and indirect benefit in pharmacy and agriculture.

Thailand is an agricultural country with agricultural produce exports accounting for the bulk of Thailand's foreign exchange earnings. Thus, farm inputs such as pesticides increase steadily and in 2001, Thailand imported approximately 70,158 tons of pesticides (Baht 9.2 billion).[1] Among these, herbicides are the most important part of the total.

Thailand, being one of the countries in Southeast Asia with tropical rain forests, has a large biodiversity of flora and fauna. Long-lasting tropical rain forests serve as the center of evolution, plant variation and various biodiversity.[2] Human rely on plants for staying healthy, increasing the quality of life and extending life span. One quarter of the prescription drugs marketed in the world today contains active ingredients derived from plants. However, many plant species have not been thoroughly studied for their utilization. Some plants have no value for use and are considered as weeds. A weed is an undesirable plant, which grows in the same planting area of crops. But they may be useful in other fields. Weeds usually spread very rapidly and are highly competitive. Some weeds produce chemicals that affect growth of other plants. This phenomenon is well known as allelopathy, the chemicals being called allelochemicals. Allelopathy, which studies interaction between plants or microorganism including positive and negative effects[3], has been proposed as a possible alternative for weed management strategy[4] (replacing synthetic organic pesticides by allelochemicals).

1.1 Scope of allelopathy

Some plants produce chemicals and release them into the environment which affect the growth of other plants. This phenomenon is known as an allelopathy.[5] This effect could be utilized to the benefit of agriculture and weed management.

Allelopathy is derived from the Greek word *allelon* (of each other), and *pathos* (to suffer). The term "*allelopathy*" has undergone several changes over time. It was first proposed by Molish.[6] His definition referred to both the detrimental and beneficial biochemical interactions among all classes of plants, including microorganisms. Rice[7] defined allelopathy as "any direct or indirect harmful or beneficial effect by one plant (including microorganism) on another through the production of chemical compounds that escape into environment".

Allelopathy has been discussed in detail by Rice[3,7,8], Thompson[9] and Putnam and Tang.[10] They classified allelochemicals produced by higher plants and microorganisms into the following major categories: simple water soluble organic acids, straight chain alcohols, aliphatic aldehydes and ketones, simple unsaturated lactones, naphthoquinones, anthraquinones and complex quinones, simple phenols, benzoic acid and derivatives, cinnamic acid and derivatives, flavonoids, tannins, terpenoids and steroids, amino acids and polypeptides, alkaloids and cyanohydrins, sulfides and glucosides and purines and nucleotides.

Examples of some chemical structures are shown as the following:

Long chain fatty acids:

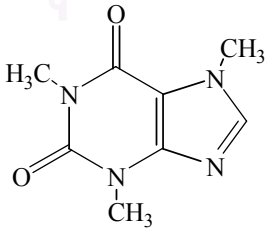
$CH_3(CH_2)_{12}CO_2H$

Myristic acid

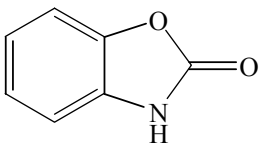
$CH_3(CH_2)_{18}CO_2H$

Arachidic acid

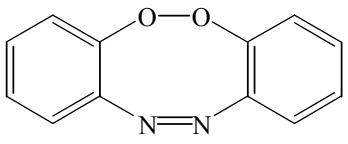
Alkaloids:



Caffeine

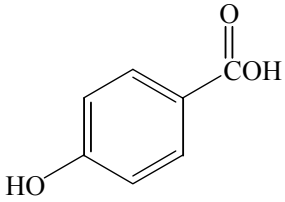


2(3)-Benzoxazolinone (BOA)

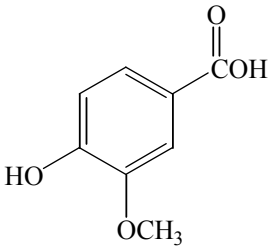


2,2-Oxo-1,1-azobenzene (AZOB)

Benzoic acid derivatives:

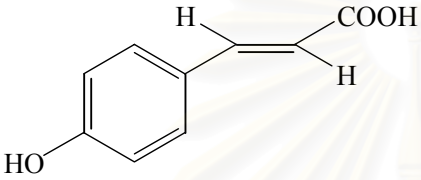


p-Hydroxybenzoic acid

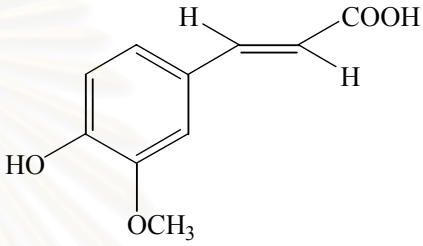


Vanillic acid

Cinnamic acid derivatives:

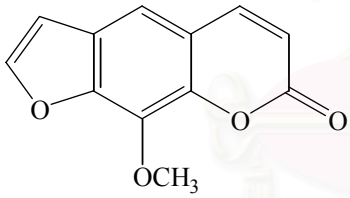


p-Coumaric acid

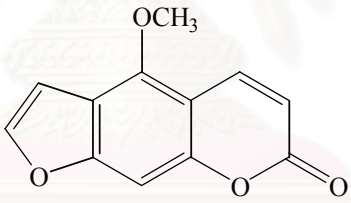


Ferulic acid

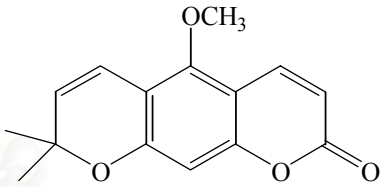
Coumarins:



Xanthotoxin

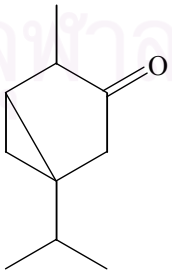


Bergapten

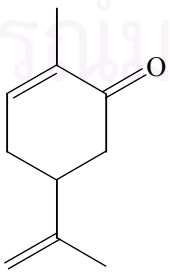


Xanthoxyletin

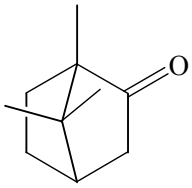
Monoterpenes:



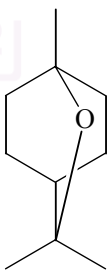
Tujone



Carvone

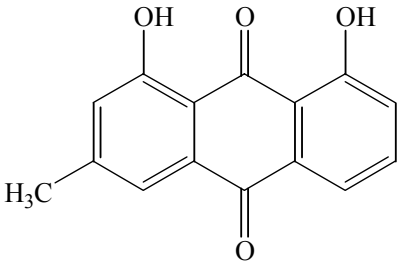


Camphor

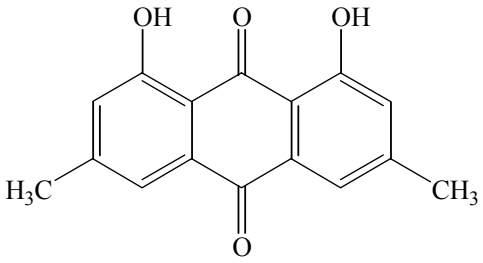


1,8-Cineol

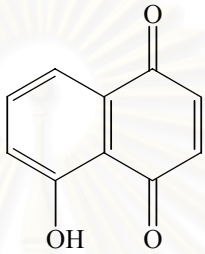
Quinones :



Emodin

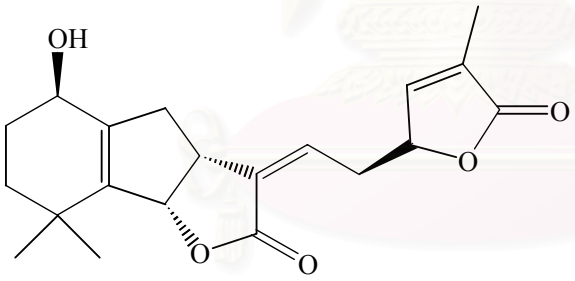


Physcion

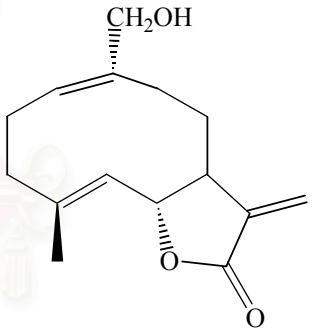


Juglone

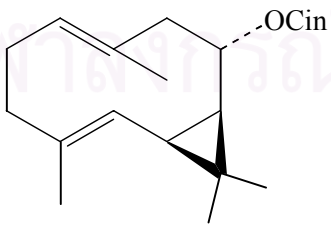
Sesquiterpenes:



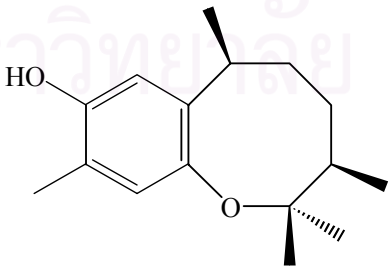
Strigol



Soulangianolide A



Guayulin A



Heliannuol A

Allelopathy is utilized for increasing crop productivity and the quality of food for humans, decreasing our reliance on synthetic herbicides and improving the environment. As demand increases for sustainable agriculture and concern grows regarding the extensive use of synthetic chemicals (*e.g*. contamination of the environment, herbicide resistance, increasing cost), attention is focused on reducing reliance upon synthetic herbicides and finding alternative strategies for weed management. Allelopathy holds great prospect for meeting some of those demands. Allelopathic potential can be used in several ways in agroecosystems.

Observations found that wherever *Hydrocotyle umbellata* grows, it can form pure stands and soon, no other plant can grow in its dense growth, not even *Panicum repens* or *Cyperus rotundus* which are one of the world's worst weeds. This suggests that *H. umbellata* produce chemicals which inhibit the growth of other plants. The purpose of this study is thus to investigate for chemical constituents with allelopathic effect of *H. umbellata*.

1.2 Botanical description

1.2.1 Characteristics of *Hydrocotyle* species

The genus *Hydrocotyle* consists mostly of small, inconspicuous marsh herbs. Plants are both aquatic and marginal, often are found as weed in mud and mire. The generic characters are small flowers, in simple umbels, 5 petals, white, the points not inflexed, calyx margin wanting, fruit consist of 2 carpels, which are flattened laterally, 5 ribbed, and not furnished with oil – tube.[11]

1.2.2 Characteristics of *Hydrocotyle umbellata* Linn.

Hydrocotyle umbellata Linn. is an aquatic macrophyte Umbelliferae family, common name is pennywort or dollar weed, known as "Waen kaew" in Thai (Figure 1.1). It is a perennial aquatic weed similar to *Centella asiatica* Urban., spreads out horizontally forming dense mats in shallow water, mud, or in marshes. *H. umbellata* also reproduces by seeds.[12]

Its long creeping stems often form dense mats, stems approximately 0.5 cm in diameter. Leaves are alternately arranged, round in outline, smooth, and shiny with petioles that may reach 15 cm in length. Leaf blades may be 5 cm in diameter and are dark green in color with veins originating from the central point of leaf. Flowers form

clusters in umbrella–like head. Individual floret is very small and consist of white petals. Many florets join together to form a single cluster.

The characteristics of *H. umbellata* is the umbrella–like heads of small white flowers. The distinctive feature helps to distinguish this plant from most others.

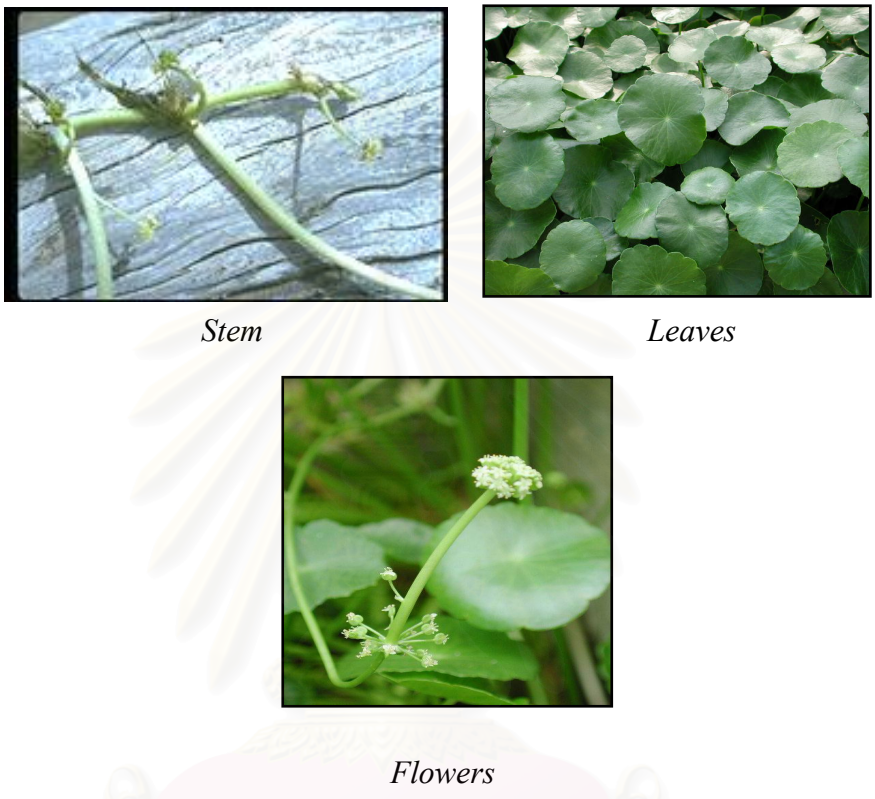


Figure 1.1 Stem, leaves and flowers of *H. umbellata* Linn.

1.2.3 Weed description

Mimosa pigra Linn. (Figure 1.2), with a common name of giant mimosa or giant sensitive plant, is a perennial shrub of sub-family Mimosaceae, a native to Mexico, Amazonia and Venezuela. It is known as ไมยราบยักษ์, ไมยราบต้น, จี่ยอบหลวง in Thai.

Figure 1.2 *Mimosa pigra* Linn.

M. pigra Linn.[13] is a perennial leguminous shrub, growing up to 2-4 m high on a wide variety of soils. Its leaves are bipinnate and sensitive to the touch, through movements of the petiole and pinnules. Petioles bear a slender prickle at the junction of each of the 6 to 16 pairs of pinnate leaves and sometimes have stouter prickles between each pair. The stems bear broad–based, sharp thorns up to 7 mm long. Mature plants have many branches growing from the base. The flowers are mauve to pink, massed in globular heads 1 cm in diameter, with each head containing about 100 florets.

M. pigra was introduced to Mae-teang and Chiang-dao district, Chiangmai province around 1947. The plant grew out of control into the Mae-ping River in which it established itself and thrived very well for many years.[14] Three to four years later, the plant has spread to 3 other nearby provinces, Chiangrai, Lampoon and Lampang. Large infestations could also be seen in Burma, Laos and along the Mae-kong River.[15]

Average seed production is about 9,000 seeds per plant, while a single plant under normal conditions can produce an average of 95,000 seeds per year.[13] Seeds

can remain viable under water for at least 2 years.[16] Giant mimosa can thrive on dry land, in swamps, along the banks of rivers or canals and also in the rivers or ponds. This weed is a serious problem in irrigation canals and reservoirs where it obstructs the water flow and causes large amount of sediment to deposit at the bottom of ponds or canals.

1.3 Relationship studies of *H. umbellata* on growth of some selected plants

Some selected plants used for examining chemical relationship studies between *H. umbellata* and the growth of other plants are listed as follows: *Lactuca sativa* Linn., lettuce (ผักกาดหอม); *Pennisetum polystachyon* Trin., ayasuwa grass (หญ้าขจรจบดอกเล็ก); *Echinochloa crus-galli* Beauv., barnyard grass (หญ้าข้าวนก); *Trianthema portulacastrum* Linn., horse purselance (ผักเบี้ยหิน); *Bidens pilosa* Linn., hairy begger-ticks (ก้นจ้ำขาว) and *Dactyloctenium aegyptium* Willd., crowfoot grass (หญ้าปากควาย).

All above species could be grouped into dicotyledon (*L. sativa, B. pilosa* and *T. portulacastrum*) and monocotyledon (*P. pedicellatum*, *E. crus-galli* and *D. aegyptium*). These plants are shown in Figure 1.3.

E. crus-galli Beauv.

L. sativa Linn.

B. pilosa Linn.

*P. polystachyon*Trin.

T. portulacastrum Linn.

D. aegyptium Willd.

Figure 1.3 Selected species for bioassay

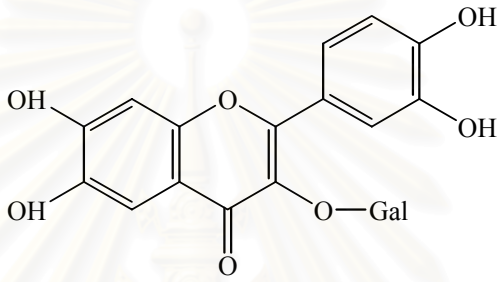
1.4 Chemical constituent studies of *Hydrocotyle* genus

The genus *Hydrocotyle* is an extensive family, comprising of nearly 100 species found throughout the temperate world. Literature survey on *H. umbellata* revealed that no phytochemical and biological activity study has been addressed. Chemical constituents of other species have been reported as shown below.

Flavonoid constituents have been reported from some plants of this genus. Nakaoki[17] isolated flavonoids from *H. wilfordi* Maxim. and identified as quercetin 3-galactoside (hyperoside). Later, in 1982, Shigematsu[18] examined the composition of

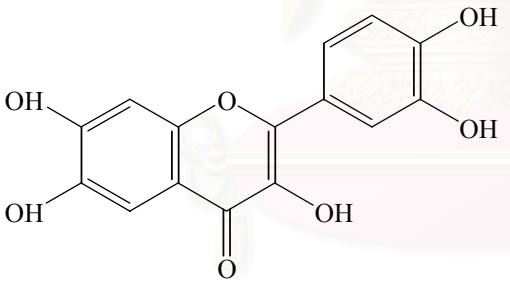
H. sibthorpioides Lam. (หญ้าเกล็ดหอย) and reported the presence of quercetin, methyl caffeate and a new caffeoyl galactoside as quercetin 3-*O*-β-D-(6″-caffeoylgalactoside). Furthermore, Gabriele *et al*[19] also found flavonoids from *H. vulgaris* Linn. They were kaempferol *O*-robinobioside and quercetin glycosides, of which quercetin-3-*O*-β-D-galactopyranoside (hyperoside) and quercetin-3-*O*-(6-*O*-arabinopyranosyl)-β-D-galactopyranoside proved to be the major constituents of this species. All isolated compounds are depicted as shown below.

Flavonoid from H. wilfordi:

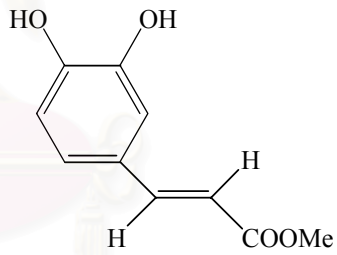


Quercetin 3-galactopyranoside (Hyperoside)

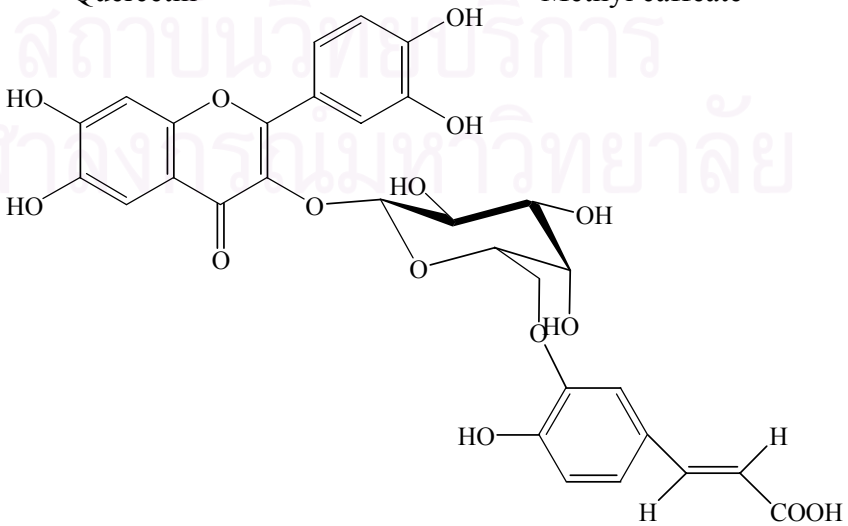
Flavonoids and cinnamic acid from H. sibthorpioides:



Quercetin

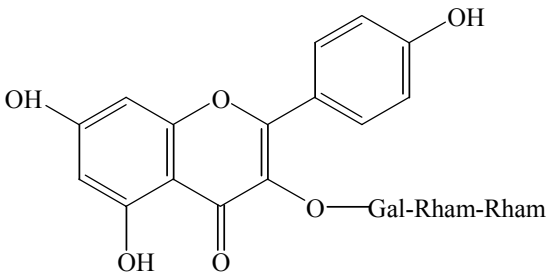


Methyl caffeate

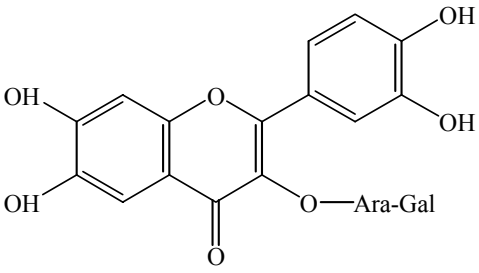


Quercetin 3-*O*-*β*-D-(6″-caffeoylgalactoside)

Flavonoids from H. vulgaris:



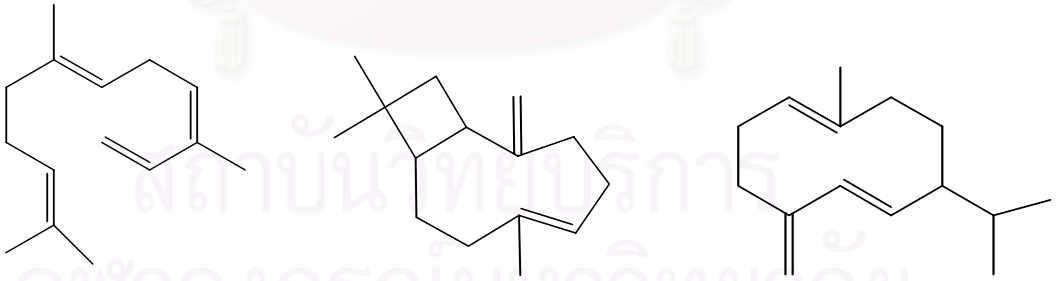
Kaempferol *O*-robinobioside



Quercetin 3-*O*-(6-*O*-arabinopyranosyl)-β- D-galactopyranoside

Typically, *Hydrocotyle* spp. produce unique essential oils throughout the whole plant. In 1982, Asakawa and coworkers studied the chemical constituents of *H. sibthorpioids* Lam., *H. maritima* Honda. and *Centella asiatica* Urban. (บัวบก).[20] They reported the distribution of monoterpenoids and sesquiterpenoids in the above three species. The major component of *H. sibthorpioids* and *H. maritima* was *trans*-*β*-farnesene whereas of *C. asiatica* is an unidentified terpenic acetate. *β*-Caryophyllene, *trans*-*β*-farnesene and germacrene D have been detected in *C. asiatica* in respectable amount.

Mono- and sesquiterpenes from Hydrocotyle spp.:



trans-*β*-Farnesene

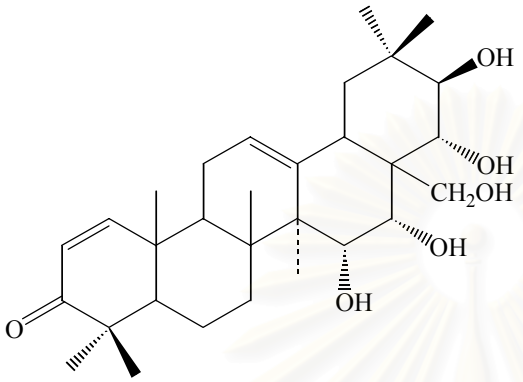
β-Caryophyllene

Germacrene D

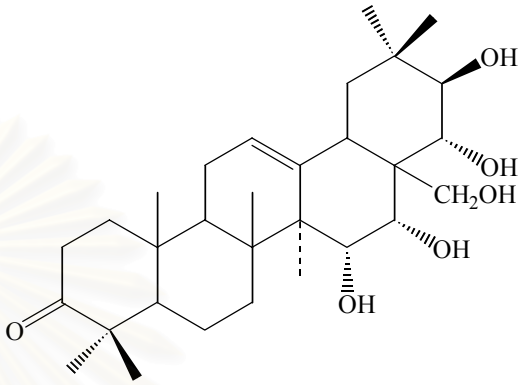
Furthermore, in 1994, Della Greca *et al* reported a search for antialgal compounds from aquatic plants.[21] They reported on the isolation and characterization of three new oleanane triterpenens, 3-oxo-15α, 16α, 21β, 22α, 28-pentahydroxy-$\Delta^{1,12}$-oleanane, 3-oxo-15α, 16α, 21β, 22α, 28-pentahydroxy-Δ^{12}-oleanane and 17, 22-*seco*-3-oxo-22, 23-dihydroxy-$\Delta^{12,16}$-oleanoic acid, along with the already known

R_1-barrigenol, barringtogenol C and hederagenic acid from *H. ranunculoides.*[22] Later, they found six new oleanane glycosides; named ranuncosides I-VI, which were obtained from a methanolic extract of this species.[23] The structures are shown below:

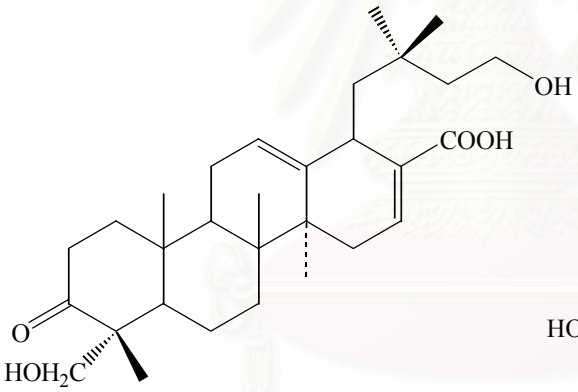
Polyoxygenated oleanane triterpenes from H. ranunculoides:



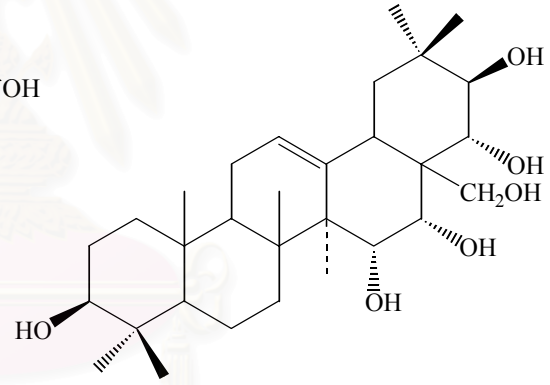
3-oxo-15α, 16α, 21β, 22α, 28-pentahydroxy-$\Delta^{1, 12}$-Oleanane



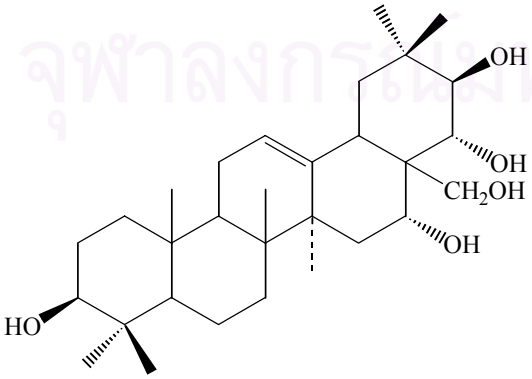
3-oxo-15α,16α, 21β, 22α, 28-pentahydroxy-Δ^{12}-Oleanane



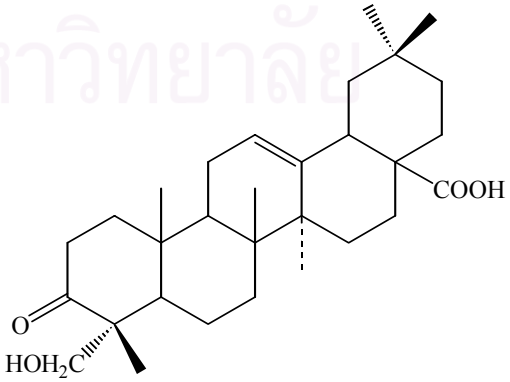
17, 22-*seco*-3-oxo-22, 23-dihydroxy-$\Delta^{12, 16}$-Oleanoic acid



R_1-Barringenol

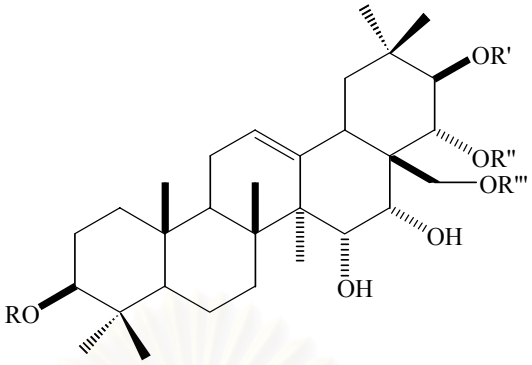


Barringtogenol C



Hederagenic acid

Oleanane glycosides from H. ranunculoides:



Ranuncoside I R = α-L-arap (1→6) β-Dglcp R′ = R″ = R‴ = H

Ranuncoside II R = [α-L-arap(1→6] [β-D-glcp (1→2)] β-D-glcp
R′ = R″ = R‴ = H

Ranuncoside III R = [α-L-arap(1→6] [β-D-glcp (1→2)] β-D-glcp
R′ = tigloyl R″ = H R‴ = acetyl

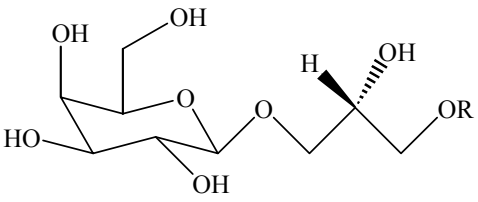
Ranuncoside IV R = [α-L-arap(1→6] [β-D-glcp (1→2)] β-D-glcp
R′ = tigloyl R″ = acetyl R‴ = H

Ranuncoside V R = [α-L-arap(1→6] [β-D-glcp (1→2)] β-D-glcp
R′ = 2-methylbutyroyl R″ = H R‴ = acetyl

Ranuncoside VI R = [α-L-arap(1→6] [β-D-glcp (1→2)] β-D-glcp
R′ = 2-methylbutyroyl R″ = acetyl R‴ = H

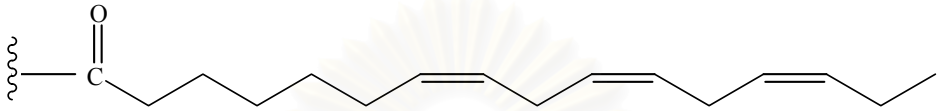
In 1998, Kwon *et al*[24] first studied chemical constituents of *H. ramiflora*, a perennial herb commonly found in Korea. It has been used in traditional Chinese herbal medicine.[25,26] They reported two new monogalactosylacylglycerols I (2*S*)-1-*O*-(7*Z*, 10*Z*, 13*Z*-hexadecatrienoyl)-3-*O*-*β*-galactopyranosylglycerol, II (2*S*)-1-*O*-(9*Z*, 12*Z*-octadecadienoyl)-3-*O*-*β*-galactopyranosylglycerol and two known com-pounds, capsidiol 3-acetate and α-spinasterol. The anti-inflammatory activity of galactosyl-acylglycerol derivatives were studied.[27]

Monogalactosyl acylglycerols, sesquiterpene, and steroid from H. ramiflora:

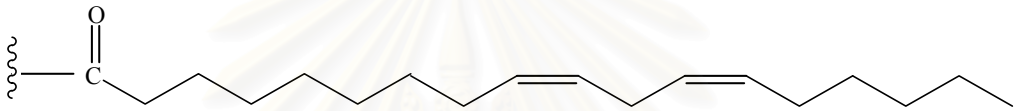


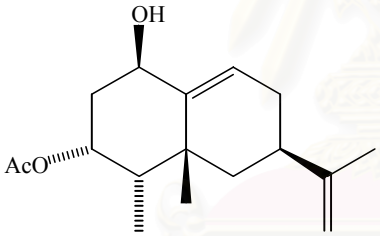
Monogalactosylacylglycerol

I: R =

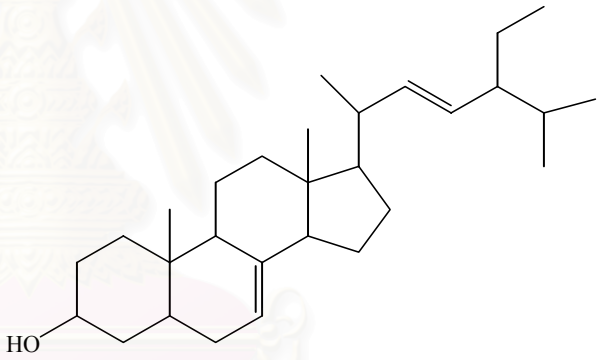


II: R =





Capsidiol 3-acetate



α-Spinasterol

1.5 Objectives

Since it is the first study on the chemical constituents of *H. umbellata* Linn., the objectives of this research could be summarized as follows:

1. To extract *H. umbellata* and to preliminarily screen and test for inhibitory activity of crude extracts on the growth of *M. pigra.*

2. To isolate and elucidate the structures of isolated substances from active fractions of the crude extracts.

3. To test for plant growth inhibitory activity of isolated substances on *M. pigra* and some selected plants.

CHAPTER II

EXPERIMENTAL PROCEDURE

2.1 Plant materials

Hydrocotyle umbellata Linn. was collected from Botany and Weed Science Division, Department of Agriculture, Bangkok, (first collection) during October 2000 and from a mango orchard in Sri-Pra-Chun district, Suphan Buri province (2nd collection, location) in July 2002. It was identified by S. Sripen.[28] A voucher specimen has been deposited in the Herbarium of the Princess Sirindhon, Ministry of agriculture and Cooperatives, Bangkok, Thailand (BK 45391).

Whole plants of *H. umbellata* (collected from Bangkok) was air-dried and crushed to coarse powder for extraction while the sample from Suphan Buri of second collection was collected as a fresh sample for hydrodistillation.

2.2 Instrumental and equipment

The melting points were determined with a Fisher – Johns melting point apparatus and are uncorrected. The Infrared (IR) spectra were recorded on a Fourier Transform Infrared Spectrophotometer model Impact 410 (KBr pellet). The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker ACF 200 and a Jeol JNMA 500 spectrometer using tetramethylsilane (TMS) as an internal reference. The mass spectra were obtained on Fission Instrument model Trio 2000 operating at 70 eV ionization voltage. The gas chromatography – mass spectrometry spectra were recorded on Varian star 3400 CX and Varian saturn 4D. The High Performance Liquid Chromatography (HPLC) was performed on Gilson model 803C. The chromatotron was conducted on Harrison Research, model 7924T, Ser. No W34 Patented, made in U.S.A. Silica gel Merck Kieselgel 60 no.7749, 7738, 7734 and 7731 were used for chromatotron, column chromatography, flash column chromatography and quick column chromatography, respectively. Thin Layer

Chromatography (TLC) was performed with aluminum sheets precoated silica gel (Merck Kieselgel 60 PF_{254}) and spots on the plate were observed under UV light or visualized by spraying with 10% H_2SO_4 in ethanol followed by heating.

2.3 Chemical reagents

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

2.4 Chemical tests

In addition to 10% H_2SO_4 in EtOH which was routinely used for detecting spots of compounds, the following reagents were used to detect certain functional groups or classes of compounds.

2.4.1 Liebermann – Burchard test

This is a general test for steroid or triterpenoid compound. To a solution of the sample to be tested (2-3 mg) in dry chloroform (0.5 mL) was added a few drops of acetic anhydride with shaking followed by one drop of concentrated sulfuric acid. The color change was observed after a few minutes. The deep green color suggested the presence of steroidal ring system while the purple color indicated a triterpenoidal skeleton.

2.4.2 2,4–Dinitrophenylhydrazine reagent (DNP)

This reagent was used for the detection of aldehyde and ketone on TLC plate.[29] It could be prepared by dissolving 0.1 g of 2,4-DNP in 100 mL of MeOH, followed by the addition of 1 mL of 36 % HCl. The plate was dipped in this reagent and evaluated immediately in visible light. Aldehyde or ketone would give a deep red spot.

2.4.3 Aniline hydrogen phthalate (AHP)

This reagent for sugar analysis[30] was prepared in ether-butanol. Sugar extracts are run one-dimentionally by descent in BuOH-EtOH-H_2O (4 : 1 : 2.2) on Whatman no.1 paper. The dried papers are dipped in AHP and redried. Finally, the papers are heated at 105°C for 5 min in order to develop the distinctive colors. Glucose, galactose and mannose give a brown color, arabinose and xylose show a red spot and rhamnose displays yellow brown.

2.5 Chemical reactions

2.5.1 Acetylation

A selected compound was dissolved in dry pyridine and acetic anhydride was added. The mixture was kept at room temperature overnight with occasional shaking. The reaction mixture was then poured, with vigorous stirring, into ice water. Stirring until the excess acetic anhydride was hydrolyzed. The crude precipitated acetate was filtered off, washed throughout with cold water until no more pyridine remained and then purified by recrystallization to obtain acetylated compound.

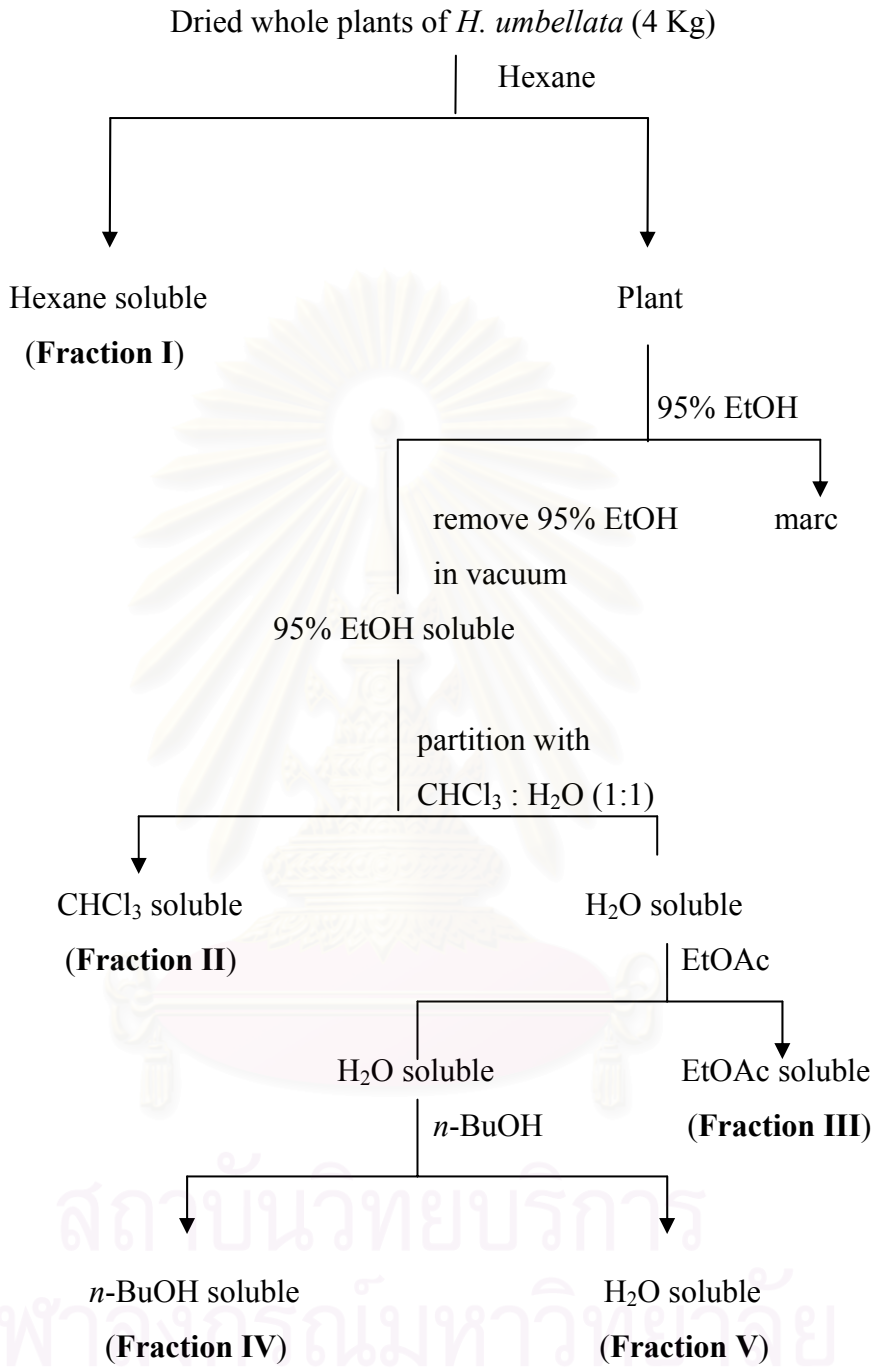
2.5.2 Hydrolysis

A selected compound was acid hydrolyzed by refluxing with excess 7% HCl in ethanol on steam bath. Evaporation of ethanol gave a solid which was extracted by diethyl ether. The etheral layer is aglycone and the water layer is a glycone part.

2.6 Extraction procedure

Whole plants of *H. umbellata*, 4 kg dry weight, were milled to fine powder which was then extracted by soaking in hexane for five days at room temperature. The residue was repeatedly extracted by hexane three times. Evaporation of the solvent affording the crude hexane extract, fraction I, as a greenish crude weighing 37.92 g or 0.95% yield (based on 4 kg dried plants). The marc was then similarly extracted with 95% ethanol. The ethanolic extract was evaporated by vacuum to a 549.80 g viscous greenish crude, 13.76% yield (based on 4 kg dried plants). The concentrated 333.58 g ethanolic crude was partitioned with chloroform : water, 1:1 (v/v) in separatory funnel to gain chloroform soluble and water soluble parts. After separation of both chloroform soluble and water soluble parts, chloroform was removed to provide a dark greenish crude 84.41 g in weight or 2.11% yield, labelled as fraction II. Another part, the aqueous layer, was further extracted with ethyl acetate to give a greenish viscous solution of ethyl acetate crude extract weighing 4.74 g or 0.12% yield, labelled as fraction III. Further extraction was performed by extracting the water soluble portion with *n*-butanol. The evaporation of organic layer *in vacuo* produced a greenish crude 45.2 g in weight or 1.13% yield, labelled as fraction IV and the water soluble portion 70.14 g in weight or 1.75% yield, fraction V. The overview of general procedure for extraction is presented in Scheme 2.1.

Scheme 2.1 Extraction procedure for the whole plants of *H. umbellata*



2.7 Hydrodistillation procedure

The essential oil was obtained by hydrodistillation of 5 kg of the crushed *H. umbellata* fresh plant in round-bottom flask with distilled, deionized water. The distillation period was approximately 2 hours. The vapor mixture consisting of water vapor and oil vapor was condensed in a receiver. Repeat distillation of the plants and extract the condensed vapor mixture with diethyl ether to obtain the essential oils, 200 mg in weight or 4.00% yield, based on fresh plants. The oil was dried over anhydrous sodium sulfate and analyzed by GC-MS for identification of its components.

2.8 Experiments for bioassays[31]

Plant growth study was used as a main bioassay to follow bioactive compounds present. *Mimosa pigra* Linn. was used as a plant model in this research.

2.8.1 Experiment for crude extract

Crude extract of 0.5, 1.0, 2.5 and 5.0 g equivalent to dried materials was dissolved in 3 mL of appropriate solvent such as dichloromethane, acetone, methanol or water and poured into Petri dishes (diameter 9 cm), each containing a filter paper. The equal amount of the same solvent to dissolve crude extract was added instead of extracts as control. Leave overnight to remove solvent by air drying, then 5.0 mL of distilled water was added to each plate. Thirty seeds of *M. pigra* were put on each filter paper. Petri dishes were closed and place at room temperature to observe the growth for 7 days. Five seedlings were randomly selected to measure the shoot and root elongation (mm) compared with the control experiment. Calculation of inhibitory effect of substance by using equation shown below. Perform each experiment in three replications.

$$\% \text{ Inhibition} = 100 - (\%C)$$

$$\%C = (A / C) \times 100$$

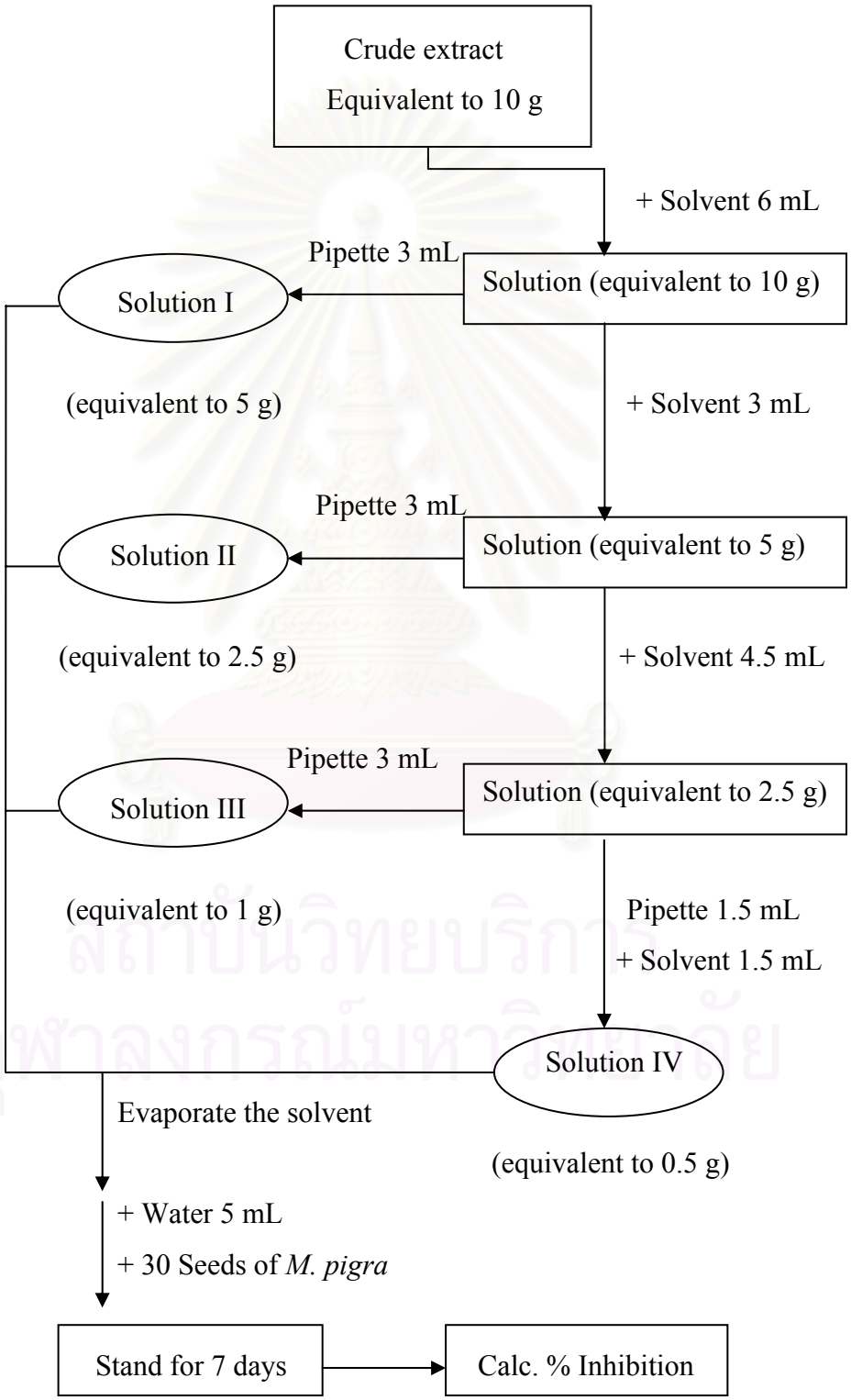
where A is the means of root or shoot length of tested sample

C is the means of root or shoot length of control sample

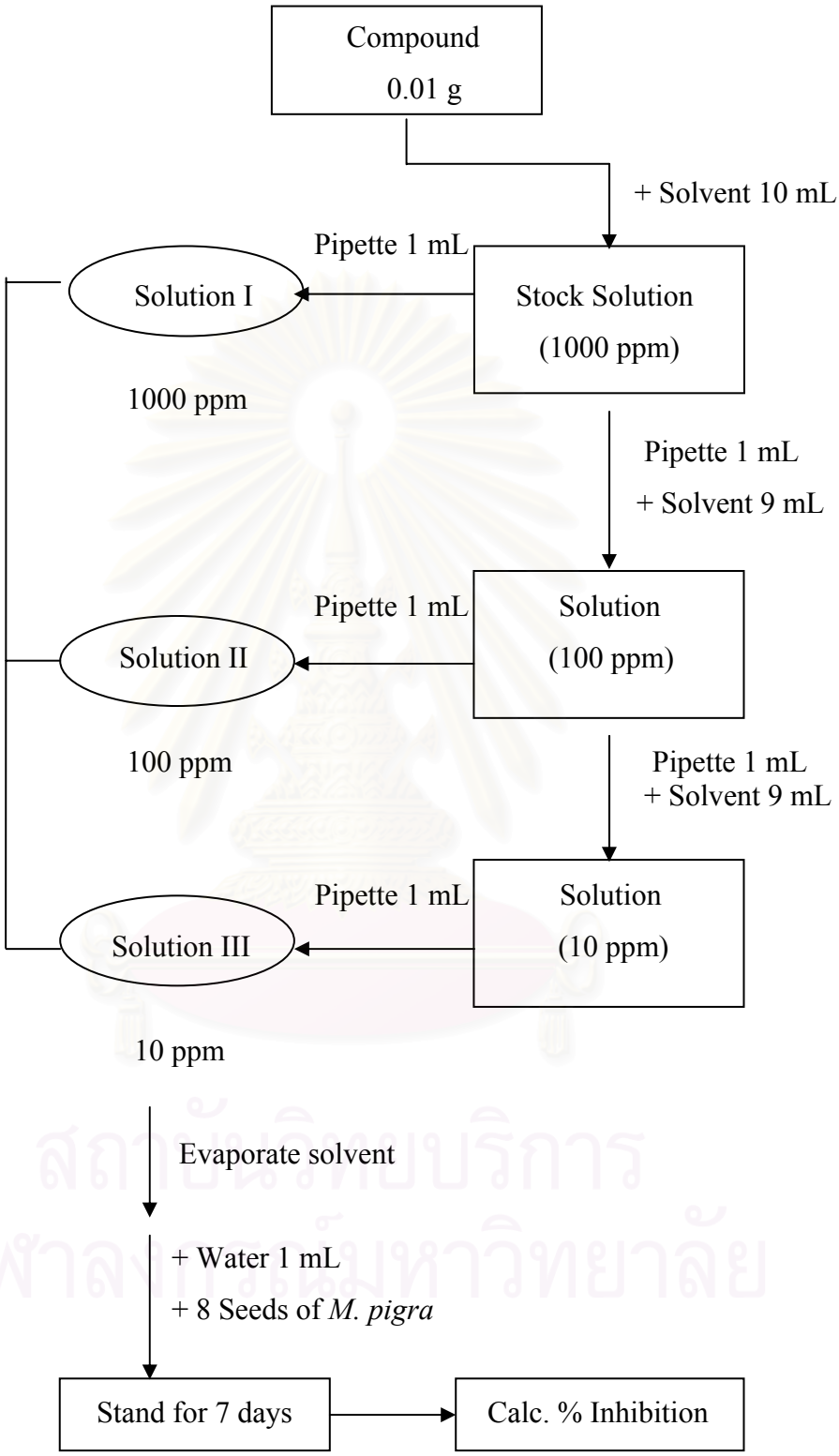
2.8.2 Experiment for pure compounds

The bioassay procedure was performed exactly the same as that for crude extract. These procedures could be summarized in Schemes 2.2 and 2.3.

Scheme 2.2 Bioassay test procedure for crude extract of *H. umbellata.*



Scheme 2.3 Bioassay test procedure for isolated compounds from *H. umbellata*



CHAPTER III

RESULTS AND DISCUSSION

Hydrocotyle umbellata Linn., an aquatic plant, was selected for chemical constituents investigation in conjunction with biological activity. The main objective is to search for bioactive compounds for plant growth inhibition. *Mimosa pigra* Linn. (dicotyledon) was chosen as a main bioassay species. In addition, the investigation on inhibitory effect of isolated substances against other dicotyledon weeds including *Lactuca sativa*, *Trianthema portulacastrum* and *Bidens pilosa*, and monocotyledon weeds, namely *Echinochloa crus-galli* and *Pennisetum polystachyon* were examined.

3.1 The results of extraction

The whole plants of *H. umbellata* were extracted following the procedures described in Scheme 2.2, Chapter 2. The results of extraction are summarized as shown in Table 3.1.

Table 3.1 The results of extraction of the whole plants of *H. umbellata* (4 Kg).

Solvent	Fraction	Weight (g)	%w/w
Hexane	I	37.92	0.95
Chloroform	II	84.41	2.11
Ethyl acetate	III	4.74	0.12
n-Butanol	IV	45.20	1.13
Water	V	70.14	1.75

The results revealed that most of chemical constituents of *H. umbellata* could dissolve in chloroform and water, 2.11% and 1.75% w/w of dried plants 4 kg,

respectively. The components soluble in chloroform possessed medium polarity, whereas those in water fractions had high polarity.

3.2 Bioassay results

3.2.1 Effect of crude extract on the growth of *M. pigra*

The haxane and ethanolic crude extracts of *H. umbellata* were preliminarily screened to observe the effect on the growth of *M. pigra*. The results are demonstrated in Table 3.2 and Figure 3.1.

Table 3.2 Effect of hexane and ethanolic crude extracts of *H. umbellata* on the growth of *M. pigra*

Fraction	Part	% Inhibition			
		0.5 g	1.0 g	2.5 g	5.0 g
Hexane	Root	2	16	40	52
	Shoot	8	19	40	49
EtOH	Root	56	87	95	95
	Shoot	60	86	95	96

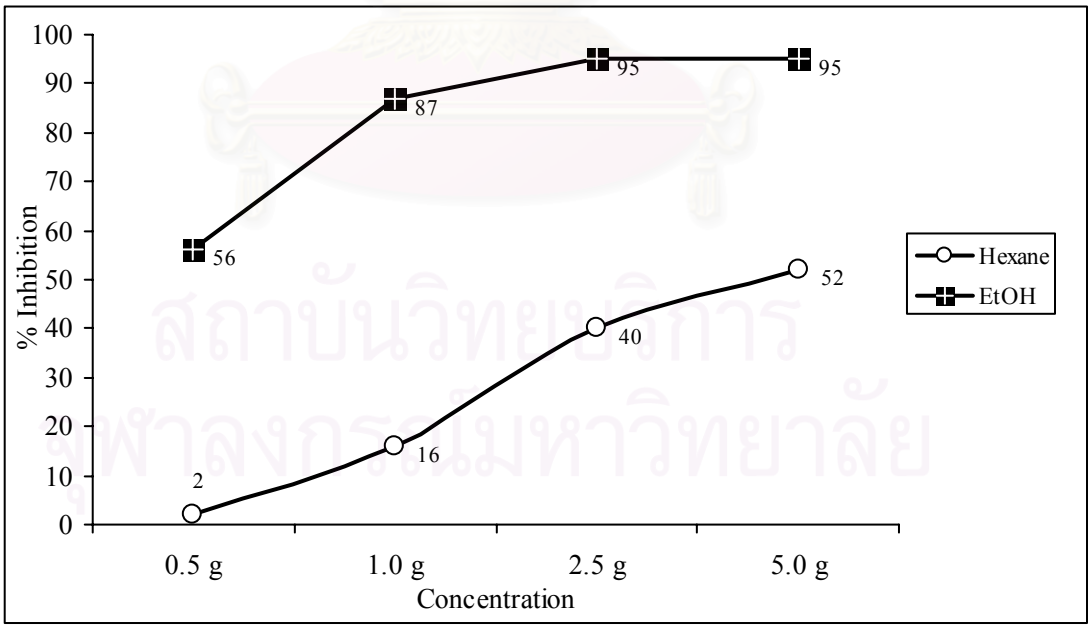


Figure 3.1 Effect of hexane and ethanolic crude extracts of *H. umbellata* on the growth of *M. pigra*

The inhibition of root is thought to be more essential for considering than that of percent shoot growth inhibition. This is because the roots are directly contacted to the tested chemical while the shoot growth is contributed from root and attributed to accumulated food from seed. Therefore, the profound effect of tested substance in this plant part might be misinterpreted. The inhibitory effect on the root length is guided for further fractionation.

In accordance with the testing result, the ethanolic extract revealed attractive results. Consequently, this crude was further partitioned with chloroform, ethyl acetate and *n*-butanol, respectively. The general extraction procedure is summarized in Scheme 2.1. Ultimately all fractions were resubmitted to test for the growth effect of *M. pigra*. The results are reported as shown in Table 3.3 and Figure 3.2.

Table 3.3 Effect of crude extracts on the growth of *M. pigra*

Fraction	Part	% Inhibition			
		0.5 g	1.0 g	2.5 g	5.0 g
$CHCl_3$	Root	50	65	79	87
	Shoot	31	44	63	80
EtOAc	Root	4	16	23	27
	Shoot	-11	-4	2	12
n-BuOH	Root	9	19	39	47
	Shoot	-2	-1	14	23
H_2O*	Root	-	-	-	-
	Shoot	-	-	-	-

*(-) Become mouldy

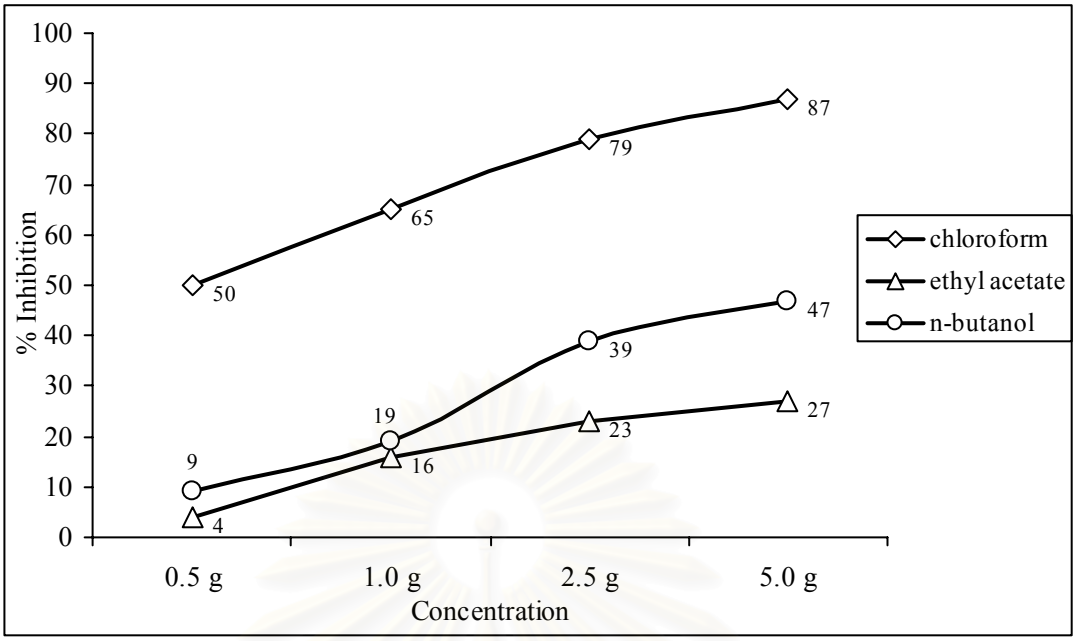


Figure 3.2 Effect of crude extracts on the growth of *M. pigra*

According to the above results (Tables 3.2 and 3.3), chloroform crude extract displayed strong inhibitory activity against *M. pigra*, followed by hexane extract. Both extracts were thus selected for further investigation for the chemical constituents which were responsible for the bioactivity.

3.2.2 The effect of *H. umbellata* on other plants

The effect of crude extracts of *H. umbellata* on the growth of some selected plants was further investigated. Those selected plant samples included *E. crus-galli* Beauv., *P. polystachyon* Trin., *D. aegyptium* Willd., representatives for monocotyledon and *L. sativa* Linn., *T. portulacastrum* Linn., and *B. pilosa* Linn. for dicotyledon plants. The criterion of species selection is based on seed size and high germination. The results are shown in Table 3.4 and Figure 3.3.

Table 3.4 Effects of crude extracts of *H. umbellata* on root and shoot of some selected plants

Plants	Concentration equivalent to dried weight of *H. umbellata*(g)	% Inhibition of Root				
		Hexane	Chloroform	Ethyl acetate	Butanol	Water
Echinochloa crus-galli	0.5	83	39	8	16	-*
	1.0	97	42	21	25	-*
	2.5	97	81	38	57	-*
	5.0	97	98	61	94	-*
Pennisetum polystachyon	0.5	63	95	-5	-38	15
	1.0	91	100	7	36	55
	2.5	97	100	11	80	86
	5.0	100	100	67	91	100
Dactyloctenium aegyptium	0.5	67	100	-48	-98	-97
	1.0	76	100	-14	-44	52
	2.5	100	100	67	61	61
	5.0	100	100	100	100	100
Lactuca sativa	0.5	52	69	14	-18	-*
	1.0	65	100	16	6	-*
	2.5	90	100	41	88	-*
	5.0	100	100	84	100	-*
Trianthema portulacastrum	0.5	19	48	7	38	40
	1.0	90	100	16	74	53
	2.5	100	100	48	93	100
	5.0	100	100	51	100	100
Bidens pilosa	0.5	79	100	37	30	87
	1.0	83	100	51	58	99
	2.5	94	100	54	95	100
	5.0	96	100	94	100	100

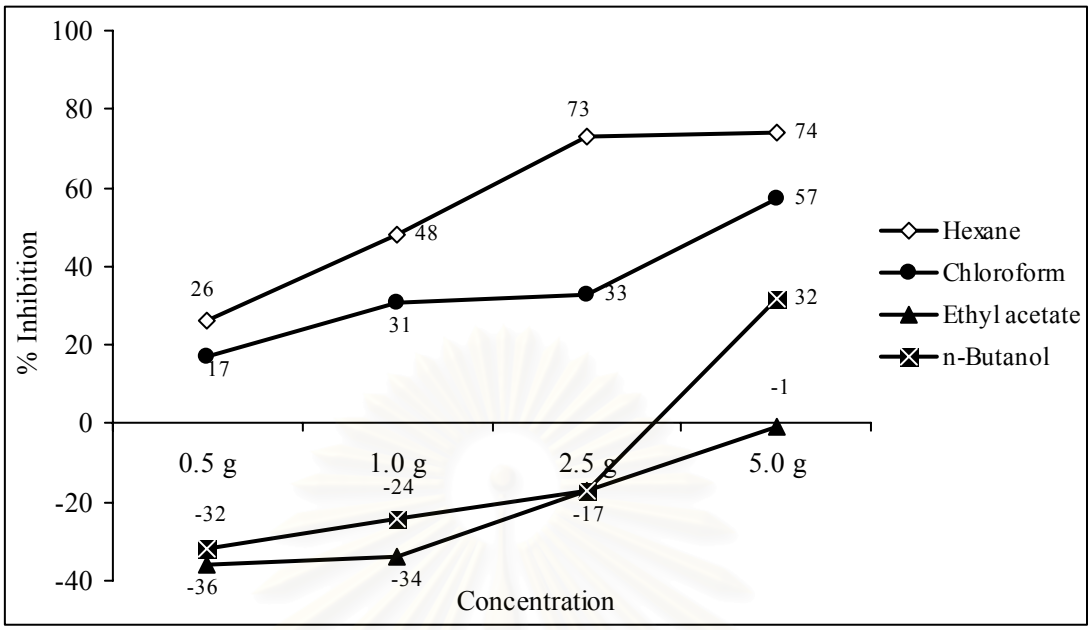
(-* Become mouldy)

Table 3.4 (Cont.)

Plants	Concentration equivalent to dried weight of *H. umbellata* (g)	% Inhibition of Shoot				
		Hexane	Chloroform	Ethyl acetate	Butanol	Water
Echinochloa crus-galli	0.5	26	17	-36	-32	-*
	1.0	48	31	-34	-24	-*
	2.5	73	33	-17	-17	-*
	5.0	74	57	-1	32	-*
Pennisetum polystachyon	0.5	70	75	-9	-23	-31
	1.0	79	100	-7	-12	-12
	2.5	88	100	-6	-7	53
	5.0	100	100	-5	30	100
Dactyloctenium aegyptium	0.5	70	100	-57	56	44
	1.0	77	100	-51	58	61
	2.5	100	100	-48	64	62
	5.0	100	100	-32	100	100
Lactuca sativa	0.5	52	22	-10	-31	-*
	1.0	59	100	12	-22	-*
	2.5	70	100	43	38	-*
	5.0	100	100	56	100	-*
Trianthema portulacastrum	0.5	25	31	-7	-33	-53
	1.0	60	100	-3	-7	-17
	2.5	100	100	-2	42	100
	5.0	100	100	-1	100	100
Bidens pilosa	0.5	81	100	42	33	59
	1.0	82	100	51	39	93
	2.5	96	100	55	95	100
	5.0	97	100	98	100	100

-* (Become mouldy)

a) *E. crus-galli*



b) *P. polystachyon*

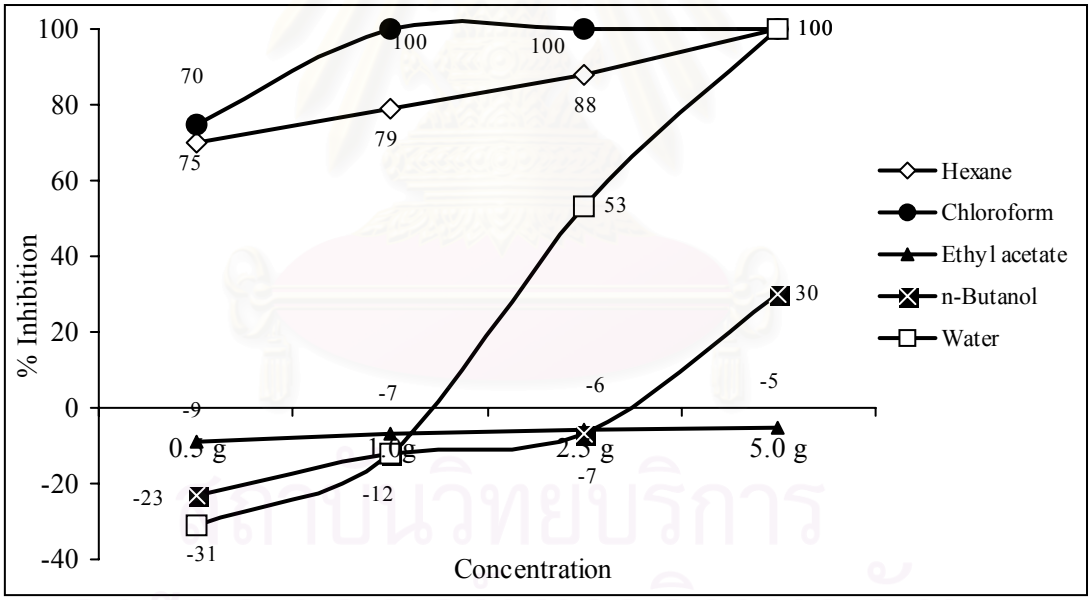
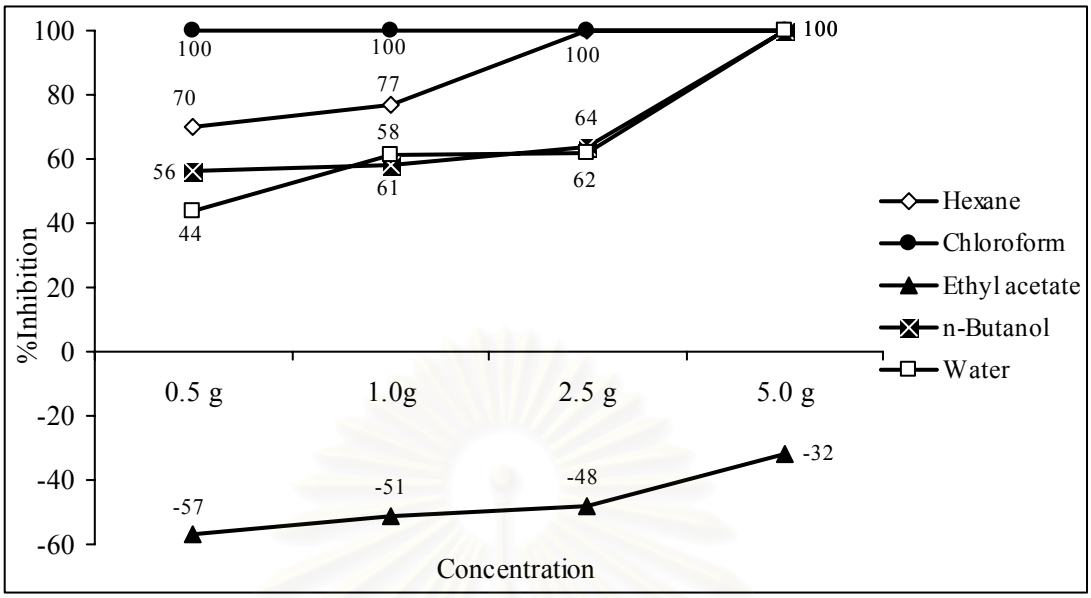


Figure 3.3 Effects of crude extracts of *H. umbellata* on the roots of a) *E. crus-galli,* b) *P. polystachyon*, c) *D. aegyptium*, d) *L. sativa,* e) *T. portulacastrum* and f) *B. pilosa*

c) *D. aegyptium*



d) *L. sativa*

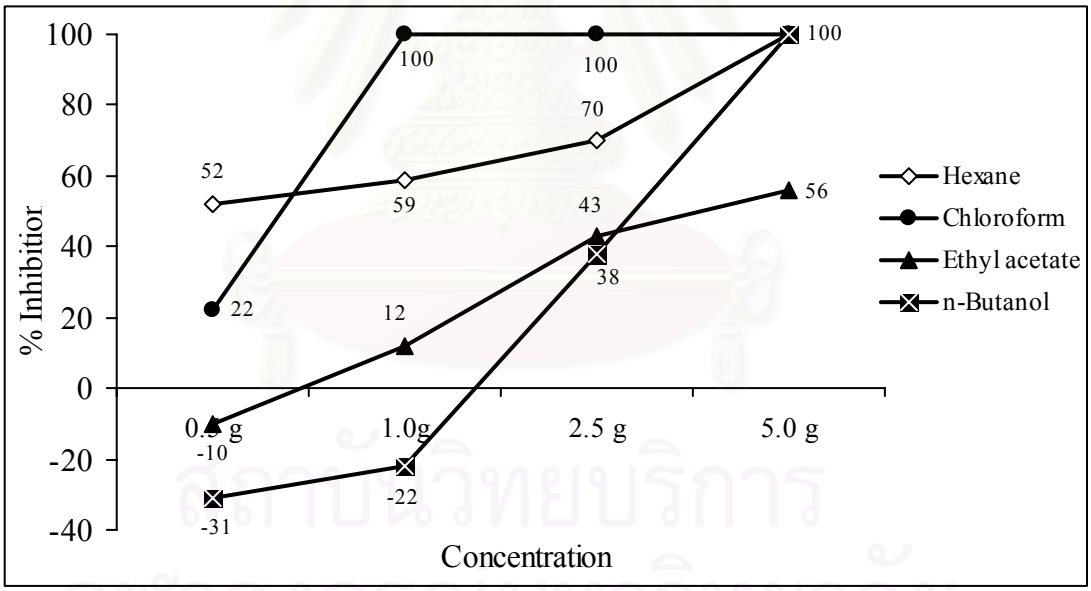
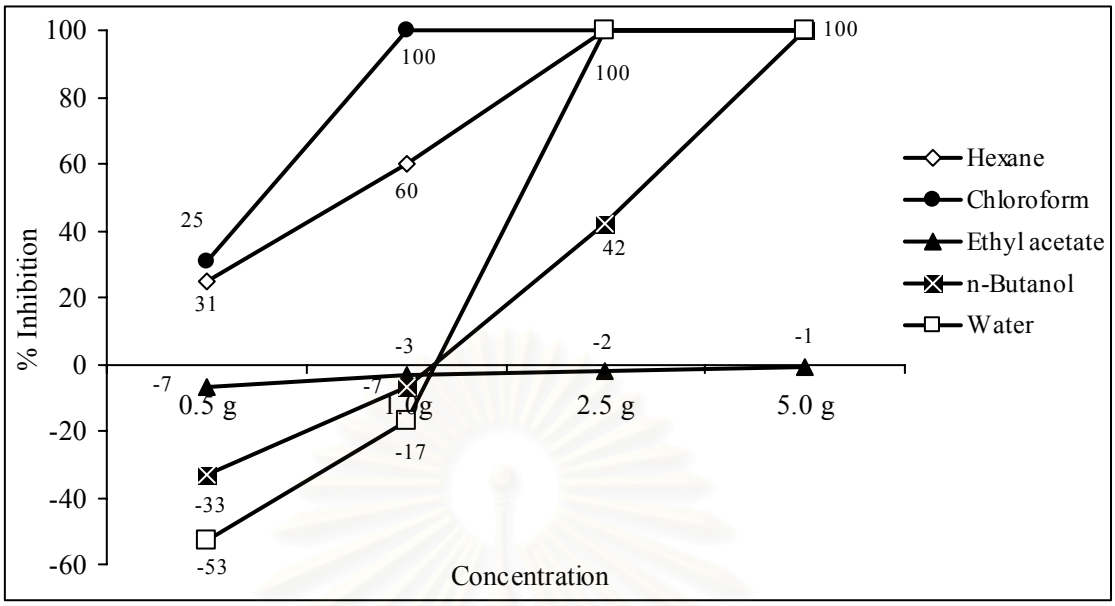


Figure 3.3 (cont.)

e) *T. portulacastrum*



f) *B. pilosa*

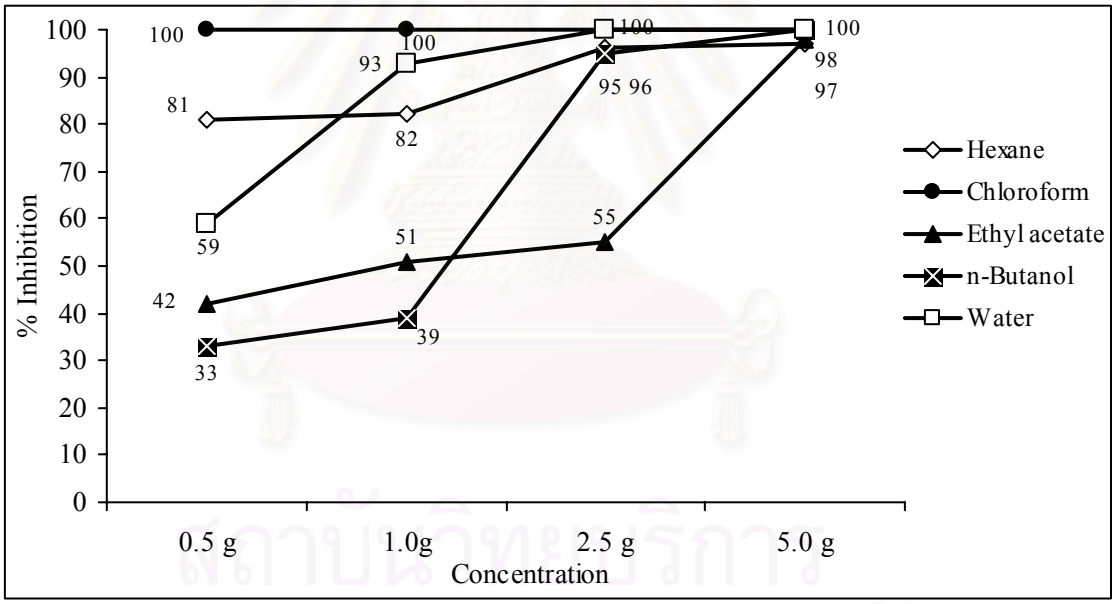


Figure 3.3 (cont.)

From the preliminary results, hexane and chloroform extracts showed high inhibitory effect against plants selected. Especially, the chloroform extract inhibited 100% for the growth of both roots and shoots of *D. aegyptium* and *B. pilosa* at concentration equivalent to dried weight 0.5 g. The root growth of *P. polystachyon*, *L. sativa* and *T. portulacastrum* was totally inhibited at 1.0 g equivalent. Hexane extract inhibited the root of each plant more than 90% at 2.5 g equivalent. Thus, hexane and chloroform extracts were considered for further study.

3.3 Fractionation

3.3.1 Fractionation of hexane extract (fraction I)

The hexane extract, fraction I, was concentrated and yielded 37.92 g of greenish sticky liquid (0.95% wt by wt). Thin–layer chromatography of the concentrated extract showed that there were at least five components in this crude (solvent system: 20% EtOAc - hexane). The portion of the concentrate 26 g was chromatographed on silica gel column chromatography. The column was initially eluted with hexane and increasing polarity by mixing with EtOAc. The results of fractionation of fraction I are shown in Table 3.5.

Table 3.5 Fractionation of fraction I

Eluents (V/V)	Fraction no.	Remarks	Weight (g)
hexane	1 - 8	Pale yellow oil	4.66
5% EtOAc – hexane	9 - 16	Pale yellow oil **(Mixture 1)**	3.94
10% EtOAc – hexane	17 - 31	Greenish sticky liquid **(Compound 1)**	5.29
20% EtOAc – hexane	32 - 52	Brown semi solid **(Mixture 2)**	4.72
30% EtOAc – hexane	53 - 63	Brown semi solid **(Mixture 3)**	3.11
40% EtOAc – hexane	64 - 77	Brown residue	2.24
50% EtOAc – hexane	78 - 90	Brown residue	1.93

3.3.2 Fractionation of chloroform extract (fraction II)

The chloroform extract (50 g), as dark green crude, was separated into subfractions by silica gel quick column chromatography technique. Mixed crude extract with silica gel, and was subjected on the top of column then initially eluted by hexane, followed by a mixture of hexane and EtOAc, EtOAc and MeOH and 100% MeOH, respectively. Eluting solvent approximately 1 L for each fraction was collected. Fractions eluted from the column were concentrated under *vacuo*. The results of fractionation are shown in Table 3.6.

Table 3.6 Fractionation of fraction II

Eluents (V/V)	Fraction	Remarks	Weight (g)
hexane	II A	Yellow oil	1.63
25% EtOAc – hexane	II B	Dark green gum	10.15
50% EtOAc – hexane	II C	Dark green gum	3.82
75% EtOAc – hexane	II D	Dark green gum	3.36
EtOAc	II E	Brown residue	2.55
25% MeOH - EtOAc	II F	Brown residue	16.44
50% MeOH - EtOAc	II G	Brown residue	9.71
75% MeOH - EtOAc	II H	Brown residue	1.64
MeOH	II I	Brown residue	0.26

Each fraction was examined for its bioactivity on the growth of *M. pigra* in order to follow the biological activity. The results are shown in Table 3.7 and Figure 3.4.

Table 3.7 Effect of fractionated $CHCl_3$ extract on the growth of *M. pigra*.

Fraction	Parts	% Inhibition			
		Equivalent to dry weight (g)			
		0.5 g	1.0 g	2.5 g	5.0 g
II A	Root	16	17	11	36
	Shoot	-4	-2	-11	3
II B	Root	36	42	40	40
	Shoot	1	6	18	19
II C	Root	37	47	56	58
	Shoot	3	4	13	21
II D	Root	35	33	41	50
	Shoot	-4	-1	9	23
II E	Root	34	36	42	42
	Shoot	-1	2	-2	18
II F	Root	34	45	53	59
	Shoot	-4	15	8	21
II G	Root	42	58	66	73
	Shoot	8	7	11	24
II H	Root	29	45	36	31
	Shoot	2	-3	2	-2
II I	Root	6	1	-1	11
	Shoot	-11	-5	-10	-6

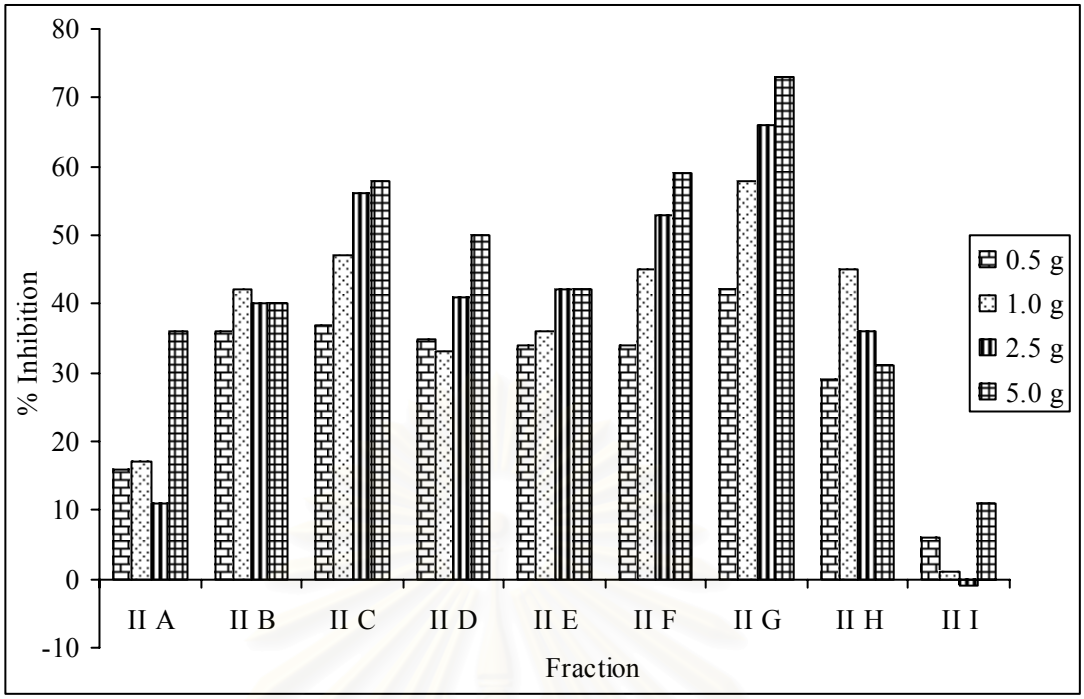


Figure 3.4 Effect of fractionated $CHCl_3$ extract on the root growth of *M. pigra*

Fraction IIG showed the highest inhibition (73%), followed by fractions IIF, IIC, IID and IIE with the inhibitory values of 59, 58, 50 and 42%, respectively at concentration 5.0 g equivalent. Accordingly, these fractions were separated to search for active ingredients. Fractions IIA, IIH and III did not show good activity even at concentration equivalent to 5.0 g of dry weight.

3.3.2.1 Separation of fraction IIB

Fraction IIB, dark green gum, 10.4 g was obtained from quick column chromatography of the chloroform extract (fraction II). Thin – layer chromatography of this fraction showed that there were at least four components (solvent system: 25% EtOAc - hexane). This fraction was rechromatographed on silica gel column chromatography. The column was initially eluted with hexane and increased polarity by gradient mixing of EtOAc. The eluted solution was collected approximately 50 mL for each fraction. Each eluted portion was monitored by thin – layer chromatography and the equivalent ones were combined. The results of the separation fraction IIB are presented in Table 3.8.

Table 3.8 The separation of fraction IIB

Eluents (V/V)	Fraction no.	Remarks	Weight (g)
hexane	-	-	-
10% EtOAc – hexane	1 - 5	Yellow oil	1.23
20% EtOAc – hexane	6 - 11	Brown semi solid **(Mixture 4)**	1.87
30% EtOAc – hexane	12 - 18	Greenish sticky liquid	2.59
	19 - 25	**(Mixture 5)**	1.45
40% EtOAc – hexane	26 - 50	Greenish gum **(Mixture 2)**	1.60
	51 - 61	Greenish gum	0.87
60% EtOAc – hexane	62 - 75	Greenish gum	0.91

3.3.2.2 Separation of fraction IIC

Fraction IIC was obtained as a dark green gum, 3.9 g from quick column chromatography of fraction II which was eluted by 50% EtOAc – hexane. Thin – layer chromatography of fraction IIC revealed at least six components in this fraction (solvent system: 55% EtOAc – hexane). Fraction IIC was reseparated by silica gel column using 120 g of adsorbent. Hexane was used as an initial eluent, followed by a mixture of hexane – EtOAc, EtOAc and EtOAc – MeOH, respectively. Each fraction was collected about 50 mL. The results of the separation of this fraction are shown in Table 3.9.

Table 3.9 The separation of fraction IIC

Eluents (V/V)	Fraction no.	Remarks	Weight (g)
10% EtOAc – hexane	1 - 14	Pale yellow oil	trace
20% EtOAc – hexane	15 - 17	Pale yellow oil	0.27
30% EtOAc – hexane	18 - 25	Yellow oil	0.15
40% EtOAc – hexane	26 - 54	White ptt in yellow oil **(Mixture 2)**	1.19
50% EtOAc – hexane	55 - 80	Dark yellow semi solid	0.71
60% EtOAc – hexane	81 - 120	Brown residue	0.53
70% EtOAc – hexane	121 - 144	Brown residue	0.99

3.3.2.3 Separation of fraction IID

Fraction IID, 3.36 g of dark green semisolid was obtained, after eluted with 75 % EtOAc – hexane. The TLC of this fraction presented at least four spots (solvent system: 75% EtOAc – hexane). It was further purified by flash column chromatography using silica gel as an adsorbent. The results of the separation are shown in Table 3.10.

Table 3.10 The separation of fraction IID

Eluents (V/V)	Fraction no.	Remarks	Weight (g)
20 % EtOAc - hexane	1 - 8	Yellow oil	trace
30% EtOAc - hexane	9 - 12	Yellow oil	trace
40% EtOAc - hexane	13 - 24	Yellow oil	trace
50% EtOAc - hexane	25 - 35	Yellow wax	0.71
60% EtOAc - hexane	36 - 39	Yellow wax	0.27
75% EtOAc - hexane	40 - 46	Yellow wax	0.83
100% EtOAc	47 - 57	Dark brown oil	trace
10 % MeOH - EtOAc	58 - 65	Dark brown solid	0.27
20 % MeOH - EtOAc	66 - 72	Dark brown solid	0.52
30 % MeOH - EtOAc	73 - 78	Dark brown solid	0.42
45 % MeOH - EtOAc	79 - 87	Dark brown solid	trace
60 % MeOH - EtOAc	88 - 93	Dark brown solid	trace

3.3.2.4 Separation of fraction IIE

Fraction IIE, dark brown residue (2.55 g), was gained after elution with 100% EtOAc. Thin layer chromatography indicated that there were at least three components in this fraction (solvent system: 10% EtOAc – hexane). This fraction was then separated by recolumn chromatography on silica gel using EtOAc and a mixture of EtOAc containing increasing proportions of MeOH as eluents. The results of the separation are shown in Table 3.11.

Table 3.11 The separation of fraction IIE

Eluents (V/V)	Fraction no.	Remarks	Weight (g)
100 % EtOAc	1 - 5	Yellow oil	trace
5% MeOH - EtOAc	6 - 10	Yellow oil	0.45
10% MeOH - EtOAc	11 - 17	Yellow oil	0.27
15% MeOH - EtOAc	18 - 23	White ppt in yellow oil **(Compound 2)**	0.75
20% MeOH - EtOAc	24 - 35	Dark brown viscous oil	0.36
30% MeOH - EtOAc	36 - 52	Dark brown viscous oil	0.22
40% MeOH - EtOAc	53 - 61	Dark brown viscous oil	trace

3.3.2.5 Separation of fraction IIF

Fraction IIF, 16.8 g was afforded from chloroform extract eluted with 25% MeOH – EtOAc. The crude was mixed with silica gel no. 7738 and then subjected to flash column chromatography. The column was initially eluted with EtOAc, followed by increasing amount of MeOH in EtOAc. The results are displayed in Table 3.12.

Table 3.12 The separation of fraction IIF

Eluents (V/V)	Fraction no.	Remarks	Weight (g)
100 % EtOAc	1 - 35	Yellow viscous oil **(Compound 2)**	1.21
10% MeOH - EtOAc	36 - 41	Dark brown semi solid	1.01
15% MeOH - EtOAc	42 - 84	White ppt in yellow oil **(Compound 4)**	5.62
25% MeOH - EtOAc	85 - 129	Dark brown viscous oil	2.00
50% MeOH - EtOAc	130 - 147	Dark brown viscous oil	1.47
70% MeOH - EtOAc	148 - 163	Dark brown viscous oil	0.95
90% MeOH - EtOAc	164 - 192	Brown solid **(Compound 3)**	1.15

3.3.2.6 Separation of fraction IIG

Fraction IIG, 9.77 g, brown solid, was separated by silica gel column chromatography. Spots revealed on TLC plate indicated at least three components present. The column was initially eluted by 15% EtOAc – hexane, followed by increasing polarity of solvent. Eluting solvent was collected for each fraction approximately 50 mL and then concentrated. Each fraction was monitered by TLC and fractions showing a similar profile were combined. The results are shown in Table 3.13.

Table 3.13 The separation of fraction IIG

Eluents (V/V)	Fraction no.	Remarks	Weight (g)
15% EtOAc - hexane	1 - 15	Dark brown liquid	0.31
30% EtOAc - hexane	16 - 30	Dark brown liquid	0.28
45% EtOAc - hexane	31 - 47	Dark brown liquid	0.19
60% EtOAc - hexane	48 - 65	Yellow ppt in brown liquid **(Compound 2)**	3.07
75% EtOAc - hexane	66 - 80	Dark brown liquid	2.10
100% EtOAc	81 - 98	Dark brown liquid	0.71
15% MeOH - EtOAc	99 - 120	Dark brown liquid	1.12
30% MeOH - EtOAc	121 - 150	Brown semi solid	0.59
40% MeOH - EtOAc	151 - 169	Brown semi solid **(Compound 3)**	1.46

3.4 Properties and structural elucidation of isolated substances from *H. umbellata*

3.4.1 Properties and structural elucidation of mixture 1

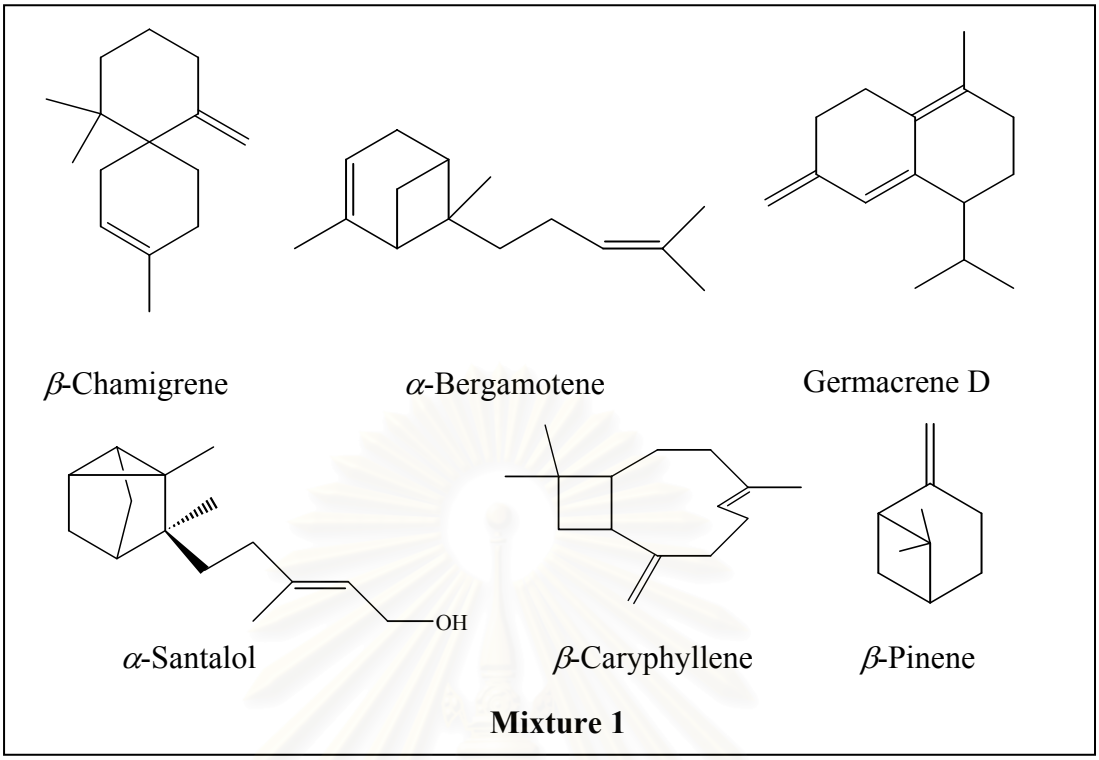
Mixture **1**, pale yellow oil, *Eucalyptus* oil like odor, was obtained from fraction no 9 – 16 of the hexane extract (fraction I) which was further fractionated by silica gel column chromatography by eluting with 15% EtOAc – hexane. This fraction was repurified by chromatotron to yield mixture **1**, 1.3 g (5% w/w of fraction I).

The gas chromatogram (Figure 3.5) exhibited six peaks. From library search (NIST database) of MS pattern (Figure 3.6), these compounds were well-matched to known essential oils: β-chamigrene, α-bergamotene, α-santalol, germacrene D, β-caryophyllene and β-pinene. The list of possible composition of mixture **1** is tabulated as presented in Table 3.14.

Table 3.14 The composition of mixture **1**

Peak no	R_t (min)	MW	Compounds	% Composition
1	6.76	204	β-chamigrene	46.3
2	6.86	204	α-bergamotene	3.4
3	7.16	220	α-santalol	2.7
4	7.36	204	germacrene D	4.7
5	7.44	204	β-caryophyllene	37.5
6	7.73	136	β-pinene	5.4

The constituents found in this mixture were mainly sesquiterpenes except for β-pinene, the monoterpene. Among them, β-chamigrene and β-caryophyllene were detected as major compositions, 46.3% and 37.5%, respectively. From the literature surveys, *Hydrocotyle* genus has been known to contain unique essential oils throughout the whole plant. For instance, in 1982, Y. Asakawa *et al* reported the distribution of mono- and sesquiterpenoids in *H. sibthorpioides*, *H. maritima* and *C. asiatica*. The major component of *H. sibthorpioides* and *H. maritima* was *trans*-β-farnesene (32.5% and 20.9% respectively), while the major composition of *C. asiatica* was an unidentified terpenic acetate (M^+274, base peak 157) (36.4%), trans-β-farnesene (17.7%), germacrene D (16.0%) and β-caryophyllene (12.5%). With the information attained from this research, it was observed that *H. umbellata* and above three species mentioned produced β-pinene, β-caryophyllene and germacrene D as common constituents. The latter component, however, has not been detected in *H. sibthorpioides*.



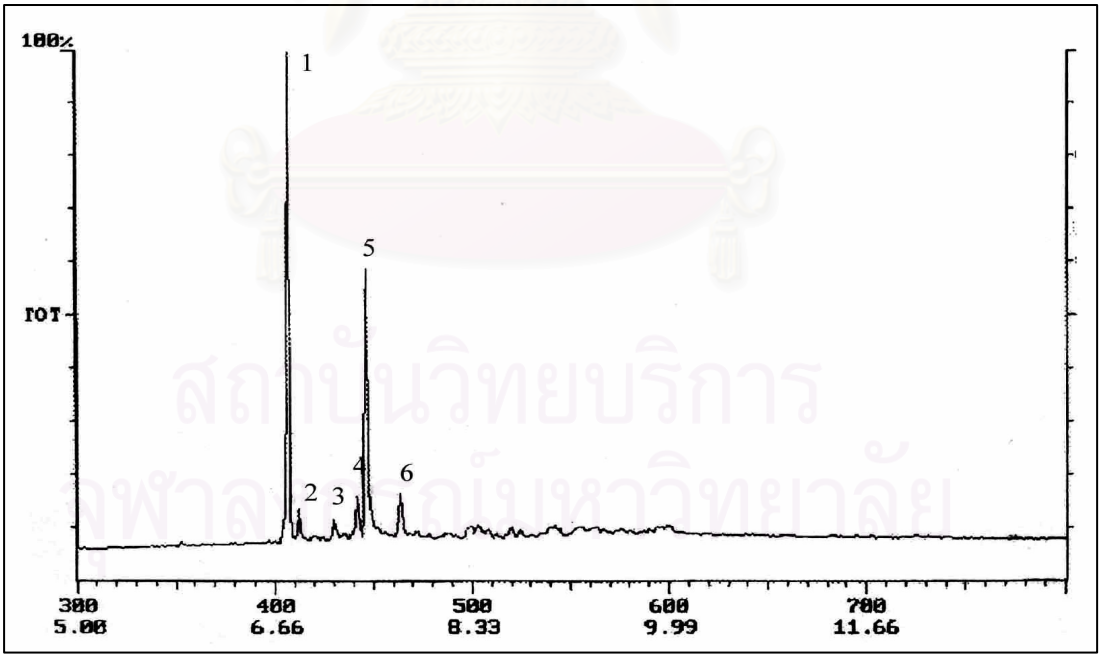


Figure 3.5 The GC of mixture **1**

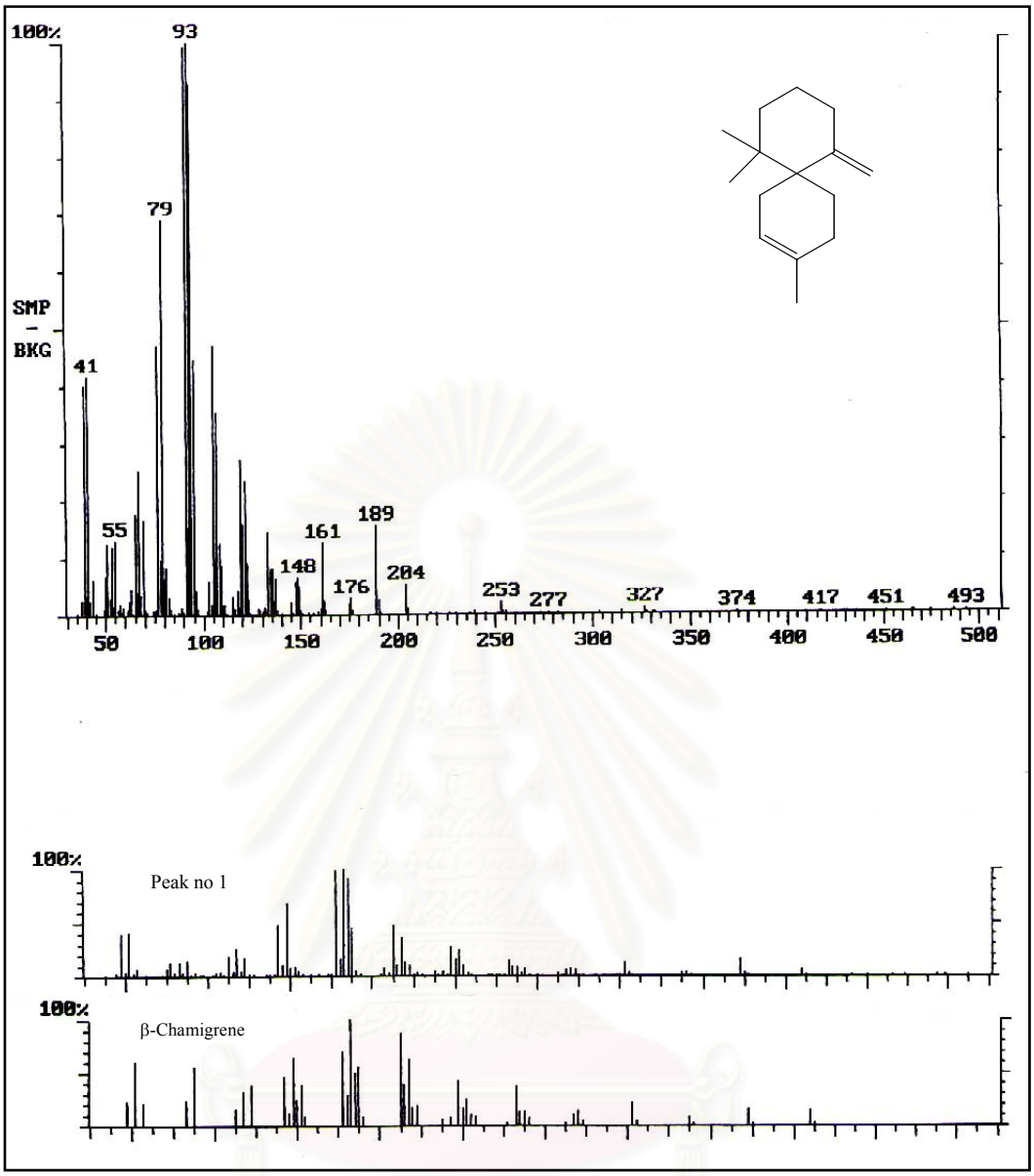
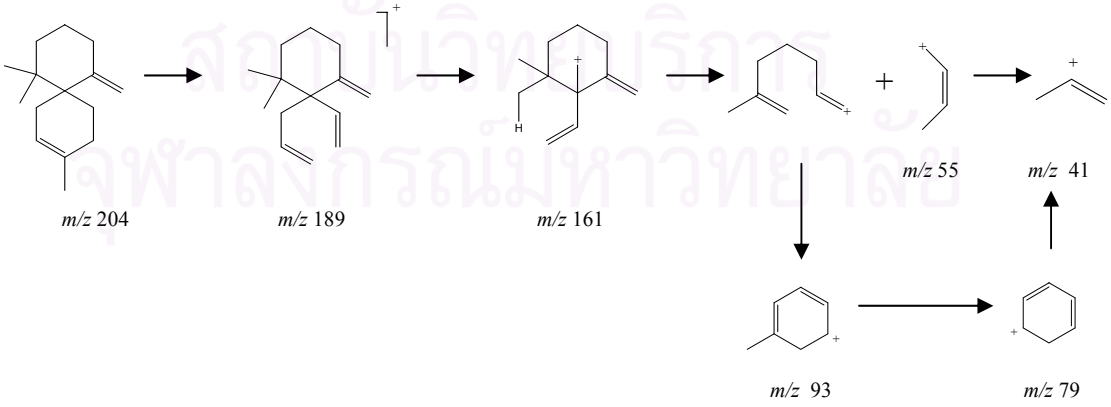


Figure 3.6 The mass spectrum of mixture **1** (peak no.1)



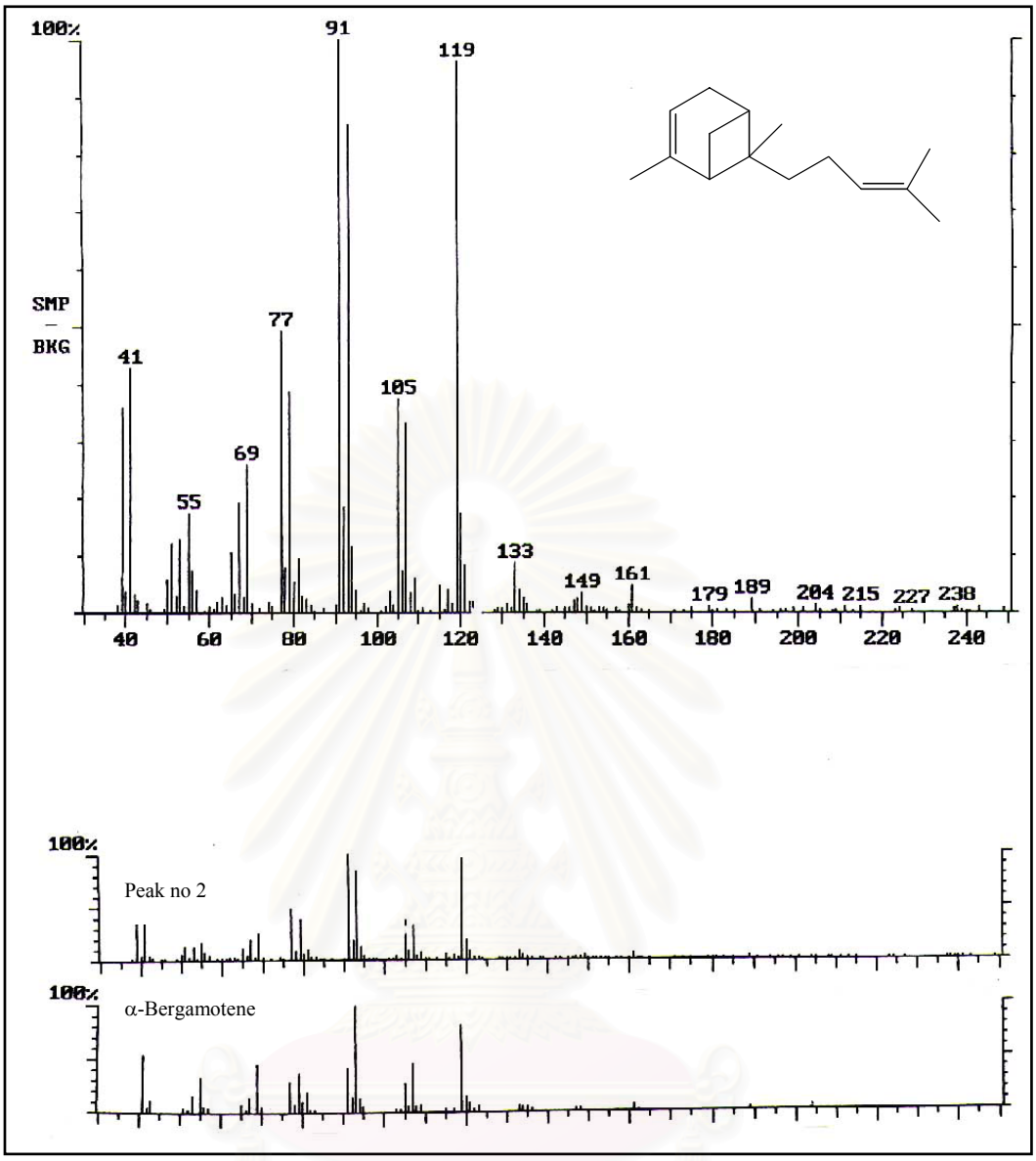
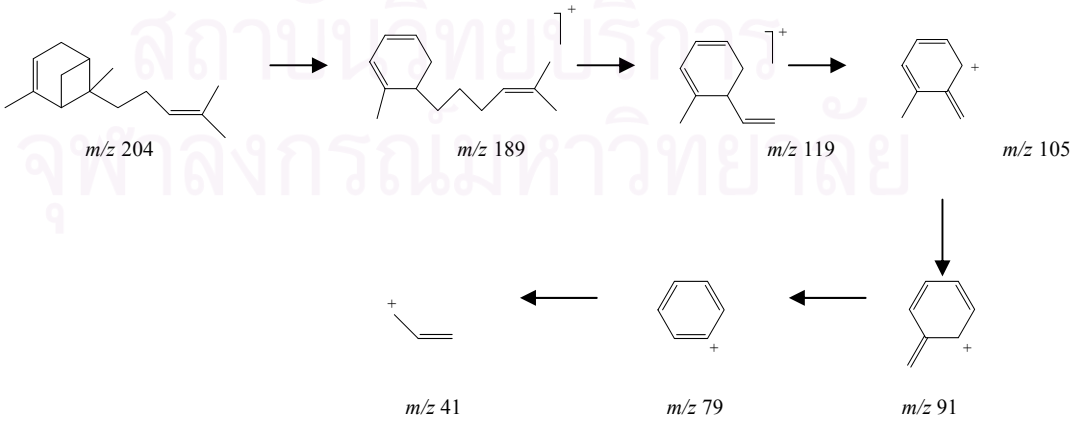


Figure 3.6 Cont. (peak no.2)



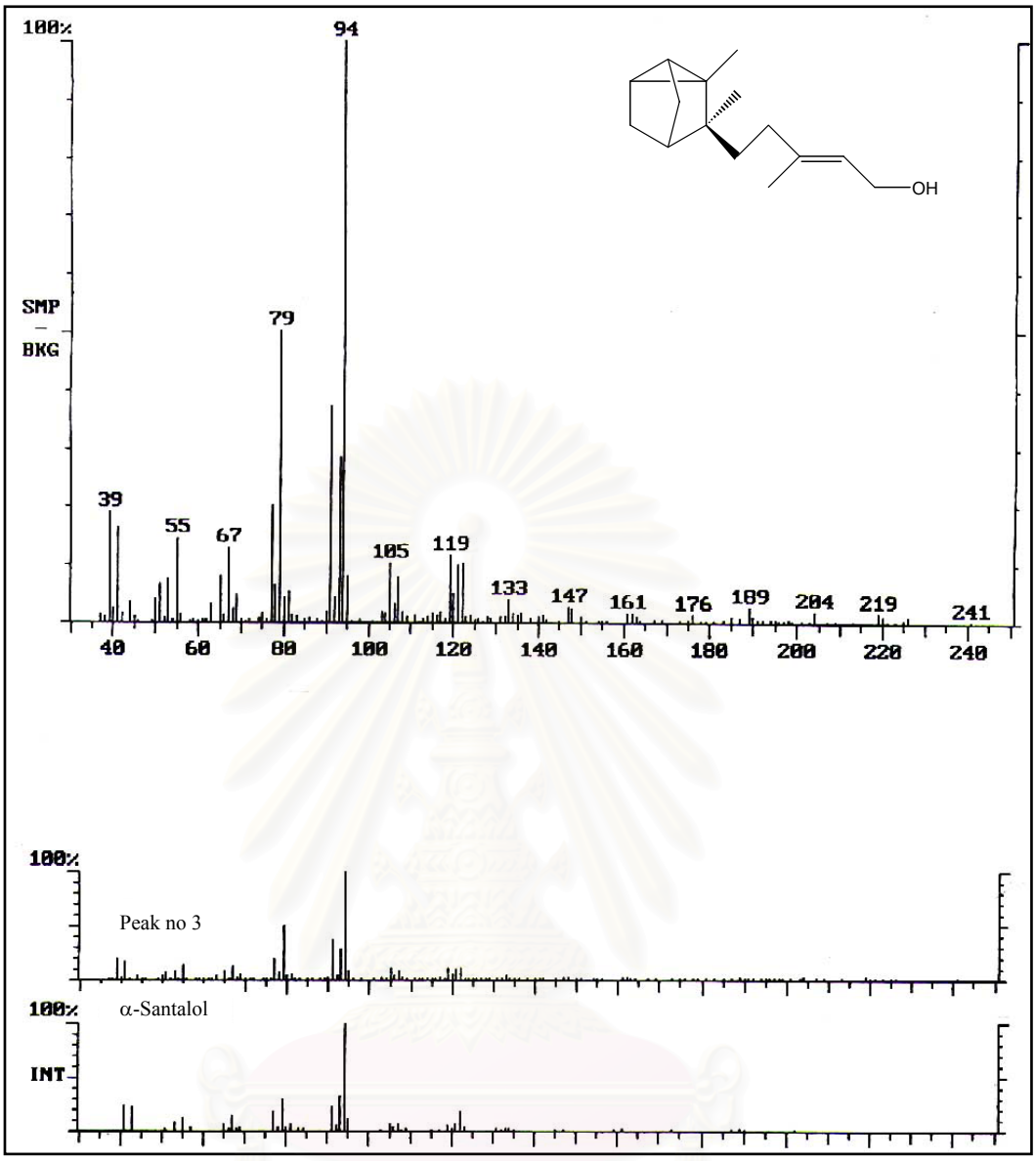
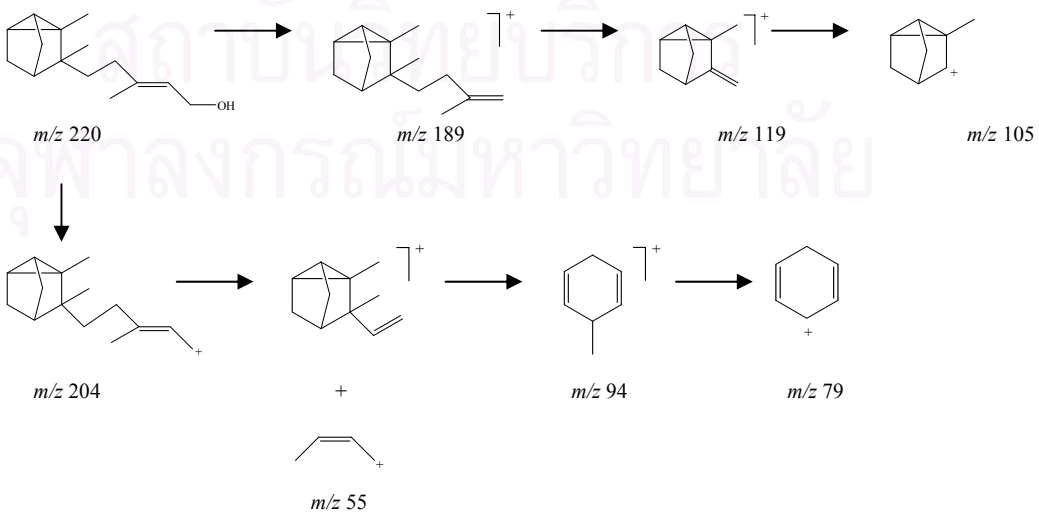


Figure 3.6 Cont. (peak no 3)



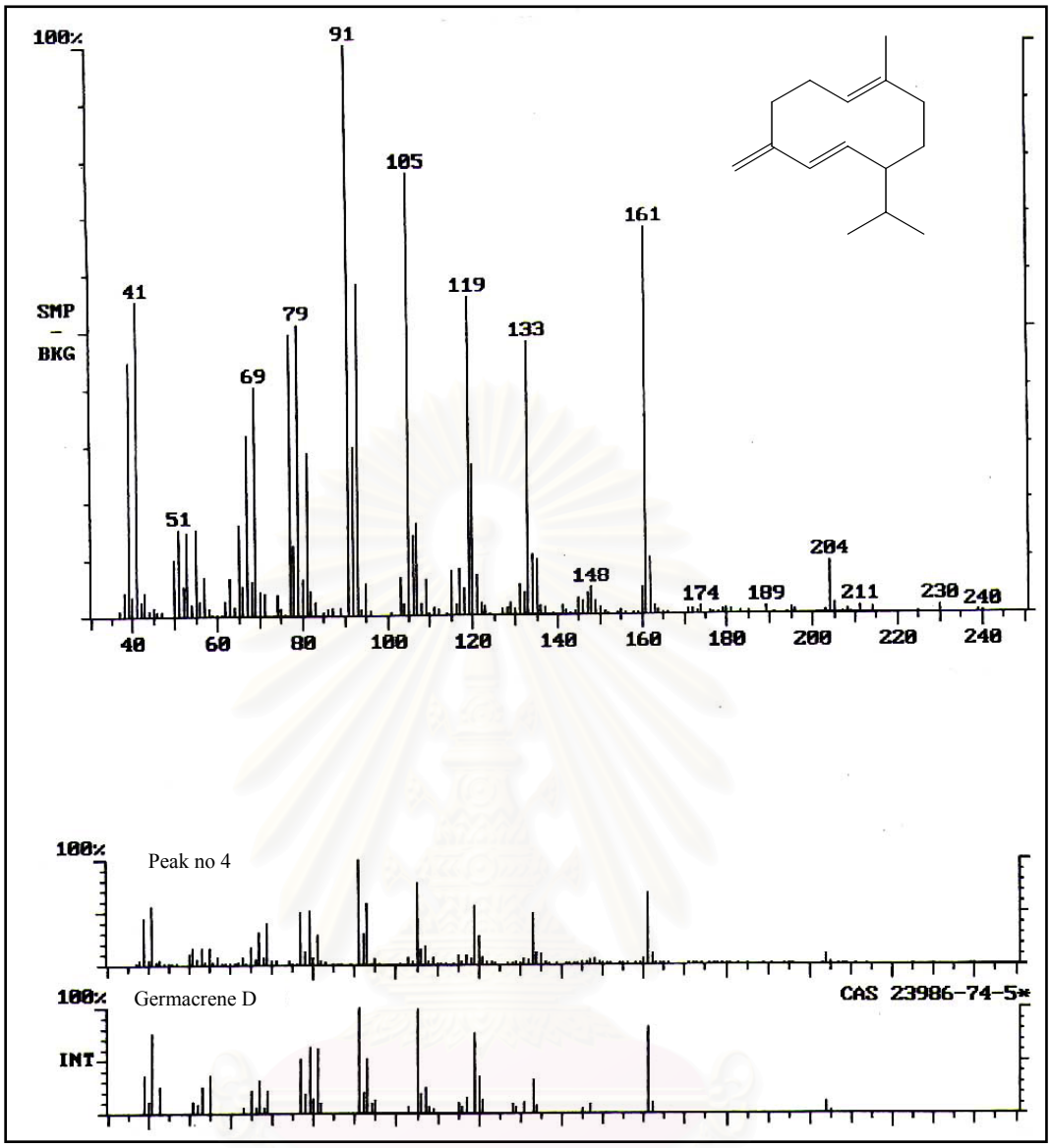
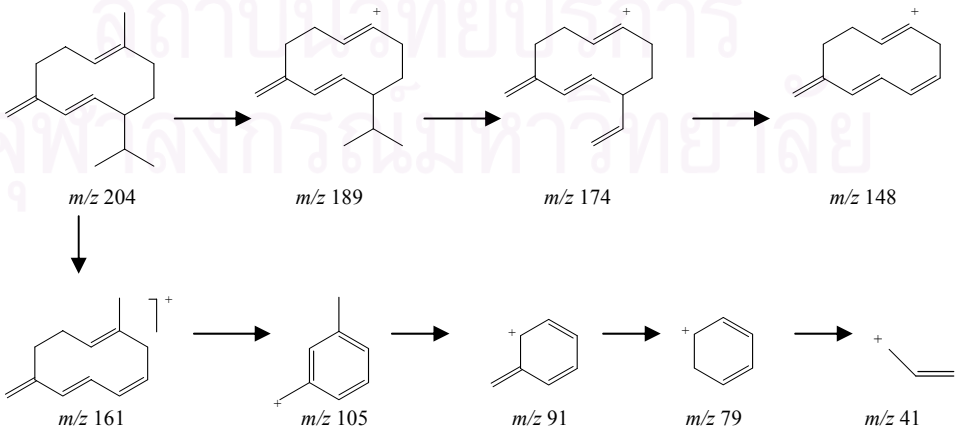


Figure 3.6 Cont. (peak no.4)



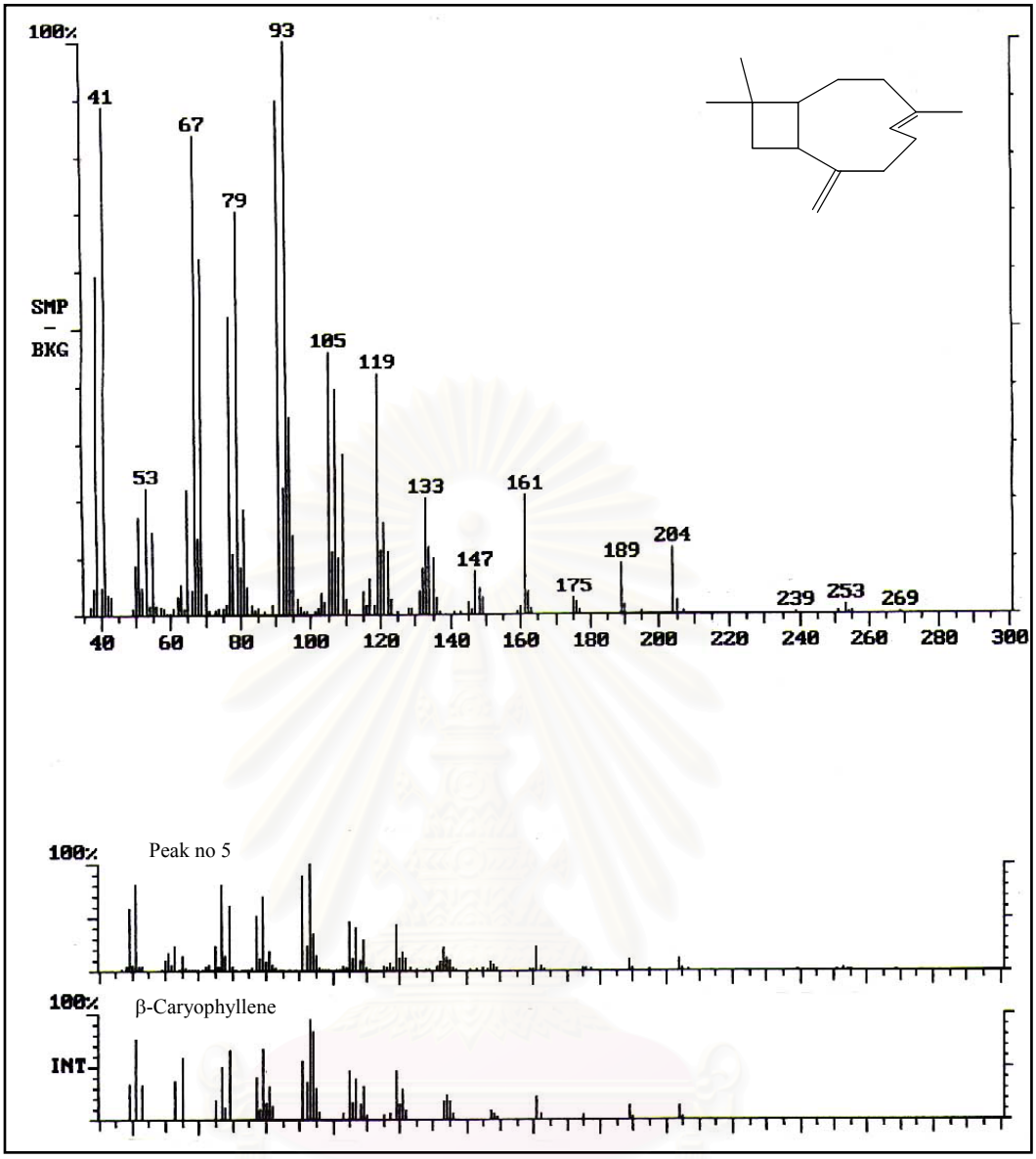
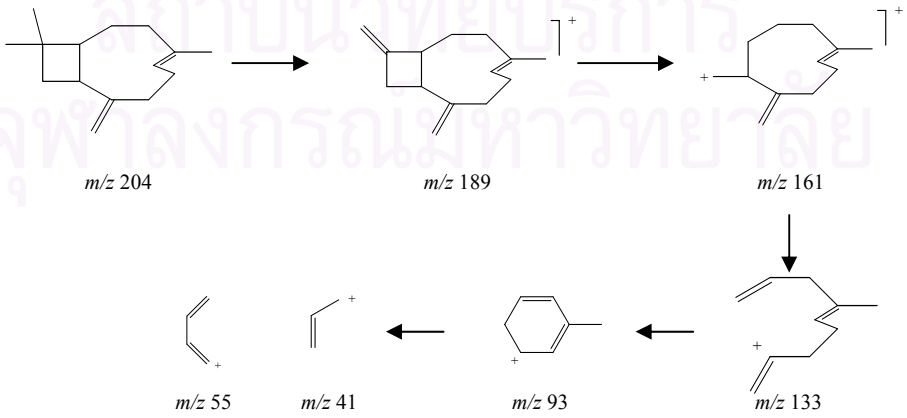


Figure 3.6 Cont. (peak no. 5)



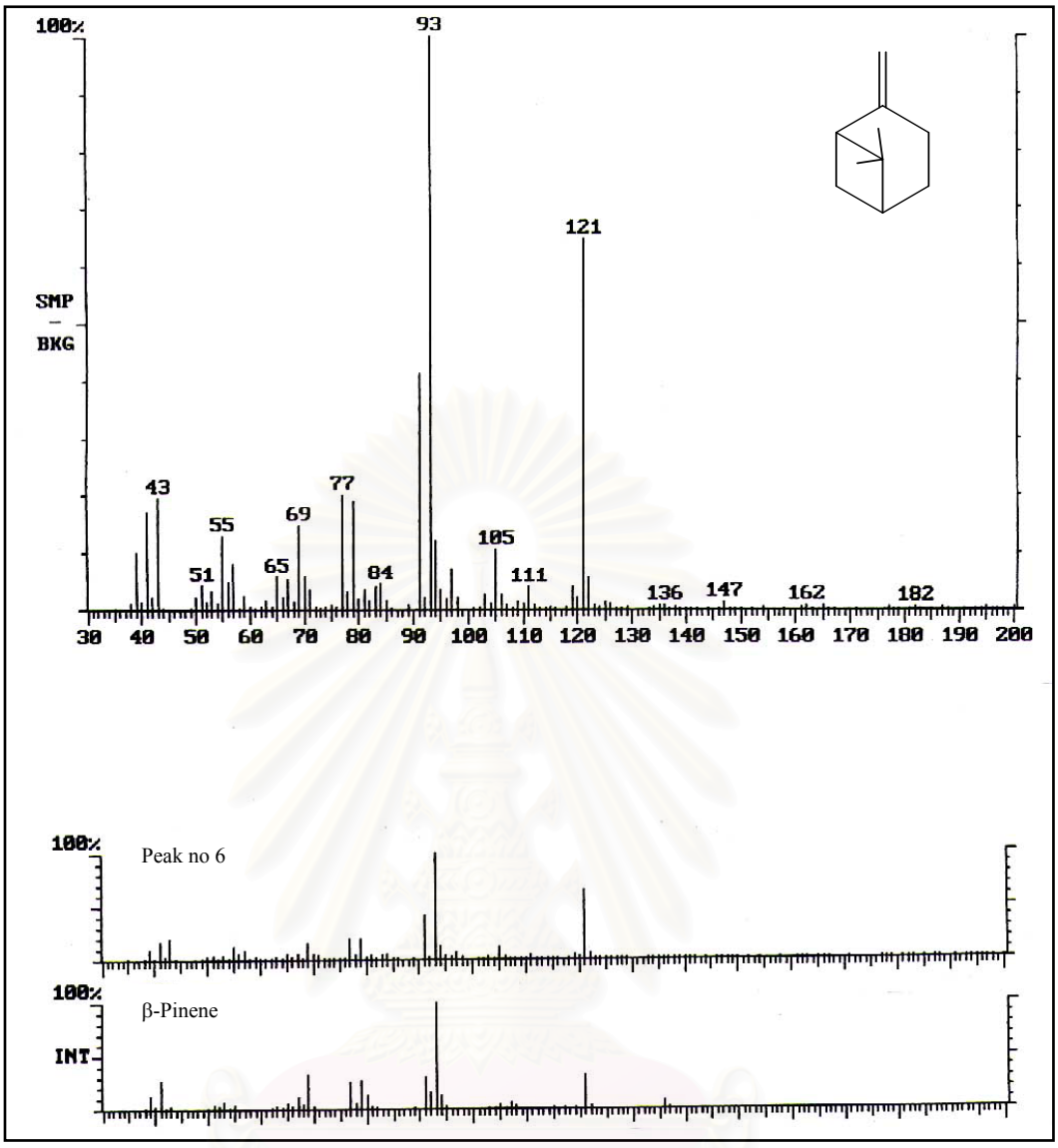
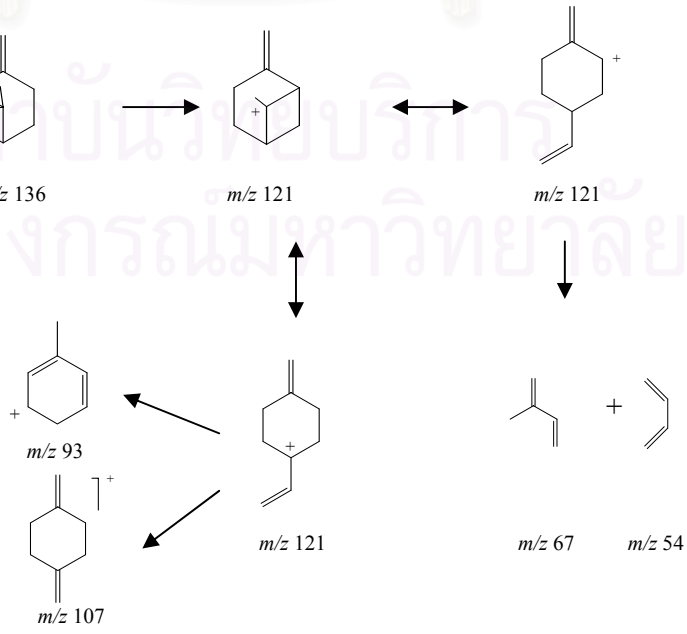


Figure 3.6 Cont. (peak no. 6)



3.4.2 Properties and structural elucidation of mixture 2

White amorphous powder was obtained from both fraction I (37 mg, 0.14% w/w of fraction I) and fraction II (20 mg, 0.04% w/w of fraction II) by eluting with 20% EtOAc – hexane. This solid was recrystallized several times using EtOAc as solvent. Its melting range was 65 – 70^{o}C; a broad range of melting point could indicate that this solid should be a mixture. This mixture was hardly soluble in various organic solvents but slightly soluble in hot acetone.

The IR spectrum of mixture **2** is depicted as shown in Figure 3.7 and the tentative IR absorption band assignments of mixture **2** are presented as shown in Table 3.15.

Table 3.15 IR absorption band assignments of mixture **2**

Absorption band (cm^{-1})	Band type	Assignments
3436	b	O-H stretching vibration of R-COOH
2918, 2844	s	C-H stretching vibration of $–CH_3$, $-CH_2-$
1708	s	C=O stretching vibration of R-COOH
1470	s	C-H bending vibration of $–CH_3$, $-CH_2-$
920	w	O-H stretching vibration out of plane
720	m	C-H rocking mode of $-(CH_2)_n-$

From IR spectrum, absorption bands belonging to carboxylic functional group displayed at 3436, 1708, 1470, 920 cm^{-1} and chain of methylene groups $-(CH_2)_n-$ appeared at 720 cm^{-1}. Owing to slightly low melting point and characteristic absorption bands of carboxylic and straight chain hydrocarbon, mixture **2** was tentatively ascribed to a mixture of long chain carboxylic acids.[32]

$CH_3-(CH_2)_n-COOH$

Saturated long chain carboxylic acids

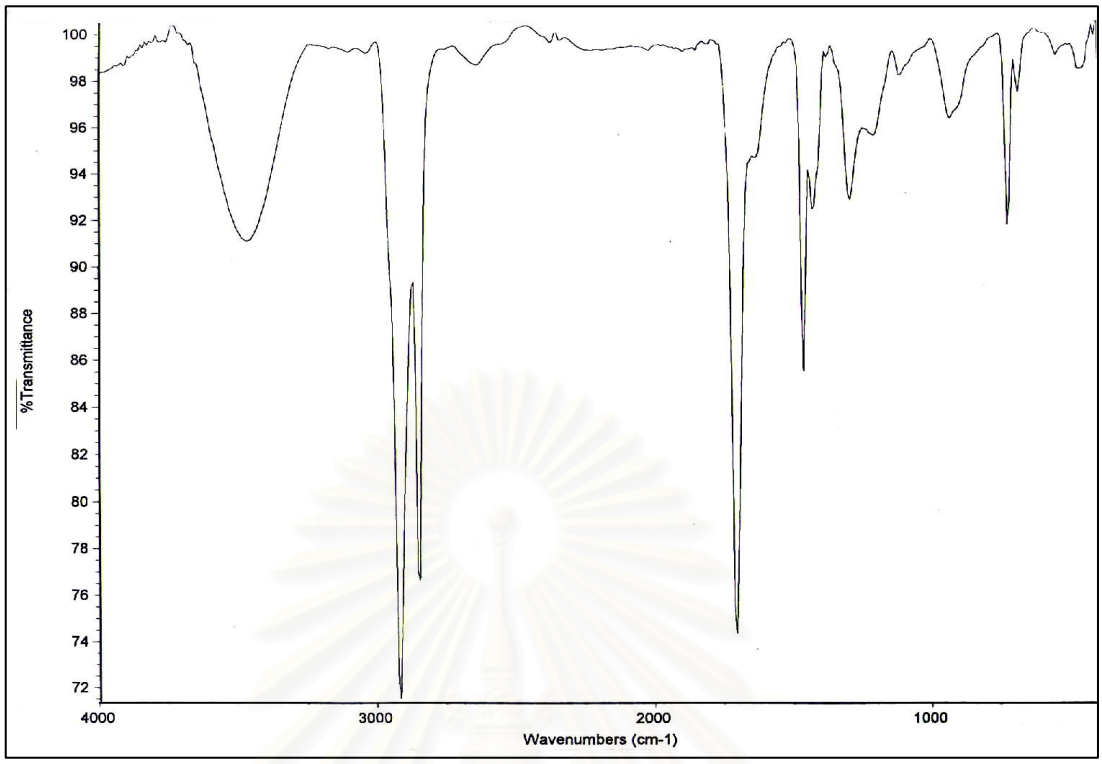


Figure 3.7 The IR spectrum of mixture **2**

3.4.3 Properties and structural elucidation of mixture 3

Mixture **3** was obtained from hexane extract (fraction I), fraction no 32 – 52 eluting with 20% EtOAc – hexane. This mixture, 30 mg, as white solid (0.12% w/w of fraction I) melted at 72 – 75^{o}C. It was purified by recrystallization in EtOAc. It was UV inactive and showed a purple spot when dipped in vanillin – H_2SO_4 reagent and heated at 100 ^{o}C. This mixture was not easily soluble in various organic solvents but soluble in hot methanol.

The IR spectrum of mixture **3** is depicted as shown in Figure 3.8 and the tentative IR absorption band assignments of mixture **3** are shown in Table 3.16.

Table 3.16 IR absorption band assignments of mixture **3**

Absorption band (cm^{-1})	Band type	Assignments
3200-3600	b	O-H stretching vibration of ROH
2848, 2914	s	C-H stretching vibration of $–CH_3$, $-CH_2-$
1466, 1470	m	C-H bending vibration of $–CH_3$, $-CH_2-$
1060	m	C-O stretching vibration of R-OH
725	m	C-H rocking mode for chain of $-(CH_2)_n-$

The IR spectrum clearly indicated the absorption band characteristic of aliphatic alcohol. Thus, the structure of mixture **3** was proposed as saturated long chain aliphatic alcohols.[32]

CH_3-$(CH_2)_n$-OH

Saturated long chain aliphatic alcohols

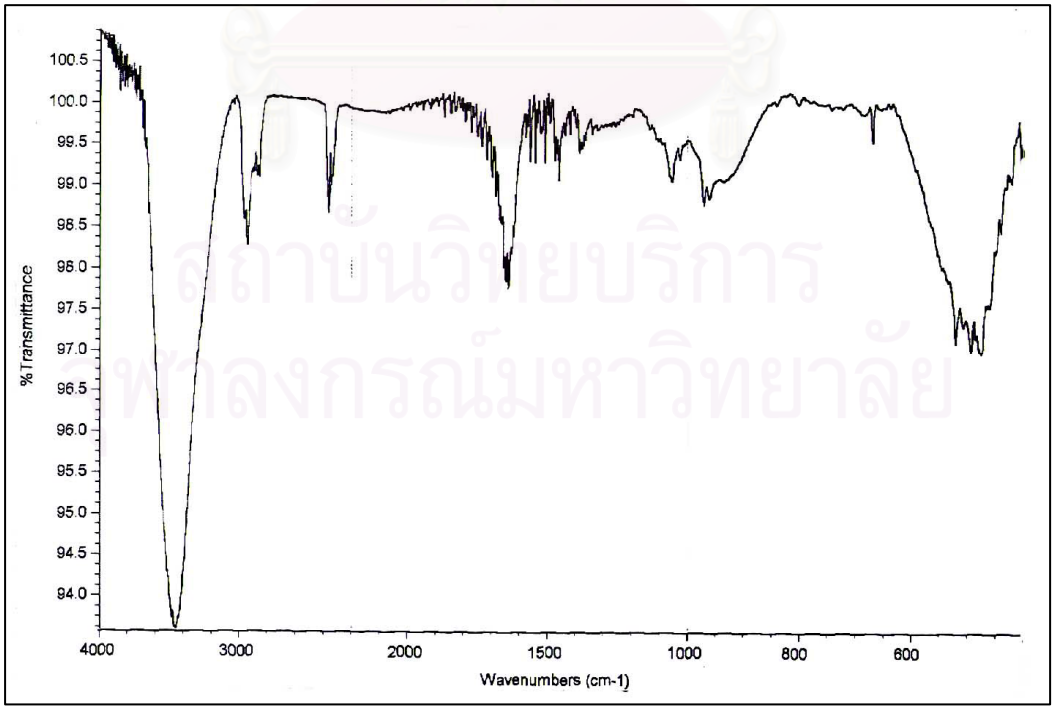


Figure 3.8 The IR spectrum of mixture **3**

3.4.4 Properties and structural elucidation of mixture 4

Mixtures **4** was isolated from fraction IIB by silica gel column chromatography and recrystallized with EtOAc several times. It was white amorphous powder, 48.4 mg (0.10% w/w of chloroform crude), m.p. 79 - 80°C. This mixture gave negative results to Liebermann-Burchard, $FeCl_3$, Br_2 in CCl_4 which indicated that it was not composed of steroidal or triterpenoidal skeleton, phenolic group and unsaturation, respectively.

The IR spectrum (Figure 3.9) and its absorption band assignments of mixture **4** are given in Table 3.17.

Table 3.17 IR absorption band assignments of mixture **4**

Absorption band (cm^{-1})	Band type	Assignments
2924, 2842	s	C-H stretching vibration of $–CH_3$, $-CH_2-$
1720	s	C=O stretching vibration of ester
1470, 1466	m	C-H bending vibration of $–CH_3$, $-CH_2-$
1265	w	C-O stretching vibration of ester
717	w	$-CH_2-$ rocking for chain, 4 carbons

The mass spectrum of mixture **4** (Figure 3.10) displayed fragmentation ions at *m/z* 480, 466, 452, 438, 424 and so on. This data indicated that this substance was lost $-CH_2-$ group (*m/z* 14) step by step. This characteristic revealed the lost of long chain methylene group.[32]

From this spectroscopic data, mixture **4** was proposed as a mixture of saturated long chain aliphatic ester.

$$\mathbf{CH_3\text{-}(CH_2)_m\text{-}COO\text{-}(CH_2)_n\text{-}\ CH_3}$$

Saturated long chain esters

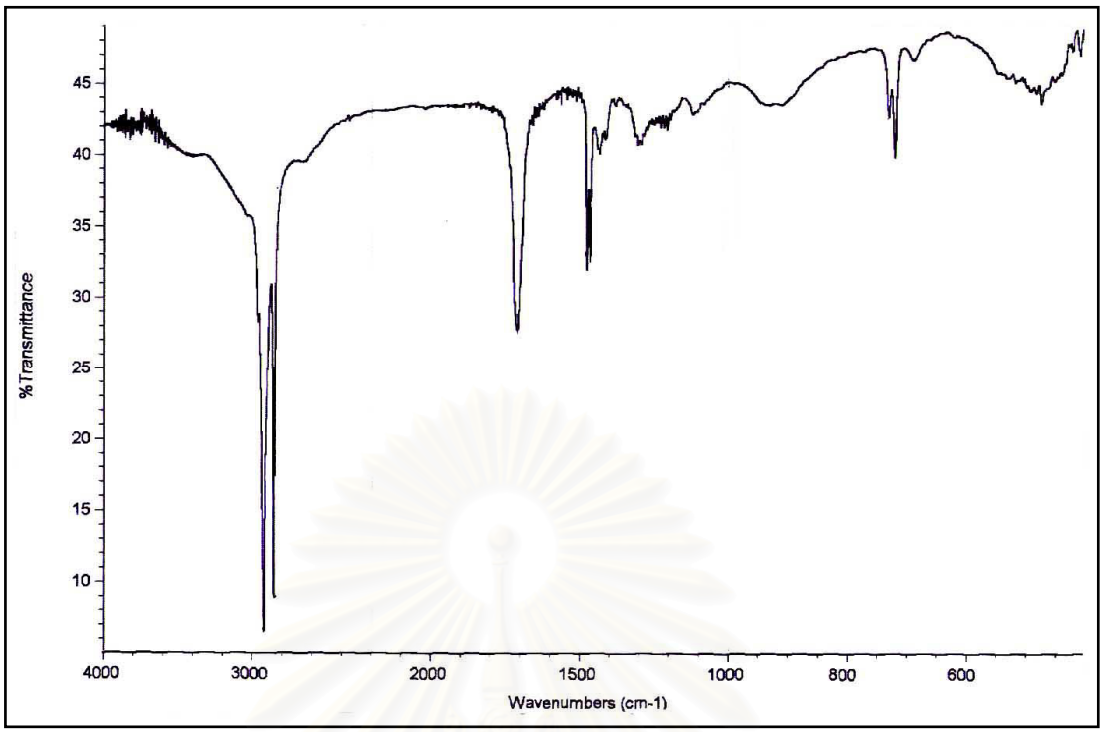


Figure 3.9 The IR spectrum of mixture **4**

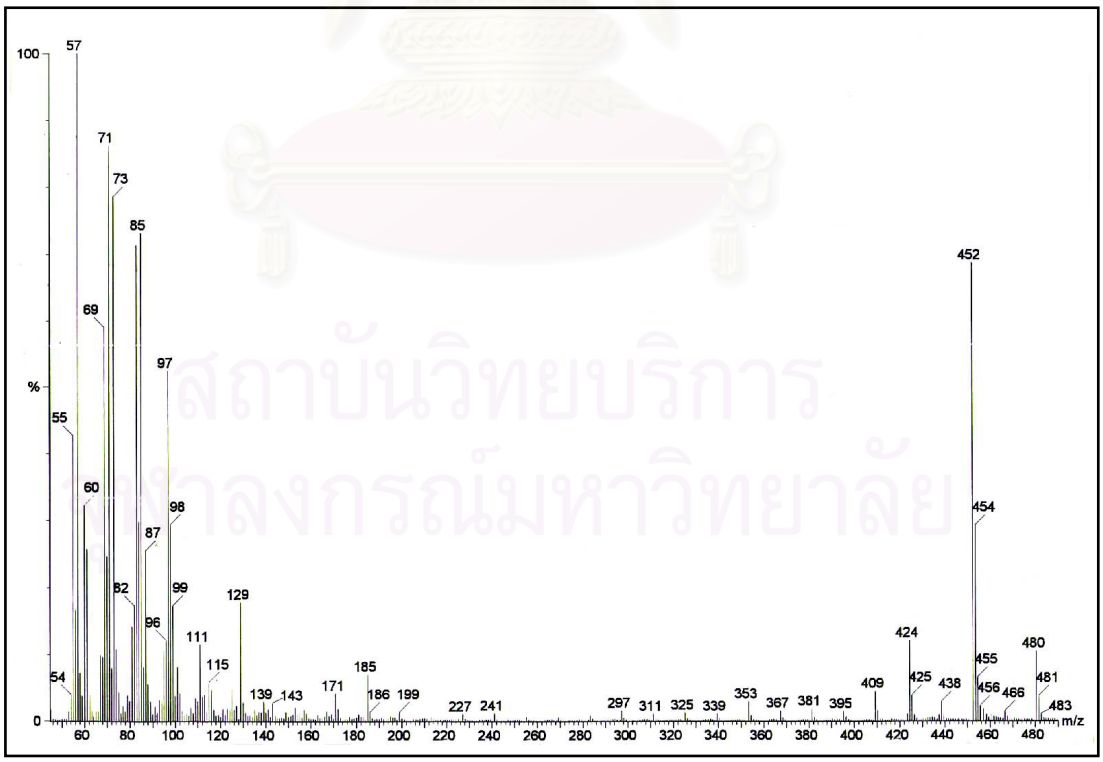


Figure 3.10 The mass spectrum of mixture **4**

3.4.5 Properties and structural elucidation of mixture 5

Mixture **5**, a white needle crystal of melting point 133 – 136°C (48.4 mg, 0.1% w/w of chloroform extract), was obtained from fraction II with 25% EtOAc – hexane. It was purified by recrystallization several times with MeOH. Mixture **5** was soluble in CH_2Cl_2 and $CHCl_3$. Its TLC profile displayed R_f value of 0.55 (solvent system: 50% EtOAc – hexane). It showed a dark purple spot when dipped in 10% H_2SO_4 in EtOH and heat to 100°C. A deep green solution was shown after reacting with Liebermann – Burchard reagent indicating of a steroid skeleton present in this molecule.

The IR spectrum of mixture **5** is shown in Figure 3.11 and the IR absorption band assignments are given in Table 3.18.

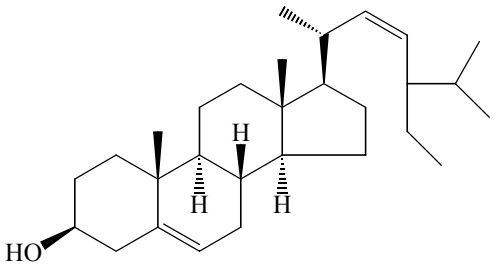
Table 3.18 IR absorption band assignments of mixture **5**

Absorption band (cm^{-1})	Band type	Assignments
3447	b	O-H stretching vibration of alcohol group
3010	s	C-H stretching vibration of =CH-
2931, 2846	s	C-H bending vibration of $-CH_3$, $-CH_2-$
1015	w	C-O stretching vibration of $\rangle$CH-OH

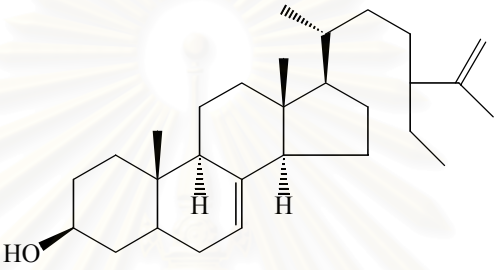
The ^1H NMR spectrum of mixture **5** (Figure 3.12) showed signals at δ 0.63 – 2.49, which were the signals of methyl, methylene and methine groups of steroids ($-CH_3$, $-CH_2-$, and -CH-, respectively).

The GC chromatogram (Figure 3.13) showed two peaks at retention time 7.64 and 8.28 min. The mass spectrum (Figure 3.14) of this mixture was well-matched with two standard steroids: stigmasterol and stigmasta–7,25–dien–3-ol.

According to the above information, this mixture should be a mixture of stigmasterol (61%) and stigmasta–7,25–dien–3-ol (39%).



Stigmasterol



Stigmasta-7,25-dien-3-ol

Mixture 5

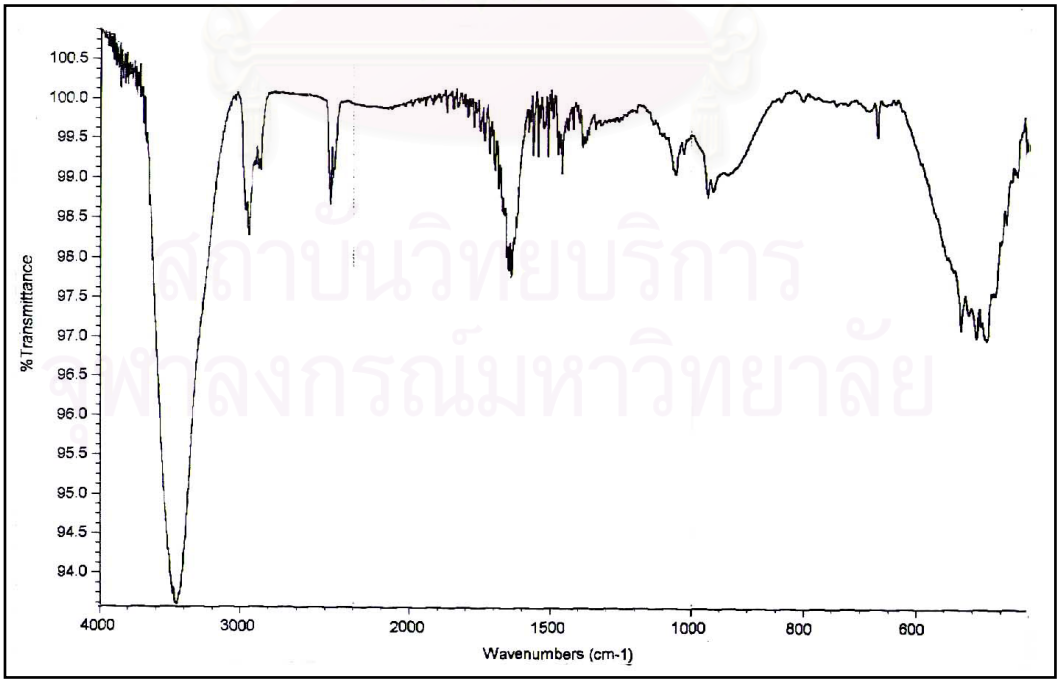


Figure 3.11 The IR spectrum of mixture **5**

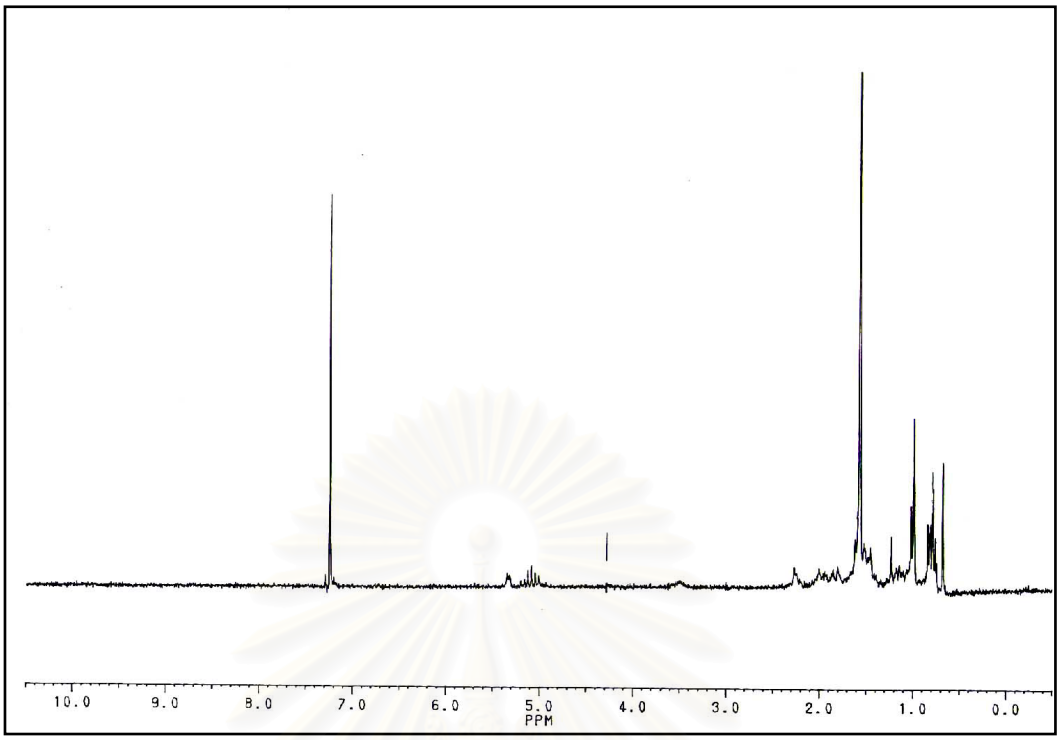


Figure 3.12 The ^{1}H NMR spectrum of mixture **5**

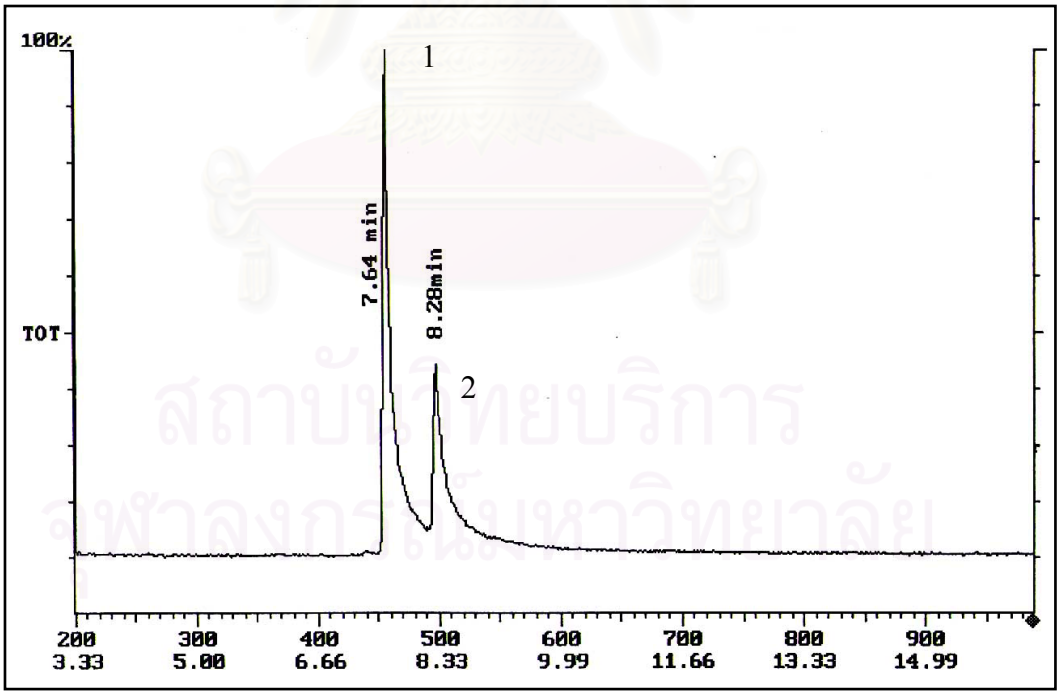


Figure 3.13 The GC spectrum of mixture **5**

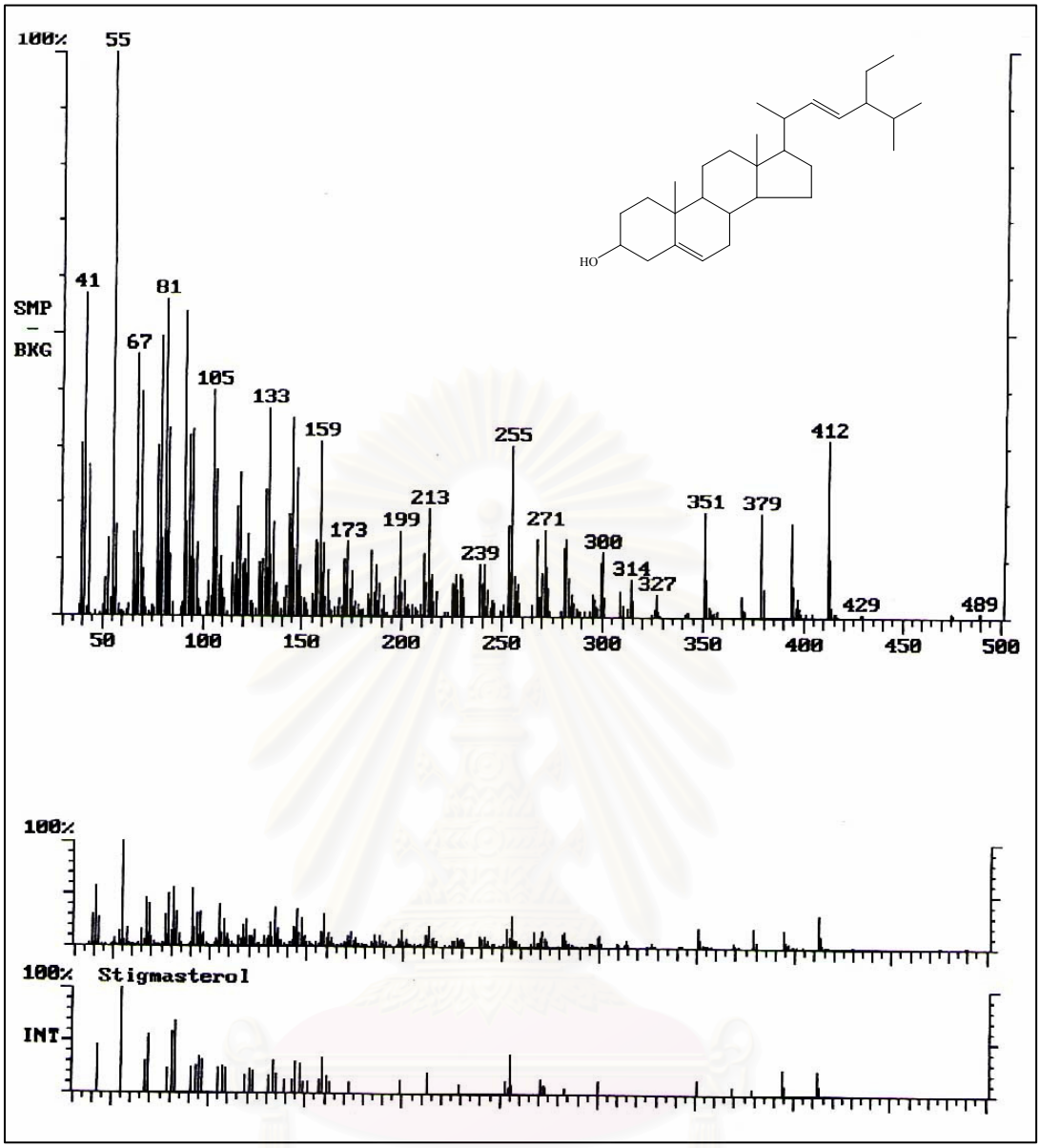
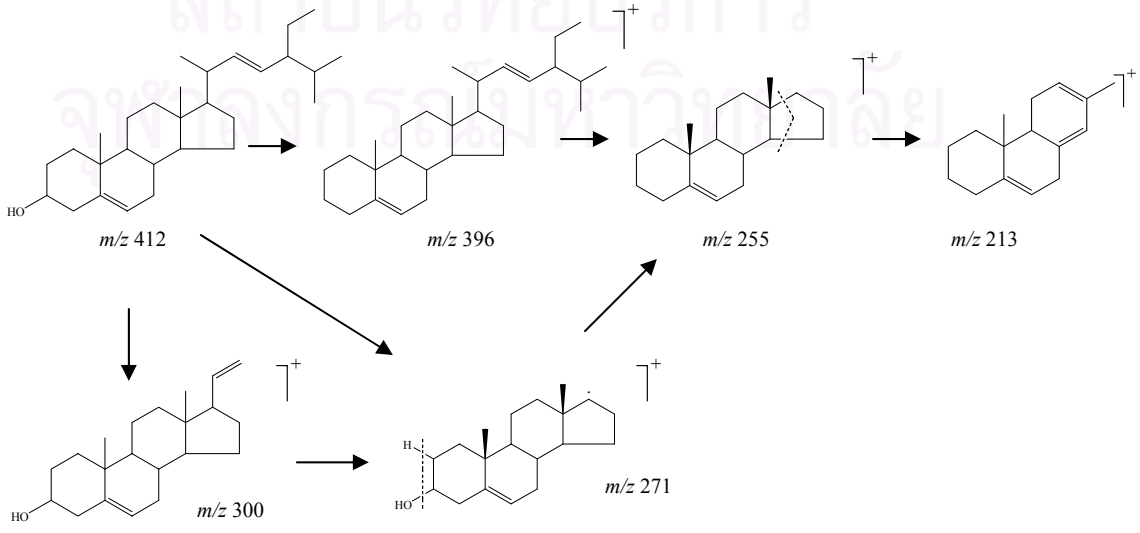


Figure 3.14 The mass spectrum of mixture **5**, component 1



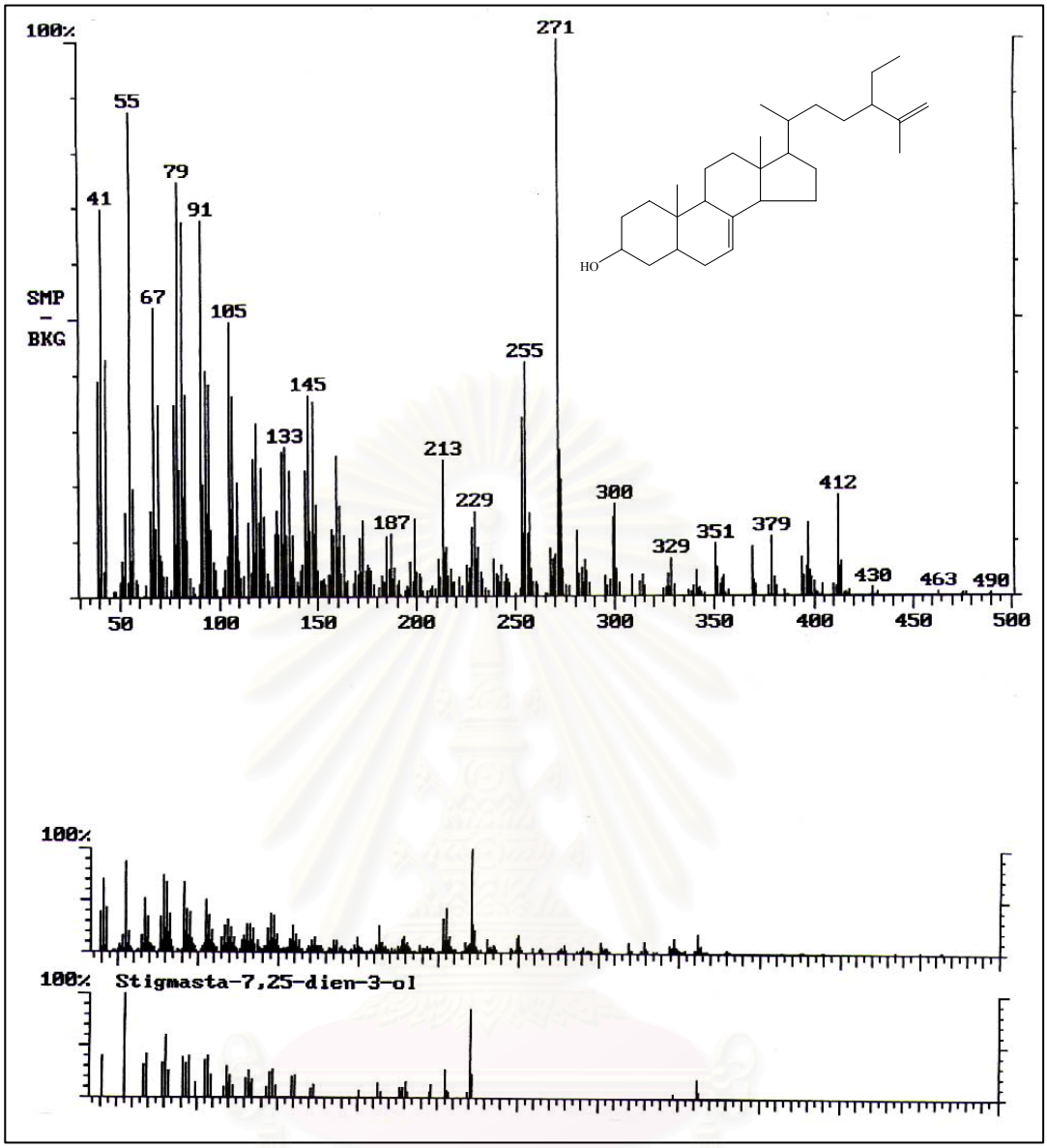
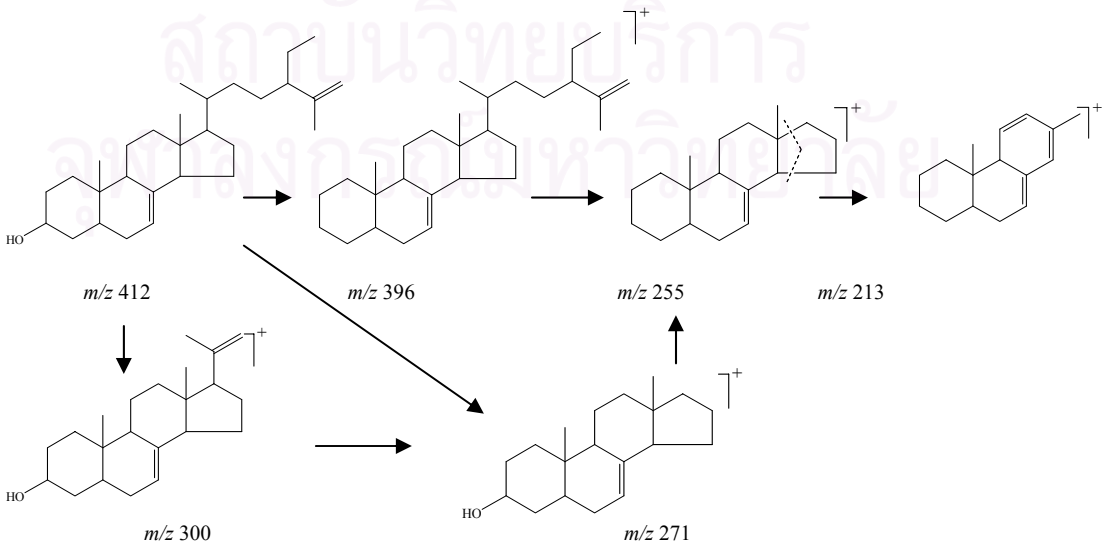


Figure 3.14 (Cont.) The mass spectrum of mixture **5**, component 2



3.4.6 Properties and structural elucidation of compound 1

Compound **1** was bright white needle crystals in yellow oil separated from fraction I. This compound was recrystallized by a mixture of 50% MeOH – $CHCl_3$ several times to obtain approximately 103 mg (0.40% w/w of fraction I). Its melting point range was 163 – 164°C. This compound was UV inactive but gave a dark purple spot on TLC plate after dipped in 10% H_2SO_4 in EtOH and heated at 100°C. R_f value of this compound was 0.51 (solvent system: 40% EtOAc – hexane). It was soluble in CH_2Cl_2 and $CHCl_3$. The color test with Liebermann–Burchard reagent showed a blue color, which means that the steroidal structure may be present in this compound.

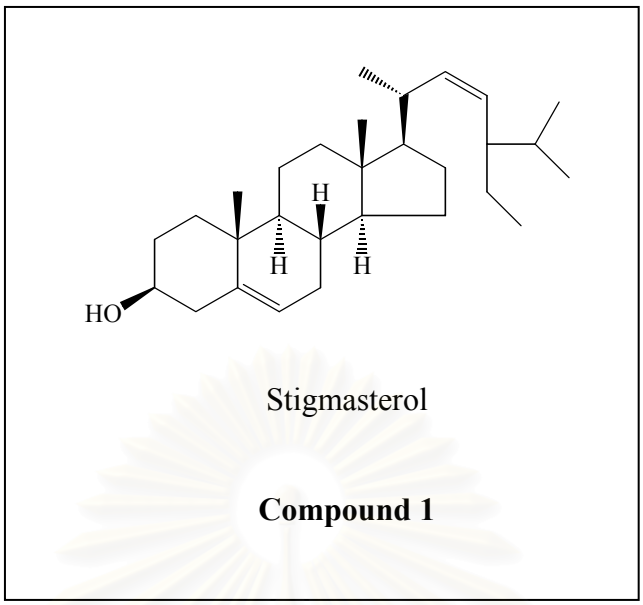
The IR spectrum (Figure 3.15) of compound **1** showed a broad absorption band at 3040 - 3600 cm^{-1} which was a characteristic of a hydroxy group. The strong absorption band at 2942 and 2868 cm^{-1} suggested the presence of methyl and methylene groups. The IR absorption bands were assigned as shown in Table 3.19.

Table 3.19 IR absorption band assignments of compound **1**

Absorption band (cm^{-1})	Band type	Assignments
3600 – 3040	b	O-H stretching vibration of –OH group
2942, 2868	m	C-H stretching vibration of $–CH_3$, $-CH_2-$
1455, 1377	m	C-H bending vibration of $–CH_3$, $-CH_2-$
1050	m	C-O stretching vibration of alcohol

The ^1H NMR spectrum ($CDCl_3$) (Figure 3.16) displayed signals of $-CH_3$, $-CH_2-$ and –CH of steroid at δ 0.50 – 2.50 and a hydroxy group at 3.50. The multiplet signal at δ 5.07 was assigned as disubstituted vinyl protons (CH=CH). The last signal at δ 5.33 was the signal of trisubstituted vinyl proton (-CH=C-).

Compound **1** was further analyzed by GC-MS technique and compared its chromatogram with the standard steroids. The GC showed only one peak at retention time 7.64 min (Figure 3.17). The mass spectrum (Figure 3.18) of compound **1** exhibited a molecular ion peak at *m/z* 412. From the library search, the mass fragmentation pattern of this compound was coincided with stigmasterol. All of these results manifestly indicated that compound **1** was stigmasterol.



Stigmasterol

Compound 1

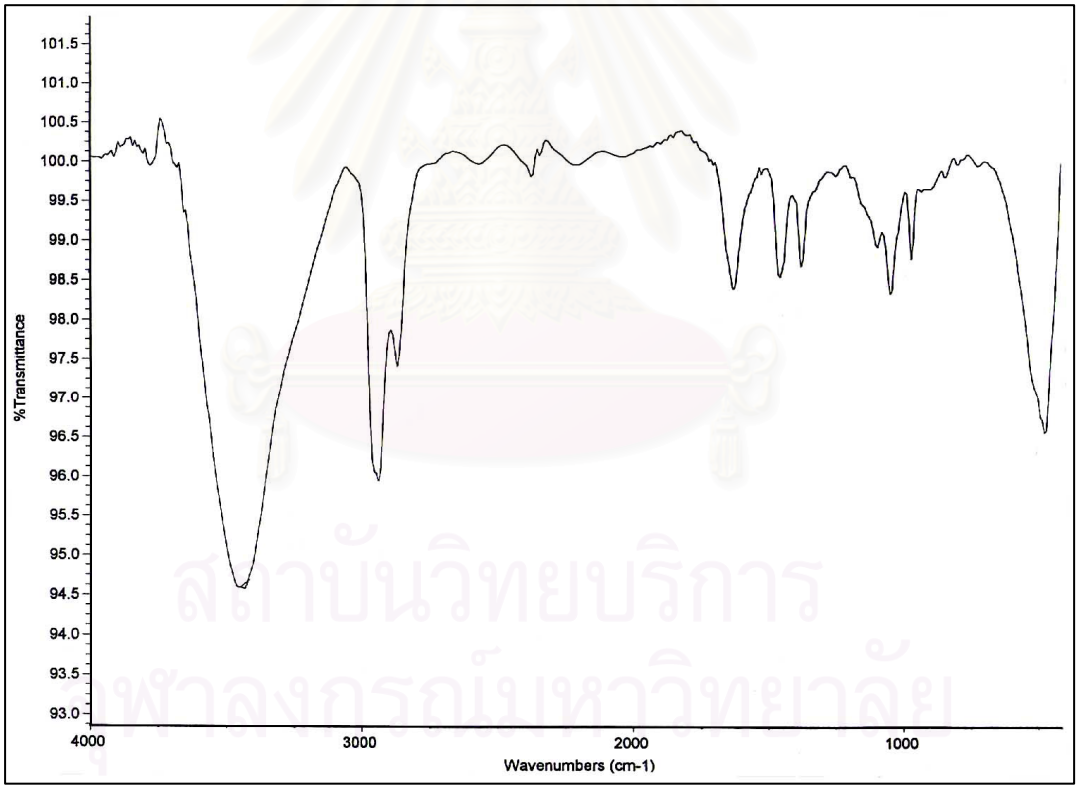


Figure 3.15 The IR spectrum of compound **1**

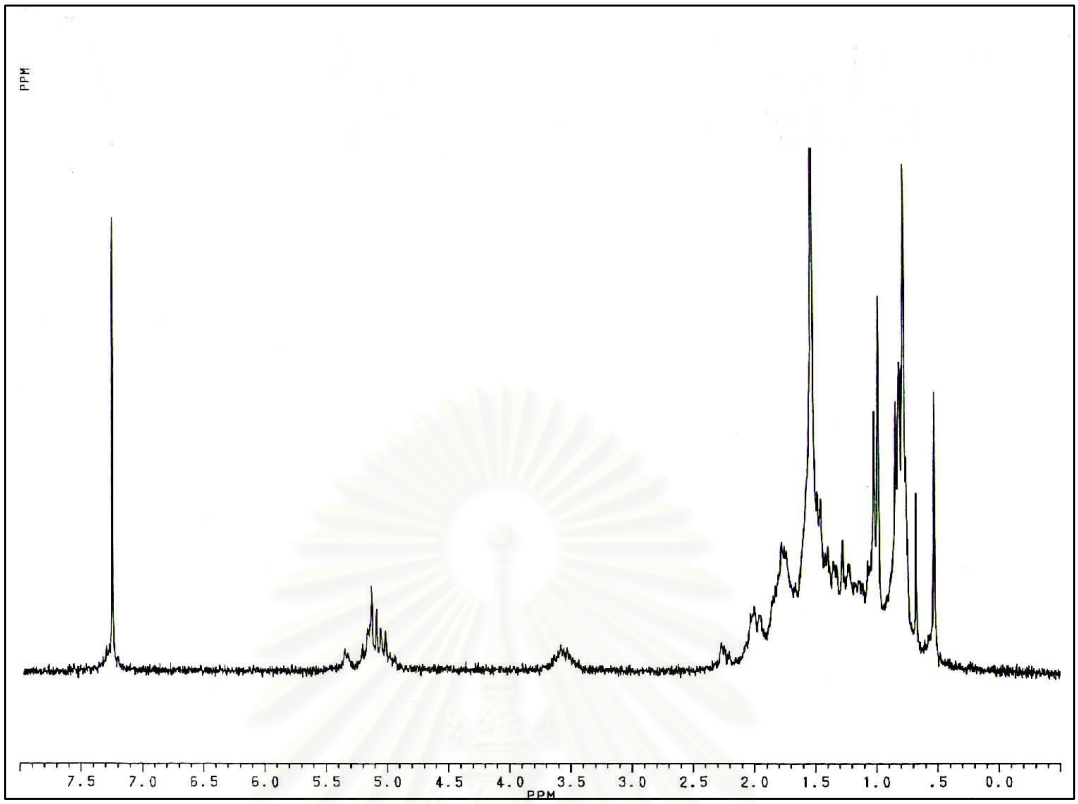


Figure 3.16 The ^{1}H NMR spectrum of compound **1**

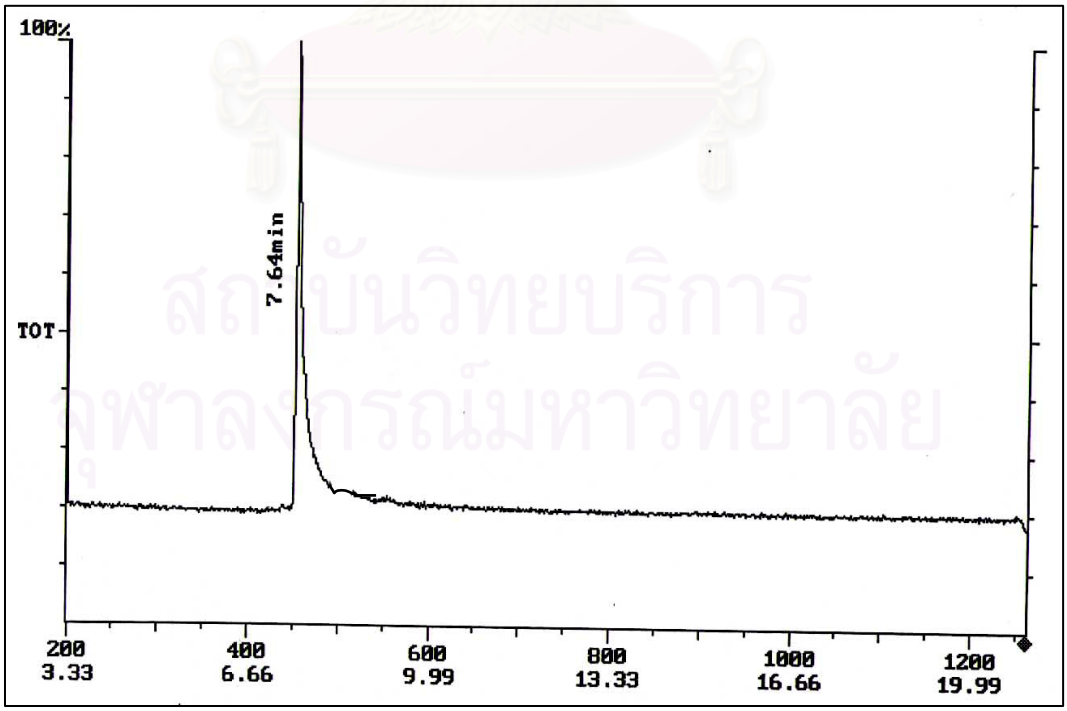


Figure 3.17 The GC of compound **1**

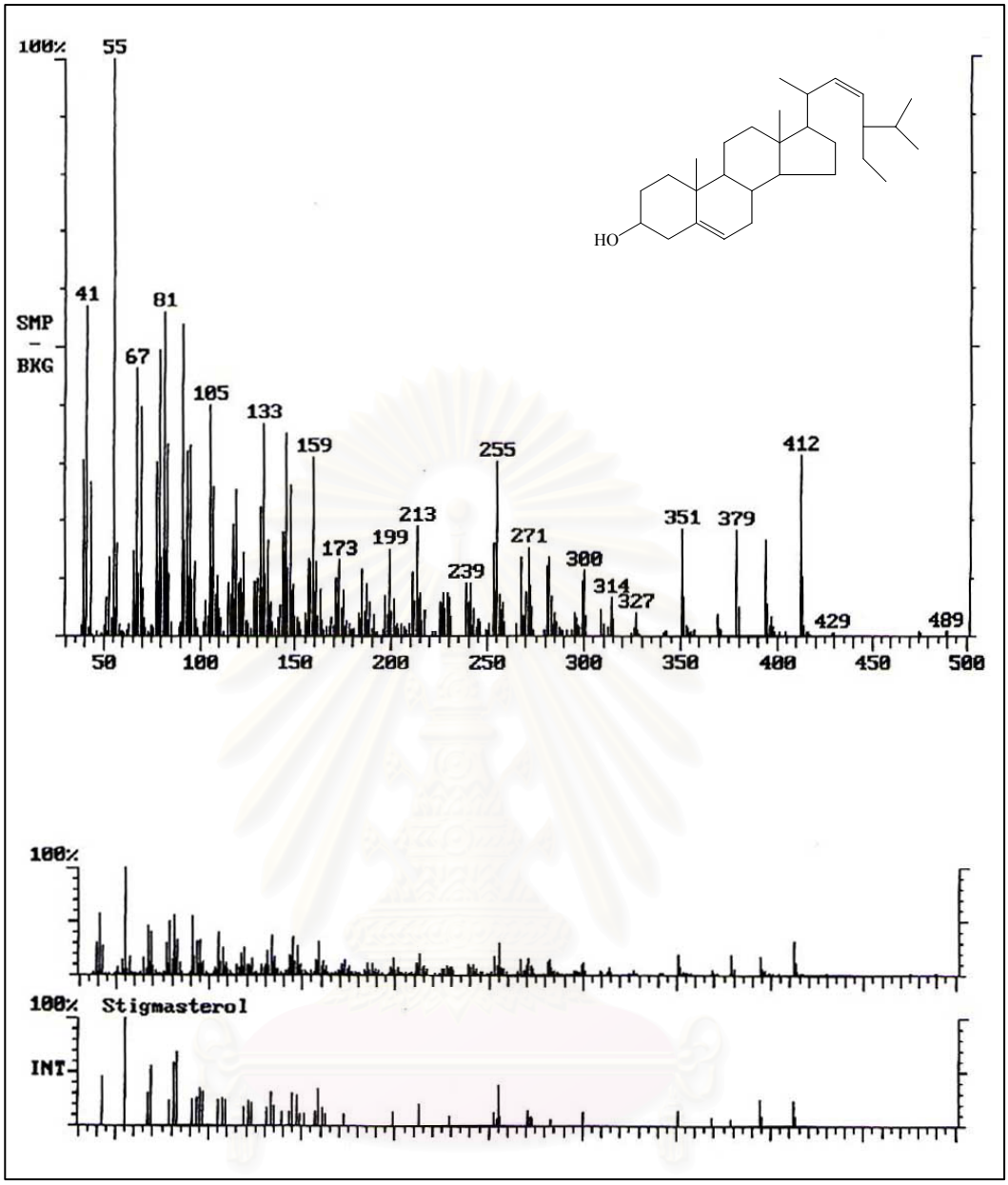
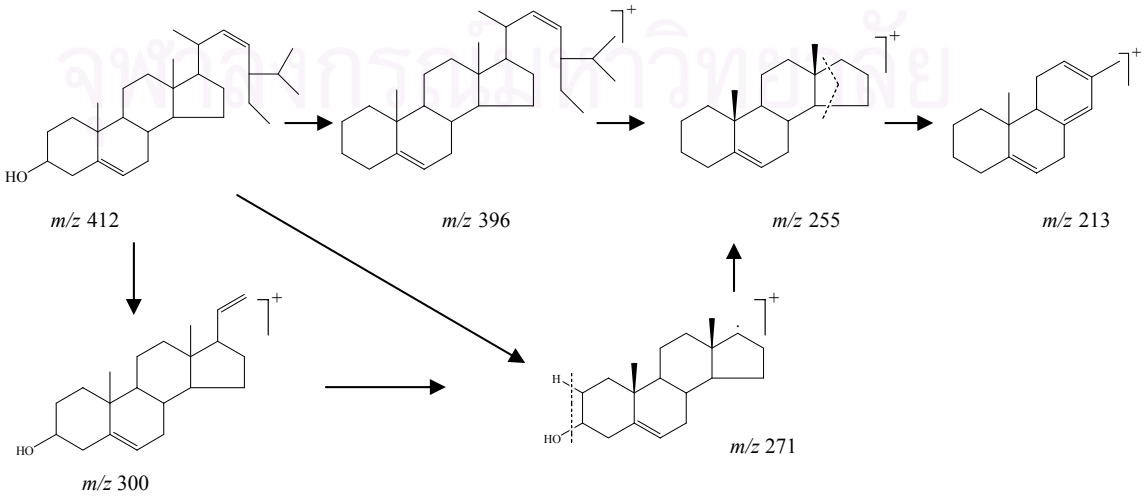


Figure 3.18 The mass spectrum of compound **1**



3.4.7 Properties and structural elucidation of compound 2

Compound **2** was obtained from both fractions IIF and IIG by eluting with 25% and 50% MeOH – EtOAc of chloroform extract, respectively. The separation of these fractions afforded brown viscous crude. An attempt to purify this compound was carried out by using Sephadex LH 20 as an adsorbent and 50% MeOH - $CHCl_3$ was used as an eluent to furnish white precipitate in yellow solution. After filtration, the white amorphous powder was recrystallized from hot MeOH to yield compound **2**, 241 mg (0.50% w/w of chloroform extract), m.p. 257-260°C (dec.), R_f 0.62 (solvent system: 25% MeOH – EtOAc). Two distinctive attributions of compound **2**, the less solubility in various solvents and UV-inactive character implied that it seemed to be a steroid or a triterpenoid glycoside.[29] Therefore, it was tested with Liebermann-Burchard reagent, and a deep green solution was gradually visible. This provided clear evidence that compound **2** was a steroid glycoside.

The IR spectrum (Figure 3.19) also showed the characteristic absorption bands of steroid glycosides: O-H stretching due to hydroxy group of sugar, C-H deformation of *β*-sugar and C-O stretching vibration of glycosidic linkage at 3421, 890 and 1071, respectively.[32] The IR absorption band assignments are listed in Table 3.20.

Table 3.20 IR absorption band assignments of compound **2**

Absorption band (cm^{-1})	Band type	Assignments
3421	b	O-H stretching vibration of alcohol group
2931, 2867	m	C-H stretching vibration of $–CH_3$, $-CH_2-$
1456, 1377	m	C-H bending vibration of $–CH_3$, $-CH_2-$
1071	s	C-O stretching vibration of alcohol
890	w	C-H deformation of *β*-sugar

The ^1H NMR spectrum (DMSO-d_6) (Figure 3.20) showed the signals in the range of δ 0.53-2.49, which were the signals of CH_3, CH_2 and CH groups of steroid. The multiplet signals at δ 2.85-3.15 were assigned to the protons of a sugar moiety. The proton on carbon attached to a hydroxyl group appeared as the multiplet signal at δ 3.53 and the signal at δ 4.20 belonged to the anomeric proton. The multiplet signal

at δ 5.11 was the signal of disubstituted vinyl protons (CH=CH). The last signal at δ 5.30 was the signal of trisubstituted vinyl proton (-CH=C-).

The mass spectrum (Figure 3.21) showed the molecular ion peak of this compound and the fragmentation ion of a steroid aglycone was detected at *m/z* 574 and 412, respectively. Other fragmentation ions at *m/z* 394, 255 and 213 were similar to that of stigmasterol.[33] Additionally, the lack of peaks at *m/z* 414 and 400 also provided a useful information that *β*-sitosterol and campesterol did not utterly contaminate in this compound.

The ^{13}C NMR spectrum (DMSO-d_6) (Figure 3.22), revealed a group of six carbon signals resonated at δ 100.8, 76.7, 76.9, 73.5, 76.7 and 61.3 exactly resembled to a distinctive manner of D-glucose[32] which were assigned for C1′, C2′, C3′, C4′, C5′ and C6′, respectively. Table 3.21 shows the ^{13}C NMR assignment of compound **2** compared with stigmasteryl-3-O-*β*-D-glucopyranoside.

Table 3.21 The ^{13}C NMR assignment of compound **2** and stigmasteryl-3-O-β-D-glucopyranoside[32]

Carbon	Chemical Shift (ppm)	
	Compound **2**	Stigmasteryl-3-O-β-D-glucopyranoside
C1	36.9	37.4
C2	29.3	31.7
C3	70.2	71.8
C4	42.8	42.4
C5	140.5	140.0
C6	121.3	121.7
C7	31.4	31.9
C8	31.4	31.9
C9	49.7	50.3
C10	36.6	36.6
C11	21.1	21.1
C12	38.4	39.8
C13	41.8	42.4
C14	56.3	57.0
C15	23.9	24.4
C16	28.6	28.9
C17	55.4	56.0
C18	12.2	12.2
C19	19.2	19.4
C20	40.8	40.5
C21	21.0	21.1
C22	138.2	138.4
C23	128.9	129.4
C24	50.7	51.3
C25	33.9	31.9
C26	19.0	19.0
C27	20.6	21.1
C28	25.0	25.4
C29	11.9	12.0

Acetylation of compound 2

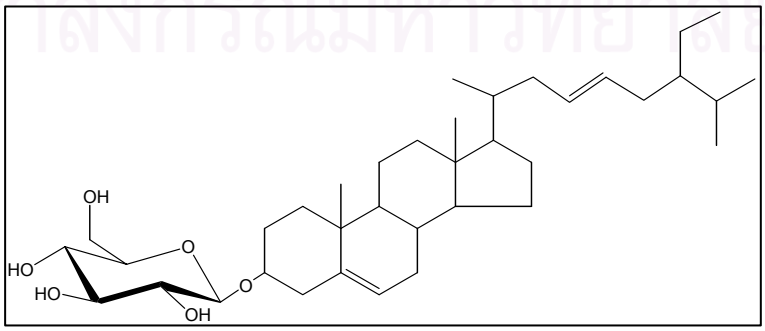
To compound **2** (38 mg) in dry pyridine (1 mL), acetic anhydride (4 mL) was added and the reaction mixture was stirred at room temperature for 24 hr. Then the mixture was poured onto ice water (10 mL). The precipitate was filtered off, washed with water and recrystallized from a mixture of MeOH and chloroform to yield white powder 36 mg (95% yield), m.p. 127–128°C, R_f 0.70 (solvent system: 25% MeOH–EtOAc), designated as compound **2A**.

The IR spectrum (Figure 3.19) showed the significant absorption band at 1752 cm^{-1} of C=O stretching vibration of ester, suggesting the presence of an acetyl group. Absorption bands at 1225 and 1045 cm^{-1} were compatible to C-O vibration of ester group. The absence of the absorption band of OH group indicated that reaction was completed.

The ^1H NMR spectrum of peracetylated derivative ($CDCl_3$) (Figure 3.20) revealed the proton signal of four acetoxy groups (-OCOC$\underline{H}_3$) at δ 1.99, 2.00, 2.01 and 2.03. On the comparison of proton signals of compound **2** with those of compound **2A**, downfield chemical shifts from δ 2.85-3.15 to δ 3.38-4.65 were observed for the signal assignable to the protons of a sugar.

The ^{13}C NMR spectrum ($CDCl_3$) (Figure 3.21) showed the signals of four carbonyls of acetoxy groups at δ 169.3, 169.4, 170.4 and 170.7. The signal at δ 96.8 was compatible with the C-3 of steroid attaching to oxygen of sugar molecule. The ^1H and ^{13}C NMR data strongly indicated that compound **2** possessed four hydroxyl groups in its molecule.

According to the above results and literature comparison, the structure of compound **2** was defined as stigmasteryl-3-O-*β*-D-glucopyranoside.



Compound 2

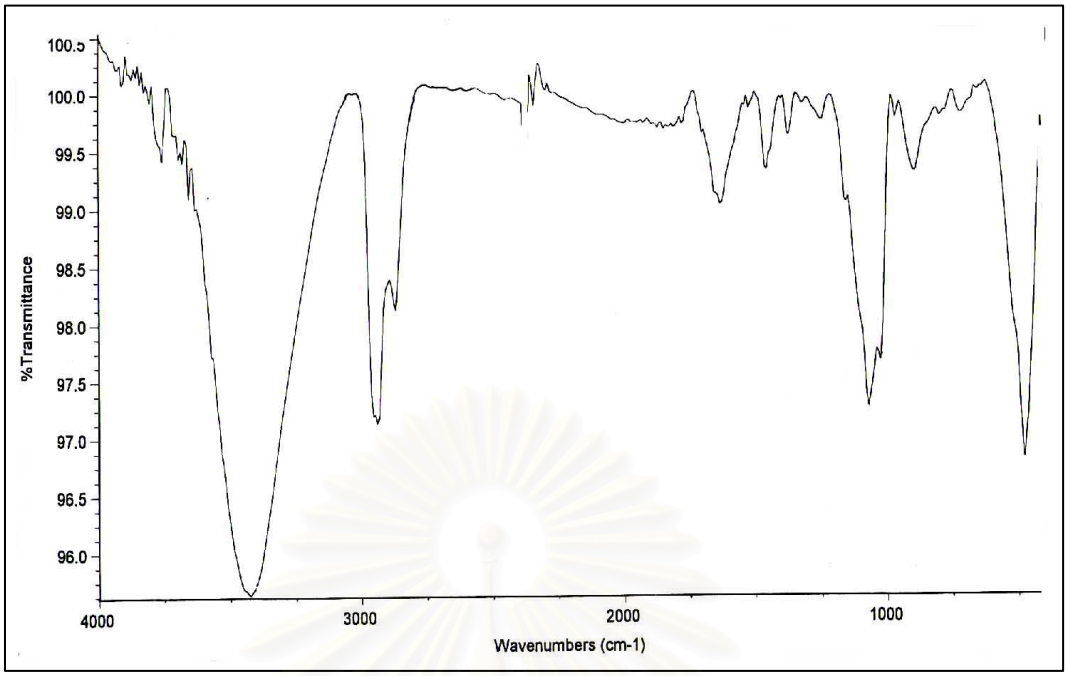


Figure 3.19 The IR spectrum of compound **2**

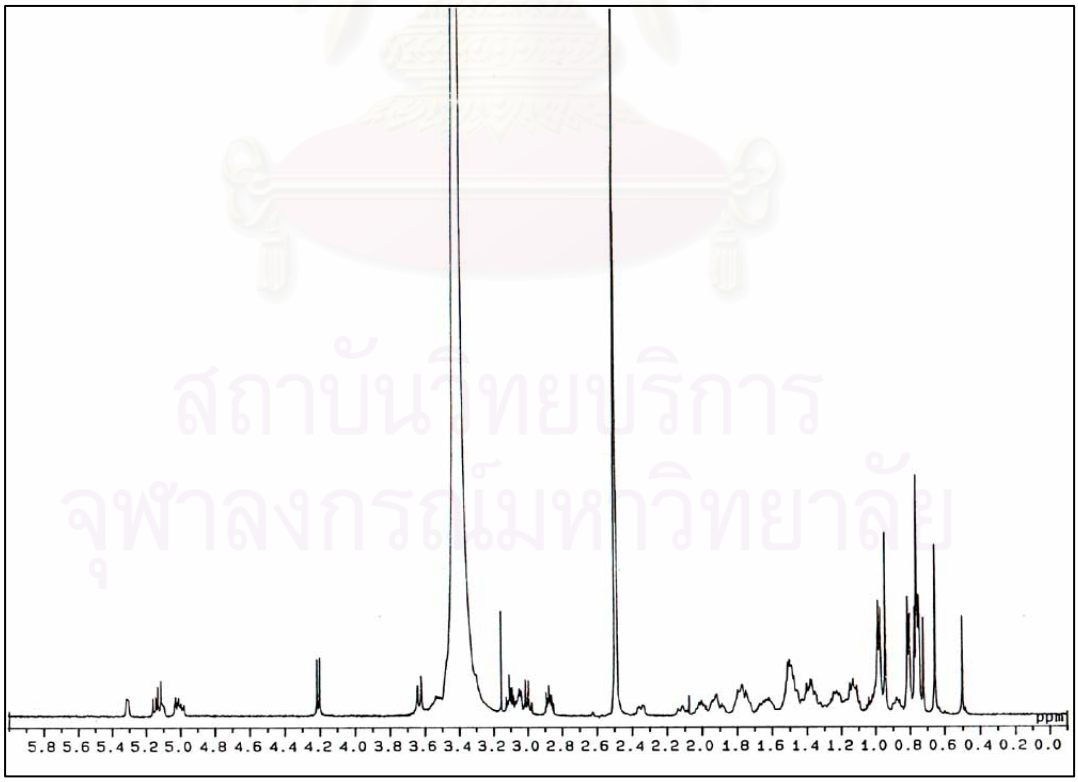


Figure 3.20 The ^{1}H NMR spectrum of compound **2**

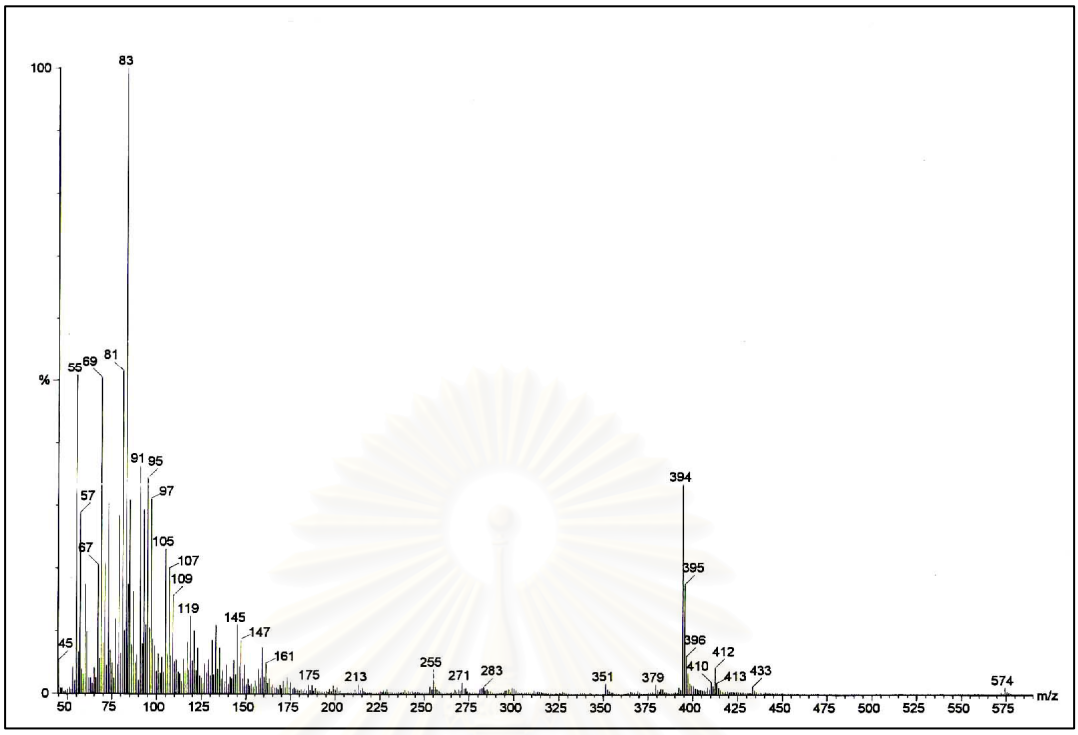
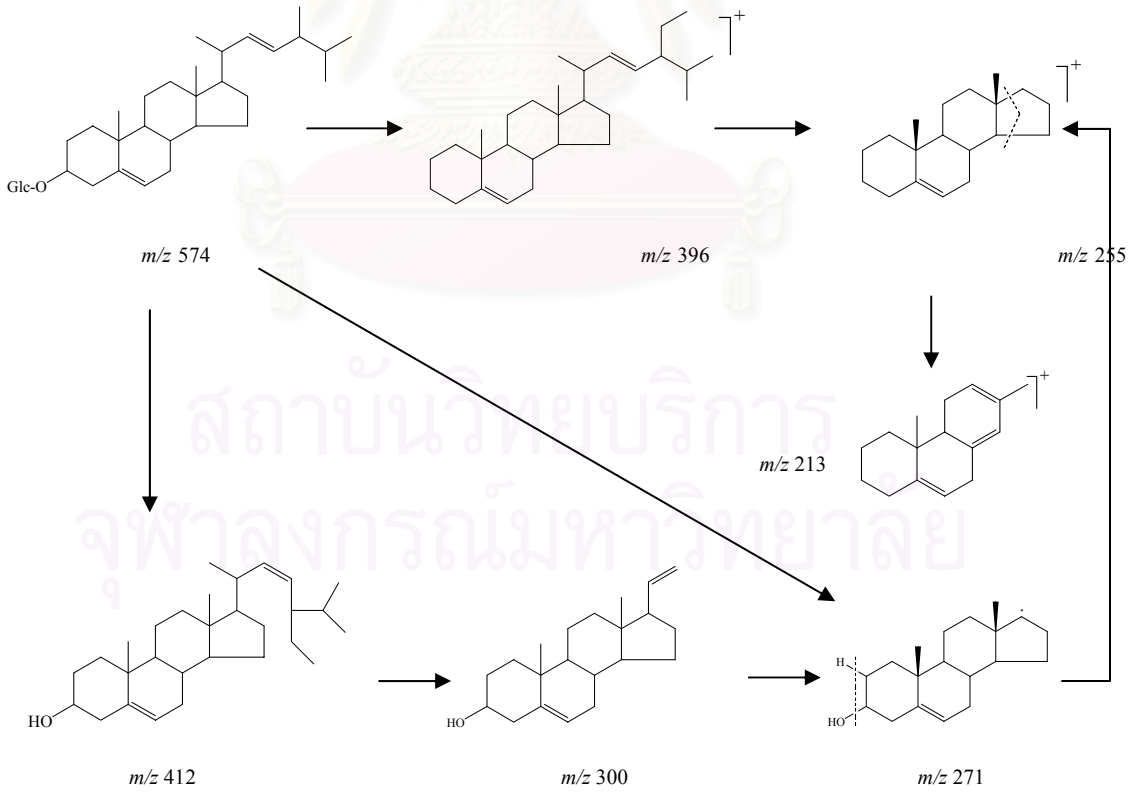


Figure 3.21 The mass spectrum of compound **2**



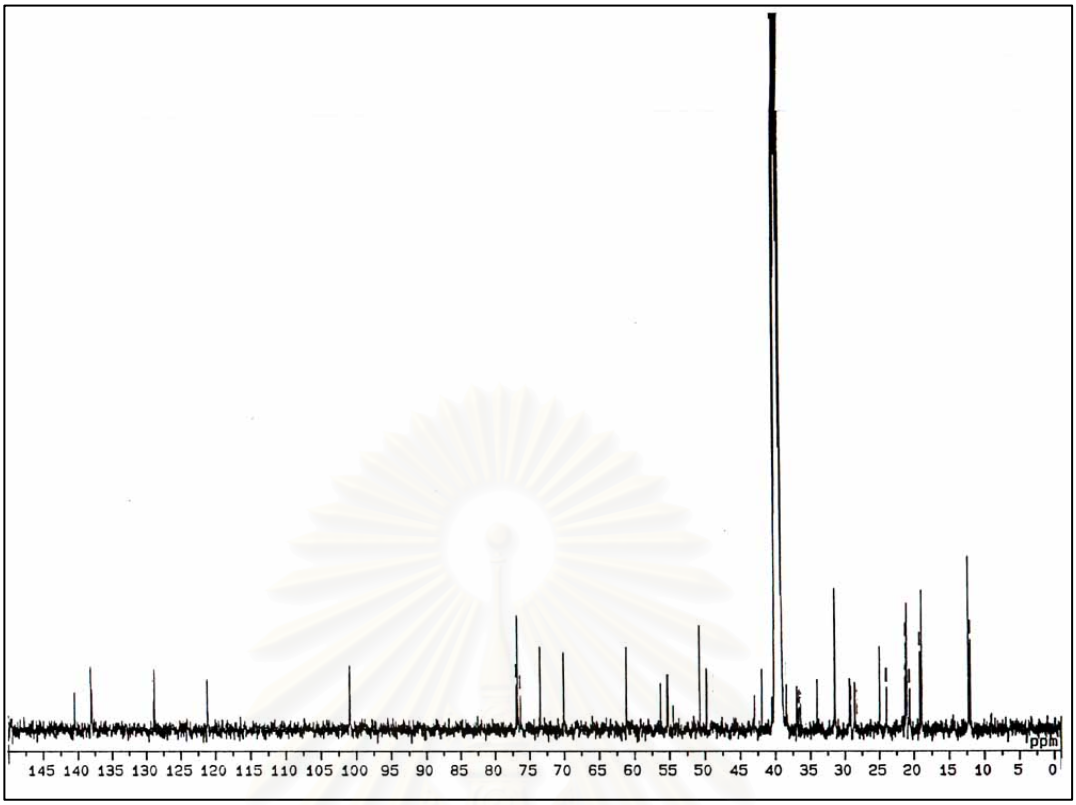


Figure 3.22 The ^{13}C NMR spectrum of compound **2**

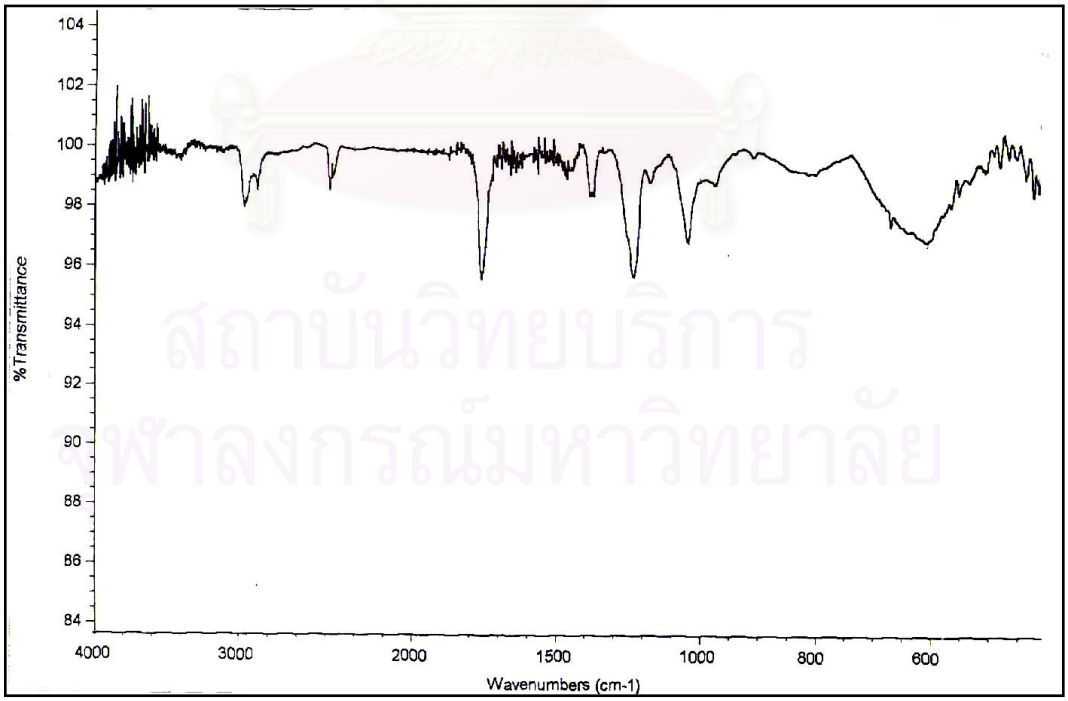


Figure 3.23 The IR spectrum of compound **2A**

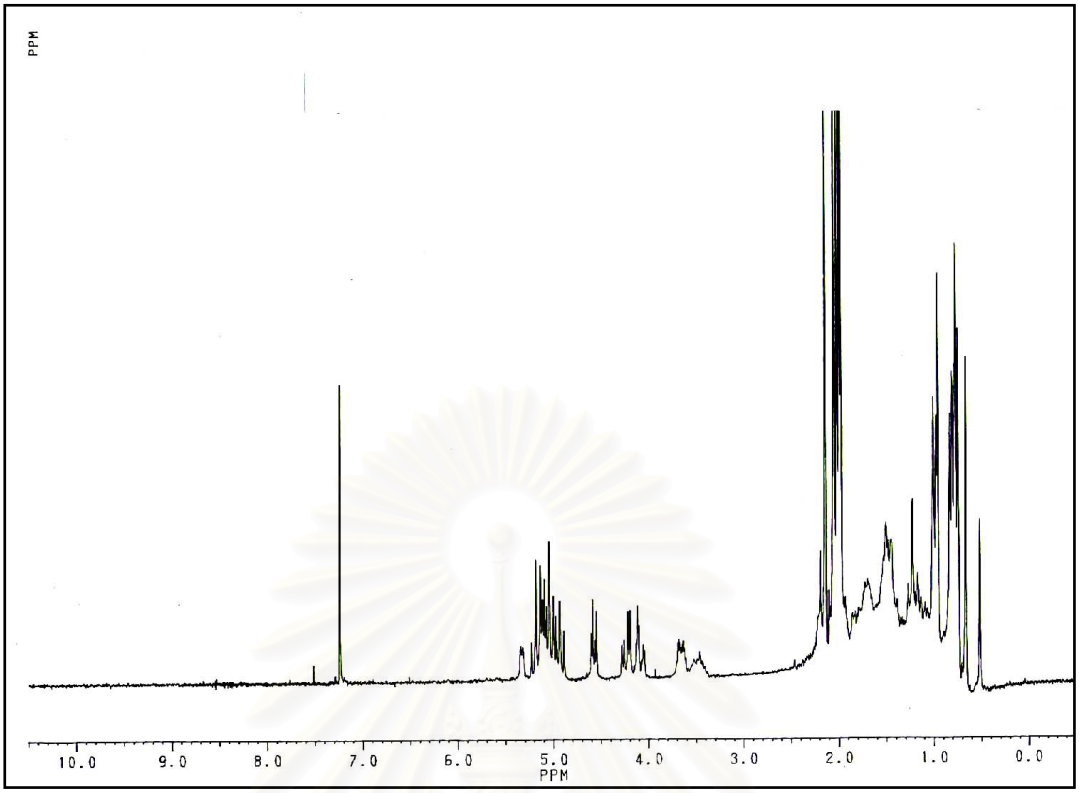


Figure 3.24 The ^{1}H NMR spectrum of compound **2A**

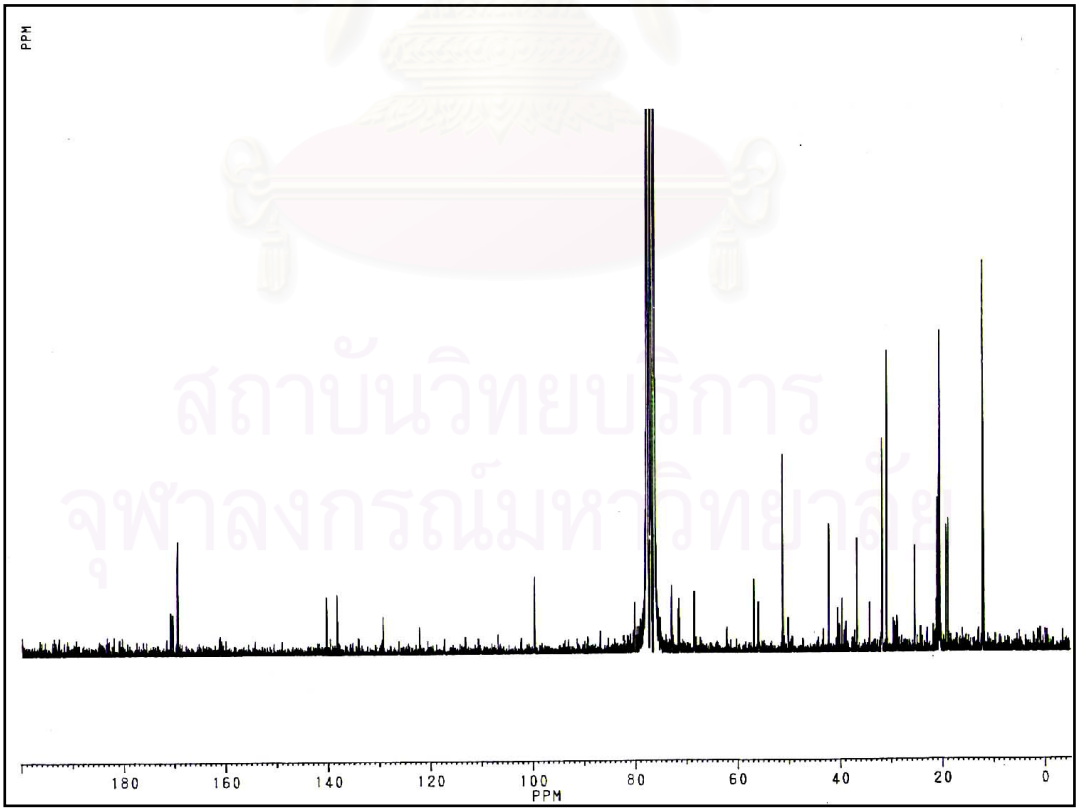


Figure 3.25 The ^{13}C NMR spectrum of compound **2A**

3.4.8 Properties and structural elucidation of compound 3

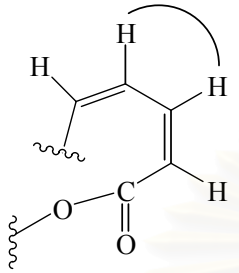
Compound **3** was isolated from silica gel flash column chromatography of fraction IIF in fraction no. 42-84 by 15% MeOH-EtOAc. After purification by recrystallization with hot EtOAc, white amorphous solid 376 mg (0.45% w/w of fraction II), m.p. 141-145°C, R_f 0.68 (solvent system: 24% MeOH-EtOAc) was obtained.

The IR spectrum of this compound reveals in Figure 3.26 and Table 3.22 presents the IR absorption band assignment. The broad absorption peak at 3400 cm^{-1} was due to the bonded hydroxyl group. The sharp peak presented at 1692 cm^{-1} should be C=O stretching vibration of conjugated ester. The methyl and methylene groups showed C-H bending vibration at 1467 cm^{-1} and 1376 cm^{-1}. The bands detected at 1084 cm^{-1} and 1034 cm^{-1} could be ascribed for the C-O stretching vibration of ester and alcohol, respectively.

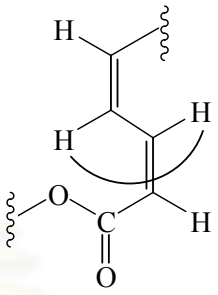
The mass spectrum of this compound (Figure 3.27) did not give the molecular ion peak, but showed very low intensity fragmentation ion peaks at *m/z* (% relative intensity): 456 (1), 412 (1), 394 (25), 354 (9), 326 (5), 294 (7), 255 (2) and the base peak at *m/z* 83 (100). Furthermore, the fragmentation ion at *m/z* 180 implied the presence of monosaccharide $C_6H_{12}O_6$ (glucose unit).[32]

The ^{1}H NMR spectrum (DMSO-d_6) (Figure 3.28) revealed an anomeric proton signal appeared as a sharp doublet at δ 4.13 (1H, d, J = 7.7 Hz, H-1′), indicative of β-D-glucosidic linkage[34], together with non-anomeric protons of sugar moiety in the range of δ 2.90-3.42. The β-configuration at the anomeric position of the glucopyranosyl unit could be confidently deduced from its relatively large $J_{1\text{-}2}$ coupling constant (7-8 Hz).[35] The high intensity signal analogous to methylene groups was detected at δ 1.25 (52H, s), the signal at δ 0.82 (3H, t, J = 6.87 Hz) should be the methyl proton signal of long chain fatty acid. The signal presented at δ 1.46 (2H, m) could be assigned as methylene protons which adjoined to terminal methyl group. [34] In addition, this spectrum also exhibited the olefinic proton signals at δ 7.53 (1H, d, J = 9.46 Hz), δ 5.59 (1H, dd, J = 9.46, 5.19 Hz) for the first double bond and at δ 4.91 (1H, dd, J = 5.19, 6.41 Hz) and δ 5.32 (2H, dd, J = 6.41, 8.24 Hz) for the second and allylic proton was detected at δ 1.90 (2H, m). The coupling constants of olefinic protons at δ 7.53 with δ 5.59 (9.46 Hz) and δ 5.32 with δ 4.91 (6.41 Hz) indicated *Z* geometry of the double bond of this compound. To illustrate this, J_{cis} was generally

observed around 4-12, while J_{trans} was around 14-19.[36] The conformation (*cisoid* or *transoid* dienes) of these two double bonds was cautiously considered from the coupling constant of protons at δ 5.59 with δ 4.19 (J = 5.19 Hz). The coupling constant of *cisoid* (0-6 Hz) usually less than *transoid* (7-12 Hz).[36] This information disclosed that the conformation of two double bonds should be *cisoid* dienes.

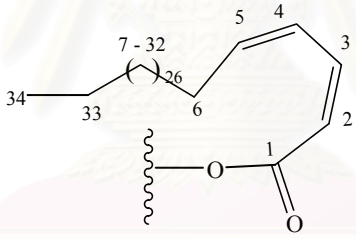


J_{cisoid} = 0-6 Hz

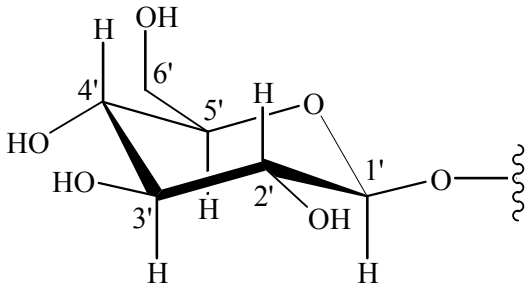


$J_{transoid}$ = 7-12 Hz

The integration value 52 protons of the sharp singlet signal at δ 1.25 indicated the number of methylene carbons in a fatty acid portion as 26. Thus, total number of this long chain acid should be 34 carbons.



The ^{13}C NMR spectrum (DMSO-d_6) (Figure 3.29) showed the carbon signal of ester at δ 173.8. The four signals detected at δ 129.5, 129.6, 129.9 and 130.3 could be assigned for olefinic carbons. The presence of one β-D-Glc was confirmed by the detection of the anomeric signal at δ 103.5. This signal supported that C-1′ must attach to an acyl group. If C-1′ bearing only the hydroxy group as a substituent, it will show the anomeric carbon signal around δ 98.4.[37] Kasai *et al* reported that in the ^{13}C NMR spectra of β-D-glucosides, carbon signals of C1′ (anomeric carbon) generally appear at the signals remarkably lower field than those of the corresponding α-anomeric configuration.[38] The chemical shift assignments of glucopyranoside unit are presented as shown in Table 3.22.



glucopyranoside unit

Table 3.22 Chemical shift assignment of glucopyranoside unit of compound **3**

Position	Chemical shift (ppm)	
	Carbon	Proton
1′	103.5	4.12 (d, *J* = 7.63 Hz)
2′	73.5	3.00 (dd, *J* = 7.6, 8.39 Hz)
3′	76.5	3.08 (m)*
4′	70.0	3.02 (m)*
5′	76.9	3.12 (m)*
6′	61.0	3.4 (dd, *J* = 5.81, 11.59 Hz)

*Obscured by other signals; couplings could not be accurately determined.

Acetylation of compound 3

Compound **3** 90 mg was acetylated in usual way to obtain compound **3A** as yellow waxy 85 mg (89.5% yield), R_f 0.60 (solvent system: 60% EtOAc-hexane).

The IR spectrum of this acetate (Figure 3.30) showed the important absorption bands at 1740 cm^{-1} of C=O stretching vibration of acetyl group, and additional bands at 2870 and 2930 cm^{-1} of C-H stretching vibration of $–CH_2$ and $-CH_3$. The significant absorption band at 1500 cm^{-1} might due to the C=C stretching vibration of olefinic structure. The band at 1070 cm^{-1} revealed the characteristic of C-O stretching vibration of glycosidic linkage.[32] The rest of this spectrum was similar to the IR spectrum of Compound **3** except for the O-H peak being absent.

The ^1H NMR spectrum ($CDCl_3$) (Figure 3.31) revealed the proton signals of four acetoxy groups ($-OCOCH_3$) at δ 2.05, 2.07, 2.09 and 2.12. The anomeric proton of sugar was detected at δ 4.41 (1H, d, *J* = 7.7 Hz). The presence of four acetoxy

groups strongly supported that compound **3** should be monosaccharide, which one hydroxy group bonded to fatty acid portion.[39]

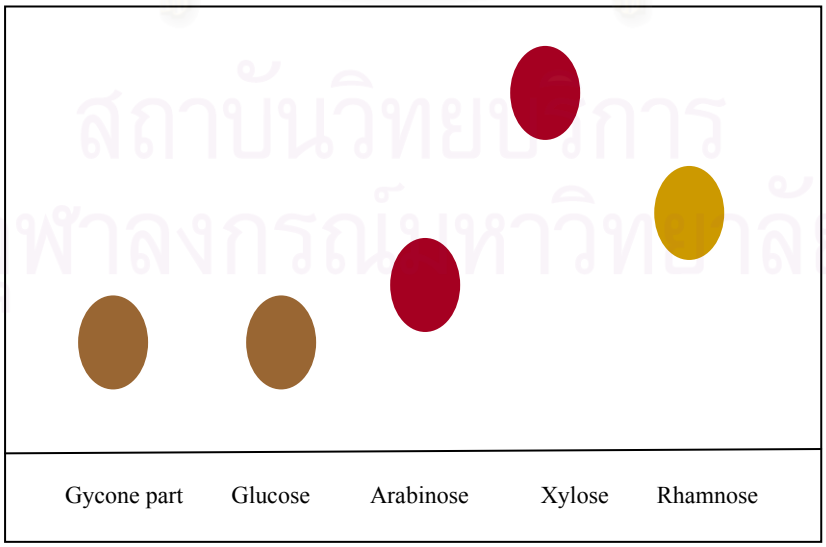
Hydrolysis of compound 3

A solution of 10% HCl in ethanol 10 mL was added to compound **3** (50 mg) and the mixture was heated under reflux for 10 hours. The mixture was then poured into water 10 mL and further extracted with EtOAc 10 mL three times. The combined EtOAc was dried over anhydrous sodium sulfate. Evaporation of the solvent furnished a solid in pale yellow oil, assigned as compound **3B** (19 mg, 3.80% yield).

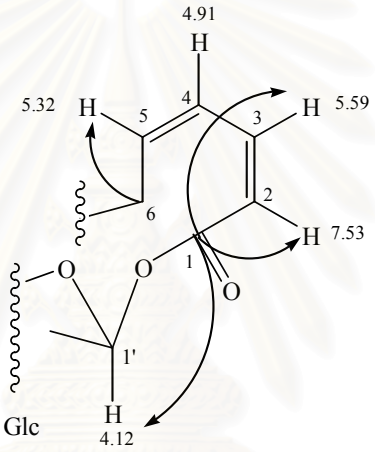
The IR spectrum (Figure 3.32) showed important absorption bands of C-H stretching vibration of $-CH_2$ and $-CH_3$ at 2850 and 2930 cm^{-1}. The bands observed at 3411 cm^{-1} and at 1739 cm^{-1} could be assigned for O-H stretching vibration of hydroxyl group and C=O stretching vibration, respectively.

The ^1H NMR spectrum ($CDCl_3$) (Figure 3.33) revealed the methyl signal at δ 0.76 and a long chain methylene signal at δ 1.66. The signals of sugar moiety around δ 3.00-4.50 were not appeared.

The glycone part of this compound (sugar moiety) was clarified by performing one-dimentionally descending paper chromatography using BuOH-EtOAc-H_2O (4 : 1 : 2.2) as an eluent.[30] It showed a single brown spot. The paper chromatography profile with four common authentic sugars was denoted that the glycone part of Compound **3** was consisted of glucose.

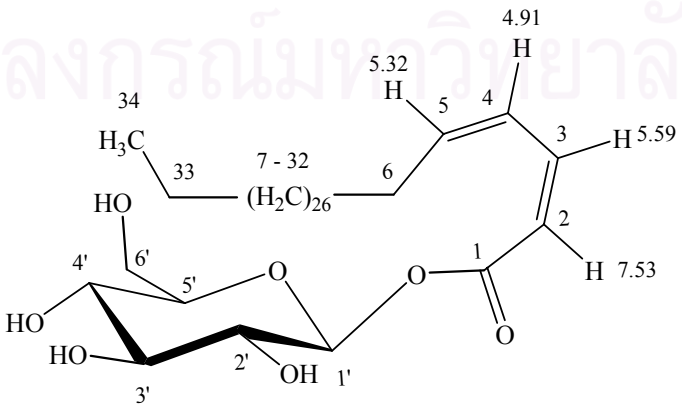


From the CIGAR spectrum (Figure 3.34) the correlation of the olefinic protons at δ 7.53, 5.59 and the anomeric proton at δ 4.12 with carbon signal at δ 173.8 was obviously detected. This was a clear evidence that the double bond present in this compound should be conjugated to the carbonyl group and this carbonyl group was connected to C1′ of glucose unit *via* ester linkage. The correlation between the allylic carbon at δ 32.1 (C6) with olefinic proton at δ 5.32 was also observed, this suggested two double bonds be conjugated dienes. Supposed there was a methylene group between two double bonds as a skipped diene, the CIGAR spectrum should be found the correlation of this methylene carbon with both protons at δ 5.59 and 4.91. However, there was only the correlation at δ 5.32.[40]



partial CIGAR correlation

From the above data, compound **3** was determined to be β-D-glucopyranosyl tetratriacontan-2,4-dienoate. The possible structure of this compound was shown below. This is the first report on the isolation of this acylated glucopyranoside in the chemical literature.



Compound 3

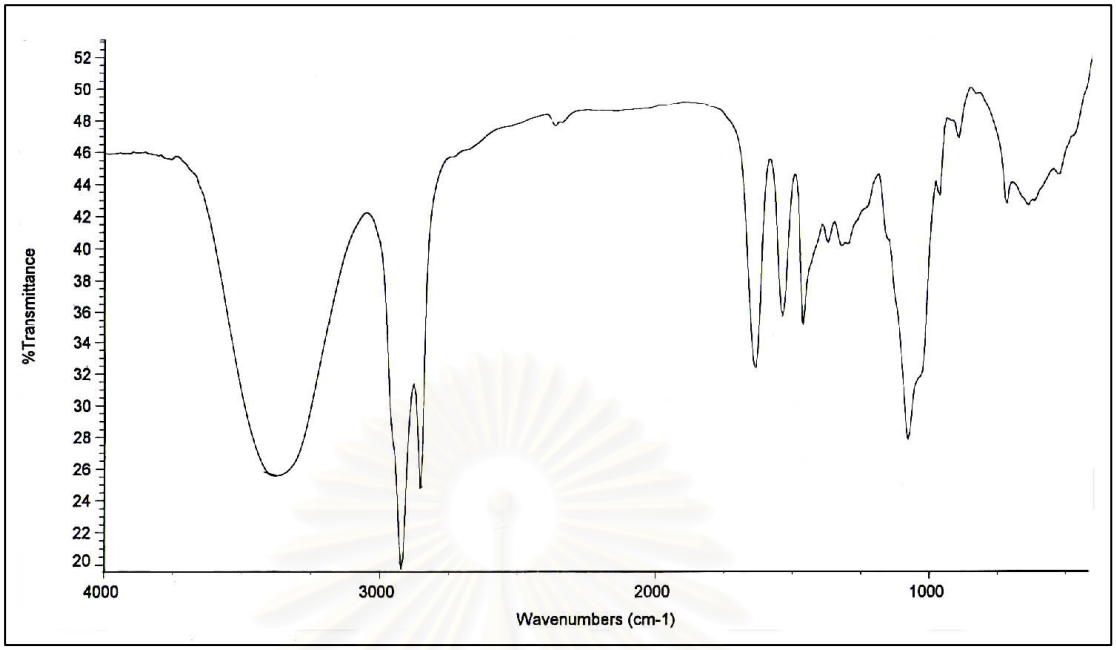


Figure 3.26 The IR spectrum of compound **3**

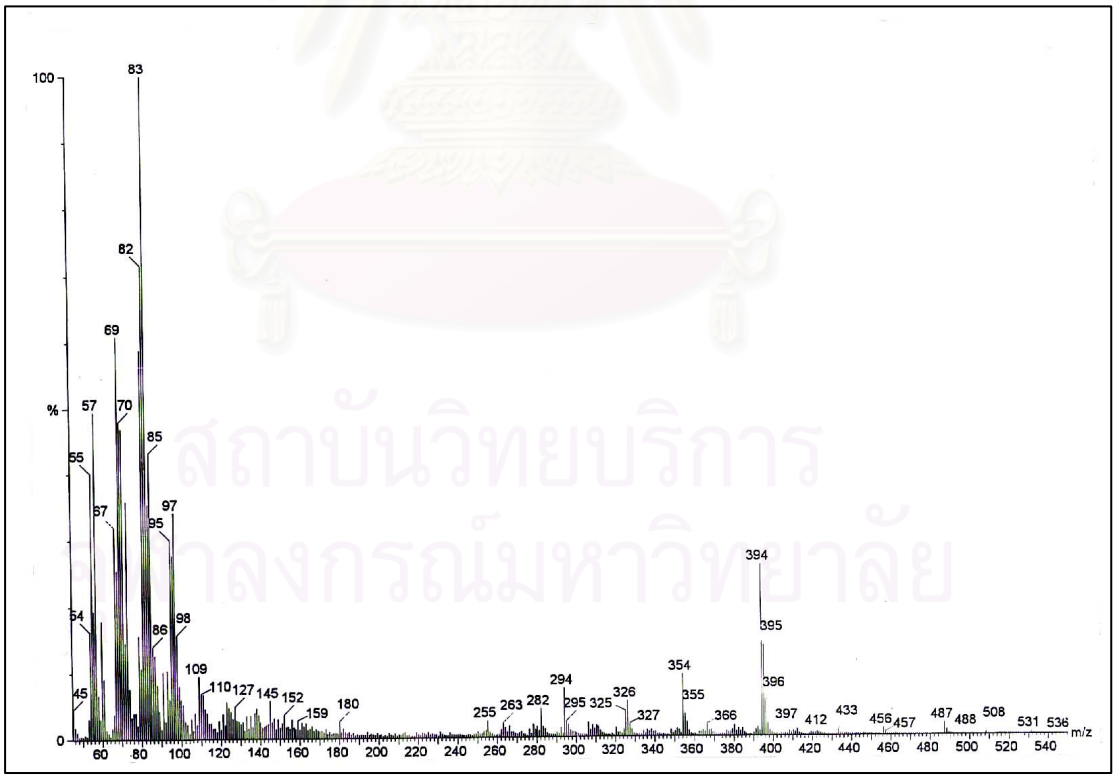


Figure 3.27 The mass spectrum of compound **3**

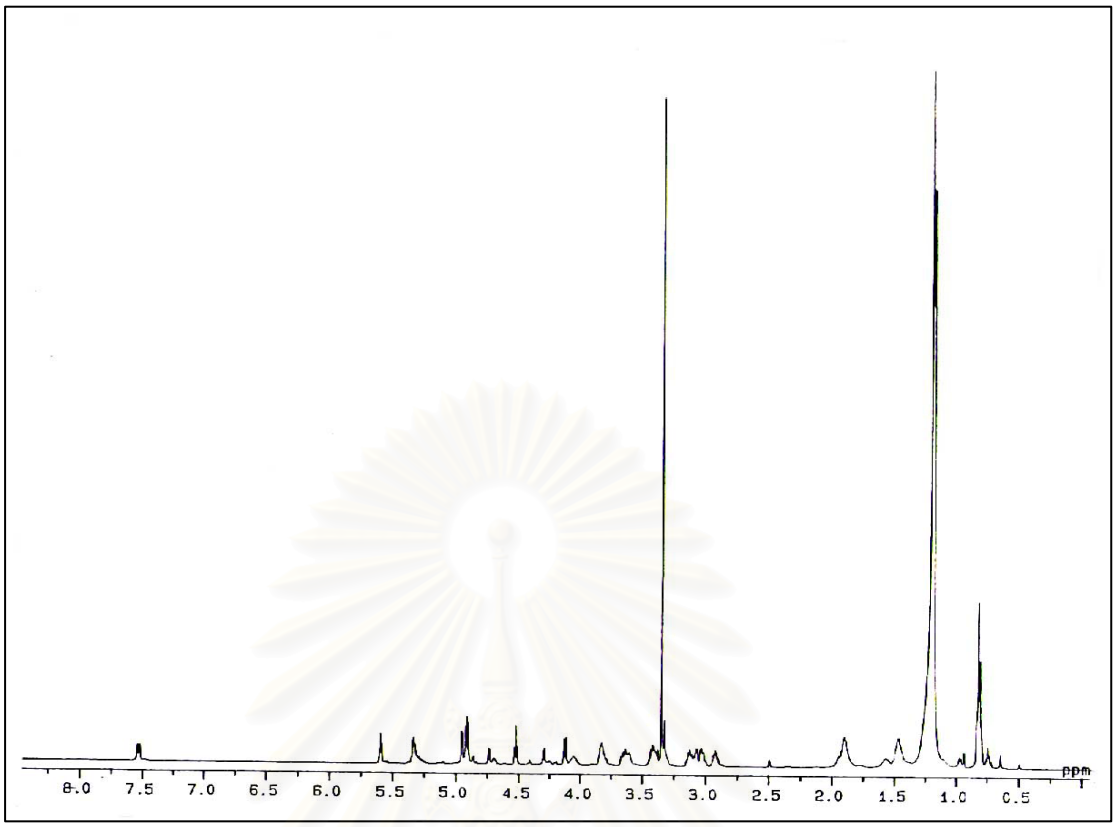


Figure 3.28 The ^{1}H NMR spectrum of compound **3**

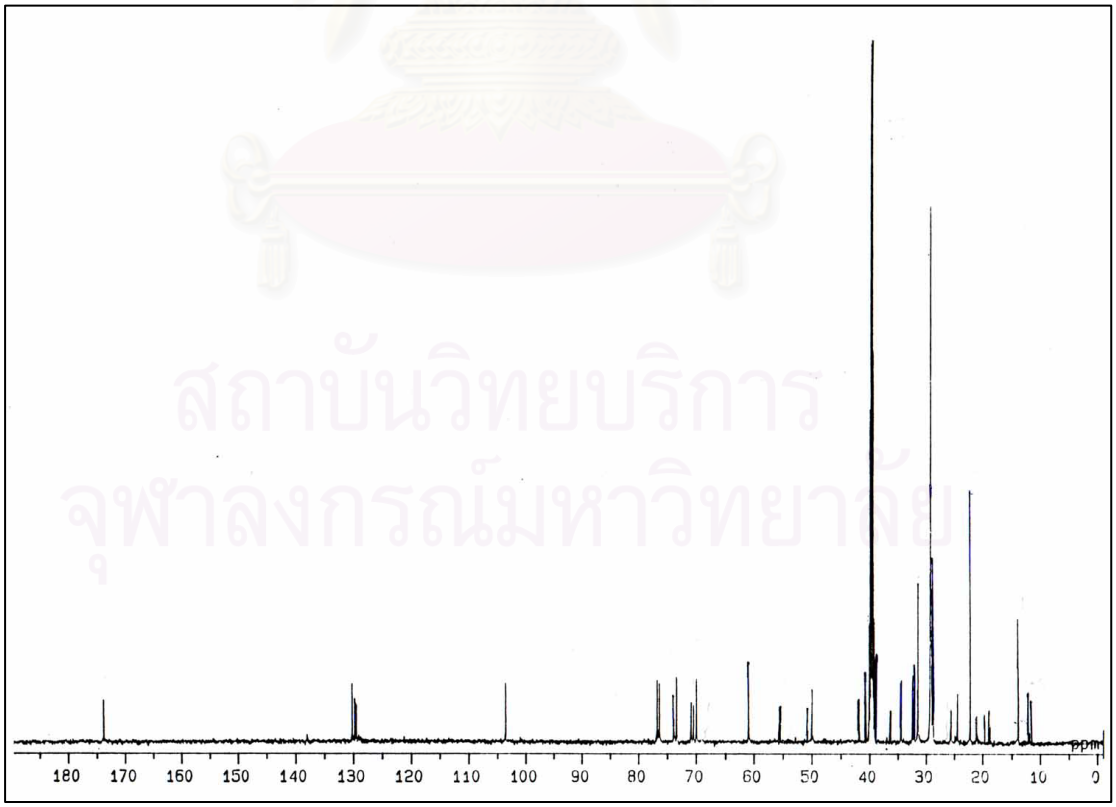


Figure 3.29 The ^{13}C NMR spectrum of compound **3**

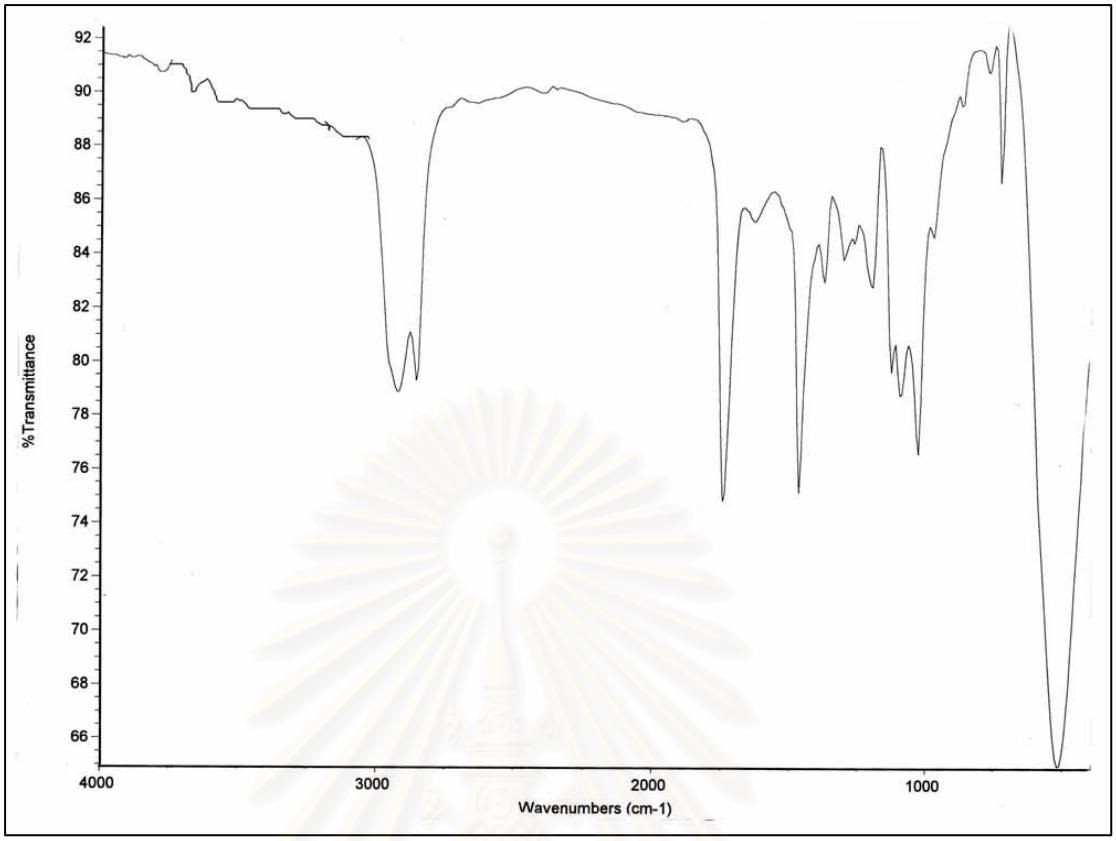


Figure 3.30 The IR spectrum of compound **3A**

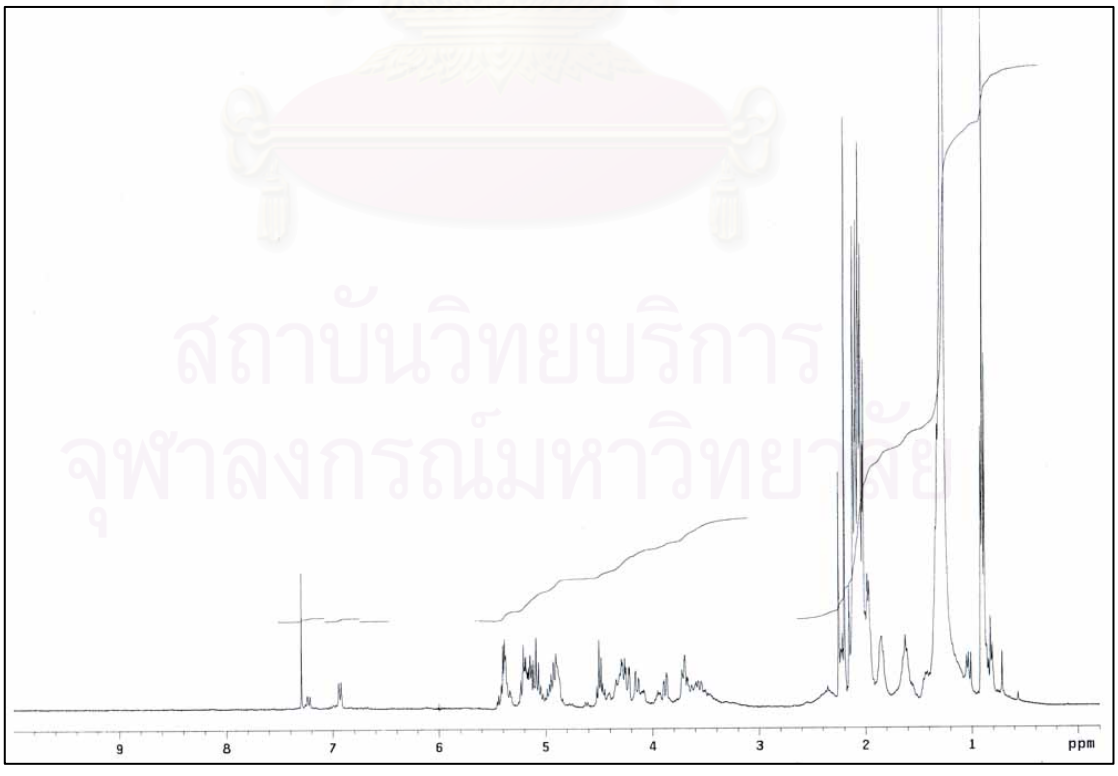


Figure 3.31 The ^{1}H NMR spectrum of compound **3A**

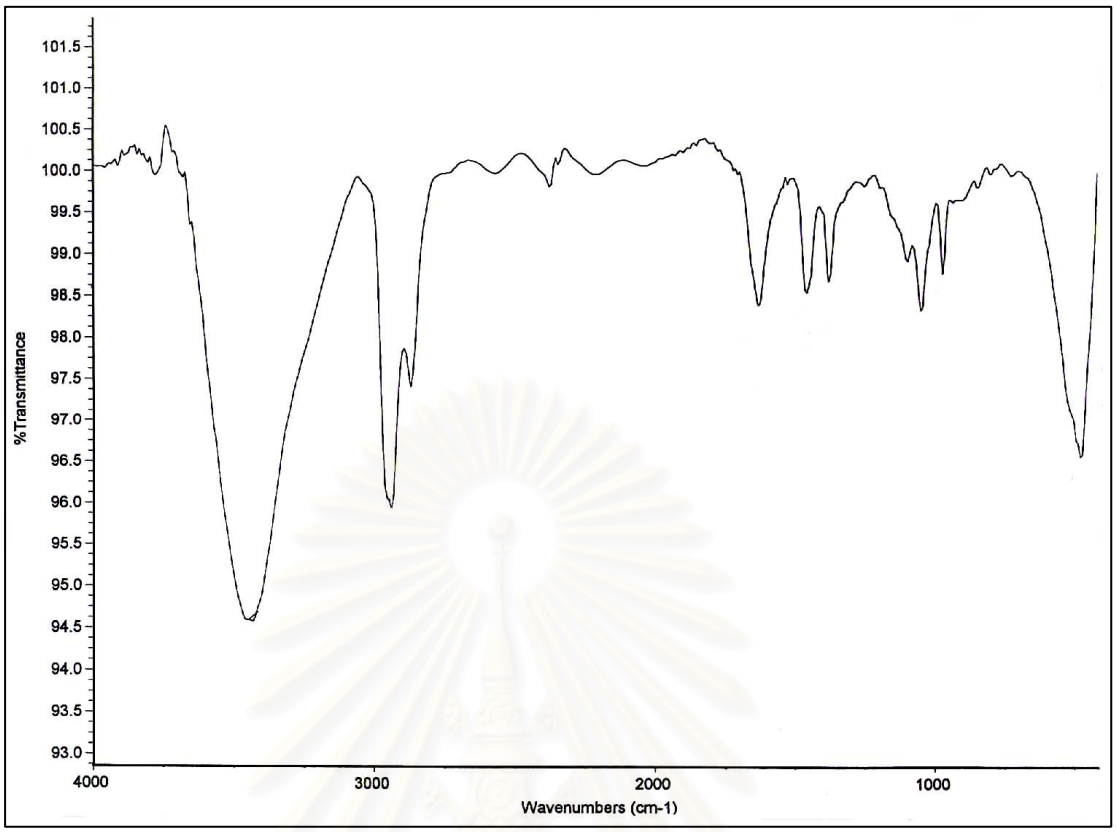


Figure 3.32 The IR spectrum of compound **3B**

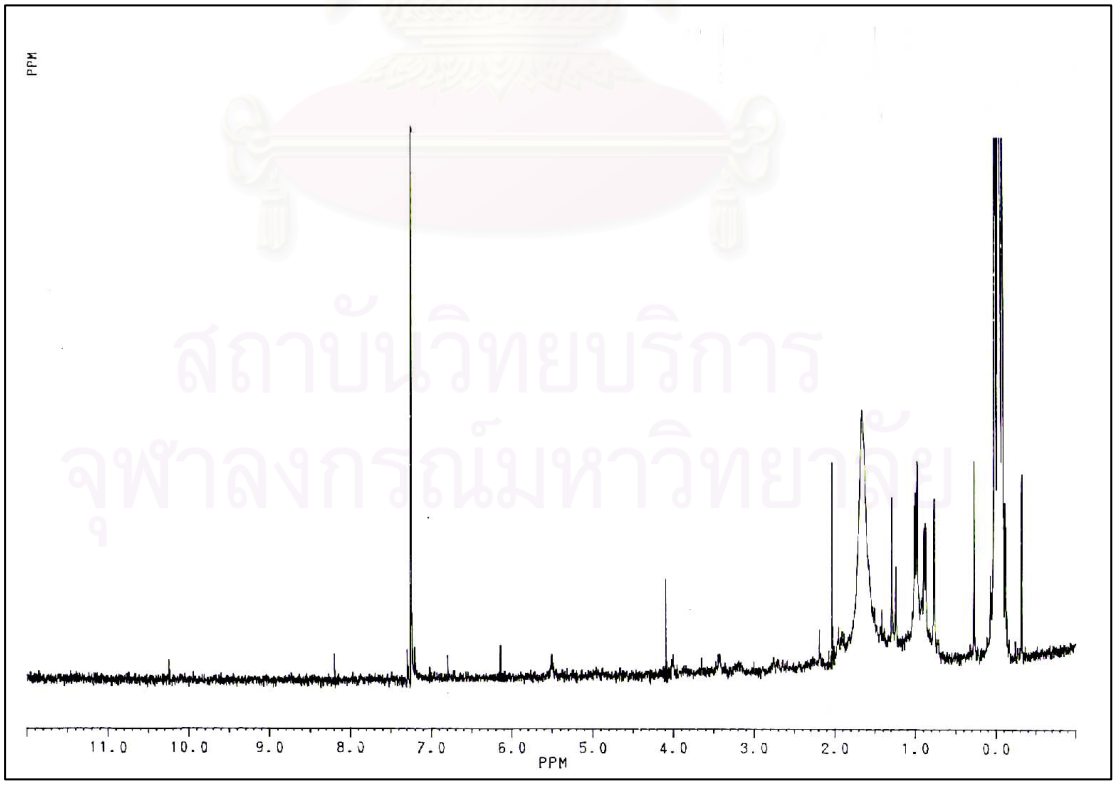


Figure 3.33 The ^{1}H NMR spectrum of compound **3B**

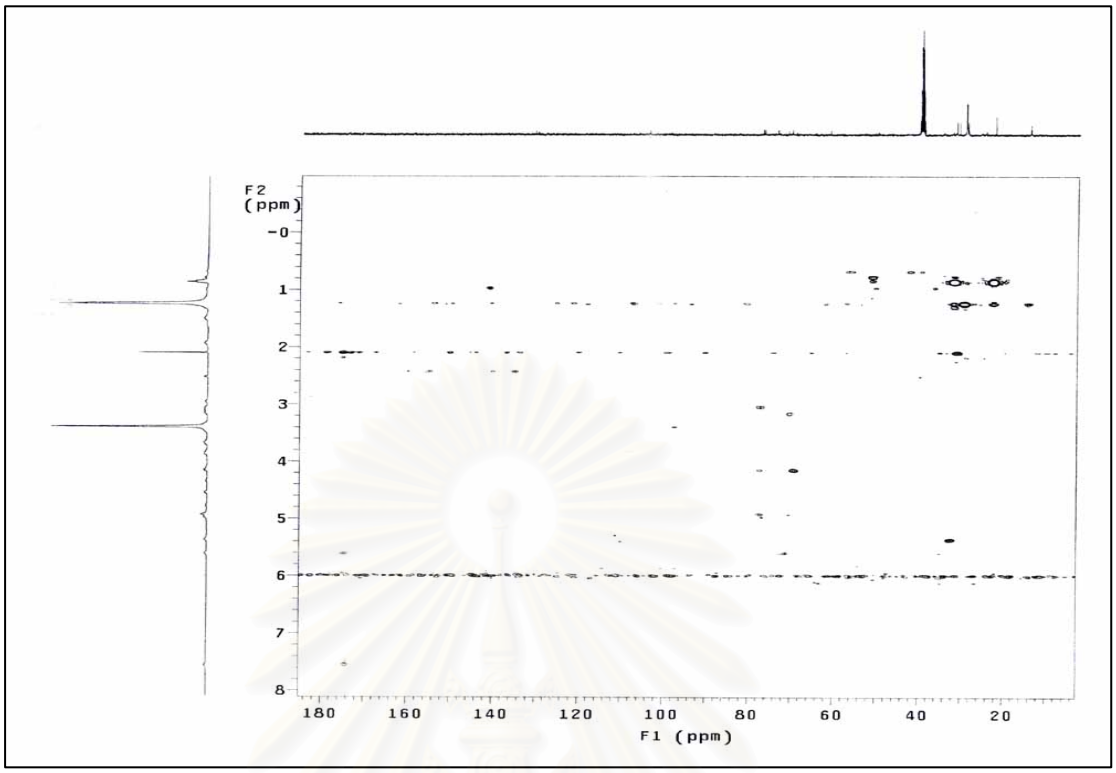


Figure 3.34 The CIGAR spectrum of compound **3**

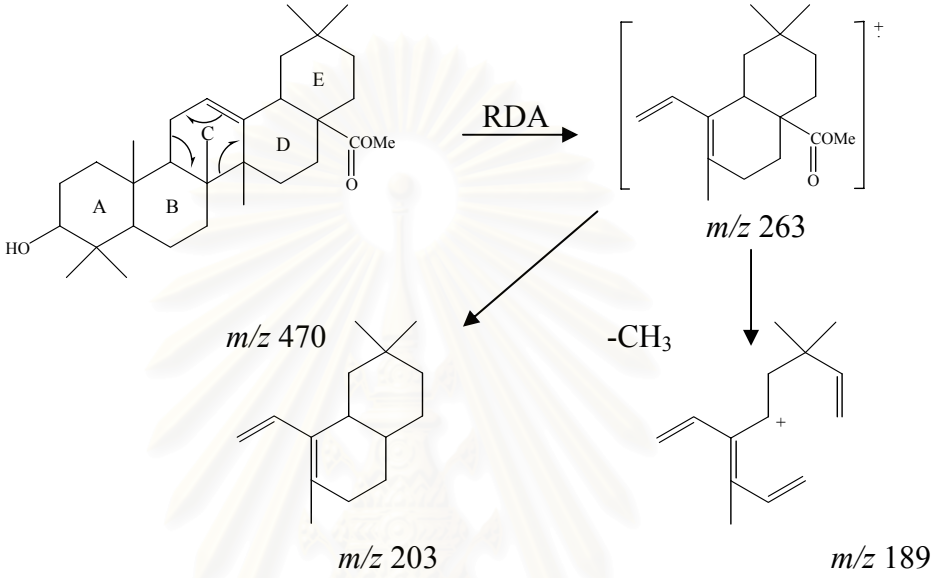
3.4.9 Properties and structural elucidation of compound 4

Compound **4** was isolated from the Sephadex LH 20 column chromatography from both fractions IIF and IIG. This compound was recrystallized by solvent-solvent precipitation[41] using a mixture of MeOH and acetone to yield compound **4** as white solid, 379 mg (0.76% w/w of Fraction II), m.p. 198-202 °C (dec.). This compound gave one spot at R_f 0.72 using 50% MeOH-EtOAc as a solvent system. Compound **4** gave a violet color to Liebermann-Burchard which indicated the presence of triterpenoidal skeleton.[42]

The IR spectrum of compound **4** (Figure 3.35) revealed a very broad absorption band at 3400 cm^{-1} due to the –OH stretching vibration of alcohol. Other characteristic absorption peaks were observed at 1745 cm^{-1} (C=O stretching), 2914, 2858 cm^{-1} (C-H stretching of CH_2, CH_3), weak signal at 1070 cm^{-1} (C-O stretching), 1670 cm^{-1} for C=C stretching vibration and two sharp peaks at 1040 and 1020 cm^{-1} for C-O stretching vibration.

The mass spectrum of compound **4** (Figure 3.36) did not show the molecular ion peak but exhibited the important fragmentation ion peak at *m/z* (% relative intensity) 470 (3) (Calcd. for $C_{31}H_{50}O_3$: MW. 470.73). In addition, the spectrum

displayed retro Diels-Alder fragmentation ion peaks at *m/z* 263 (24) and 203 (23) which were the characteristic of triterpenoid compound.[43] The formation of fragment at *m/z* 263 demonstrated the presence of methyl ester group in ring D or E,[44] as well as the proposed structure of methyl oleanolate. Other fragmentation peaks at *m/z* 280 (15), 263 (25), 135 (60), 95 (100), 55 (97) were also observed. The possible fragmentation pattern of compound **4** is presented below.



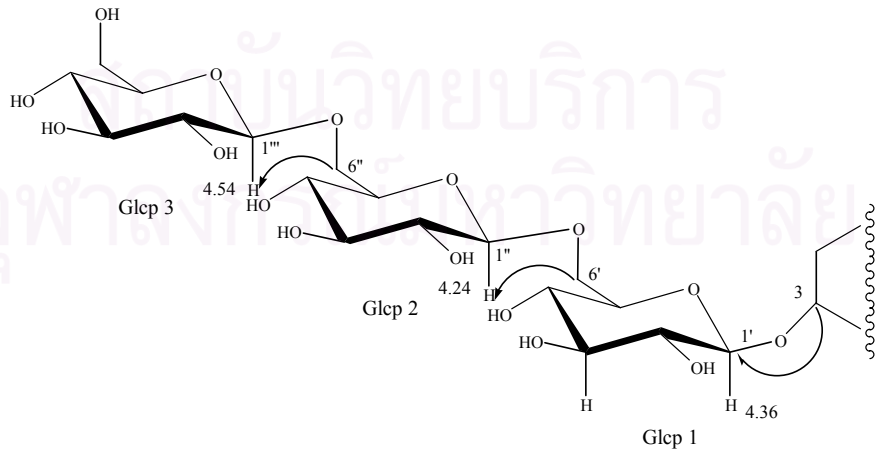
The ^{1}H NMR spectrum (DMSO-d_6) (Figure 3.37) displayed the proton signals typical of triterpenoidal compound. It showed signals for methyl groups at δ 0.72, 0.86, 0.87, 0.90, 0.93, 0.97 and 1.21. The olefinic proton appeared at δ 5.30 (1H, br, H-12). The H-3 absorbed at δ 3.08 (1H, dd, J = 9.01 and 4.27 Hz). The signals around δ 3.01-4.82 could be assigned for sugar protons.

The ^{13}C NMR spectrum (DMSO-d_6) (Figure 3.38) showed carbon signals resemble to those of certain triterpenoidal structures. To illustrate this, the ^{13}C NMR spectrum of compound **4** exhibited the carbonyl carbon of ester at δ 170.2, presumably a carbonyl group of –COOMe. The presence of methyl ester moiety could be endorsed by the detection of singlet signal of methyl group at δ 3.52 in the ^{1}H NMR spectrum. Besides, three anomeric signals at δ 102.9, 103.1 and 103.8 indicated the presence of three sugar moieties.[45] Supposed the sugar residue was attached to aglycone by ester bond, the anomeric carbon signal should appear at approximately δ 95.0 (more upfleld than ether bond). The disappearance of the anomeric signal around δ 95.0 suggested that no sugar moiety be attached to the aglycone by an ester

bond.[46] This data strongly supported that sugar moieties attached to C3 of the aglycone by a glycosidic linkage. Besides, the signal of C-3 was observed at low field δ 88.7 suggested that the hydroxyl group at C-3 attach to a sugar moiety.[46] The other signals could be assigned as: one olefenic quaternary carbon at δ 142.6 and one olefinic tertiary carbon at δ 128.3. From ^{13}C NMR data revealed that this compound belonged to olean-12-ene triterpenoids. The olean-12-ene and urs-12-ene triterpenes could be distinguished by inspection of the resonance of olefinic carbons. The chemical shifts of C-12 and C-13 in olean-12-enes normally appeared at δ 122.1 and 143.4 but those of urs-12-enes could generally be observed at δ 125.5 and 138.0.[47]

The assignments of anomeric protons, immediately permitted the assignment of the resonances of the linked carbon atom by HSQC (Figure 3.39). The correlation peaks were observed between anomeric carbon signals at δ 102.9, 103.1 and 103.8 with anomeric protons at δ 4.54 (1H, d, *J* = 7.6 Hz), 4.36 (1H, d, *J* = 7.0 Hz) and 4.24 (1H, d, *J* = 7.2 Hz), respectively. The large coupling constants ($J_{1\text{-}2}$ = 7-8 Hz) were corresponded to β-configuration at the anomeric position of glucopyranosyl units.[35]

Information concerning the sequence of saccharide chain was deduced from an CIGAR experiment. In the CIGAR spectrum (Figure 3.40), correlation peaks were observed between the anomeric proton at δ 4.36 and C-3 of aglycone (δ 88.7), anomeric proton at δ 4.24 of the second glucose unit (position 1′′) and C-6′ of the first glucose unit (δ 66.4) and anomeric proton at position 1′′′ (δ 4.54) and C-6′′ of the second unit glucose (δ 65.4).

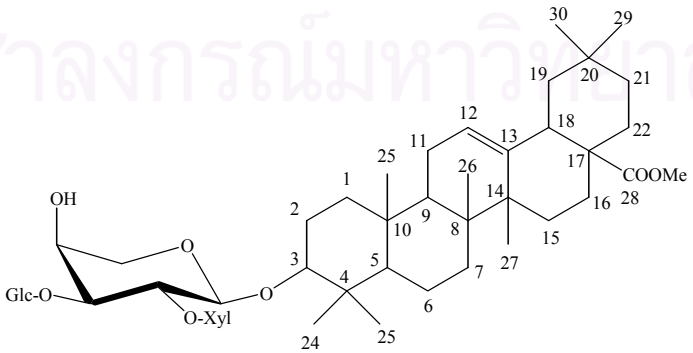


The comparison of the carbon signals of aglycone of compound **4** and that of Tarasaponin III methyl ester (methyl oleanolate as an aglycone)[48] was exhibited in

Table 3.23. Compound **4** had the same aglycone, methyl oleanolate, likewise to that of Tarasaponin III methyl ester. Notwithstanding, the sugar moieties were different.

Table 3.23 The ^{13}C NMR assignment of aglycone of compound **4** compared with that of Tarasaponin III methyl ester[48]

Carbon	Chemical Shifts (ppm)		Carbon	Chemical Shifts (ppm)	
	aglycone of cpd **4**	aglycone of tarasaponin III		aglycone of cpd **4**	aglycone of tarasaponin III
C1	38.7	39.0	C17	46.8	47.4
C2	25.8	27.0	C18	40.6	42.3
C3	88.7	89.4	C19	46.0	46.5
C4	38.7	40.1	C20	27.6	31.3
C5	60.8	56.2	C21	29.3	34.5
C6	18.0	19.0	C22	40.6	33.3
C7	36.3	33.5	C23	29.1	28.6
C8	38.5	40.2	C24	17.0	17.5
C9	46.3	48.3	C25	15.5	16.0
C10	38.8	37.4	C26	16.2	17.7
C11	20.2	24.0	C27	23.2	26.7
C12	124.5	122.8	C28	170.2	177.6
C13	142.7	144.8	C29	29.1	33.6
C14	41.3	42.8	C30	25.8	24.2
C15	27.6	28.7	$-CO_2\underline{Me}$	54.8	51.9
C16	23.2	24.2			



Tarasaponin III methyl ester

Acetylation of compound 4

Compound **4** 50 mg was acetylated in usual way to obtain compound **4A** as pale yellow solid, 45 mg (90% yield), R_f 0.48 (solvent system: 40% MeOH-EtOAc), m.p 147-150°C.

The IR spectrum (Figure 3.41) of this acetate showed the characteristic absorption peaks at 1740 cm^{-1} of C=O stretching vibration of an ester group. The significant absorption band at 1490 cm^{-1} might due to the C=C stretching vibration of olefinic structure. The band at 1050 cm^{-1} revealed the characteristic absorption band of C-O stretching vibration of glycosidic linkage.[32] The reminder of this spectrum was similar to the IR spectrum of compound **4** except the O-H peak being absent.

The ^1H NMR spectrum of this acetate ($CDCl_3$) (Figure 3.42) showed the multiplet signals around δ 1.98 – 2.23 revealed the protons of acetyl groups. This data supported that compound **4** should be of three sugar moieties. The signals around δ 0.75-1.95 exhibited the characteristic of triterpenoidal compound. The signals around δ 3.50-4.80 could be assigned for proton signals of sugar moieties. The signals of olefenic protons appeared at δ 4.90-5.50.

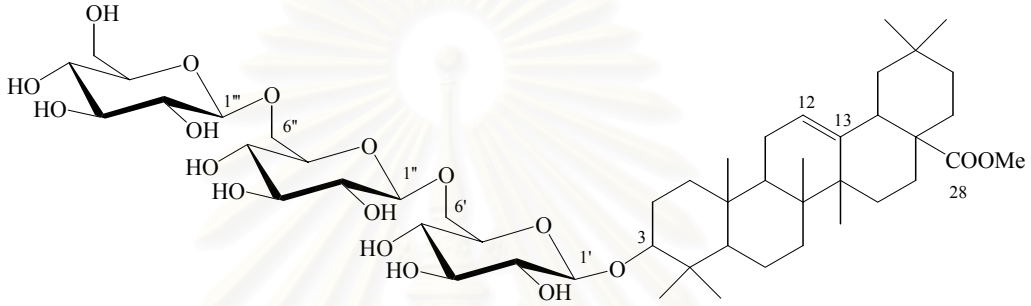
Hydrolysis of compound 4

Compound **4**, 35 mg was acid hydrolyzed by 10% HCl in ethanol 10 mL. It was refluxed for 10 hours. Evaporation of ethanol and the residue was further extracted with diethyl ether:water (1:1) twice. The combined diethyl ether was dried over sodium sulfate and evaporated the solvent to yield pale yellow wax which then recrystallized by a mixture of chloroform and methanol to afford compound **4B**. This compound was identified by *co*-TLC with oleanolic acid authentic sample. It showed a single spot at R_f 0.66, solvent system 100% EtOAc. The water part was concentrated and the remaining material was paper chromatographed. After sprayed with AHP reagent,[30] it showed a single brown spot with R_f value of 0.30 (solvent system: BuOH:EtOAc:H_2O (4:1:2.2)) identical with D-glucose, an authentic sample.

The IR spectrum (Figure 3.43) showed an important absorption band of O-H stretching vibration at 3450 cm^{-1}. The additional bands were observed at 2850 and 2910 cm^{-1} of C-H stretching vibration of $–CH_2$ and $-CH_3$, 1710 cm^{-1} of C=O stretching of acid and 1490 cm^{-1} for C=C stretching vibration of olefin.

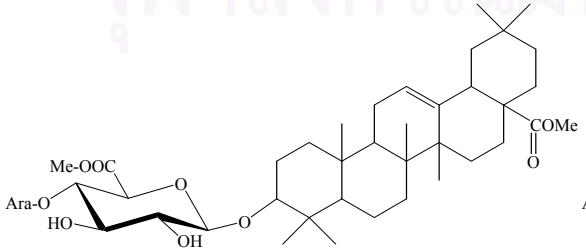
The ^{1}H NMR spectrum ($CDCl_3$) (Figure 3.44) exhibited the signal at δ 5.43 which could properly be assigned to olefinic carbons and multiplet signals around δ 0.72-2.00 indicative of methyl, methylene and methine protons of triterpenoidal skeleton.

According to all information obtained including physical properties, color test and spectroscopic data, compound **4** was characterized as methyl oleanolate 3-*O*-[β-D-glucopyranosyl (1→ 6)][-β-D-glucopyranosyl (1→ 6)]-β-D-glucopyranoside. The possible structure of compound **4** was shown below.

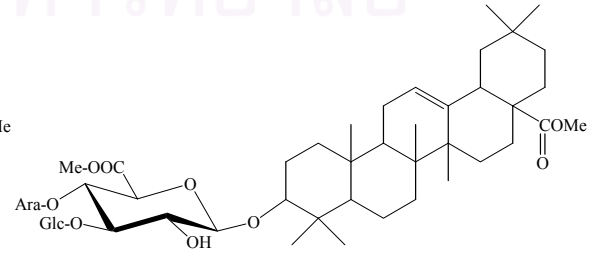


Compound **4**

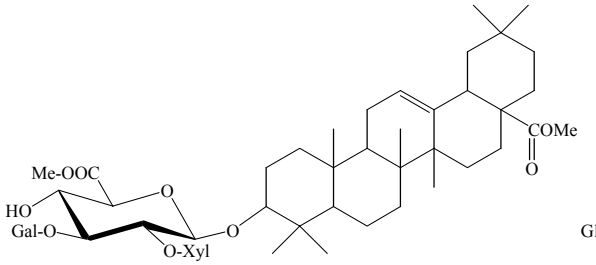
Throughout the chemical literatures, this compound had never been reported. Therefore, compound **4** was a new triterpenoid glycoside isolated from *H. umbellata*. However, the triterpenoid saponins contained methyl oleanolate as aglycone had been reported. In 1994, *Sakai et al* reported four methyl oleanolates from the dried root-bark of *Arilia elata*, which has been used as a folk medicine for diabetes, hepatitis, stomach ulcer, *etc*.[48,49] These are 28-Desglucosyl-chikusetsusaponin IV, Tarasaponin I dimethyl ester, Tarasaponin II dimethyl ester and Tarasaponin III methyl ester. In addition, the compounds of this type had never been reported on agricultural base activity, particularly plant growth regulator. The structures of triterpenoid compounds from *A. elata* are shown as follows.



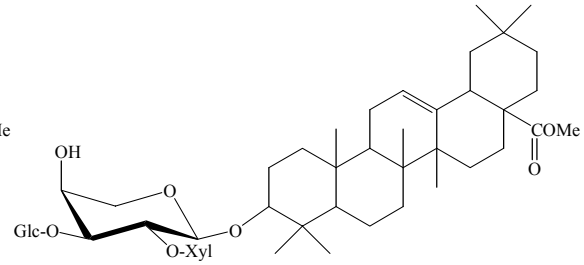
28-Desglucosyl-chikusetsusaponin IV



Tarasaponin I dimethyl ester



Tarasaponin II dimethyl ester



Tarasaponin III methyl ester

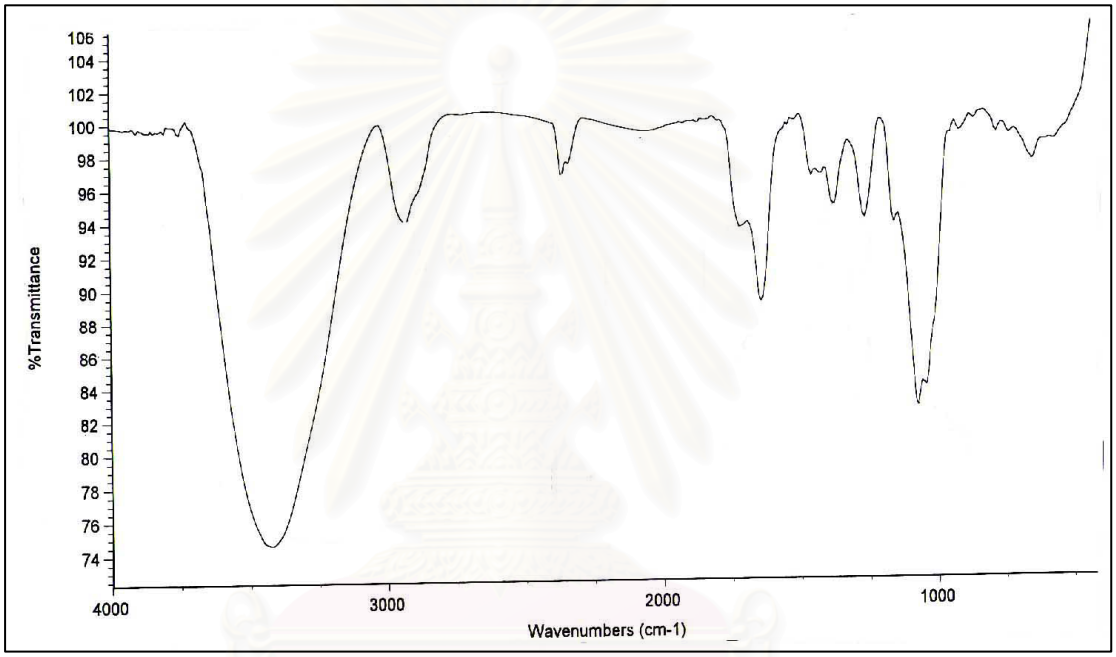


Figure 3.35 The IR spectrum of compound **4**

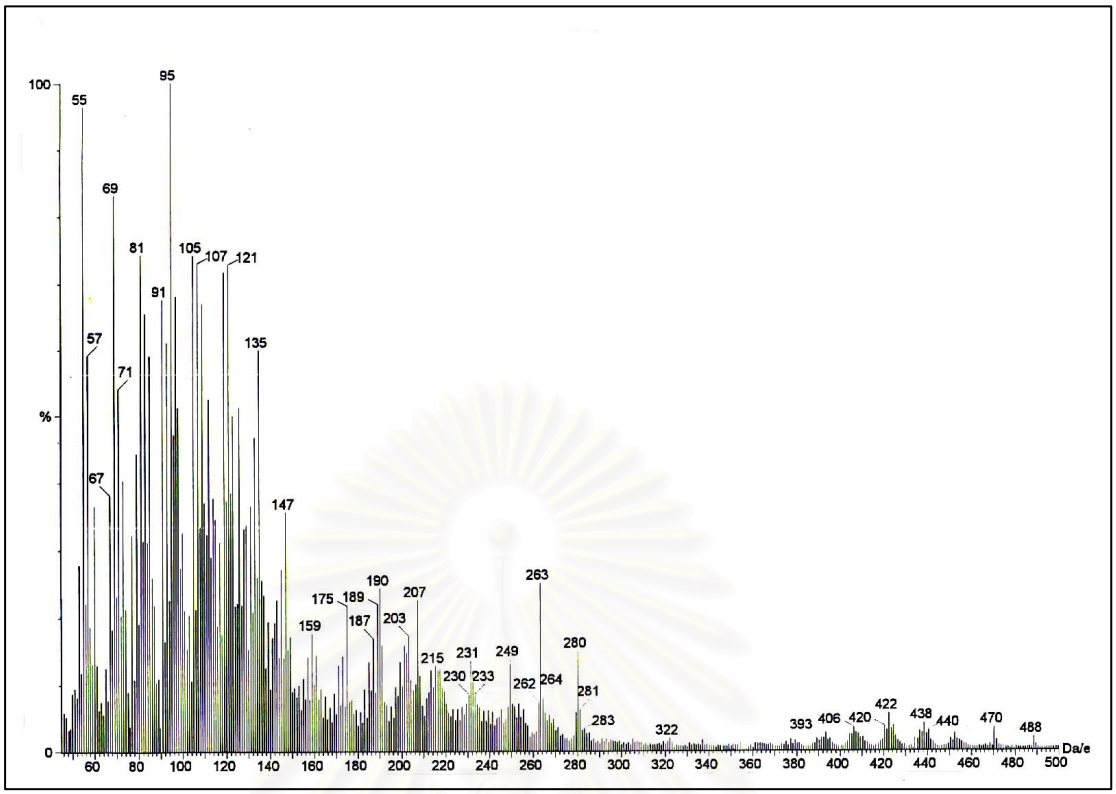


Figure 3.36 The mass spectrum of compound **4**

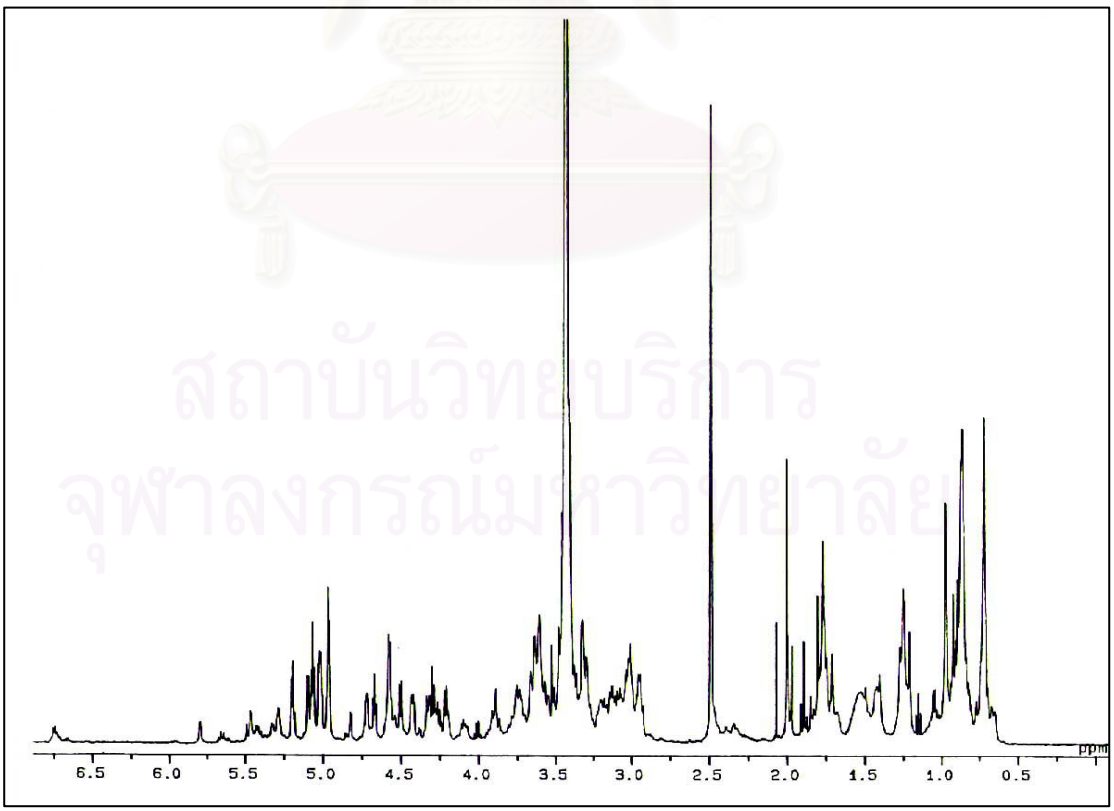


Figure 3.37 The ^{1}H NMR spectrum of compound **4**

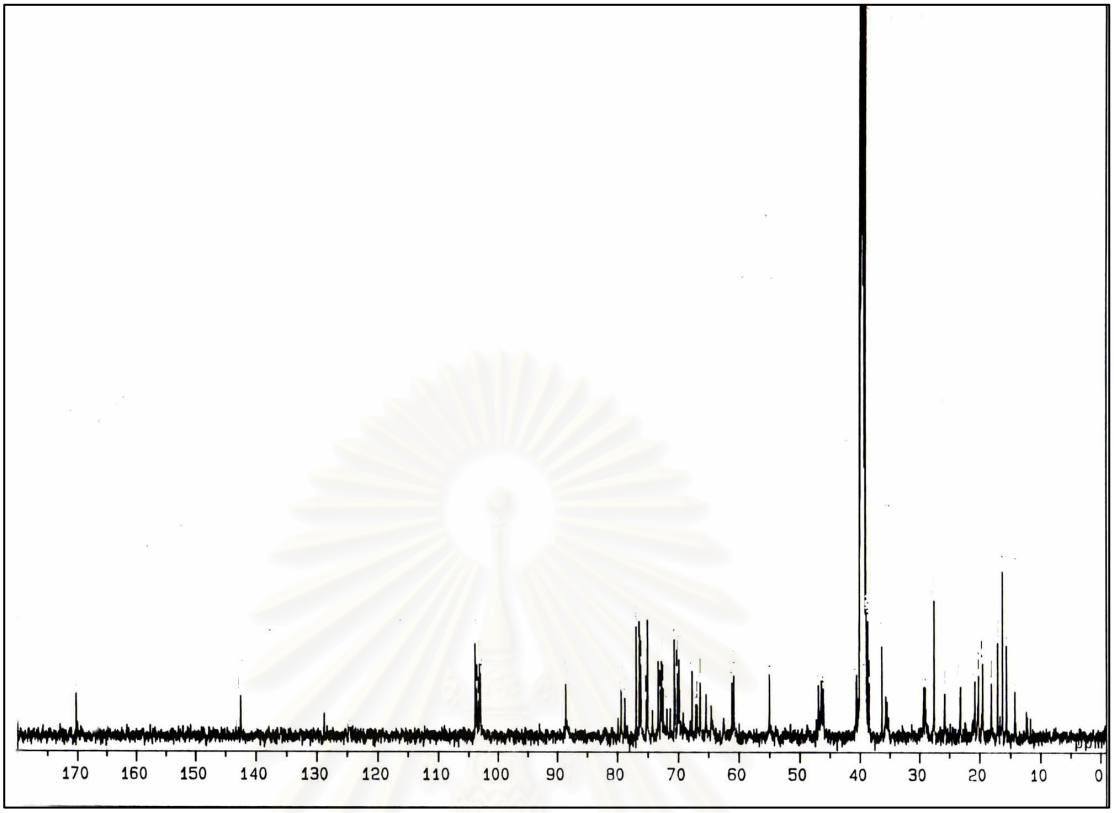


Figure 3.38 The ^{13}C NMR spectrum of compound **4**

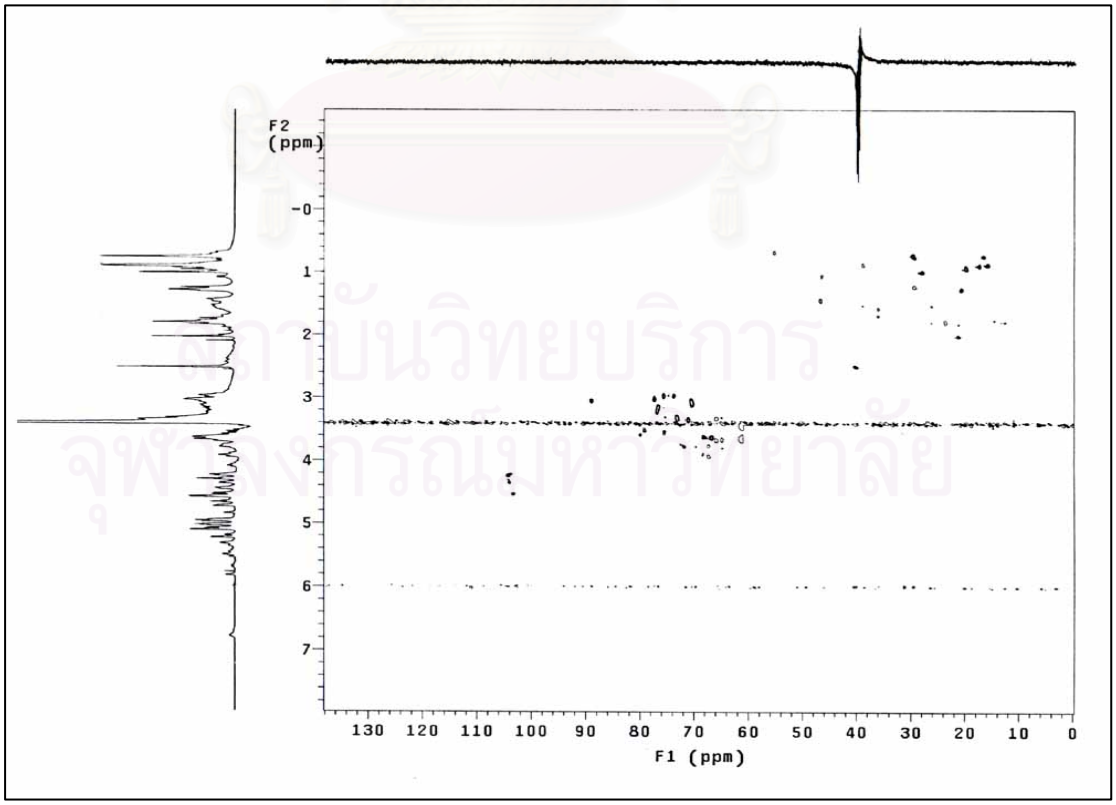


Figure 3.39 The HMQC spectrum of compound **4**

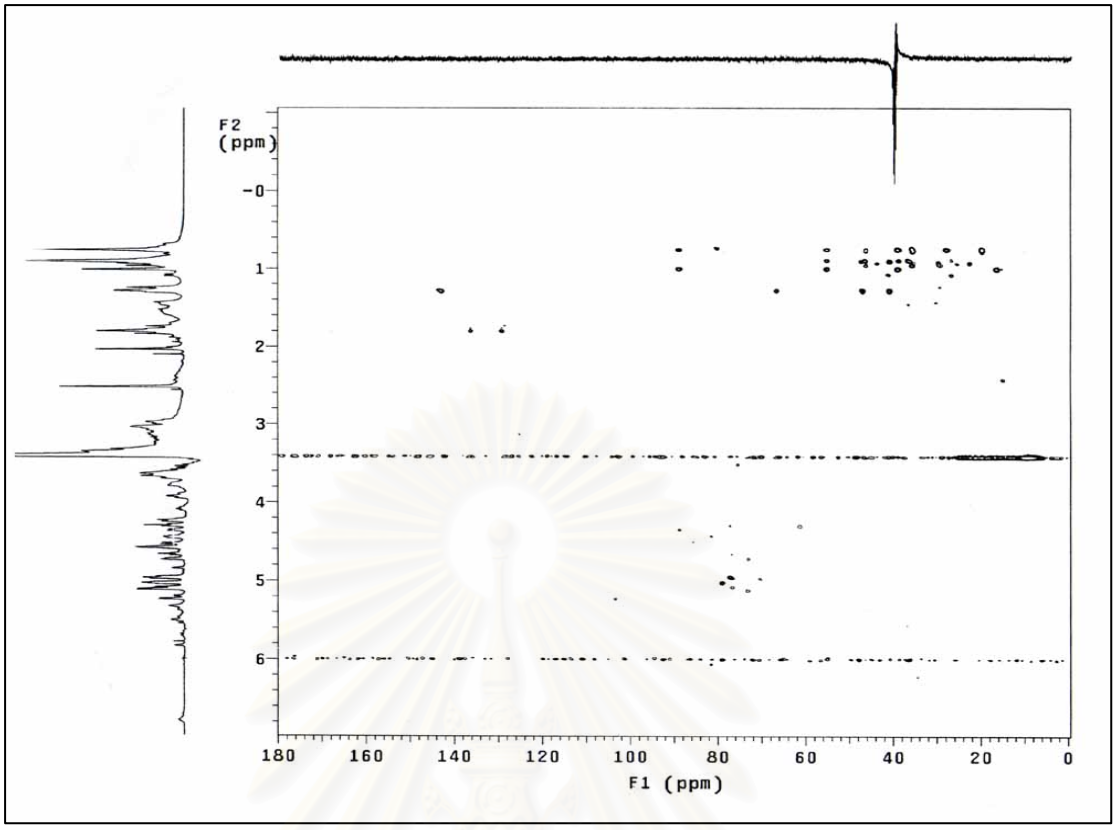


Figure 3.40 The CIGAR spectrum of compound **4**

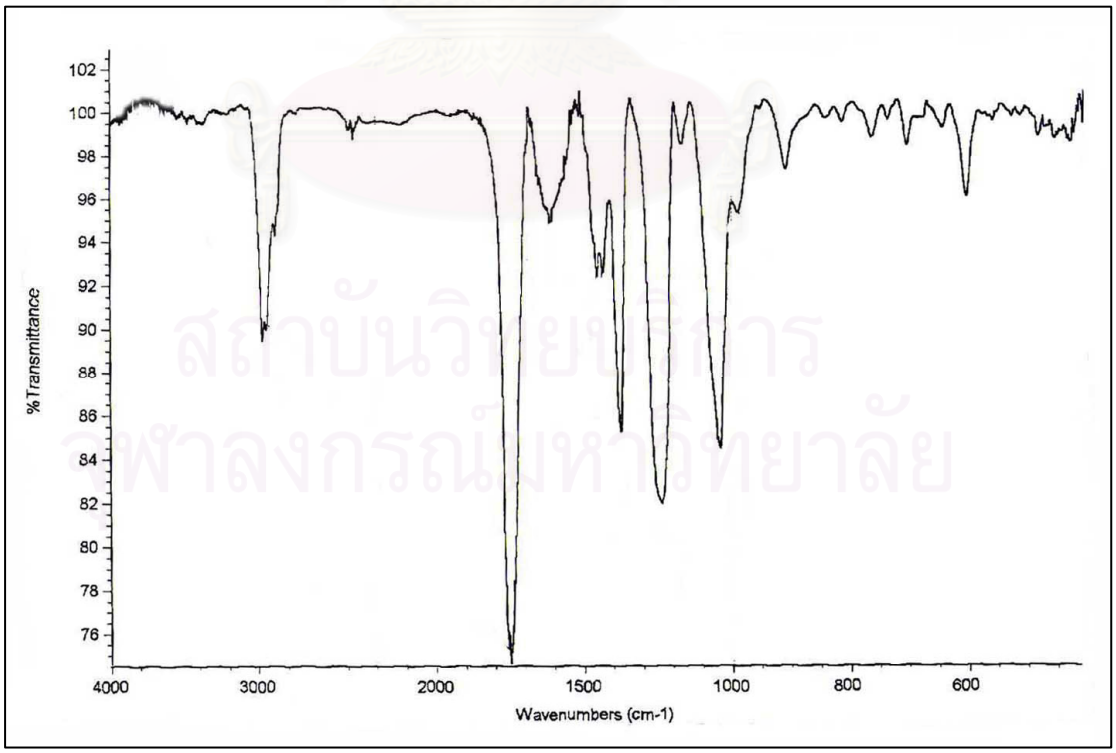


Figure 3.41 The IR spectrum of compound **4A**

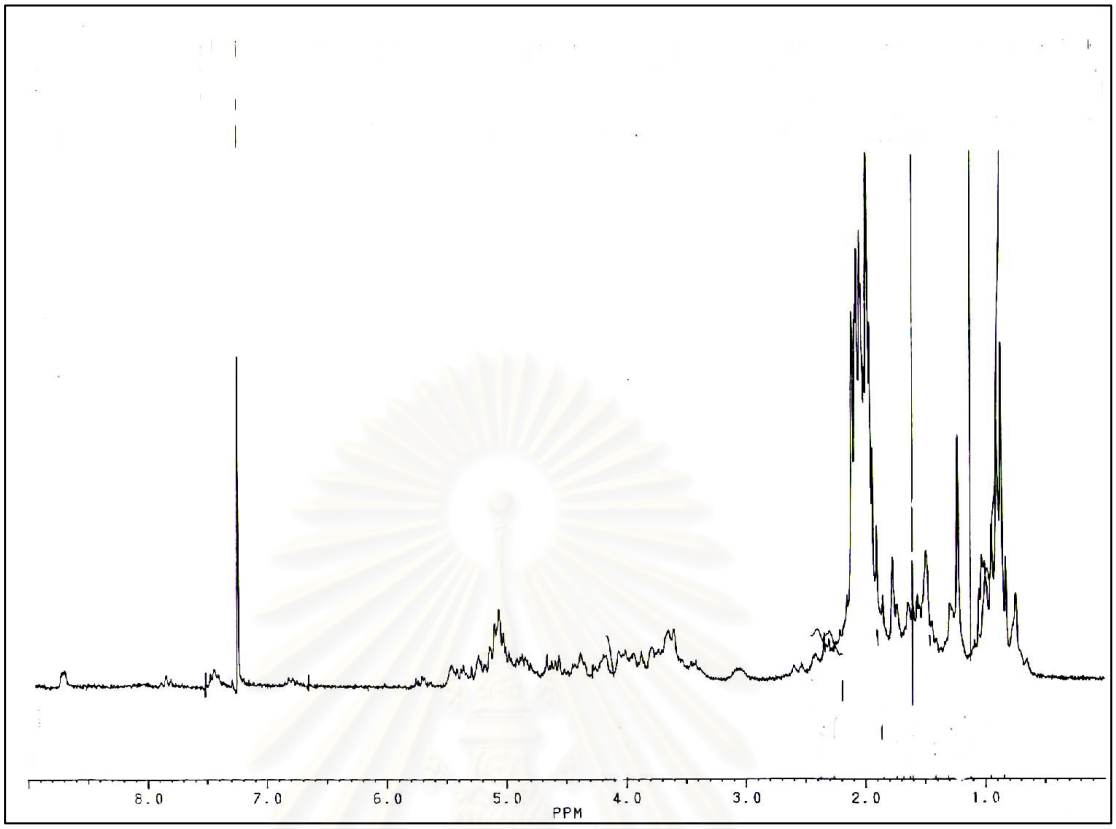


Figure 3.42 The ^{1}H NMR spectrum of compound **4A**

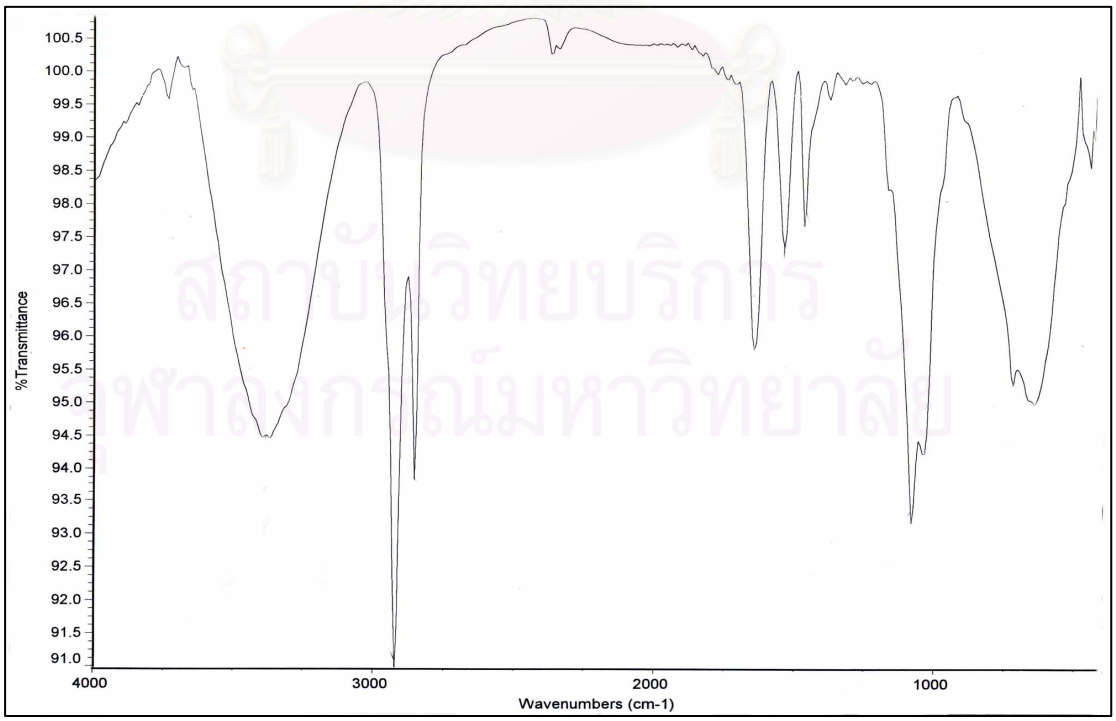


Figure 3.43 The IR spectrum of compound **4B**

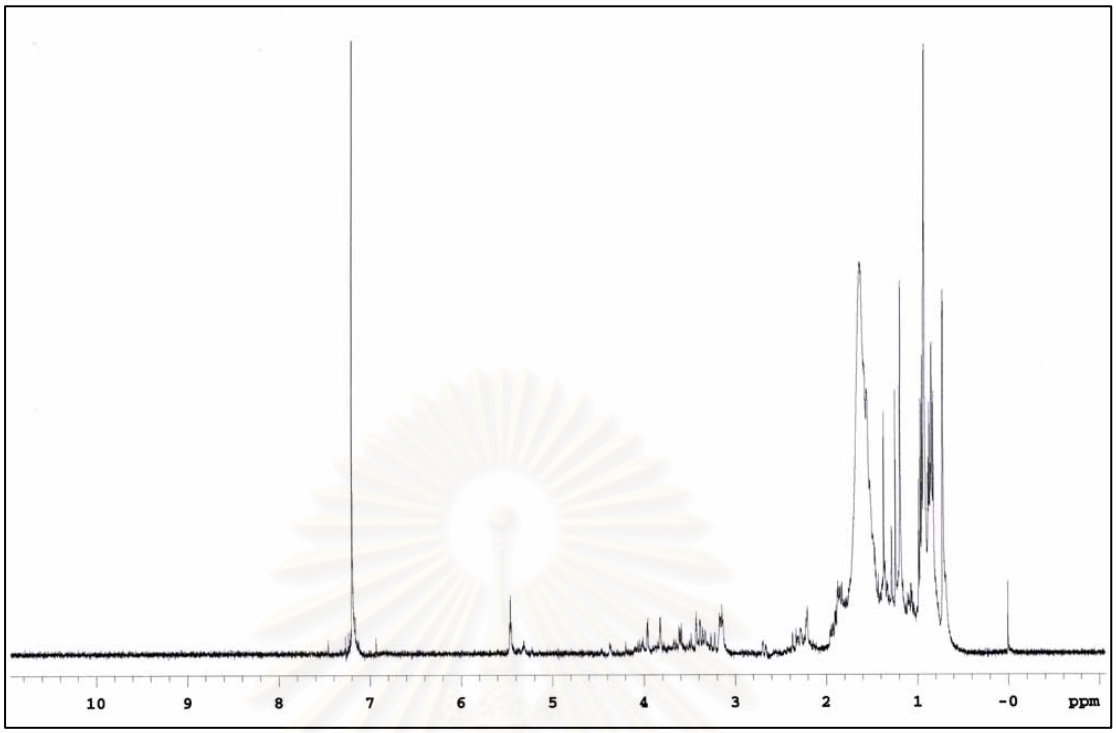


Figure 3.44 The ^{1}H NMR spectrum of compound **4B**

3.5 The result of hydrodistillation

The essential oil as pale yellow oil 2.0 g (0.04 % w/w of fresh plants 5 Kg) was obtained after the hydrodistillation of the fresh whole plants of *H. umbellata*. Identification of its composition was analyzed by gas chromatograph / mass spectrometry (GC/MS).

3.6 Chemical composition of the essential oil

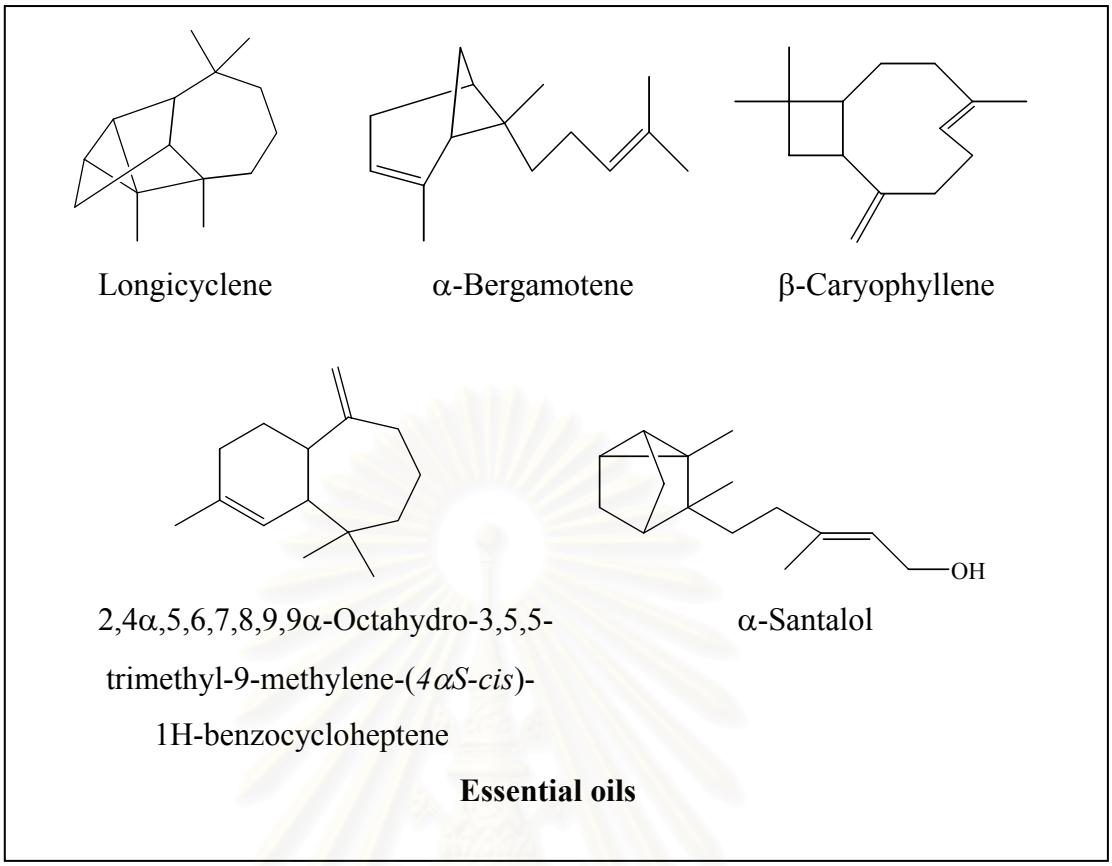
The components of the oil were analyzed and identified by GC-MS analysis using a 30 m DB-5 glass capillary column. The column was programmed at 110°C for 1 min to 270°C at a rate of 4°C/min. The chromatogram (Figure 3.45) displayed seven peaks at retention time (R_t): 8.56, 8.91, 9.45, 9.56, 10.94, 11.23 and 11.38 min. The list of R_t, molecular weight and name of these possible components are presented in Table 3.24.

The mass spectrum (Figure 3.46) of each peak was compared with the standard compounds accumulated in the library (NIST database).

Table 3.24 The retention time, molecular weight, name and % composition of components of essential oil

Peak no	R_t (Min)	Molecular weight	Name	Composition (%)
1	8.56	204	Longicyclene	31
2	8.91	204	α-Bergamotene	4
3	9.45	204	β- Caryophyllene	22
4	9.56	278 (base peak 165)	Unidentified (Alicyclic methyl ether)	7
5	10.94	204	2,4α,5,6,7,8,9,9α-Octahydro-3,5,5-trimethyl-9-methylene-(*4S-cis*), 1H-benzocycloheptene	25
6	11.23	220	α-santalol	5
7	11.38	204 (base peak 91)	Unidentified (Alicyclic hydrocarbon)	7

Longicyclene, β-caryophyllene and 2,4α,5,6,7,8,9,9α-octahydro-3,5,5-trimethyl-9-methylene-(*4αS-cis*)-1H-benzocycloheptene were detected as major compositions: 31, 22 and 25%, respectively.



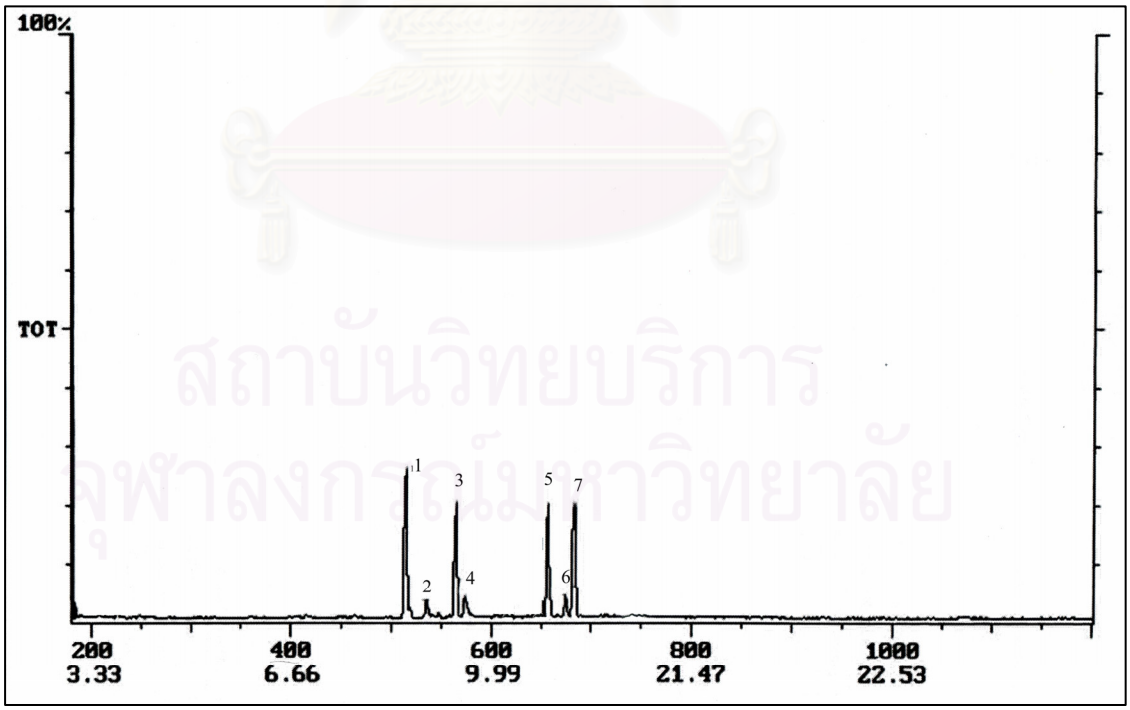


Figure 3.45 The GC of essential oil from *H. umbellata*

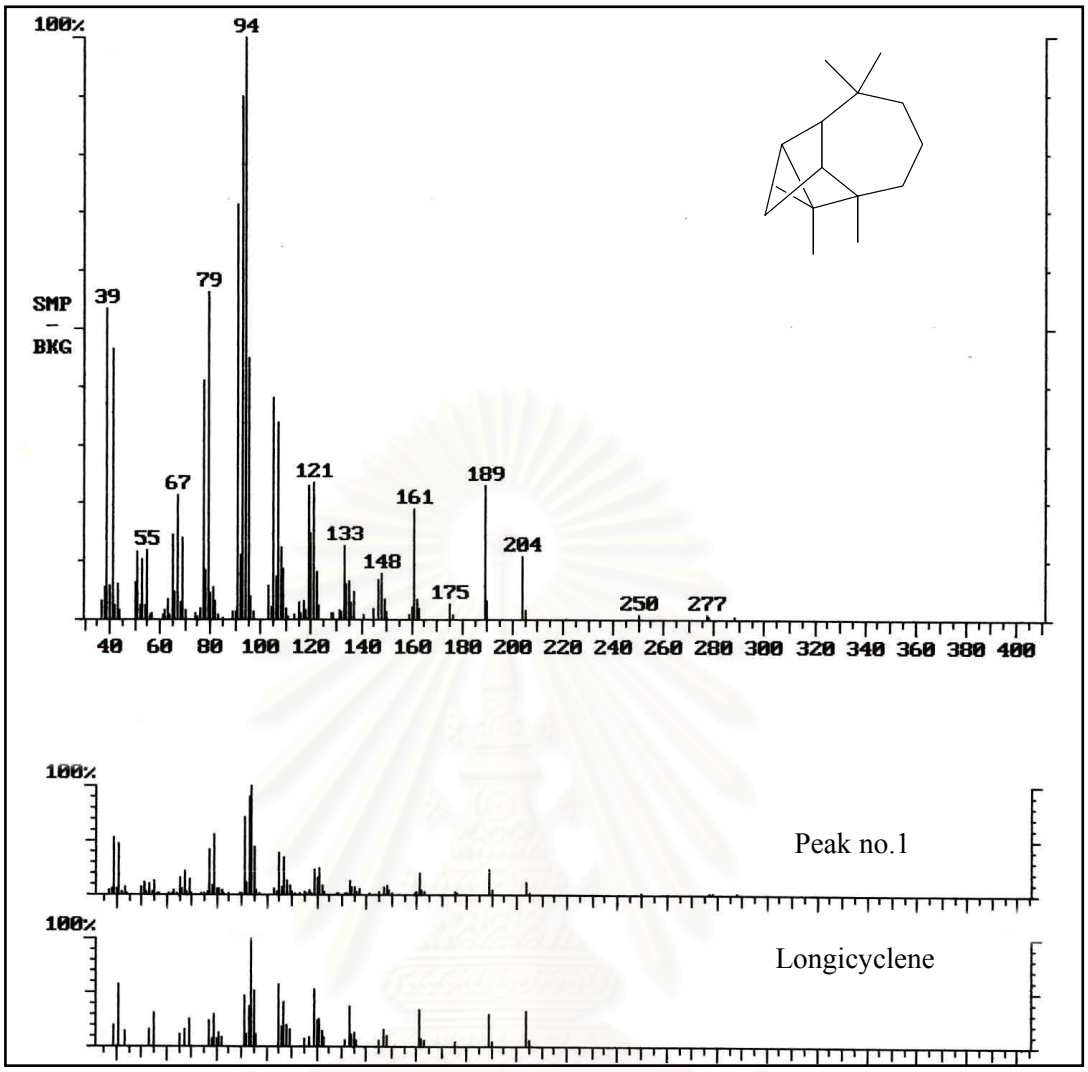
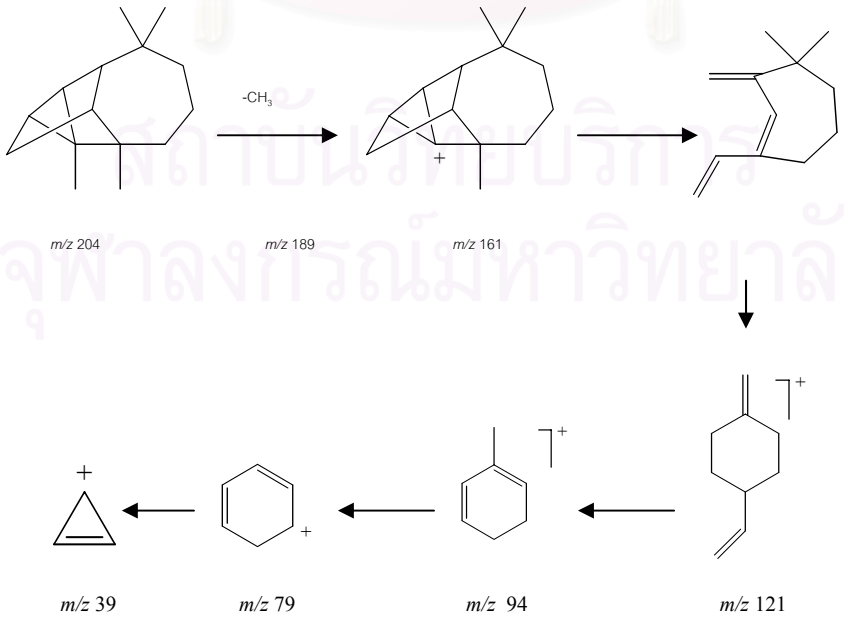


Figure 3.46 The mass spectrum of component of essential oil (peak no.1)



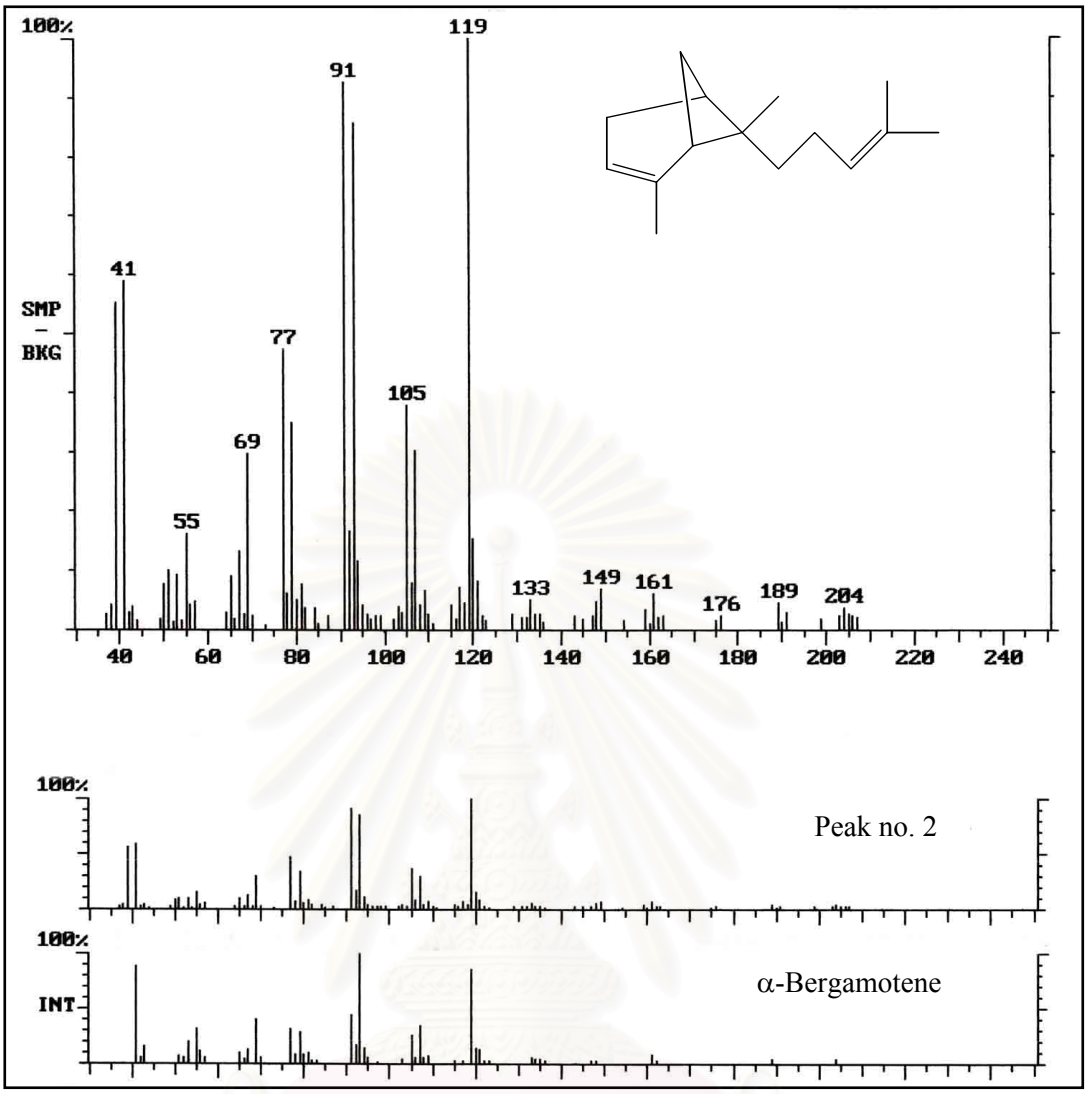
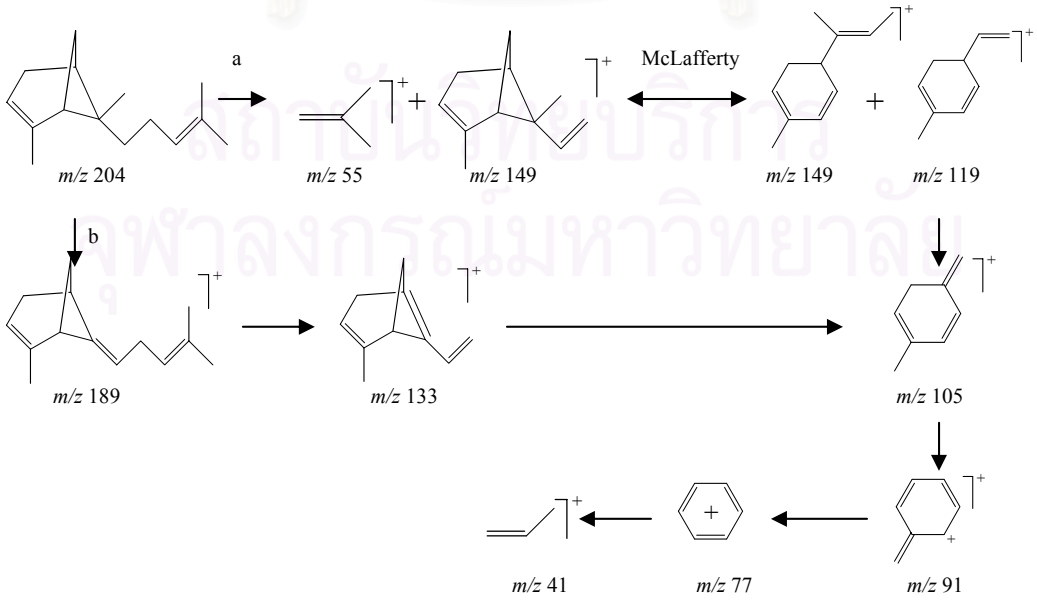


Figure 3.46 (Cont.) The mass spectrum of component of essential oil (peak no.2)



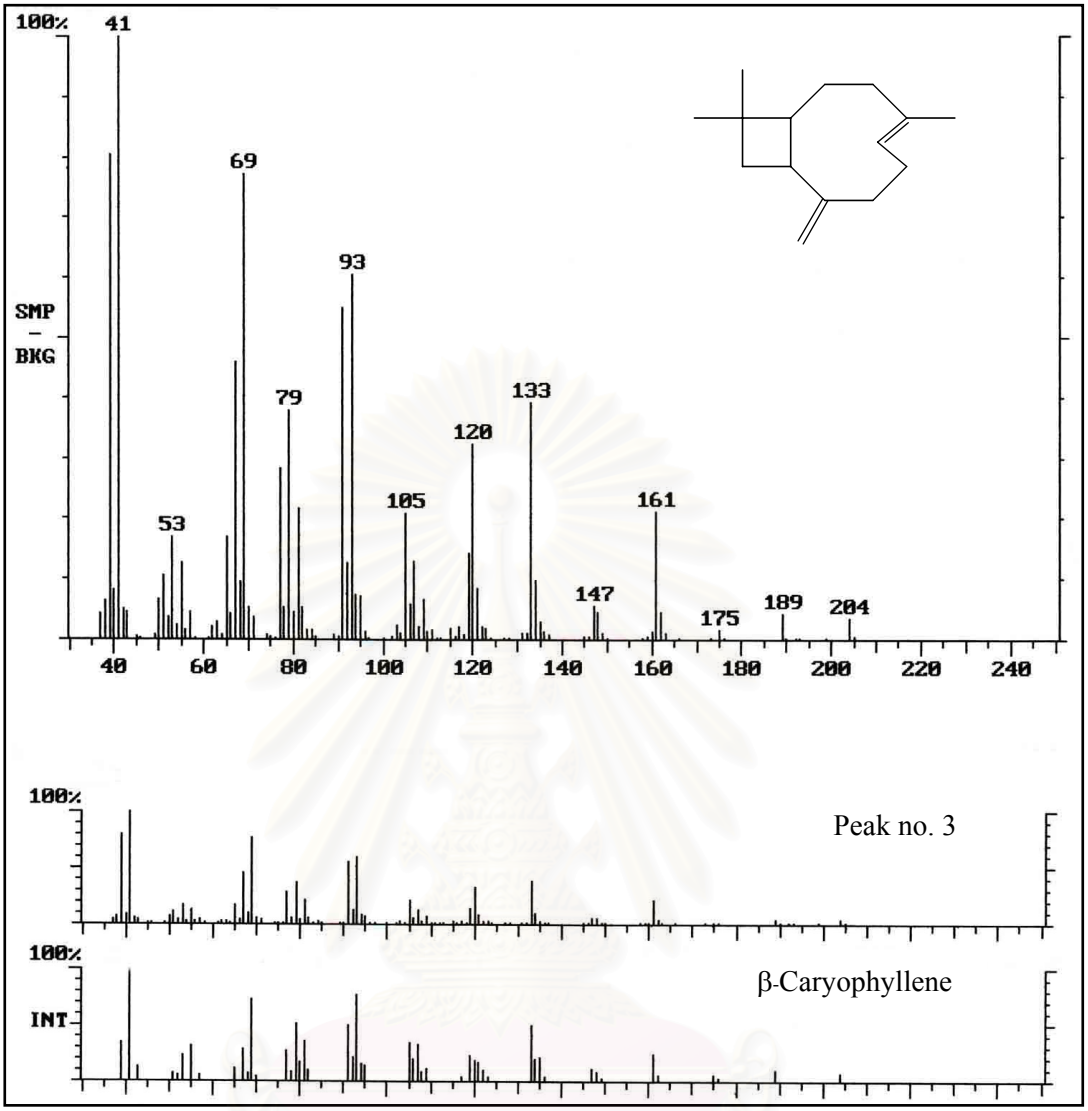
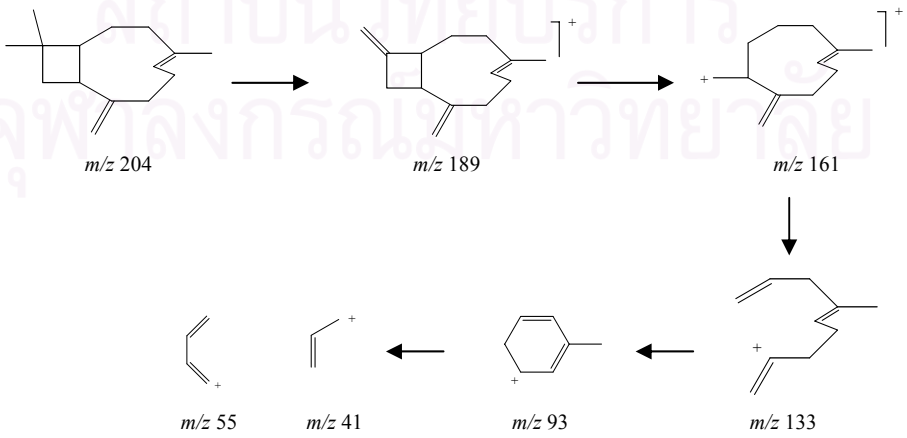


Figure 3.46 (Cont.) The mass spectrum of component of essential oil (peak no.3)



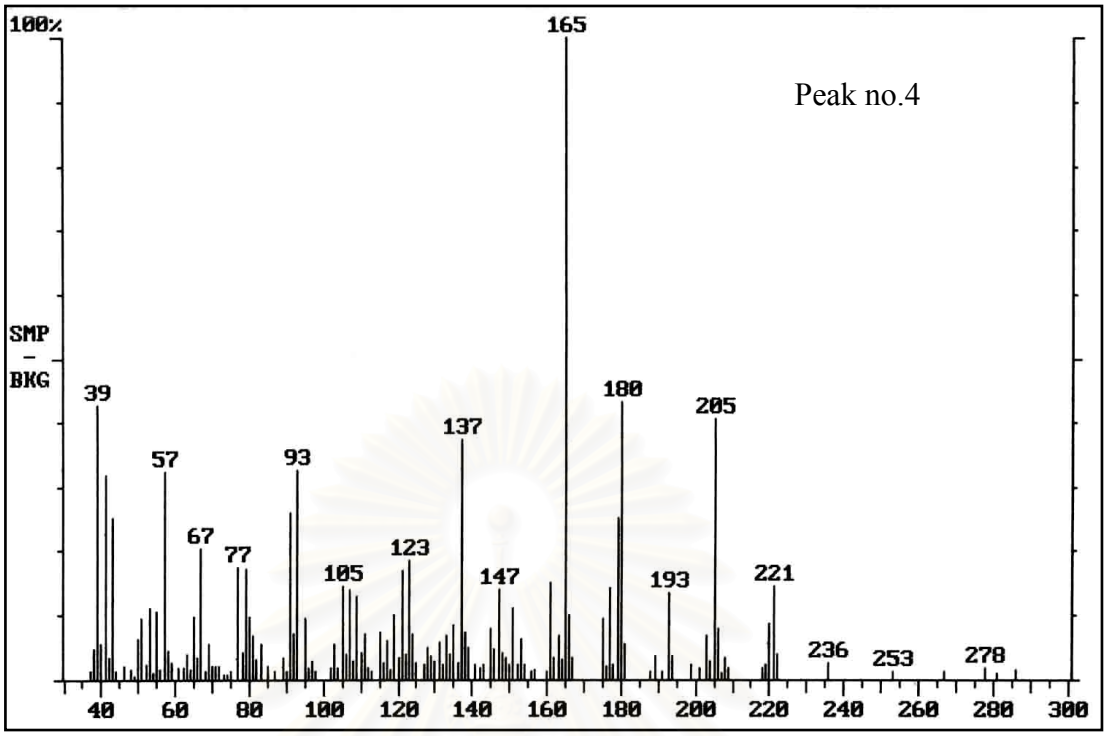
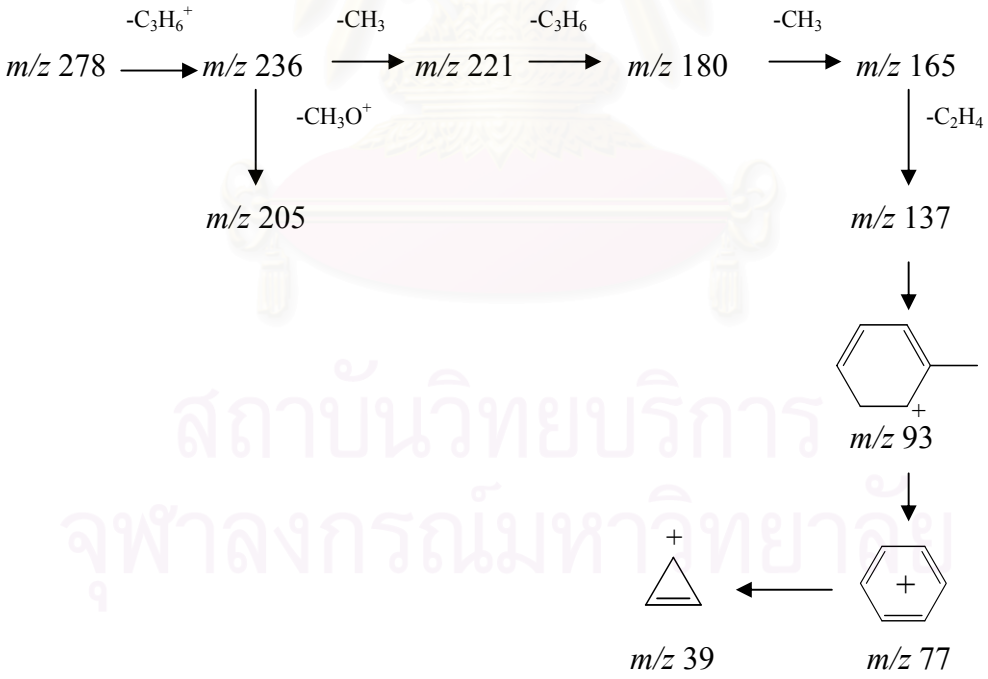


Figure 3.46 (Cont.) The mass spectrum of component of essential oil (peak no.4)



This compound showed the loss of alkyl fragment ions. The fragment ion at *m/z* 31 was due to the methoxy group. This compound may be an alicyclic compound containing a methoxy group.

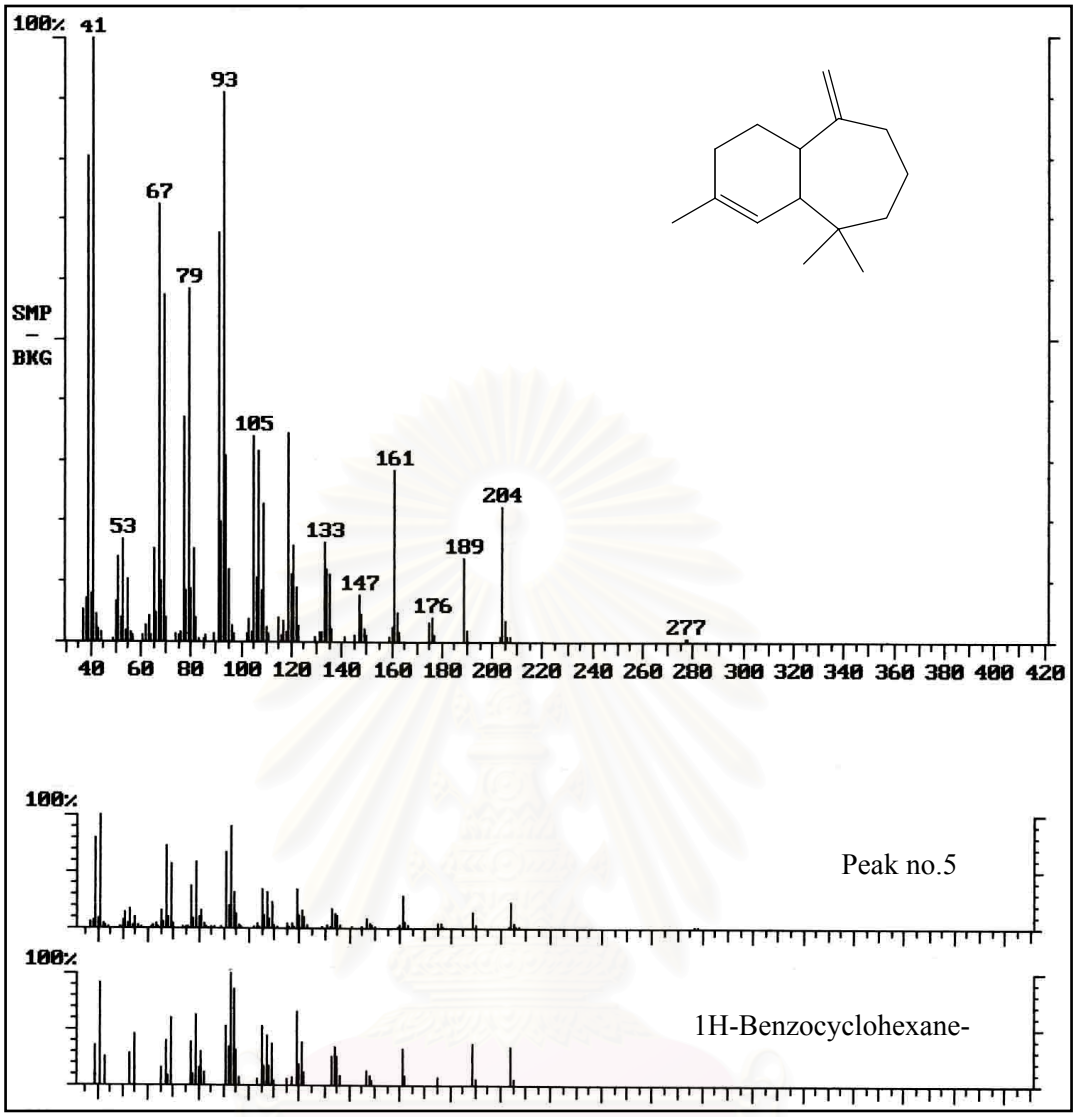
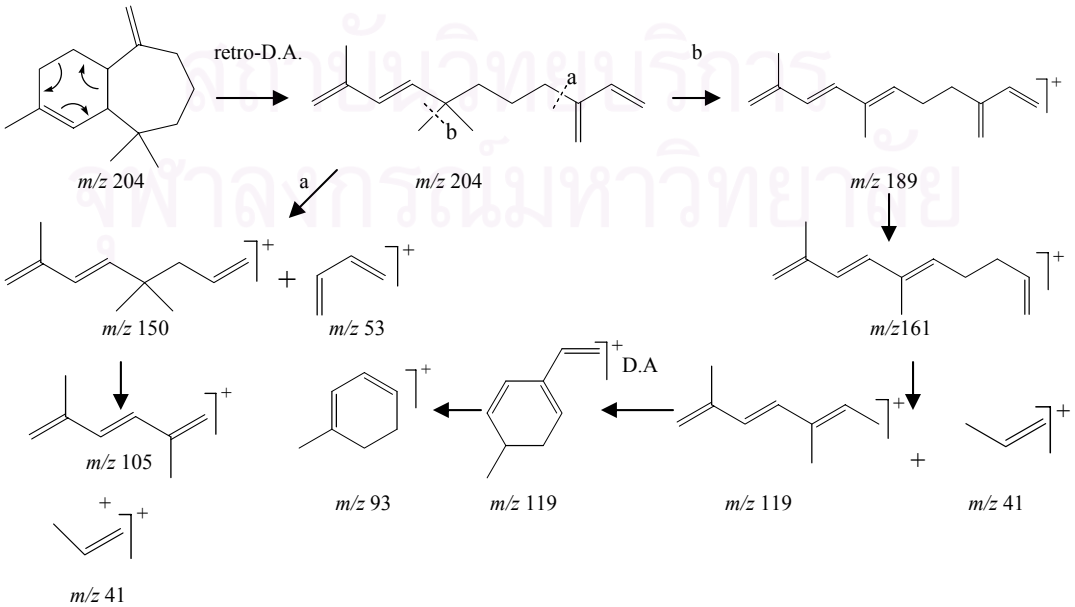


Figure 3.46 (Cont.) The mass spectrum of component of essential oil (peak no.5)



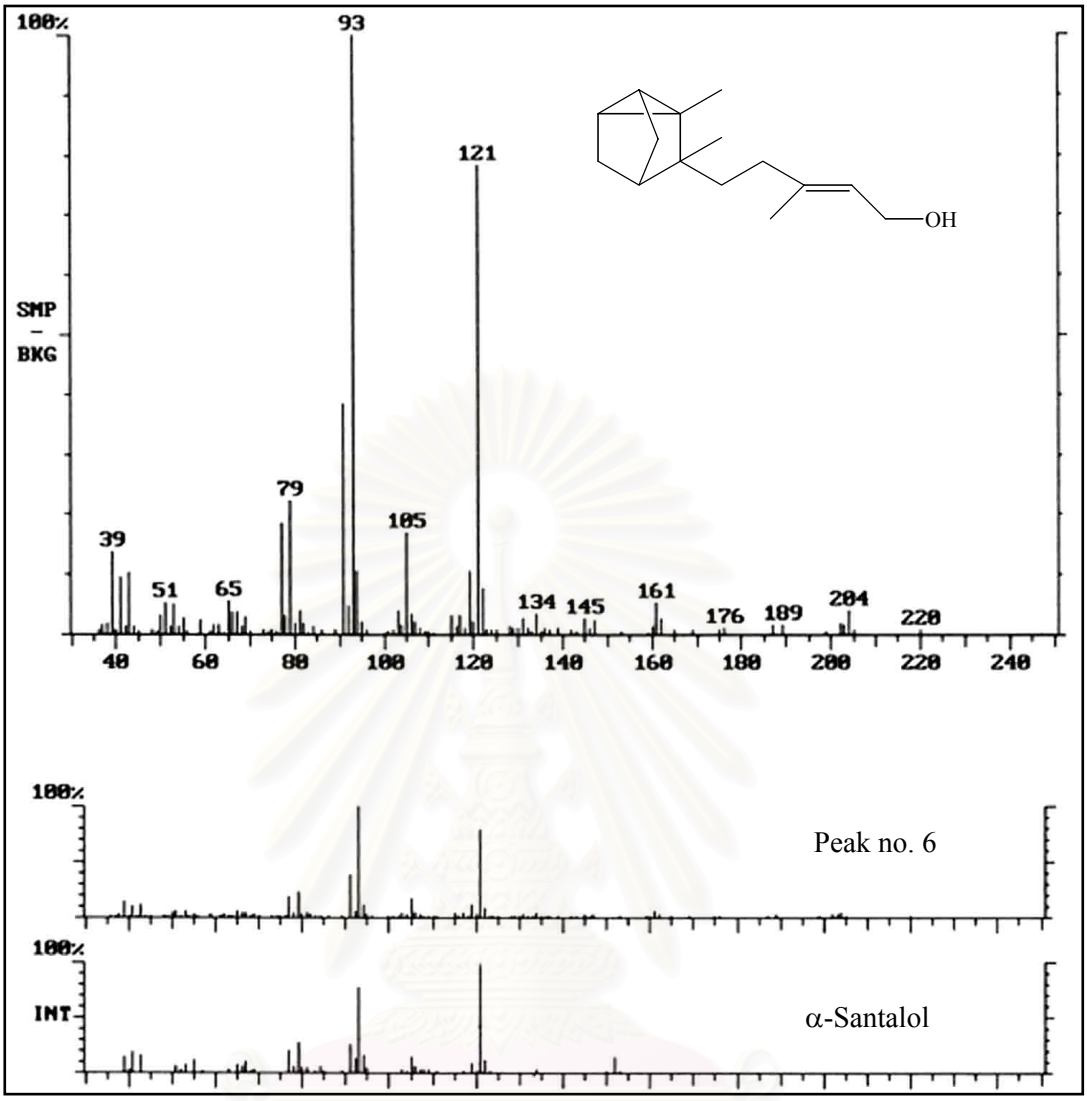
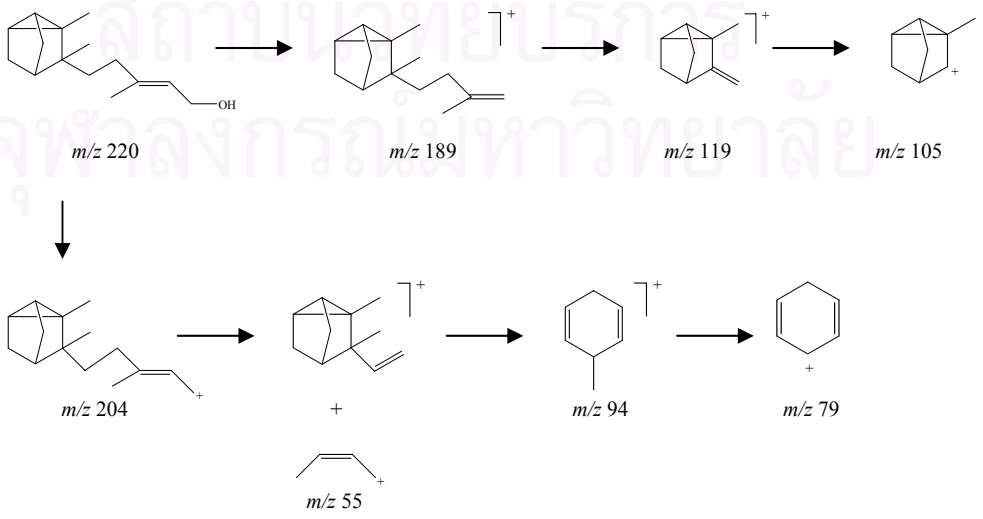


Figure 3.46 (Cont.) The mass spectrum of component of essential oil (peak no.6)



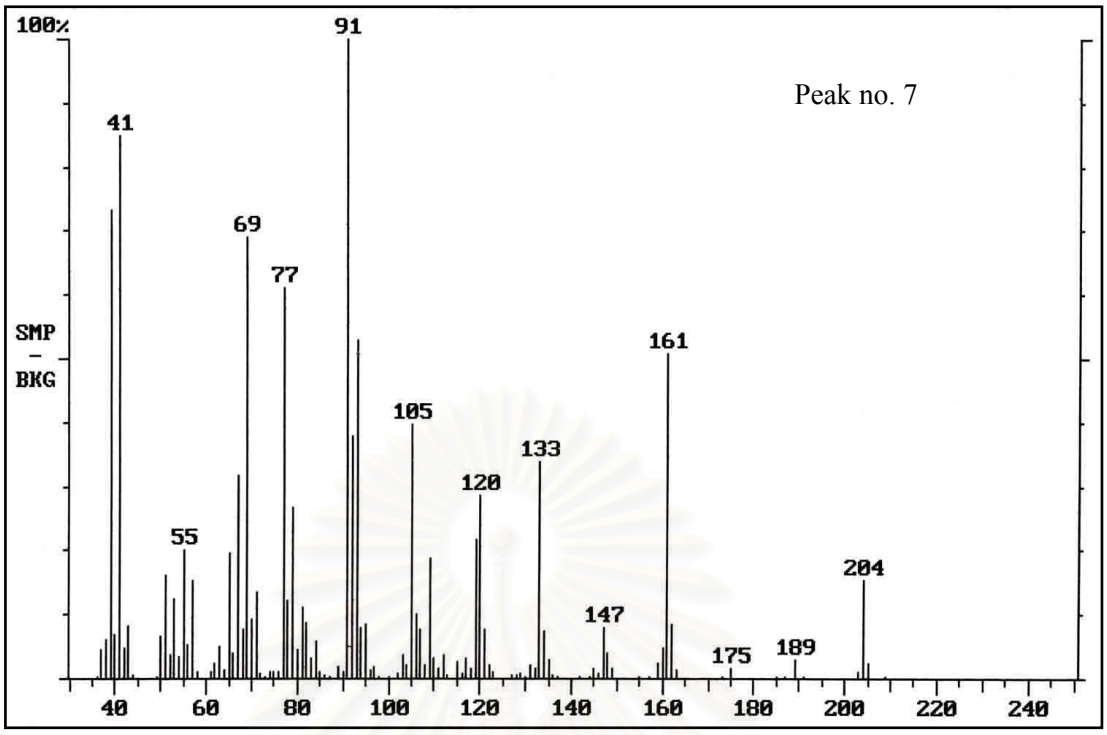
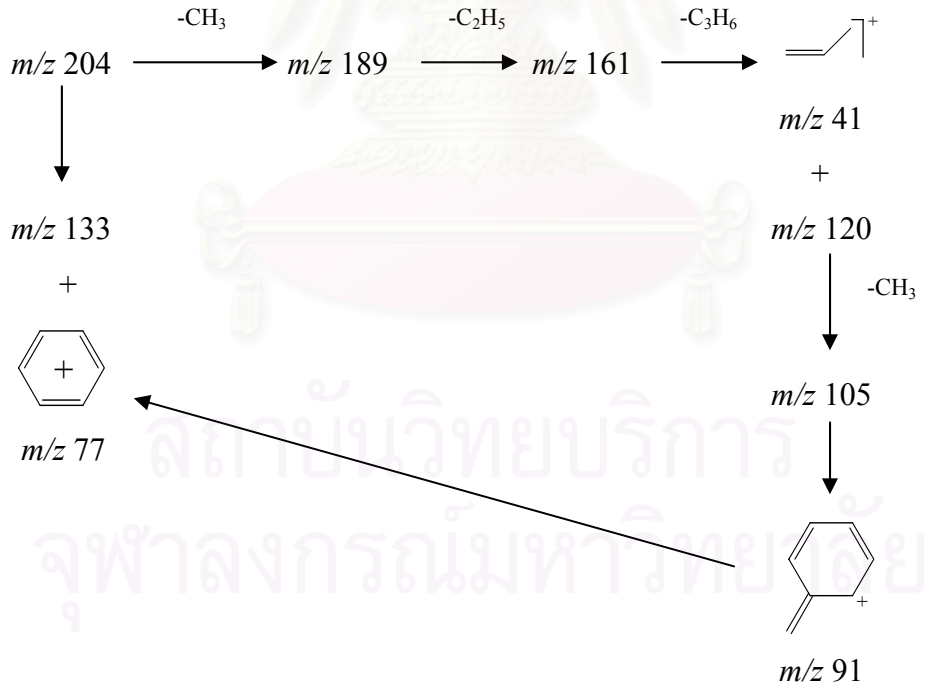


Figure 3.46 (Cont.) The mass spectrum of component of essential oil (peak no.7)



The loss of *m/z* 15 and 14 step by step and the fragmentation ion series at *m/z* 41, 55, 69 are the characteristic of alicyclic hydrocarbon. Thus, this unidentified peak was believed to be an alicyclic hydrocarbon.

3.7 Bioassay result

3.7.1 Bioassay result of isolated substances

From the fractionation and purification of *H. umbellata* crude extract, nine substances were isolated. In order to reach the goal of this research, all isolated substances were subjected to examine the effect on weed growth. The effect on the growth of *M. pigra* is presented in Table 3.25 and Figure 3.47. In addition, all isolated substances were further bioassayed for plant growth inhibition against other five selected species, *E. crus-galli*, *P. pedicellatum*, *L. sativa*, *T. portulacastrum* and *B. pilosa*.

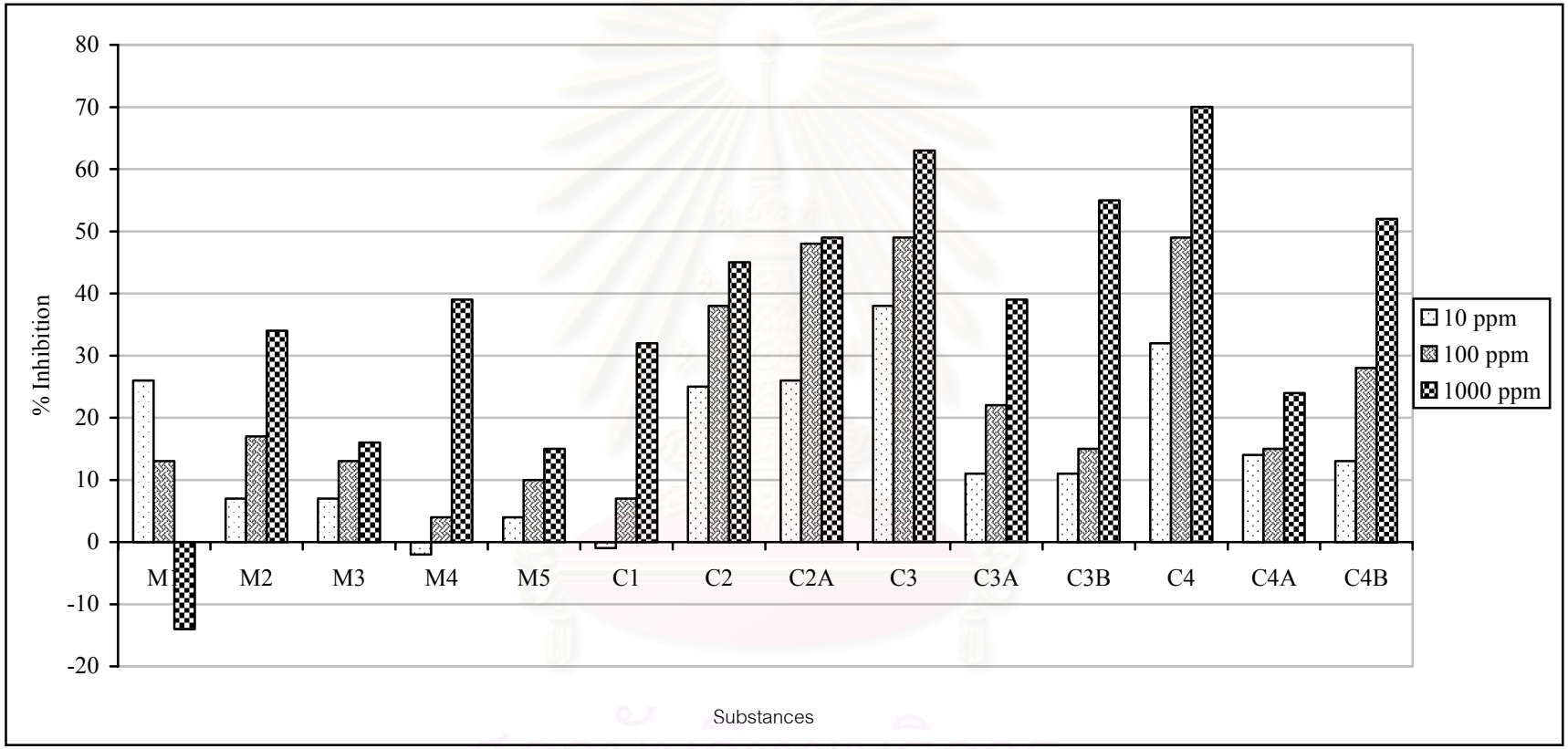
Table 3.25 The effect of isolated substances on the growth of *M. pigra*

Substances	Part	% Inhibition		
		Concentration (ppm)		
		10	100	1000
Mixture **1**	Root	26	13	-14
	Shoot	-3	7	-6
Mixture **2**	Root	7	17	34
	Shoot	8	10	26
Mixture **3**	Root	7	13	16
	Shoot	-6	-4	2
Mixture **4**	Root	-2	4	39
	Shoot	-8	-4	1
Mixture **5**	Root	4	10	15
	Shoot	-3	-11	5
Compound **1**	Root	-1	7	32
	Shoot	30	19	33
Compound **2**	Root	25	38	45
	Shoot	-5	-5	40
Compound **2A**	Root	26	48	49
	Shoot	-21	-17	-16
Compound **3**	Root	38	49	63
	Shoot	-9	1	7
Compound **3A**	Root	11	22	39
	Shoot	2	25	30
Compound **3B**	Root	11	15	55
	Shoot	14	14	50
Compound **4**	Root	32	49	70
	Shoot	-8	-2	5
Compound **4A**	Root	14	15	24
	Shoot	38	30	31
Compound **4B**	Root	13	28	52
	Shoot	27	33	50

The isolated compounds from *H. umbellata* which exhibited significant effect on the growth of *M. pigra* were compounds **2**, **3** and **4** with the inhibitory effect of 45%, 63% and 70%, respectively at 1000 ppm. Derivatives of compound **3**, **3A**

(acetate derivative) and **3B** (hydrolyzed product), and those of compound **4**, **4A** (acetate derivative) and **4B** (hydrolyzed product) showed moderate root inhibition effect 39%, 55%, 24% and 52% at 1000 ppm, respectively. Considering all isolated substances and derivatives, compound **4**, the triterpenoid containing three sugar moieties displayed the highest activity. Compound **4B** oleanolic acid, derived from the hydrolysis of compound **4**, showed the root inhibition effect lower than compound **4**, but higher than compound **4** peracetate (compound **4A**). These results implied that acetyl substituents on glucopyranosyl moieties lessened the inhibitory activity. Besides, the lack of sugar moiety on oleanolic acid also slightly reduced the root inhibition.

Taking to a consideration, compound **3** which was identified to comprise a sugar moiety and long chain fatty acid linked by an ester bond, exhibited the root inhibition of 63% at 1000 ppm. In the case of compound **3** derivatives, compound **3A**, it was found that the acetyl groups on sugar moiety exhibited low root inhibition. The same trend could be observed for compound **3B**, a long chain carboxylic acid containing conjugated dienes. From the above results, it was conceivable that the number of oligosaccharides markedly affected on the root length inhibition. In other words, the high number of sugar moieties might exhibit high root length inhibition while decreasing number of sugar units will decrease the growth inhibition.



M= Mixture, C= Compound

Figure 3.47 Effect of isolated substances on the root growth of *M. pigra*

Table 3.26 Effect of isolated substances on the root and shoot length of selected plants

Substances	Concentration (ppm)	Root growth inhibition (%)				
		Monocotyledon		Dicotyledon		
		E. crus-galli	*P. pedicellatum*	*L. sativa*	*T. portulacastrum*	*B. pilosa*
Mixture **1**	10	-11	3	1	7	30
	100	5	14	13	25	36
	1000	31	58	30	70	88
Mixture **2**	10	0	-19	40	-4	7
	100	-5	-4	54	-18	30
	1000	-15	21	39	-24	49
Mixture **3**	10	-15	26	49	15	26
	100	-23	-15	57	23	28
	1000	-33	17	39	15	56
Mixture **4**	10	22	0	-3	13	-4
	100	29	20	-18	-2	-5
	1000	15	24	5	12	15
Mixture **5**	10	-3	-17	11	3	-1
	100	-7	23	-7	32	-13
	1000	-9	-24	-25	79	-22

Table 3.26 (Cont.)

Substances	Concentration (ppm)	Root growth inhibition (%)				
		Monocotyledon		Dicotyledon		
		E. crus-galli	*P. pedicellatum*	*L. sativa*	*T. portulacastrum*	*B. pilosa*
Compound **1**	10	-11	6	30	-6	7
	100	6	1	-45	23	20
	1000	-2	-17	-16	10	22
Compound **2**	10	-63	-16	24	-7	18
	100	-54	-27	13	11	5
	1000	-47	-28	0	16	3
Compound **3**	10	-70	49	14	7	23
	100	-63	24	19	23	15
	1000	-40	3	13	29	34
Compound **4**	10	36	-6	-13	29	6
	100	50	23	16	81	32
	1000	94	90	86	93	86

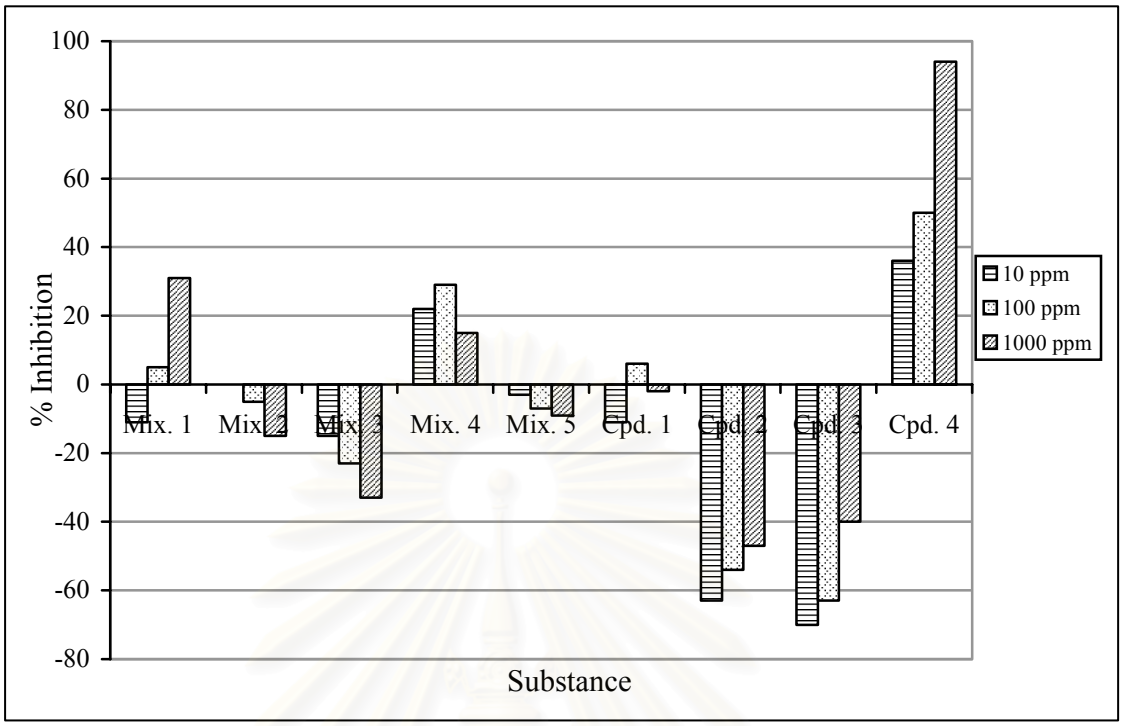
Table 3.26 (Cont.)

Substances	Concentration (ppm)	Shoot growth inhibition (%)				
		Monocotyledon		Dicotyledon		
		E. crus-galli	*P. pedicellatum*	*L. sativa*	*T. portulacastrum*	*B. pilosa*
Mixture **1**	10	3	12	-33	16	39
	100	0	2	10	48	40
	1000	-9	43	63	70	93
Mixture **2**	10	4	26	-74	34	15
	100	13	28	-51	28	40
	1000	19	37	-57	14	46
Mixture **3**	10	22	7	-16	25	18
	100	17	-7	40	26	20
	1000	25	17	-18	14	62
Mixture **4**	10	10	9	-5	-5	-12
	100	-6	17	-11	-10	-2
	1000	8	17	1	-4	-1
Mixture **5**	10	10	31	-8	10	6
	100	-1	40	-1	15	2
	1000	-6	26	2	35	-6

Table 3.26 (Cont.)

Substances	Concentration (ppm)	Shoot growth inhibition (%)				
		Monocotyledon		Dicotyledon		
		E. crus-galli	*P. pedicellatum*	*L. sativa*	*T. portulacastrum*	*B. pilosa*
Compound **1**	10	0	28	-10	5	9
	100	1	14	-13	23	22
	1000	16	14	7	37	28
Compound **2**	10	15	16	4	4	-10
	100	30	10	-7	7	8
	1000	4	10	8	19	9
Compound **3**	10	36	23	-1	13	30
	100	15	11	20	45	20
	1000	13	7	9	37	42
Compound **4**	10	12	8	-15	6	8
	100	23	14	-9	41	25
	1000	42	43	66	60	56

a) *E. crus-galli*



b) *P. pedicellatum*

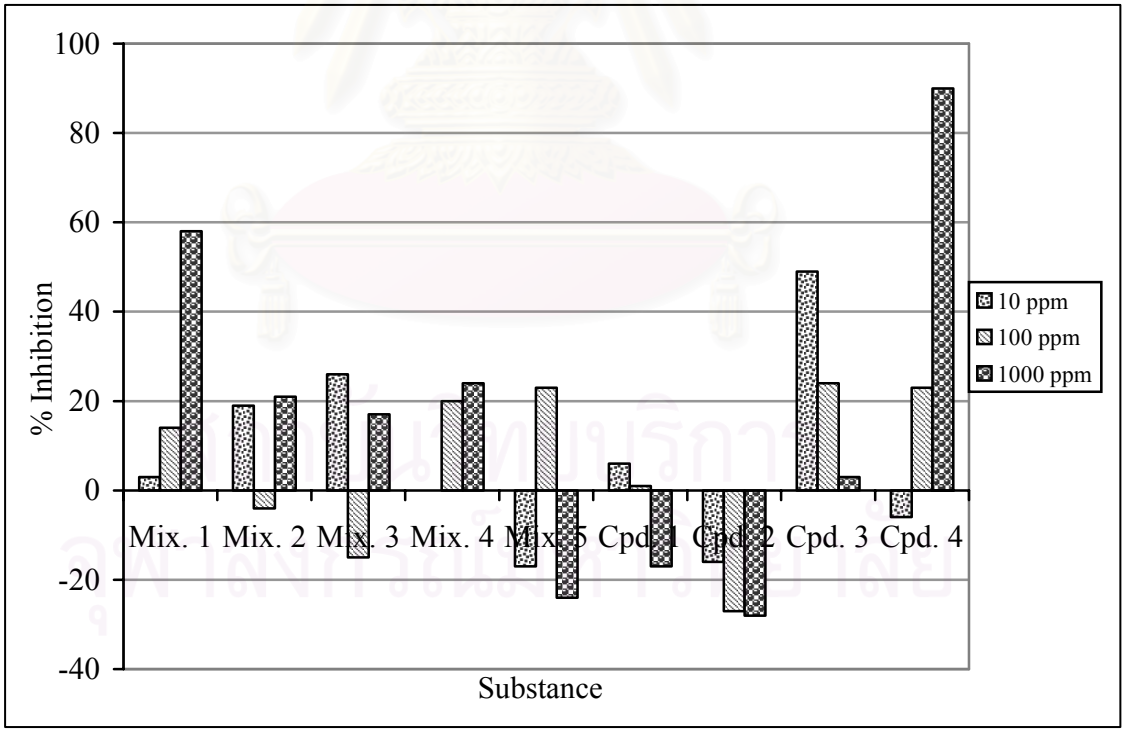
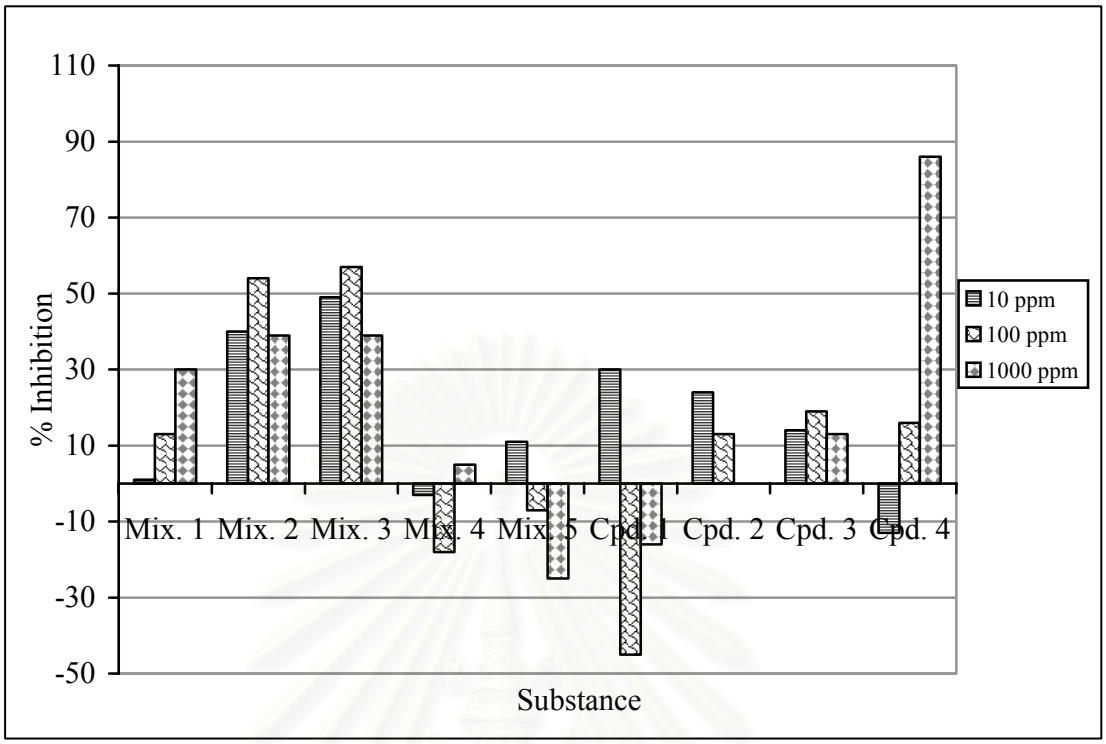


Figure 3.48 Effect of isolated substances of *H. umbellata* on the roots of a) *E. crus-galli,* b) *P. pedicellatum*, c) *L. sativa* d) *T. portulacastrum* and e) *B. pilosa*

c) *L. sativa*



d) *T. portulacastrum*

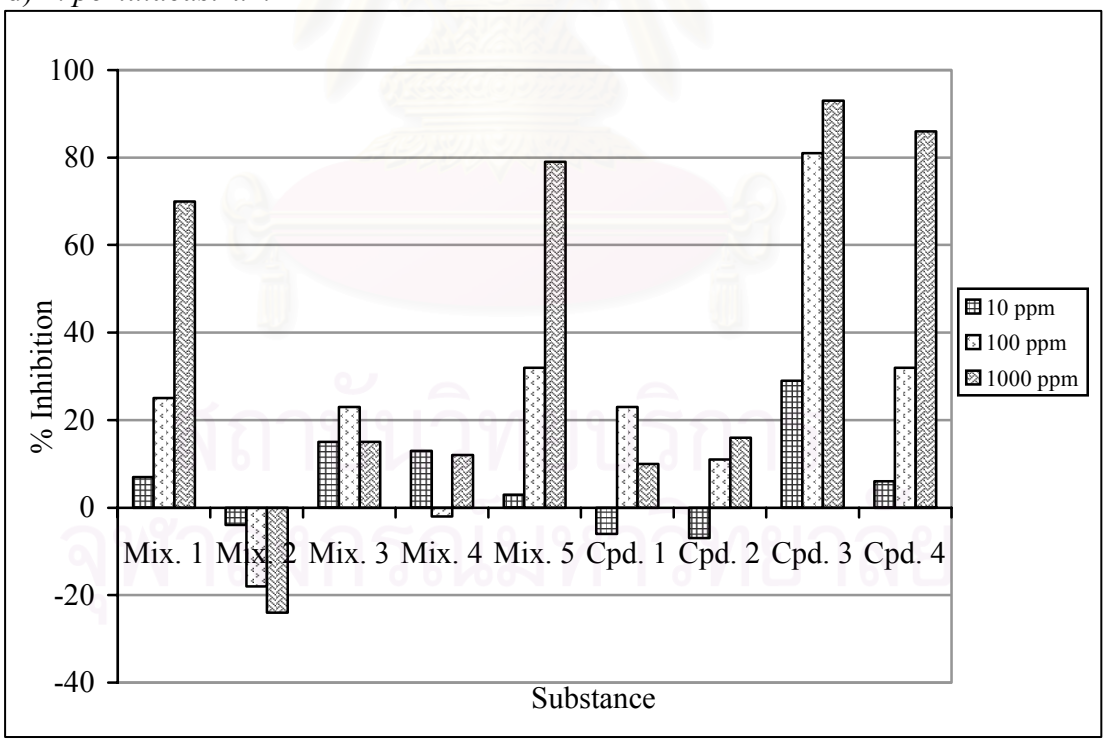


Figure 3.48 (Cont.)

e) *B. pilosa.*

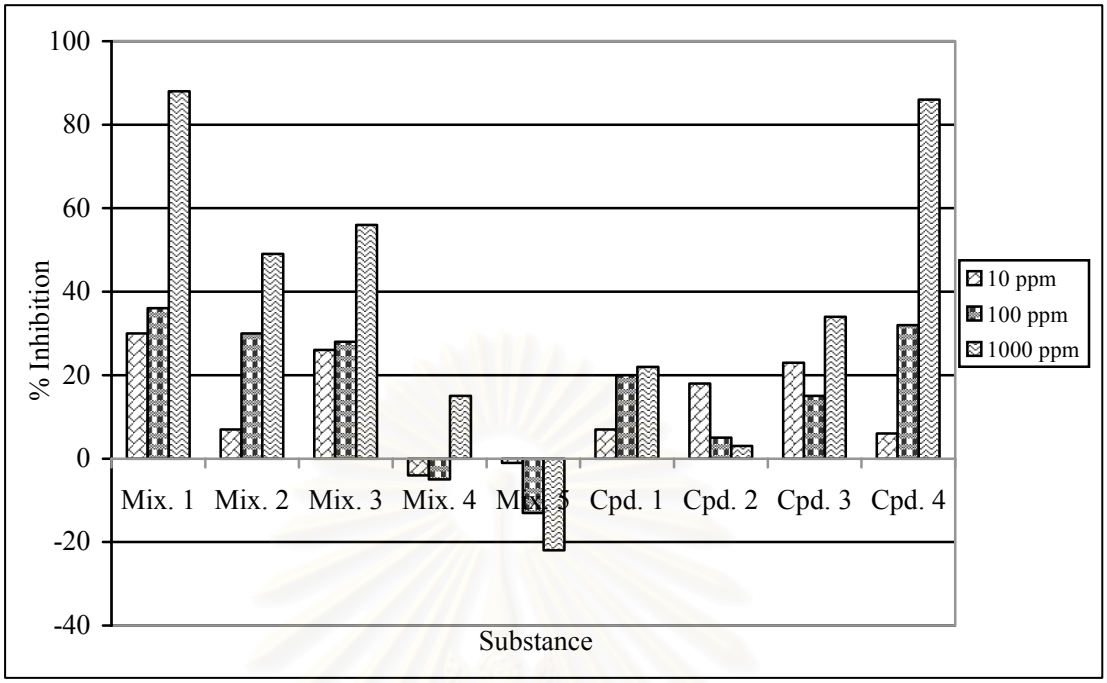


Figure 3.48 (Cont.)

As discussed earlier, compounds **3** and **4** displayed good root growth inhibitory effect against *M. pigra*. For further exploration, all isolated substances were also bioassayed for plant growth inhibition against selected species.

E. crus-galli, barnyard grass: The most intriguing result for the inhibitory effect on barnyard grass could be observed from compound **4**. It revealed the root and shoot inhibition of 94% and 42%, respectively at 1000 ppm. On the other hand, the promotion effect could be noticed from compounds **2** and **3**. They exhibited root growth promotion of 47% and 40%, respectively. When using the lower concentration such as 100 ppm and 10 ppm, the root length was found to increase. These two compounds could thus be utilized as a template for studying plant growth promoter.

P. pedicellatum, kayasuwa grass: This plant was a monocotyledon weed like barnyard grass. Compound **4** still displayed high root inhibition, 90%, at 1000 ppm, the shoot was inhibited 43% merely. Besides compound **4**, mixture **1** also showed moderate root and shoot inhibition at the same concentration with 58% and 43%, respectively.

L. sativa, lettuce: This plant was classified as a dicotyledon plant. The substance which had potent root and shoot growth inhibition was compound **4** (86% and 66%, respectively).

T. portulacastrum, horse purselance: This plant was belonged to a dicotyledon plant. Mixtures **1**, **5** and compound **4** was not only exhibited root growth inhibition with 70%, 79% and 93%, respectively at 1000 ppm, but also revealed shoot growth inhibition 70%, 60% and 60%, respectively. Noticeable results displayed that mixture **1** could inhibit the growth of this plant, but not showed the effect on lettuce. In consideration of mixture **5**, it manifestly exhibited the growth of horse purselance only.

B. pilosa, hairy begger-ticks: The substances exhibiting potent inhibition effect on the growth of this dicotyledon plant was ascribed as mixtures **1**, **2**, **3** and compound **4**. These substances attractively inhibited for the root growth inhibitory effect at the highest concentration, 1000 ppm, 88%, 49%, 56% and 86%, respectively. Besides, these substances also displayed shoot growth inhibition 93%, 56%, 62% and 46%. Remarkably, mixture **1** could inhibit both the root and shoot growth higher than other substances. Mixtures **2** and **3** showed moderately inhibition on the growth of this plant.

From the above results, the plant growth inhibitory effect of all isolated substances from *H. umbellata* against selected plants could be summarized as: compound **4** was the most active compound showing high inhibition against both mono- and dicotyledron plants, *E. crus-galli* was found to inhibit strongly for the root growth. Mixture **1** revealed growth inhibition over some monocotyledron (*P. pedicellatrum*) and dicotyledron (*T. portulacastrum* and *B. pilosa*). It showed the highest root inhibition over *B. pilosa*. Mixtures **2** and **3** only exhibited inhibition on the growth of *B. pilosa*. From the above results, compound **4** could be templated for further study on plant growth inhibitor. Considering the substances that might be use as plant growth promotor, compounds **2** and **3** fell in to this category; they presented the root growth promotion of *E. cruss-galli*.

3.7.2 Bioassay result of essential oil

The essential oil was bioassayed for plant growth inhibition activity on *M. pigra.* The results are presented in Table 3.27 and Figure 3.49.

Table 3.27 Effect of essential oil on the growth of *M. pigra*

Part	% Inhibition		
	Concentration (ppm)		
	10	100	1000
Root	13	20	46
Shoot	3	19	48

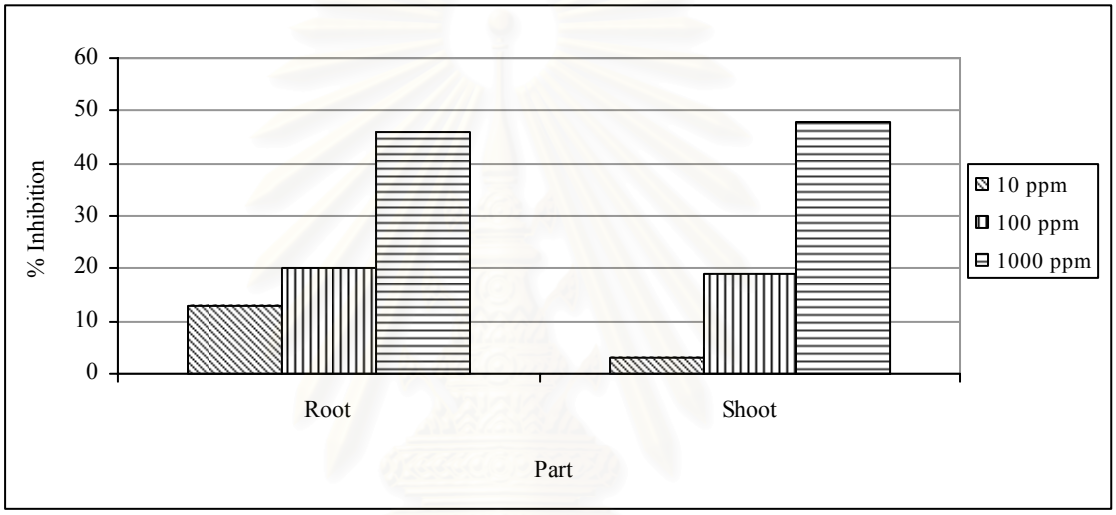


Figure 3.49 Effect of essential oil on the growth of *M. pigra*

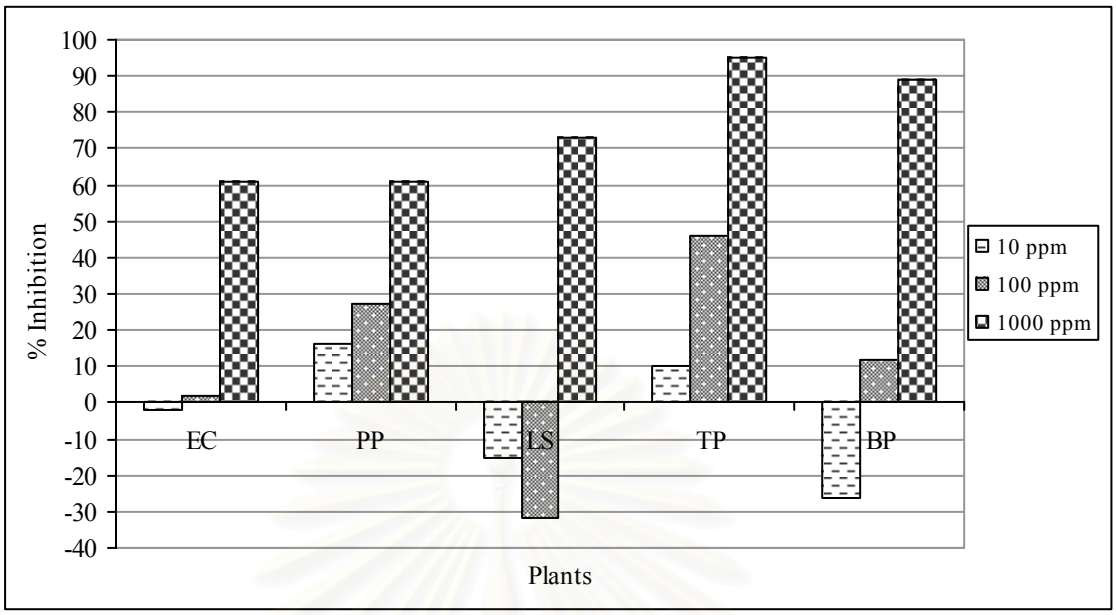
The essential oil of *H. umbellata* revealed moderate inhibitory effect on the growth of *M. pigra* for 13%, 20% and 46% at concentration of 10, 100 and 1000 ppm, respectively. It inhibitory effect was markedly depended upon the concentration, the higher concentration used, the stronger inhibitory effect expressed. This obtained essential oil was further tested with some selected species: *E. crus-galli, P. pedicellatum, L. sativa, T. potulacastrum* and *B. pilosa.* The results are shown in Table 3.28 and Figure 3.50.

Table 3.28 Effect of essential oil on the growth of other selected plants

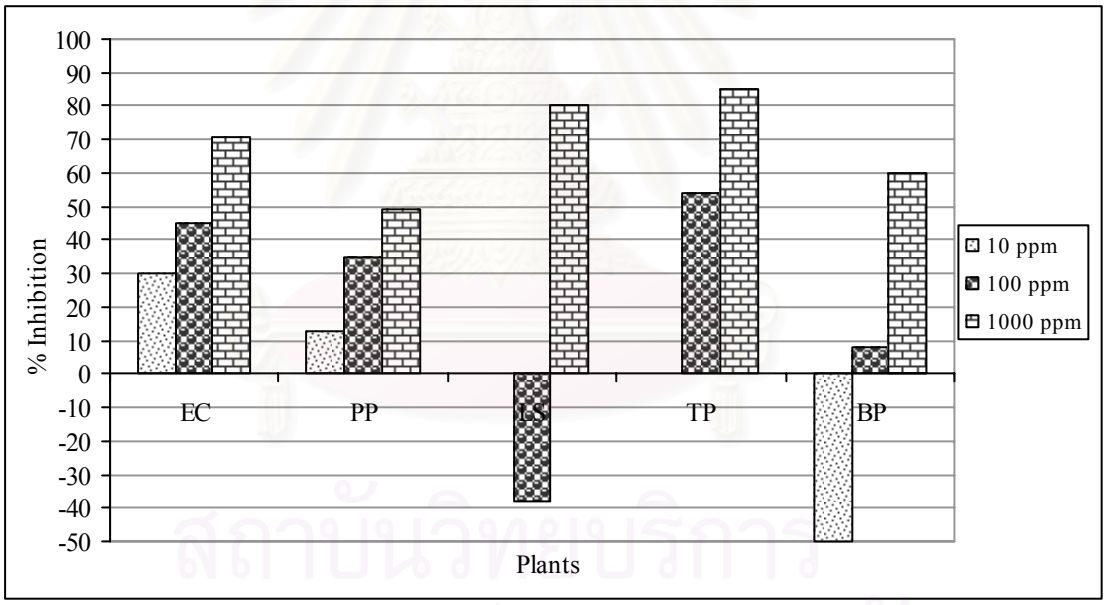
Weeds	Part	% Inhibition		
		Concentration (ppm)		
		10	100	1000
E. crus-galli	Root	-2	2	61
	Shoot	30	45	71
P. pedicellatum	Root	16	27	61
	Shoot	13	35	49
L. sativa	Root	-15	-32	73
	Shoot	0	-38	80
T. potulacastrum	Root	10	46	95
	Shoot	0	54	85
B. pilosa	Root	-26	12	89
	Shoot	-50	8	60

From the activity results obtained, essential oils showed growth inhibition over each plant at 1000 ppm. Dicotyledon plants (the last three mentioned species) were of more inhibitory effect than monocotyledon (the first two species). Noticeably, *B. pilosa* was gradually promoted when decreasing concentration to 100 and 10 ppm. The comparison inhibitory effect between essential oils and isolated substances was found that essential oils exhibited plant growth inhibitory effect more than most of isolated substances. Besides compound **4**, the essential oils showed slightly lower plant growth inhibition. However, this essential oil was a mixture, thus the study on activity of each component may clearly provide informative data of weed inhibitory effect. Furthermore, these components should be used as lead compounds for further study on plant growth regulator as inhibitor.

a) Root



b) Shoot



Note: EC = *E. crus-galli*, PP = *P. pedicellatum*, LS = *L. sativa*, TP = *T. portulacastrum*, BP = *B. pilosa*

Figure 3.50 Effect of essential oil on the growth of other plants

3.8 Discussion on biological activity

3.8.1 Biological activity discussion of isolated substances

Mixture 1

Mixture **1** was isolated from hexane extract of whole plants of *H. umbellata* and was elucidated its structure as a mixture of essential oils, namely β-chamigrene, α-bergamotene, α-santalol, germacrene D, β-caryophyllene and β-pinene. There had been recent reports on the study of mono- and sesquiterpenoids from *H. sibthorpioides*, *H. maritima* and *C. asiatica.*[20] Only β-pinene, β-caryophyllene and germacrene D were detected in *H. umbellata*.

The bioactivities cited in the literature revealed as follows. There are some previous reports concerning insecticidal activity of β-chamigrene,[49] antibacteria[50] and sedative properties[51] of α-santalol, pesticide[52] and pheromone[50] of germacrene D, antimicrobial activity of β-caryophyllene[53] and allergenic[52], antiseptic[54], herbicide[55] and insecticidal activity of β-pinene.[56]

From the bioassay results, mixture **1** slightly inhibited the root length of *M. pigra* at concentration of 10 ppm (26% inhibition). On the contrary, the root elongation was observed at higher concentration. Even though, this mixture had low affected on the growth of *M. pigra*., mixture **1** showed high root growth inhibition against *B. pilosa*, *T. portulacastrum* and *P. pedicellatum* 88%, 70% and 58%, respectively at 1000 ppm.

Mixture 2

Mixture **2** was isolated and elucidated its structure as a mixture of saturated long chain carboxylic acids derived from both hexane and chloroform extracts. This type of compound always occurs in a variety of plant species.[57] The bioassay results of this mixture showed low inhibitory activity on the root and shoot length of *M. pigra* at 1000 ppm (34 and 26%, respectively). Besides, it inhibited the root growth of *L. sativa* and *B. pilosa* 39%, and 49% at 1000 ppm, respectively. While, the shoot growth of *L. sativa* was promoted 74% at 10 ppm.

From the literature search, long chain fatty acids, behenic acid (C22:0), arachidic acid (C20:0) and myristic acid (C14:0) were reported to express inhibitory effect on the germination of *Cynodon dactylon* (L.) Pers.[4]

Mixture 3

Mixture **3** was isolated and identified as a mixture of long chain aliphatic alcohols from hexane extract. The saturated long chain aliphatic alcohols are widely distributed in the waxy fraction of the plant extract. From the bioassay results, this mixture revealed low effect on both root and shoot growth of *M. pigra*. However, it showed moderate inhibition effect against root and shoot growth of *B. pilosa* 56% and 62%, respectively at 1000 ppm. For the rest species, mixture **3** displayed slightly inhibitory activity.

Mixture 4

Mixture **4** was separated from chloroform extract and identified as a mixture of long chain aliphatic esters. Bioassay results indicated that it exhibited root growth of *M. pigra* 39% at 1000 ppm, while no effect on the shoot. Mixture **4** revealed weakly inhibition or promotion effect on the root and shoot length of the other selected species.

Mixture 5

Mixture **5** was separated from chloroform extract and elucidated its structure as a mixture of two steroids: stigmasterol and stigmasta-7, 25-dien-3-ol. In most plants, stigmasterol is an abundant phytosterol likewise to β-sitosterol and campesterol. A number of plant sterols have been reported to possess growth-regulating activity.[58]

The bioassay results revealed that mixture **5** showed weak inhibition on root and shoot growth of *M. pigra.* Nevertheless, this mixture inhibited root (79%) and shoot (35%) length of *T. portulacastrum* at 1000 ppm, but were weakly affected on growth of the rest species.

Compound 1

Compound **1** was isolated from hexane extract and was identified as stigmasterol. Bioassay results of this compound showed low inhibitory activity on root and shoot growth of *M. pigra* (32 and 33%, at 1000 ppm). Besides, it was slightly affected on the root and shoot growth of other species.

Compound 2

Compound **2** was separated from chloroform extract and its structure was characterized as stigmasteryl-3-*O*-*β*-D-glucopyranoside. The biological activity test indicated that compound **2** exhibited the inhibition of root and shoot length of *M. pigra* 45 and 40%, respectively at 1000 ppm. On the other hand, it revealed moderate promotion of root length of *E. crus-galli* 63% at 10 ppm.

Compound 3

Compound **3**, one of the major constituents of *H. umbellata*, was purified and identified as β-D-glucopyranosyl tetratriacontan-2,4-dienoate. From the bioassay results, compound **3** showed inhibitory activity over root length of *M. pigra* 63% at 1000 ppm. Compound **3** derivatives (compounds **3A** and **3B**) were also inhibited the root growth of *M. pigra* 39 and 55%, respectively at 1000 ppm.

For the effect on the growth of selected species, compound **3** revealed the root growth promotion of *E. crus-galli* 70%, whereas it showed shoot inhibition 36% at 10 ppm. The rest species were slightly inhibited on root and shoot growth.

Compound 4

Compound **4** was separated from chloroform extract and identified as methyl oleanolate 3-*O*-[β-D-glucopyranosyl (1→6)][-β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside. This compound is the major constituent of *H. umbellata*.

From the bioassay results, compound **4** showed rather high inhibitory effect against the root length of *M. pigra* (70%), but had no effect on the shoot length (5%) at 1000 ppm. Compound **4** derivatives (compounds **4A**, **4B**) showed less inhibition effect than compound **4** at 1000 ppm (24 and 31% root and shoot growth inhibition for compound **4A** and 52 and 50% inhibition of root and shoot length for compound **4B**). Furthermore, it exhibited high root growth inhibition effect on *E. crus-galli*, *P. pedicellatum*, *L. sativa*, *T. portulacastrum* and *B. pilosa* more than 85%.

3.8.2 Biological activity studies of essential oils

Essential oils were obtained from the hydrodistilation of fresh whole plants of *H. umbellata.* These composed of longicyclene, α-bergamotene, β-caryophyllene, 2,4α,5,6,7,8,9,9α-octahydro,3,5,5-trimethyl-9-methylene-(*4aS-cis*)-1H-benzoheptene, α-santalol and two unidentified compounds.

The bioassay results revealed that this obtained essential oils showed moderate inhibitory activity against root and shoot growth of *M. pigra*. In addition, it exhibited root and shoot growth against all selected species over 60% at 1000 ppm. Only β-caryophyllene had been reported on antimicrobial activity.[51]

CHAPTER IV

CONCLUSION

During this course of research, chemical constituents and biological activity of *Hydrocotyle umbellata* Linn. were thoroughly examined. Nine substances were isolated from hexane and chloroform extracts of the dried whole plants by means of column chromatography. These substances are elucidated as a mixture of essential oils (β-chamigrene, α-bergamotene, α-santalol, germacrene D, β-caryophyllene and β-pinene), a mixture of long chain carboxylic acids, a mixture of long chain aliphatic alcohols, a mixture of long chain aliphatic esters, a mixture of stigmasterol and stigmasta-7,25-dien-3-ol, stigmasterol, stigmasteryl-3-*β*-D-glucopyranoside, β-D-glucopyranosyl tetratriacontan-2,4-dienoate and methyl oleanolate 3-*O*-[β-D-glucopyranosyl (1→6)][-β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside. Furthermore, seven substances could be identified as constituents in the hydrodistillate from fresh whole plants, four of them were elucidated as longicyclene, α-bergamotene, β-caryophyllene, 1H-benzoheptene,2,4α,5,6,7,8,9,9α-octahydro-3,5,5-trimethyl-9-methylene-*(4αS-cis),* α-santalol and two unidentified components, possibly are alicylic compound containing a methoxy group and alicyclic hydrocarbon. All isolated substances were presented in Figures 4.1 and 4.2.

In terms of biological activity, the two new compounds **3** and **4** displayed inhibitory activity on the root growth of *M. pigra* at 1000 ppm (63 and 70 % inhibition, respectively). Compound **4** also exhibited highly inhibition to the roots of *E. crus-galli***,** *P. pedicellatum*, *L sativa*, *T. portulacastrum* and *B. pilosa* at 1000 ppm (94, 90, 86, 93 and 86 % inhibition, respectively). Moreover, mixture **1** and essential oil from hydrodistillation also showed significant growth inhibition against selected plant

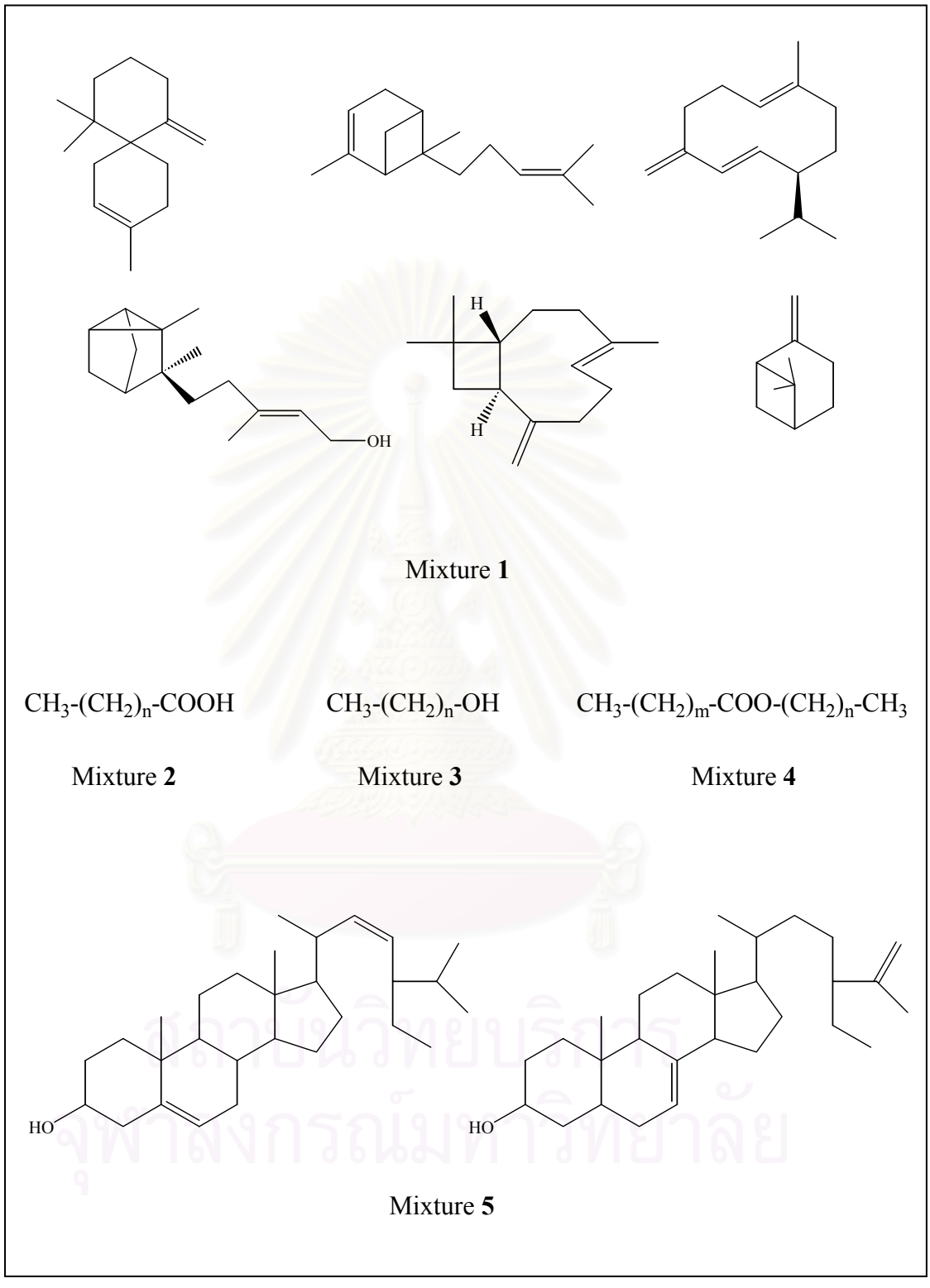


Figure 4.1 All isolated substances from hexane and chloroform extracts of the dried whole plants of *H. umbellata* Linn.

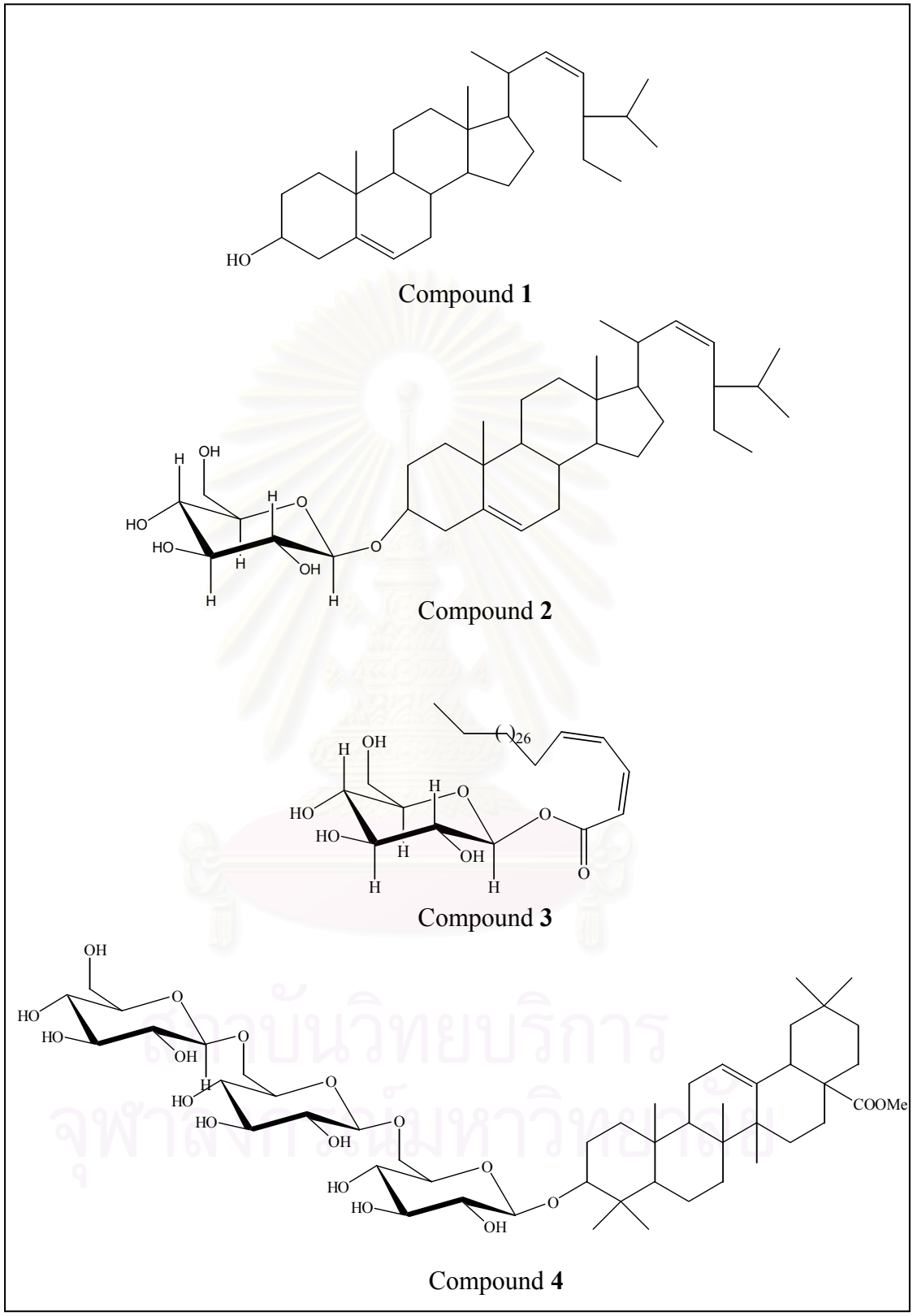


Figure 4.1 (Cont.)

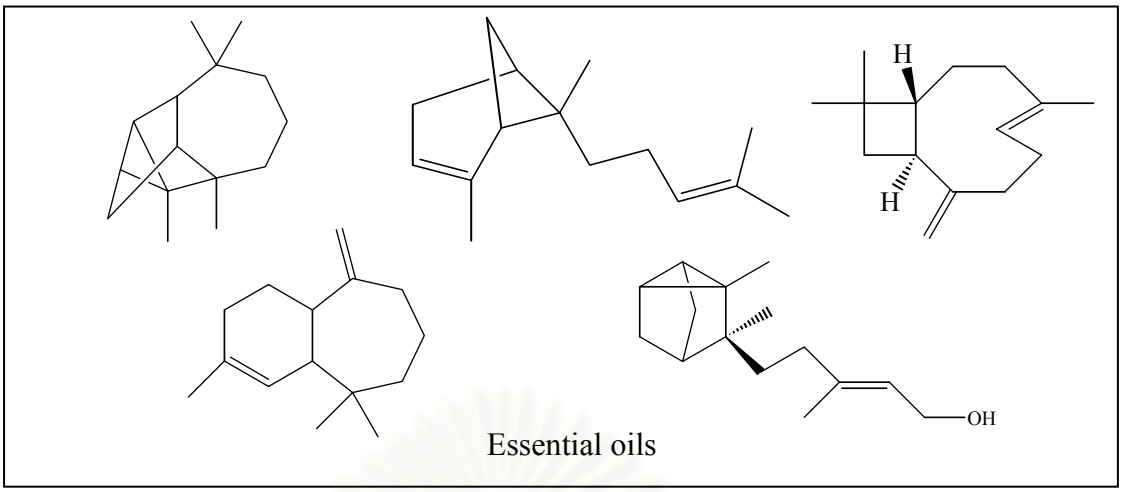


Figure 4.2 All isolated substances from the hydrodistillation of fresh whole plants of *H. umbellata*.

This is the first report of the chemical constituents and biological activity of this plant. It could be seen from this research work that *H. umbellata*, aquatic plant, mostly produced essential oils and glycoside compounds. Furthermore, based on the chemical structures, compounds **3** and **4**, two major compounds, were firstly disclosed. Especially compound **4**, a new triterpenoid glycoside, exhibited as the most active compound from *H. umbellata* and should be considered as a lead compound responsible for plant growth inhibitory activity. It exhibited high root inhibition against both mono- and dicotyledon plants. These bioactive compounds could be good templates for further study. This study endorsed the concept of the needs of biological-guided test for the discovery of new lead compounds.

4.1 Proposal for the future work

The discovery of compounds belonging to *H. umbellata* firstly reported in this thesis provided several intriguing points for future investigation. Isolated substances from this plant might be further tested for other biological activities such as antioxidant and antifungal *etc*. Compound **4** which possessed the highest plant growth inhibition against *M. pigra* and other plants studied in this research could be employed as a template for further study on plant growth inhibitor. Mixture **1** (mixture of essential oils) and essential oils from hydrodistillation could be further studied on biological activity of its individual component to observe structure activity relationship. These approaches will confidently lead to the discovery of the most potentially active compounds in the future.

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