องค์ประกอบทางเคมีของผลพิลังกาสา *Ardisia colorata* Roxb. และฤทธิ์ทางชีวภาพ

นายพิชยา ประเสริฐแสง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CHEMICAL CONSTITUENTS OF *Ardisia colorata* Roxb. FRUIT AND THEIR BIOLOGICAL ACTIVITIES

Mr. Pichaya Prasertsang

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

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By	Mr.Pichaya Prasertsang
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Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of Faculty of Science

(Associate Professor Wanchai Phothiphichitr, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Professor Udom Kokpol, Ph.D.)

..... Thesis Advisor

(Assistant Professor Warinthorn Chavasiri, Ph.D.)

..... Member

(Professor Padet Sidisunthorn, Ph.D.)

..... Member

(Associate Professor Kingkaew Wattanasermkit, Ph.D.)

..... Member

(Assistant Professor Nattaya Ngamrojnavanich, Ph.D.)

พิชยา ประเสริฐแสง : องค์ประกอบทางเคมีของผลพิลังกาสา *Ardisia colorata* Roxb. และฤทธิ์ทางชีวภาพ (Chemical constituents of *Ardisia colorata* Roxb. fruit and their biological activities) อ. ที่ปรึกษา: ผศ. ดร.วรินทร ชวศิริ, 110 หน้า. ISBN 974-17-1965-5.

ผลการศึกษาฤทธิ์ทางชีวภาพเบื้องต้นชี้ให้เห็นว่าสิ่งสกัดไดคลอโรมีเทนของผลพิลังกาสาแสดงความเป็นพิษต่อไรสีน้ำตาล *Artemia salina* ในระดับสูง และแสดงฤทธิ์ต้านอนุมูลอิสระ DPPH จึงศึกษาองค์ประกอบทางเคมีของสิ่งสกัดดังกล่าว พบสารบริสุทธิ์ 3 ชนิด ได้แก่ 2,5-dihydroxy-3-undecyl-1,4-benzoquinone (embelin), stigmasterol, octadeca-9,12-dienoic acid (linoleic acid) และของผสม 4 ชนิด ได้แก่ ของผสมแอลกอฮอล์โซ่ตรง, ของผสมของ stigmasteryl-3-O-palmitate และ β-sitosteryl-3-O-palmitate, ของผสมซึ่งมี α-amyrin เป็นส่วนประกอบ และของผสมของ 1,3-dihydroxy-5-(heptadec-8,11-dienyl)benzene และ 1,3-dihydroxy-5-(pentadec-8-enyl)benzene พบว่า embelin ซึ่งเป็นองค์ประกอบหลัก แสดงความเป็นพิษต่อไรสีน้ำตาลและแสดงฤทธิ์ต้านอนุมูลอิสระ DPPH สูงสุด ในขณะที่แสดงฤทธิ์ต้านการกินของแมลงในระดับปานกลาง แสดงฤทธิ์ยับยั้งจุลชีพ *Bacillus subtilis* และฤทธิ์การยับยั้งเอนไซม์ *p*-hydroxypyruvate dioxygenase พบว่าของผสมของ 1,3-dihydroxy-5-(heptadec-8,11-dienyl)benzene และ 1,3-dihydroxy-5-(pentadec-8-enyl)benzene แสดงฤทธิ์ยับยั้งการเจริญของเชื้อราโรคพืช *Alternaria* sp. โดยอาศัยวิธี bioautographic และแสดงฤทธิ์ฆ่าหนอนกระทู้ผัก *Spodoptera litura* สารทั้งสองชนิดแสดงฤทธิ์ยับยั้งเซลล์มะเร็งเต้านมและเซลล์มะเร็งปอดได้ในระดับสูง

ภาควิชา.....เคมี.....	ลายมือชื่อนิสิต.....
สาขาวิชา.....เคมี.....	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา.....2545.....	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....-.....

4372353823 : MAJOR CHEMISTRY

KEY WORD: ARDISIA COLORATA / CHEMICAL CONSTITUENTS / BIOLOGICAL ACTIVITIES

PICHAYA PRASERTSANG: CHEMICAL CONSTITUENTS OF *Ardisia colorata* Roxb. FRUIT AND THEIR BIOLOGICAL ACTIVITIES THESIS ADVISOR: ASSIST. PROF. WARINTHORN CHAVASIRI, Ph.D., 110 pp. ISBN 974-17-1965-5.

The preliminarily biological activity results pointed out that the dichloromethane crude extract of *Ardisia colorata* Roxb. fruits exhibited high cytotoxic activity against brine shrimp *Artemia salina* as well as scavenged the DPPH radical. The chemical constituents investigation disclosed three pure compounds: 2,5-dihydroxy-3-undecyl-1,4-benzoquinone (embelin), stigmasterol and octadeca-9,12-dieonoic acid (linoleic acid), and four mixtures: a mixture of long chain alcohols, a mixture of stigmasteryl-3-*O*-palmitate and β-sitosteryl-3-*O*-palmitate, a mixture containing α-amyrin, and a mixture of 1,3-dihydroxy-5-(heptadec-8,11-dienyl)benzene and 1,3-dihydroxy-5-(pentadec-8-enyl)benzene. Among isolated substances, embelin, the major component, exhibited the highest cytotoxic activity against brine shrimp, the highest scavenging effect on DPPH radical, moderate insect antifeedant activity, and gave positive tests for antimicrobial activity against *Bacillus subtilis*, enzymatic inhibitor on *p*-hydroxypyruvate dioxygenase. A mixture of 1,3-dihydroxy-5-(heptadec-8,11-dienyl)benzene and 1,3-dihydroxy-5-(pentadec-8-enyl)benzene displayed the growth inhibition effect on phytophatogenic fungi, *Alternaria* sp., by bioautographic method as well as showed insecticidal activity against *Spodoptera litura*. These two substances displayed strongly cytotoxic activity against the breast cancer cell and the small cell lung cancer.

Department…..Chemistry…….	Student's signature……..
Field of study…Chemistry…….	Advisor's signature……..
Academic year…2002……...	Co-advisor's signature……..-…….

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Finally, the author would like to express his gratitude to his parents and family members for their inspiration, understanding, great support and encouragement throughout the entire study.

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List of Abbreviations

br	broad
BHA	butyrated hydroxyanisole
°C	degree of celcius
CH_2Cl_2	dichloromethane, methylene chloride
$CHCl_3$	chloroform
cm^{-1}	unit of wavelength
d	doublet (NMR)
DPPH	2,2-diphenyl-1-picryhydracyl
ED_{50}	50% effective dose
EtOAc	ethyl acetate
DMSO	dimethylsulfoxide
g	gram (s)
GC	gas chromatography
J	coupling constant
IC_{50}	50% inhibitory concentration
kg	kilogram (s)
wt	weight
NMR	nuclear magnetic resonance
IR	infrared
L	liter (s)
LC_{50}	50% lethality concentration
LD_{50}	50% lethality dose
m	multiplet (NMR)
mg	milligram (s)
mL	milliliter (s)
m.p.	melting point
m/z	mass to charge ratio
MW	molecular weight
M^+	molecular ion
nm	nanometer
ppm	part per million
s	singlet (NMR)

List of abbreviations (continued)

t	triplet (NMR)
TLC	thin layer chromatography
δ	chemical shift
μg	microgram (s)
λ	wavelenght
MeOH	methanol
MS	mass spectrometry
R_f	retardation factor

CHAPTER I

INTRODUCTION

Thailand is a tropical country located in suitable landscape. Therefore, a variety of plants species is widely spread. A lot of species exhibit significance for medically useful drugs. In early age, the use of medicinal plants for medical treatments was not complicated and mainly depended on ancient belief. They were normally used in both a single form and multiple forms, which have been existed by the passage of knowledge down the generations within each ethic group.

Although several plant species have been distributed in Thailand, only small proportion has been thoroughly phytochemical and pharmacological investigation. When one considers that a single plant may contain up to thousands of constituents, the possibilities of making new discoveries become evident. Fransworth claims that 119 characterized drugs are still obtained commercially from higher plants and that of 74% were found from ethnobotanical information.[1]

At the present time, the research and development of new drugs for treatments are still main problems of medicinal development. In this research, the searching for biologically active compounds from a medicinal plant, *Ardisia colorata* Roxb., has been focused.

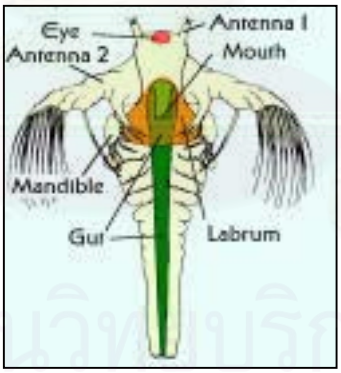
About half of useful drugs nowadays are derived from natural sources. Therefore, the natural products have served as an important source of drugs since ancient times. Rapid identification of bioactive compounds from natural product mixtures still remained as a critical factor since the development procedures for searching biologically active compounds from natural source such as plants, microorganisms, marine organisms *etc*. required a long and tedious process. For this reason, it is necessary to be of methods available which eliminate unnecessary separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or useful types of constituents with potential activities.

Screening program for biologically active natural products requires the right bioassays. Bioassays are also essential for detecting the required effects throughout activity-guided fractionation. They must be simple, inexpensive and rapid in order to cover with large number of samples.

Since most active plant principles are toxic at elevated doses, a possible approach to develop an effective general bioassay might be simply to screen for substances that are toxic to zoologic systems. Desiring a rapid, inexpensive, in-house bioassay for screening and fractionation monitoring of physiologically active plant extracts. Brine shrimp lethality cytotoxic test has been used as a bioassay for a variety of toxic substances. This method has also been applied to plant extracts in order to facilitate the isolation of biologically active compounds.

Brine shrimp *Artemia salina* Linn. as shown below is a tiny crustacean. It can response to biologically active compound comparable with mammalian system. It has also DNA-dependent RNA polymerase enzyme and ouabaine sensitive Na^+ and K^+ dependent ATPase like in mammalian system.[2]

Therefore, the results from brine shrimp assay can be used as a primary toxic representative for other mammal species.

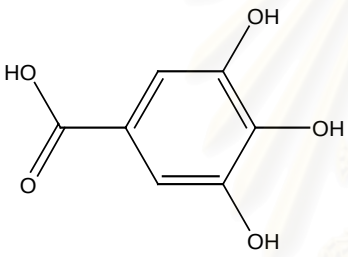


Brine shrimp

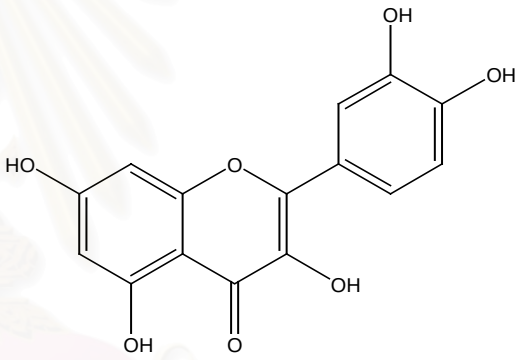
The searching for cytotoxic compound such as anticancer, antimicrobial, insecticide *etc*. has been frequently used brine shrimp as general tools by microwell method.[3,4] The eggs of brine shrimp are readily available at low cost in pet shops as food for tropical fish, and they remain viable for years in the dry state. Upon being placed in as brine solution, the eggs hatch within 24 hours, providing large number of larvae (nauplii). This method is convenient since it requires a little material, rapid,

inexpensive and reliable. For this reason, this brine shrimp assay is selected as one of primary screening methods of this study.

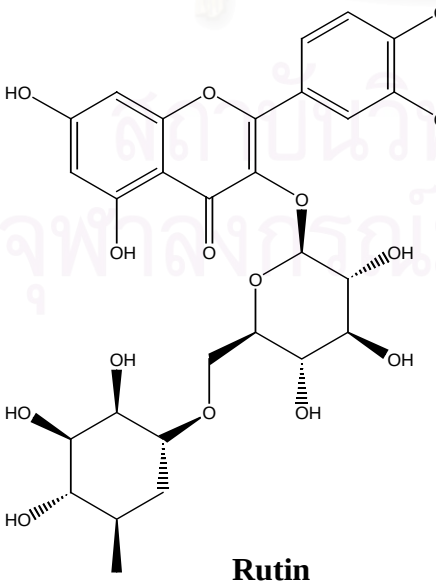
The second screening method involving to this research is an antioxidant activity. Oxidative processes are well known to be major causes of degradation of food and materials. More recently, free radicals in particular oxygen reactive species, have been recognized to be involved in many diseases, including cancer and arteriosclecrosis. Aging also may be the sum of the free radical reactions which occur continuously throughout cells and tissues. Plant contains several widespread phenolic compounds with well established antioxidant activity. Typical examples are common flavonoids such as quercetin or rutin, phenolic acid such as gallic or caffeic acids and tannins. The structures of each compound are shown below.



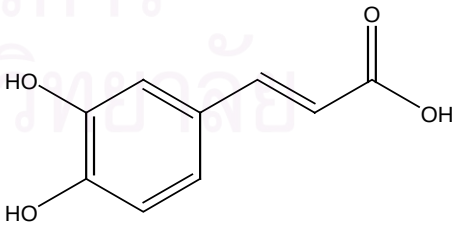
Gallic acid



Quercetin



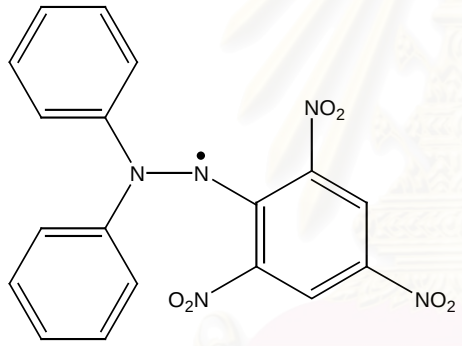
Rutin



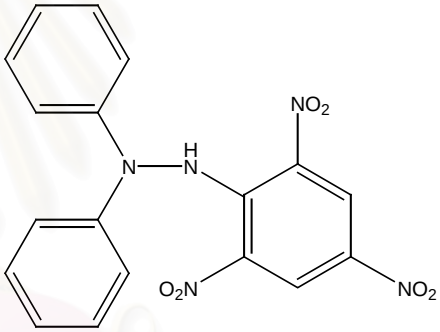
Caffeic acid

The screening strategy for searching new antioxidant compounds from crude plant extract based on simple and rapid TLC autographic assays. In 1994, Takao *et al*.[5] used 2,2-diphenyl-1-picryhydrazyl (DPPH) radical for screening antioxidants in marine bacteria as a TLC spray reagent. It was also proved to be well suited for the detection of antioxidants in crude plant extracts.

Scavenging effect towards DPPH radical consisted of two assays for qualitative and quantitative analysis.[6] The assay for qualitative analysis using TLC autographic method, and spectrophotometric assay for quantitative analysis respectively. Both of them use DPPH radical as a radical source. The different color between radical form (purple) and non-radical form (colorless) of DPPH was used for regarding. The structures of DPPH radical and non-radical forms are illustrated as shown below.



DPPH radical form

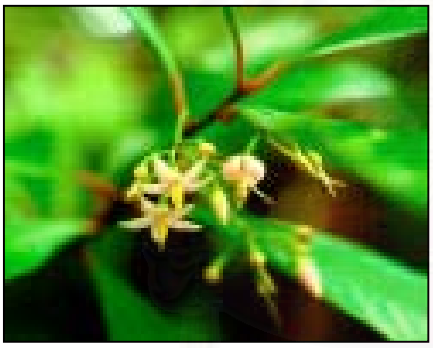


DPPH non-radical form

From primarily biological screening of Thai medicinal plants using two screening methods described above, under co-operation between Department of Chemistry and Department of Biology, Faculty of Science, Chulalongkorn University, it was found that the dichloromethane extract of dried fruits of *Ardisia colorata* Roxb. (Pi-Lang-Ka-Sa) belonging to Myrsinaceae family showed attractive results for both screening methods.

1.1 Botanical Aspects and Distribution

Ardisia colorata Roxb. Shrub or small tree to 5-10 m. The bark is brown, quite smooth and very thin. The oblong-lanceolate with point or slightly tapering tip and blunt or slightly pointed base leaf about 13-28 cm. long and 3-8 cm. wide. These plants have no teeth or gland on margin. Mature leaves without hairs but with scattered tiny rusty-brown scale and dark gland dots below. It has 15-20 pairs of side veins with many shorter intermediate ones. Stalks 0.6-1.5 cm the young twigs pale cream, densely scaly. The flower is pale pink about 0.25-0.3 cm in branched pyramidal clusters (panicles) at end of twigs, 10-18 cm. Individual stalks 0.2-0.4 cm ± scaly. Calyx ±0.1 cm spilt 2/3 into spreading lobes, not overlapping, black-dotted. The corolla deeply spilt with blunt lobes, no gland dots. Slender and projecting before petals open. The fresh fruit is pale yellow, when old is black.[7] The pictures of flower, leaf and fruit of *Ardisia colorata* Roxb. are displayed below.

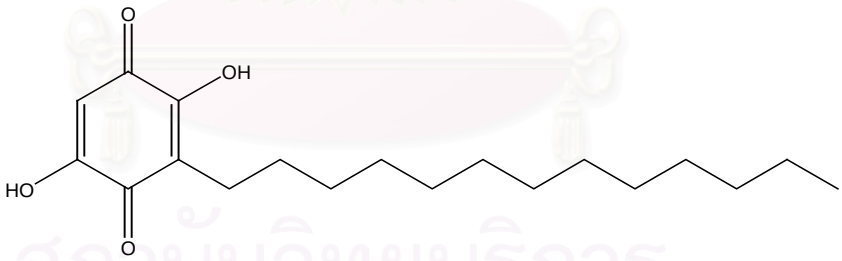


According to Thai herbal ancient believes, the leaf was used to treat for the liver diseases. The fruits showed antidiarrheal and antipyratic activity and the roots were used for treatment of gonorrheal diseases. The isolated mixtures from fruits can inhibit the growth of *Salmanella* spp. and *Shigella* spp. which cause of diarrhea diseases.[8]

In Thailand, the plants belonging to Myrsinaceae family are consisted of 6 species[7] i.e. *Ardisia colorata* Roxb. (Pi-Lang-Ka-Sa), *Ardisia nervosa* Kurz. (Gee-Par-Taek), *Ardisia viren* Kurz.(Tar-Nok), *Rapanea yunnanensis* Mez. (Lang-Ka-Tae), *Maesa ramentacea* A.Dc. (Kra-Duk-Kai) and *Maesa montana* A.Dc. (Hat-Sa-Kun-Kreua). In this research, the fruits of *Ardisia colorata* Roxb. were selected to examine for their chemical constituents.

1.2 Literature Reviews for *Ardisia colorata* Roxb.

In 1986, O. Luanratana *et al*. reported the orange pigment, rapanone which was obtained from petroleum ether, chloroform and ethanol extracts of the barks of *A.colorata* Roxb. It exhibited antileprotic activity on mice which infected with *Mycobacterium leprae*.[9]



Rapanone : 2,5-dihydroxy-3-tridecyl-1,4-benzoquinone

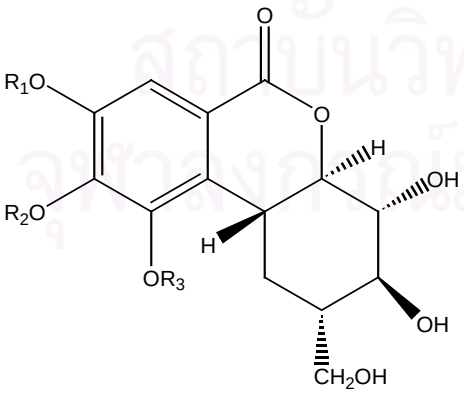
Furthermore, O. Luanratana *et al*. addressed the microbial growth inhibition results of the ethanolic extract from the barks. The ethyl acetate fraction exhibited good activity to inhibit the growth of *Aeromonas hydrophila, Vibrio cholerae* and *Shigella dysenteriae*, whereas the butanolic fraction revealed antigonorrhoeal activity against *Neisseria gonorrhoea*.[10]

1.3 Chemical Constituents Studies on *Ardisia* Genus

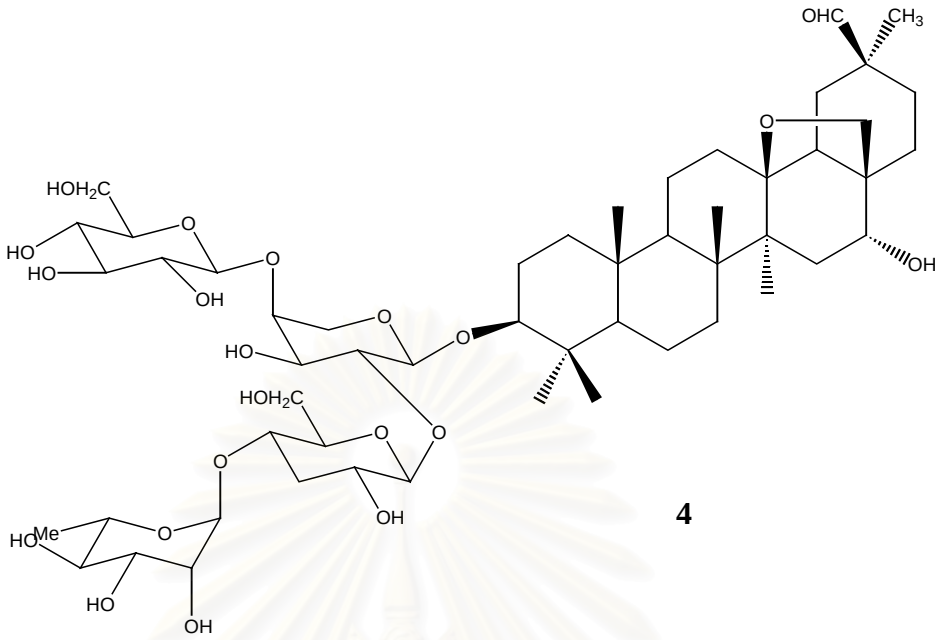
Literature surveys of chemical constituents of the plants belonging to *Ardisia* genus are presented in Table 1.1. The structures of some isolated compounds are shown below.

Table 1.1 Compounds found in *Ardisia* genus

Scientific Name	Compound	Ref.
Ardisia japonica Thunb.	Norbergenin **(1)**, Bergenin **(2)** and tri-O-methylnorbergenin **(3)** Triterpenoid saponin **(4)**, Triterpenoid glycoside,	11
Ardisia iwahigensis Elmer.	Ardisenone **(5)**	12
Ardisia cornudentata Mez.	Ardisianone **(6)**, Cornudentanone **(7)**	13
Ardisia solanacea Roxb.	Bauerenol acetate **(8)** α-Amyrin and β-Amyrin	14
Ardisia mamillata Hance.	Ardisimamillosides A-D **(9-12)**	15
Ardisia crenata	Ardisicrenoside A-F **(13-18)**	16
Ardisia sieboldii	Ardisiaquinone A-F **(19-24)**	17



	R_1	R_2	R_3
1	H	H	H
2	H	CH_3	H
3	CH_3	CH_3	CH_3



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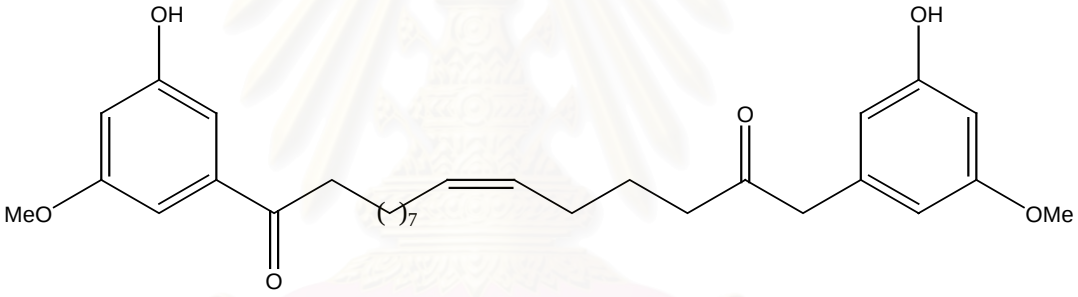

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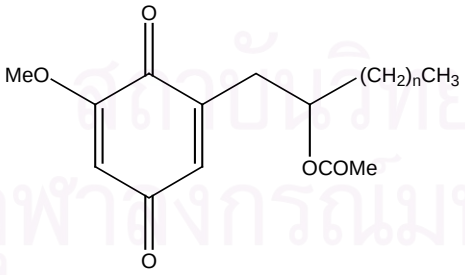
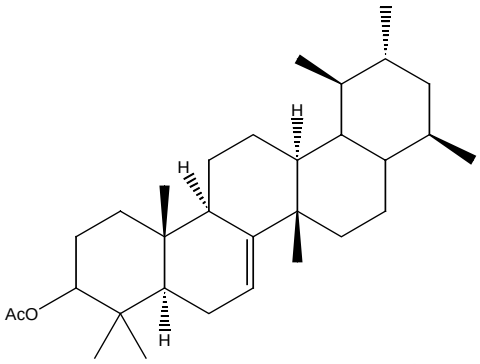


6 n=12
7 n=10



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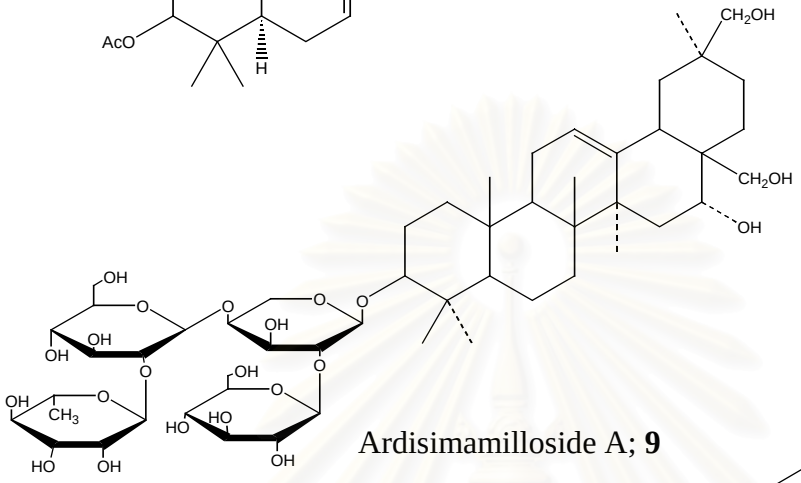
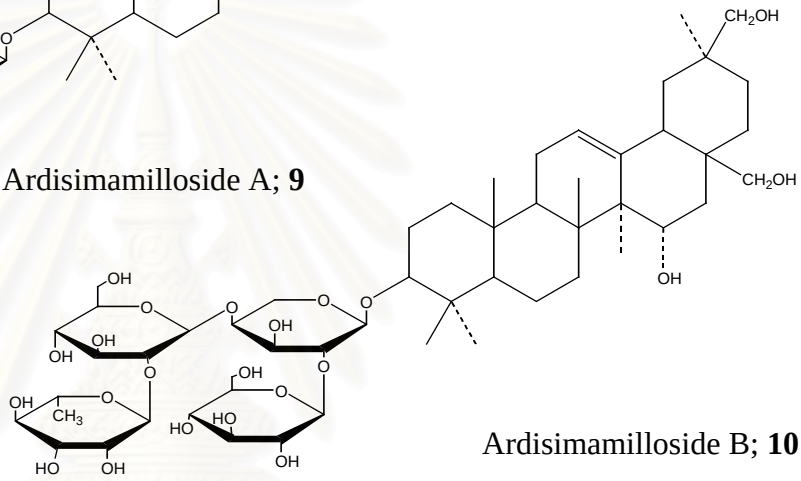
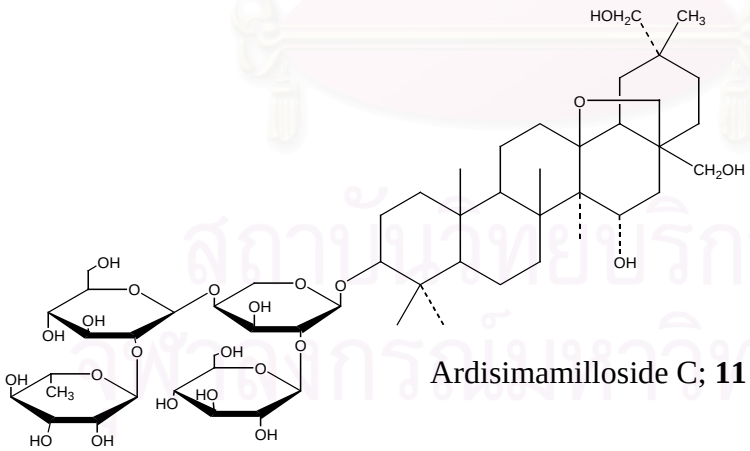


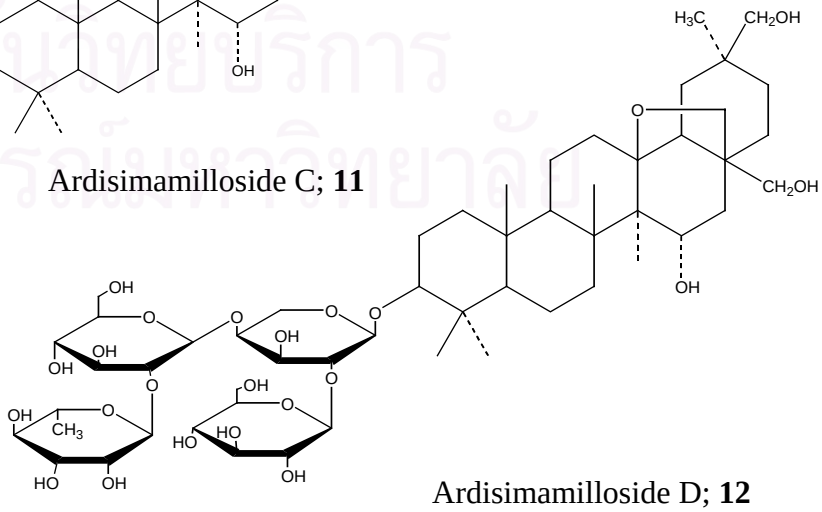
Ardisimamilloside A; **9**



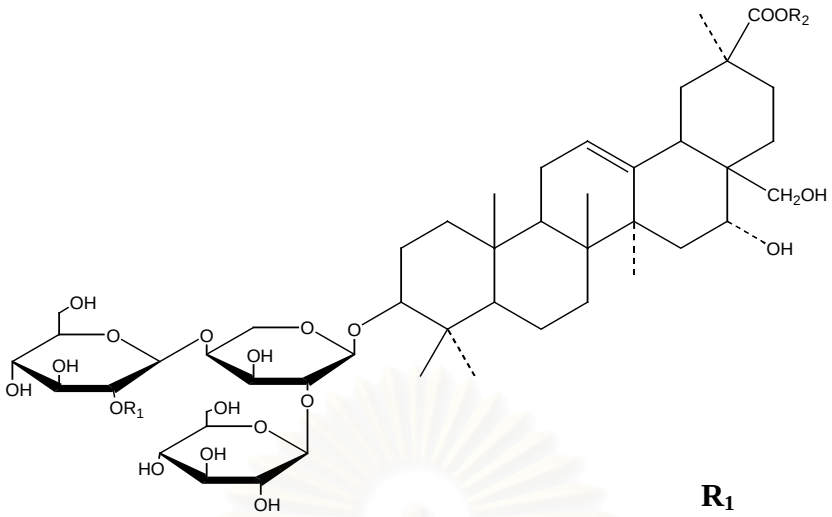
Ardisimamilloside B; **10**



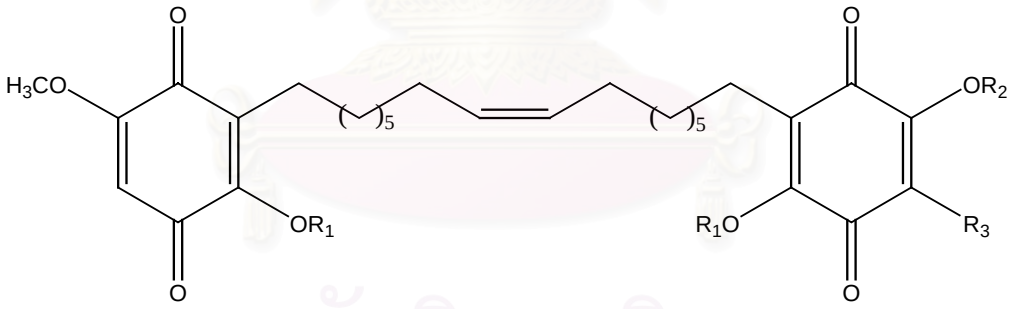
Ardisimamilloside C; **11**



Ardisimamilloside D; **12**



	R_1	R_2
Ardisiacrenoside A(13)	α-L-rham	CH_2OH
Ardisiacrenoside B(14)	β-D-xyl	CH_2OH
Ardisiacrenoside C(15)	α-L-rham	COO-β-D-glc
Ardisiacrenoside D(16)	β-D-xyl	COO-β-D-glc
Ardisiacrenoside E(17)	α-L-rham	COOH
Ardisiacrenoside F(18)	β-D-xyl	COOH



	R_1	R_2	R_3
Ardisiaquinone A(19)	H	CH_3	H
Ardisiaquinone B(20)	H	H	CH_3
Ardisiaquinone C(21)	CH_3	CH_3	H
Ardisiaquinone D(22)	H	CH_3	CH_3
Ardisiaquinone E(23)	CH_3	CH_3	CH_3
Ardisiaquinone F(24)	CH_3	H	CH_3

1.4 The Goal of This Research

The attractive preliminary results for bioassay of *Ardisia colorata* Roxb. crude extract call for intensive investigation. Therefore, the goal of this research can be summarized as:

1. To extract and isolate the chemical constituents from the dried fruit of *A. colorata* Roxb.
2. To elucidate the structures of isolated compounds.
3. To explore the biological activities of isolated compounds.

CHAPTER II

EXPERIMENTAL

2.1 Plant Materials

The dried fruits of *Ardisia colorata* Roxb. (Pi-Lang-Ka-Sa) were purchased from Vetchapong, Bangkok, Thailand, in June 2001. The plant sample has been compared with voucher specimen deposited in the Herbarium of the Princess Sirindhon, Thailand (BK 14133).

2.2 Instruments and Equipment

Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on an aluminium sheet precoated with silica gel (Merck's Kieselgel 60 PF_{254}). Column chromatography was performed on silica gel (Merck's Kieselgel 60G) and flash chromatography was proceeded on silica gel (40 μm average particle diameter). Chromatotron was performed on Harrison research, Model 7924 T, Serial No. W34 Patented, made in U.S.A.

The Fourier Transform-Infrared Spectra (FT-IR) were recorded on NICOLET IMPACT 410 FT-IR spectrometer. The ^{1}H and ^{13}C NMR spectra (both 1D and 2D) were performed in deuterated chloroform or dimethylsulfoxide with tetramethylsilane as an internal reference on Bruker Fourier Transform Nuclear Magnetic Resonance Spectrometer, model AC-F200 and a Joel, model JNM-A500 and a Bruker Avance 300 FT-NMR spectrometer. Mass spectrometry (MS) analysis was conducted on Fisson Instrument Model Trio 2000. GC analysis was carried out on a Shimadzu GC GC-9A instrument equipped with flame ionization detector with N_2 as a carrier gas. The GC-MS analysis was performed on GC model star 3400Cx and MS model saturn 4D from Varian.

2.3 Chemical Reagents

All solvents used in this research were purified by standard methodology except for those which were reagent grades. The reagents utilized for synthesizing all derivatives were purchased from Fluka Chemical Company or otherwise stated and were used without further purification.

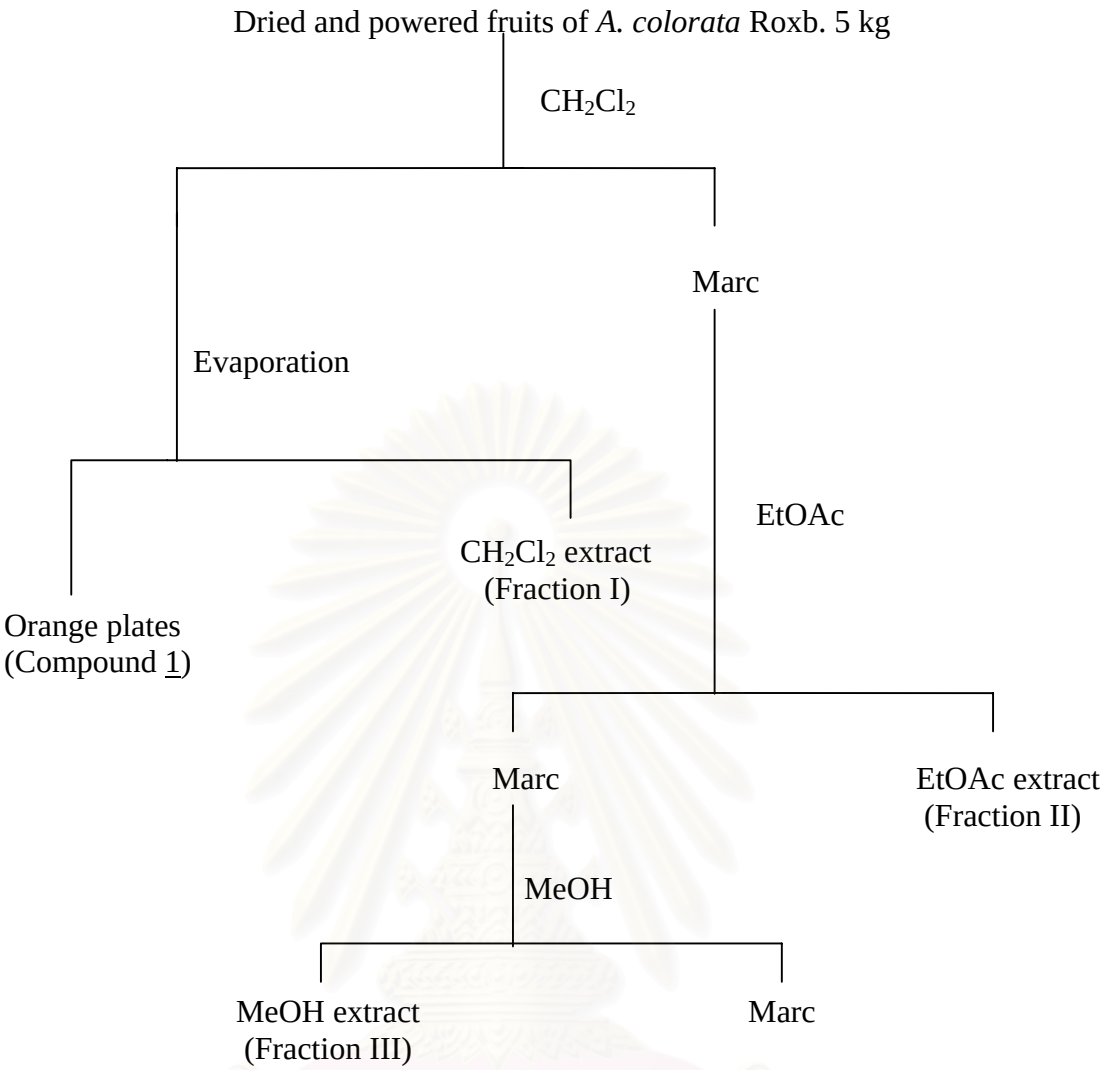
2.4 Chemical Tests

- Liebermann-Berchard's Test

The sample was dissolved in dry chloroform 0.5 mL, then slowly added 2-3 drops of acetic anhydride followed by one drop of concentrated sulfuric acid. Development of the color suggests the presence of steroids or triterpenoids. If the solution was dark blue or greenish blue, the sample may contain steroidal nucleus, whereas if the solution turned reddish, the sample should be triterpenoidal compound.

2.5 Extraction of *Ardisia colorata* Roxb.

The dried and powdered fruits of *A. colorata* 5 kg were extracted by soaking with dichloromethane for two days at room temperature. The same process was repeated twice. During the evaporation of dichloromethane from the extract, the orange plate was precipitated. This orange solid was recrystallized from methanol twice to give Compound 1. The remained portion was completely evaporated to furnish a dark brownish crude as Fraction I. The marc was similarly extracted with ethyl acetate and methanol to yield Fractions II and III as dark brownish crudes. The extraction process is summarized as shown in Scheme 2.1.



Scheme 2.1 Extraction procedure for the dried fruits of *A. colorata* Roxb.

2.6 Chemical Reactions

2.6.1 Hydrolysis of Mixture 3[18]

Mixture 3 2.0 g was dissolved with 10% ethanolic KOH 40 mL in the 100 mL round bottom flask. The solution was stirred under refluxing on a water bath for 4 hours. The reaction was monitored until the reaction was completed by thin layer chromatography compared with starting material. After the reaction was completed, the solvent was evaporated and the residue was extracted with diethyl ether 150 mL for three times. The combined diethyl ether layer was dried over anhydrous sodium sulfate. Evaporation the solvent furnished a hydrolyzed product, assigned as Mixture 3A.

The remained aqueous layer after extraction with diethyl ether was acidified with dilute hydrochloric acid to yield the acid part designated as Mixture 3B which was further analyzed as methyl ester.

2.6.2 Methylation of Mixture 3B

Mixture 3B was dissolved in a small amount of $CHCl_3$ and ethyl acetate. Then, two drops of trimethylsilyl diazomethane ($TMSCHN_2$) were added under nitrogen gas flow condition to give the permanent yellow solution. The solution was left at room temperature overnight. After the reaction was completed, the product was further analyzed by GC-MS analysis.

2.7 Bioassay Experiments

2.7.1 Brine Shrimp Cytotoxic Lethality Test[4]

This bioassay is a procedure for general toxicity screening. The advantage of this assay is rapid, reliable, convenient and inexpensive. It requires small quantities of material and is able to identify a broad spectrum of activities. Thus, it is essential as a preliminary testing for the search of bioactive compounds. There are several techniques for this assay. A microwell method has been used for this experiment and was described as follows:

2.7.1.1 Sample Preparation

The samples were prepared by dissolving 4 mg of tested compound (either crude extract or pure compound) in 80 μL of dimethyl sulfoxide (DMSO) then added artificial sea water (dissolving 38.5 g of NaCl in 1 L of distilled water and filtered by cellophane paper) to the solution to make 4000 μL and allowed to shake-well to afford solution A (1000 ppm). Serial dilution of this stock solution was made to obtain solution B (100 ppm) and solution C (10 ppm), respectively. The control solution was prepared by using only DMSO and artificial sea water.

2.7.1.2 Hatching Brine Shrimp

Brine shrimp eggs *Artemia salina* obtained locally were hatched in artificial sea water in gloomy plastic box. The box was divided into unequal two sections linked with four 2 mm diameter holes. The eggs were scattering into the larger section which was darkened with aluminum foil while the smaller section was

illuminated with the 20 watt lamp. The box was kept at 22-29 °C. After 24 hours, nauplii were collected by micropipette from the smaller section.

2.7.1.3 Bioassay

Transferred five nauplii in 100 μL of artificial sea water into each well of 24-well microplates by micropipette. Each concentration was performed for six replications. The covered plates were kept under the same conditions as hatching. The number of dead nauplii in each well was counted under binocular microscope after at 6 hours (for acute toxicity) and 24 hours (for chronic toxicity).

2.7.1.4 LC_{50} Determination

LC_{50} values were calculated by probit analysis program.[19]

2.7.2 Scavenging Effects on DPPH Radicals[6]

2,2-Diphenyl-1-picryhydrazyl (DPPH) radical is a stable radical with a purple color (λ_{max} 517 nm). Upon reduction by a scavenger, the extensive conjugation is disrupted and the compound turns yellow.

2.7.2.1 TLC Autographic Assay

After developing and drying, TLC plates were sprayed with a 0.2% DPPH in methanolic solution. The plates were examined 5 minutes after spraying. Active compounds appeared as yellow spots against purple background.

2.7.2.2 Spectrophotometric Assay

Samples of various concentrations (0.5 mL) were added to a 1 mL methanolic solution of DPPH radical (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and then left for 30 minutes. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer. All tests and analyses were run in three replicates and averaged. Calculate the percentage of radical scavenging by the following equation.

$$\text{The percentage of radical scavenging} = (1 - A_{sample}/A_{control}) \times 100$$

A_{sample} = Absorbance of sample solution with DPPH

$A_{control}$ = Absorbance of only DPPH and used solvent

2.7.3 Antifungal Activity by Bioautographic Method[20]

Direct bioautographic detection of the TLC plate is applicable to microorganisms that can grow directly on the TLC plate and suitable precautions are required. Each isolated substance was spotted on silica gel TLC plate and developed in suitable solvent system. This plate was allowed to air-dry for complete removal of solvents. The TLC plate was sprayed with a spore suspension of *Fusarium oxysporum* and *Alternaria* sp., and incubated at room temperature for 3 days. After incubation, the TLC was stained with 1% (V/V) lacto-phenol in 5% (V/V) acetic acid for 5 minutes and then destained with 5% (V/V) acetic acid for 10 minutes. The active components appeared as clear zone against blue background.

2.7.4 Insecticidal Activity by Vial Test Contact Toxicity[21]

This assay is dry film method or vial test to provide the potential substance on insecticidal activity, so it is a chance of finding substance for the phytophagous control. A polyphagous insect, the common cutworm, *Spodoptera litura* was used as a model for meanwhile investigation.

2.7.4.1 Sample preparation

The samples were prepared by dissolving 4 mg of test substances in 4 mL of acetone to provide 1000 ppm solution. Serial dilution of this stock solution was made to obtain 500, 100, 50, and 10 ppm, respectively. Then 1 mL of each concentration solution was poured into the glass vial (3 replication). Following, each vial that contained 1 mL of sample was evaporated the acetone, and the treated vial was placed in an open space for a few minutes to ensure complete removal of acetone. The test substances have already coated on the wall and bottom of vial.

2.7.4.2 Bioassay

Common cutworms *Spodoptera litura* were reared on an artificial diet in a controlled environment. The 15 of first instar larvae were placed in the vial to free movement for 6 hrs, then the larvae were transferred into the new vial containing artificial diet and were kept at 25°C for 5 days.

After the fifth day, the died cutworms were counted and converted to percentage of died larvae of *S.litura*. Finally, the LD_{50} in ppm was calculated by probit analysis program.

CHAPTER III

RESULTS AND DISCUSSION

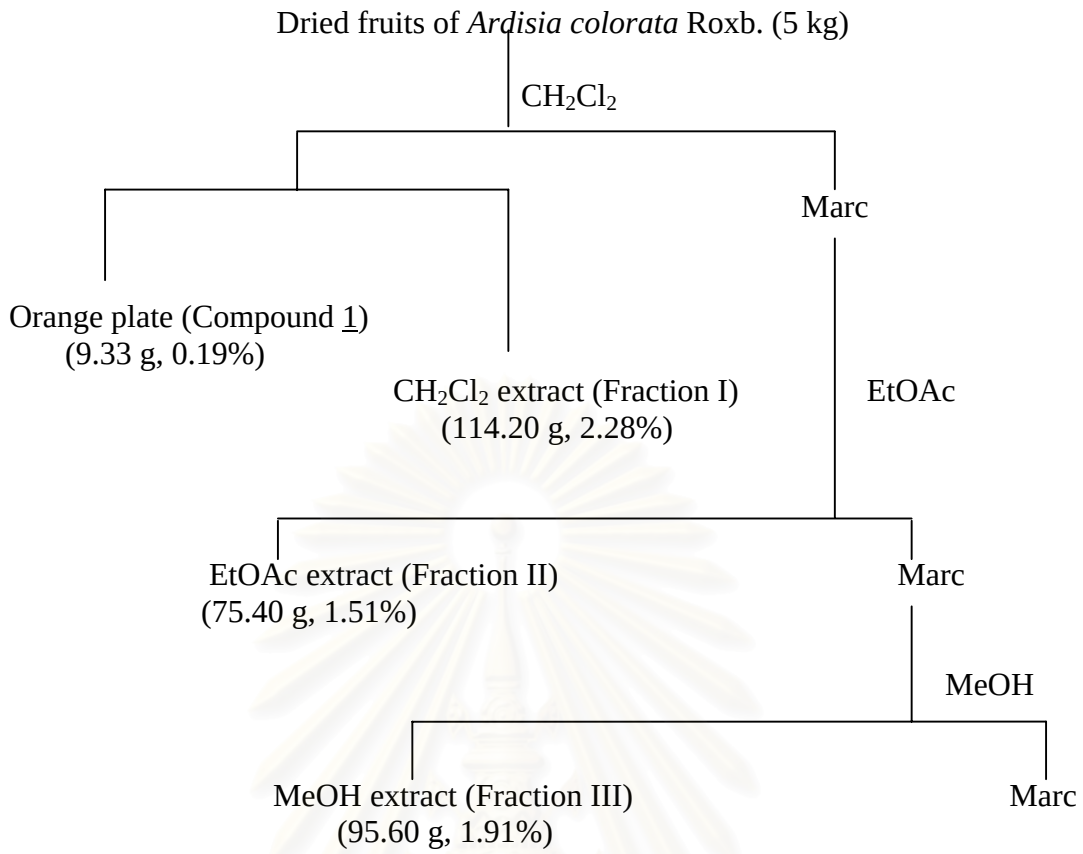
Stemmed from the preliminary results that the dichloromethane extract from *Ardisia colorata* Roxb. fruit exhibited high activity for brine shrimp cytotoxicity as well as showed positive results for radical scavenger properties toward DPPH, this extract was rationalized to further investigate for chemical constituents and their biological activities.

3.1 Results of Extraction

General Extraction

The crush dried fruits of *Ardisia colorata* Roxb. were extracted according to the procedure described in Chapter II. Each crude extract was subjected to preliminarily biological screening test followed the procedure described in Chapter II. The results of extraction are exhibited as shown in Scheme 3.1. Weight of each crude extract and percentage yield (w/w of dried plant material) are summarized as shown in Table 3.1.

From the extraction result (Scheme 3.1 and Table 3.1), it was signified that the orange plate solid (Compound 1) 9.33 g or 0.19 % (w/w of dried fruit material) was deposited form CH_2Cl_2 extract. The extraction procedure was proceeded by increasing solvent polarity. Dichloromethane extract gave the best yield 114.20 g or 2.28 %(w/w). All crude extracts including Compound 1 were further screened for the biological activity using cytotoxicity test against brine shrimp and radical scavenging effect on DPPH radical.



Scheme 3.1 The results of extraction of dried fruits of *Ardisia colorata* Roxb.

Table 3.1 Weight and percentage yield of each crude extract from the extraction procedure of dried fruits of *Ardisia colorata* Roxb.

Crude extract	Weight (g)	Percentage yield*
CH_2Cl_2 (Fraction I)	114.20	2.28
EtOAc (Fraction II)	75.40	1.51
MeOH (Fraction III)	95.60	1.91
Orange plate (Compound 1)	9.33	0.19

* The percentage yield was calculated based on dried fruit materials (5 kg)

3.2 Preliminary Study on Biological Activity Test

3.2.1 Brine Shrimp Cytotoxic Lethality Test

This bioassay was performed following the methodology described in Chapter II. For preliminary study, each crude extract as referred in Scheme 3.1 was subjected for this assay. The results are displayed in Figure 3.1.

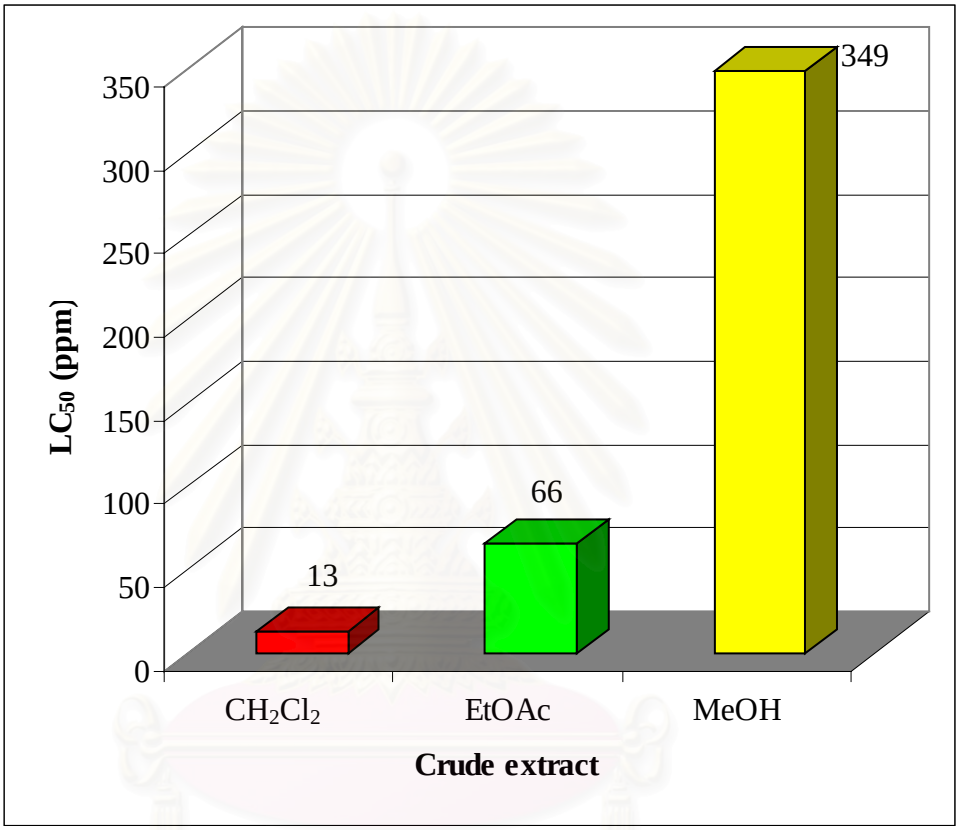


Figure 3.1 LC_{50} values from brine shrimp cytotoxic lethality test for crude extracts of *Ardisia colorata* Roxb.

Among crude extracts, the dichloromethane extract displayed high toxicity level with LC_{50} value of 13 ppm. Other crude extracts showed moderate toxicity against brine shrimp. This obtained result implied that the dichloromethane extract should contain substances responsible for cytotoxic activity.

3.2.2 Scavenging Effect on DPPH Radical

TLC Autographic Assay

Antioxidant assay was accomplished by the protocol described in Chapter II. The chromatograms of all crude extracts before and after spraying with DPPH radical reagent are demonstrated in Figure 3.2.

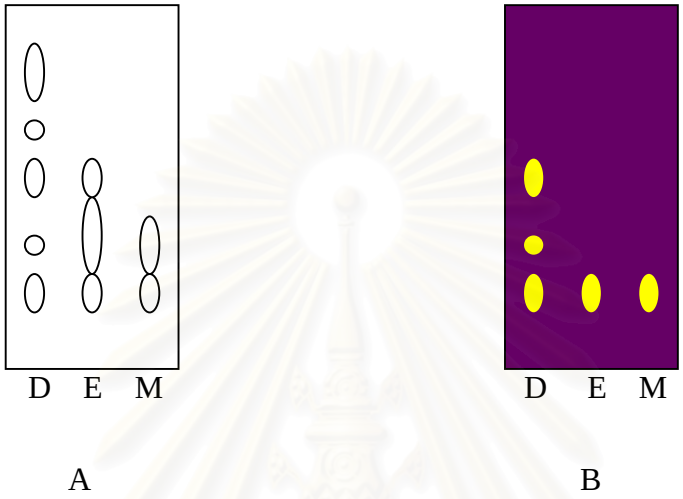


Figure 3.2 TLC autographic assay for DPPH radical scavenger assay

(A) TLC chromatogram before spraying with DPPH reagent

(B) TLC chromatogram after spraying with DPPH reagent

D : dichloromethane, E : ethyl acetate and M : methanol extracts

Figure 3.2A shows the TLC of all crude extracts which was developed in 30% ethyl acetate: hexane solvent system before spraying with DPPH. After sprayed with DPPH reagent, the active components were visualized as yellow spot against purple background (Figure 3.2B). It was found that the dichloromethane crude extract revealed at least 3 components active towards DPPH radical reagent.

From these preliminary results for biological activities of both brine shrimp cytotoxic lethality test and TLC autographic assay for DPPH radical scavenger, the most tendency potent extract was dichloromethane. The other crude extracts gave medium activity. Therefore, the dichloromethane extract was selected for further examination.

3.3 Chemical Constituents of Dichloromethane Extract (Fraction I)

During the evaporation of solvent from dichloromathane extract, it was noticed that some orange plate deposited. These solids were filtered and designated as Compound 1. The remaining dichloromethane extract was further concentrated to furnish sticky dark brown material (Fraction I), which was further examined its constituents by separation with column chromatography.

3.3.1 Structural Elucidation of Compound 1

The orange plate solid deposited upon the solvent evaporation of the dichloromethane extract was recrystallized with methanol twice to afford Compound 1 as orange plate crystal, 9.33 g (0.19% w/w of dried fruit material), m.p. 140-142 °C. This compound was soluble in dichloromethane and chloroform.

The IR spectrum of this compound (Figure 3.3) gave the absorption peaks of hydroxyl group at 3308 cm^{-1}, carbonyl group at 1613 cm^{-1} and C-O bond at 1332 cm^{-1}. Other signals were tentatively assigned as shown in Table 3.2.

The mass spectrum (Figure 3.4) showed the molecular ion peak, M^+, at *m/z* 294 (Calcd. for $C_{17}H_{26}O_4$: MW 294.18) and other important fragmentation ion peaks at *m/z* (% relative intensity) 155(40), 154(100), 153(28), 142(25) and 125(20). According to literature search, the fragmentation pattern of this compound[22] was proposed as shown in Scheme 3.2.

The ^{1}H NMR spectrum ($CDCl_3$) of this compound (Figure 3.5) displayed the proton signals as follows: protons of hydroxyl group (2-OH, 5-OH) at δ 7.67 ppm (br, 2H), aromatic proton (H-6) at δ 5.98 ppm (s, 1H), methylene group (H-1′) at δ 2.43 ppm (t, 2H, *J* = 7.05 Hz), methylene group (H-2′) at δ 1.48 ppm (t, 2H, *J* = 6.89 Hz), methylene group (H-3′-10′) at δ 1.24 ppm (s, 16H) and terminal methyl group (H-11′) at δ 0.86 ppm (t, 3H, *J* = 6.20 Hz). The ^{1}H NMR chemical shift assignment of Compound 1 is shown in Table 3.3 compared with those of embelin from previous report.[23]

The ^{13}C NMR spectrum ($CDCl_3$) of this compound (Figure 3.6) displayed the important carbon signals at 161.1 (C-1, C-2, C-4 and C-5), 116.1 (C-6), 102.2 (C-3) and 22.5 (C-1′). Others signals were detected and compared with those of embelin from previous report[23] as presented in Table 3.4.

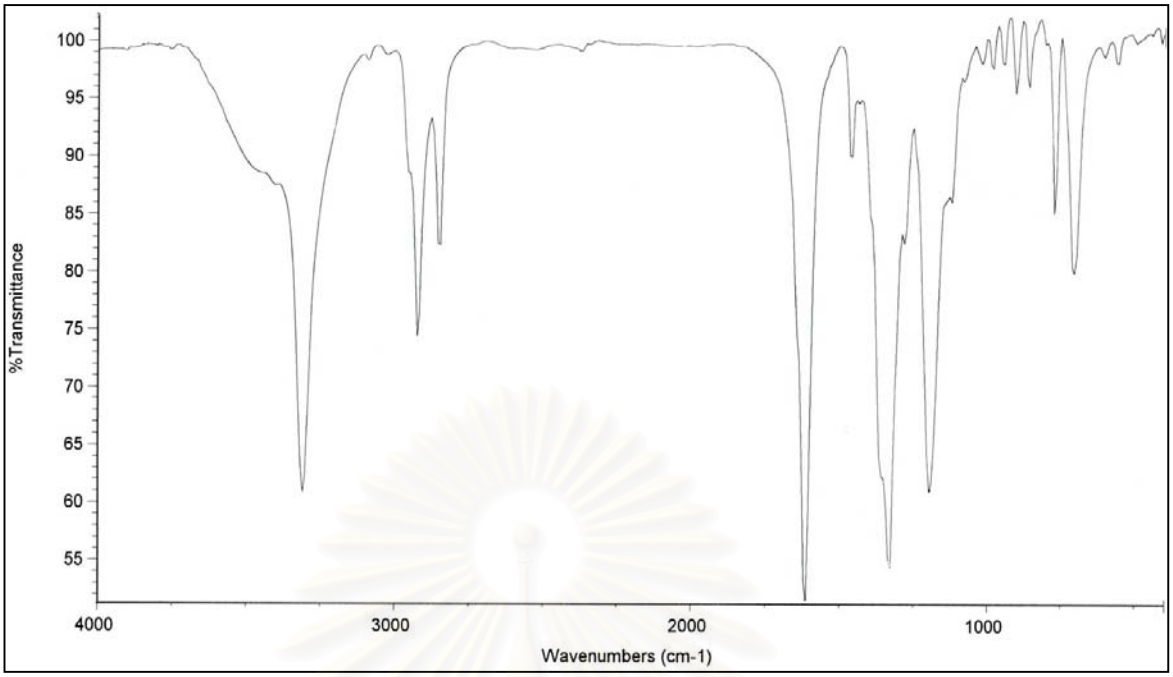


Figure 3.3 The IR spectrum of Compound 1

Table 3.2 The IR absorption band assignments of Compound 1

Wavenumber(cm^{-1})	**Intensity**	**Tentative assignments**
3308	Strong	O-H stretching vibration of hydroxyl group
2919, 2847	Medium	C-H stretching vibration of CH_2 and CH_3
1613	Strong	C=O stretching vibration of benzoquinone
1460	Weak	C-H bending vibration of CH_2
1332	Strong	C-H bending vibration of CH_3
1193	Strong	C-O stretching vibration

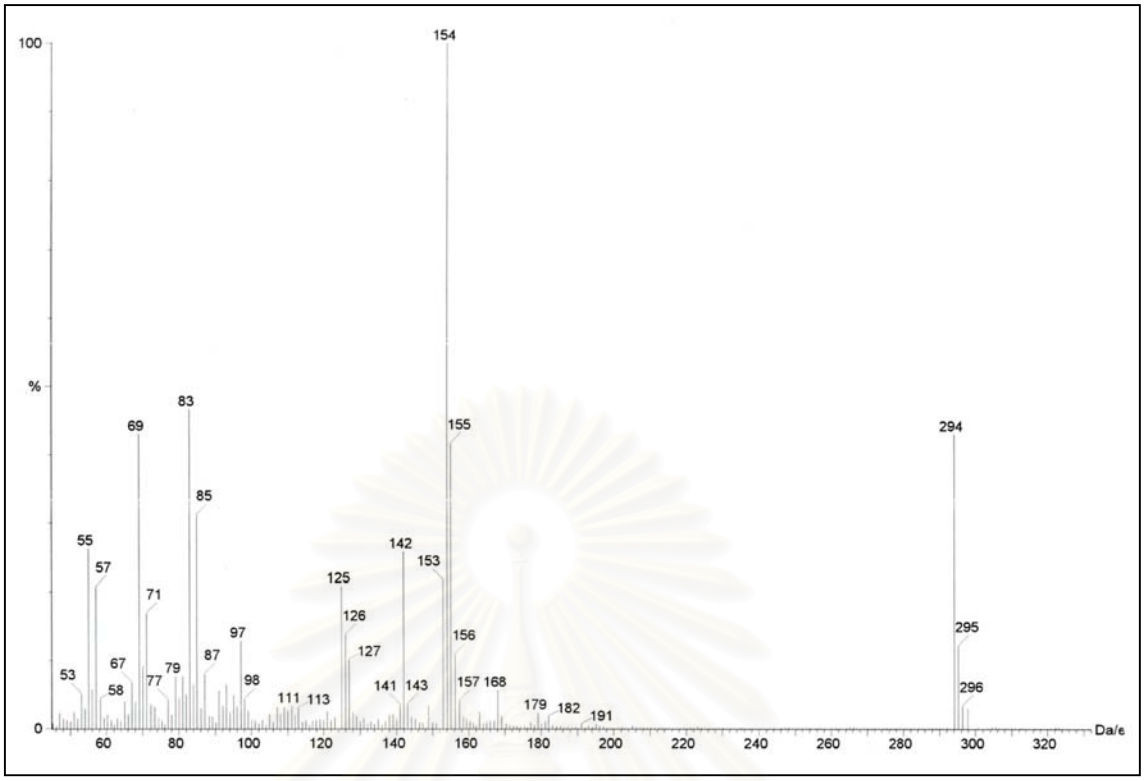
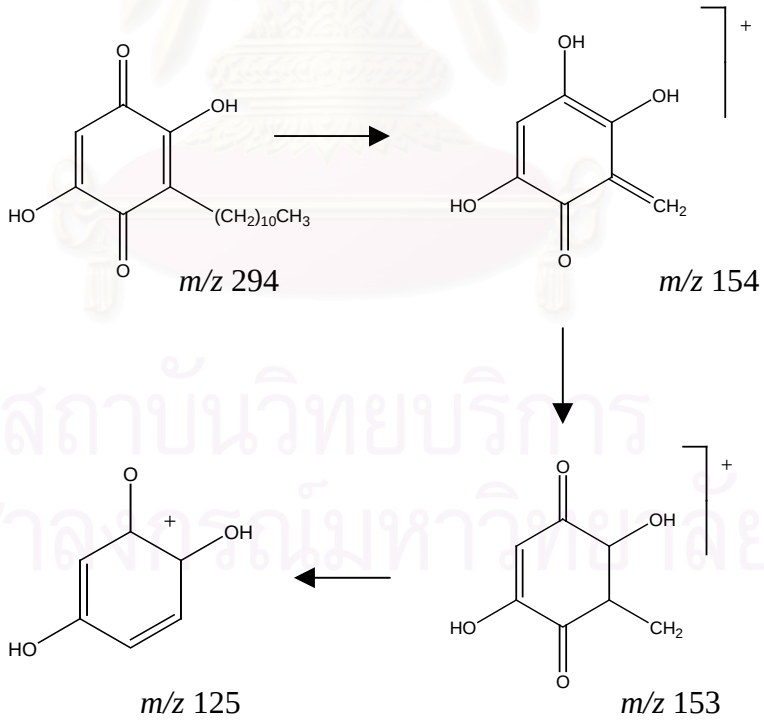


Figure 3.4 The mass spectrum of Compound 1



Scheme 3.2 The possible mass fragmentation pattern of Compound 1[22]

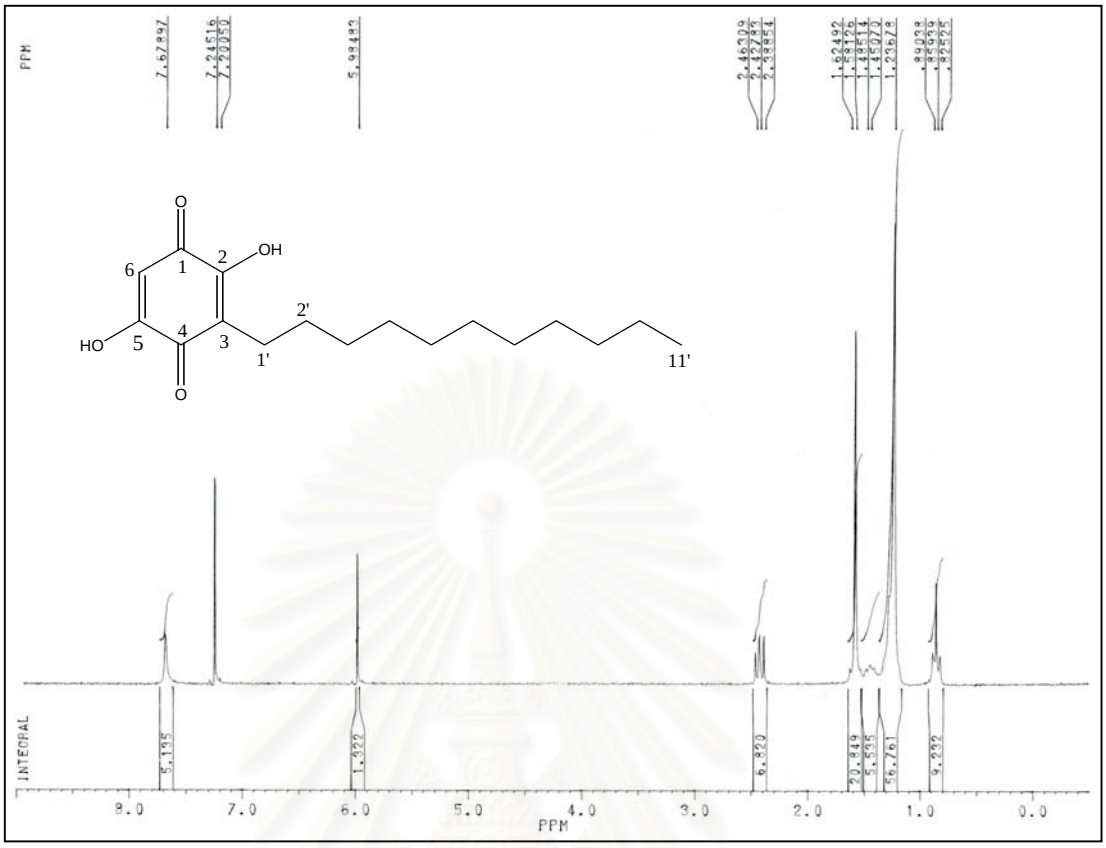


Figure 3.5 The ^{1}H NMR spectrum of Compound 1

Table 3.3 The ^{1}H NMR chemical shift assignment of Compound 1 compared with embelin[23]

Position	Chemical shift (ppm)	
	Compound 1	**Embelin**
-OH	7.67 (br, 2H)	7.72 (br, 2H)
H-6	5.98 (s, 1H)	6.01 (s, 1H)
H-1′	2.43 (t, 2H, *J* = 7.05 Hz)	2.45 (t, 2H, *J* = 7.5 Hz)
H-2′	1.48 (t, 2H, *J* = 6.89 Hz)	1.47 (m, 2H)
H-3′-H-10′	1.24 (overlapping, 16H)	1.22 (overlapping, 16H)
H-11′	0.86 (t, 3H, *J* = 6.20 Hz)	0.88 (t, 3H, *J* = 7.0 Hz)

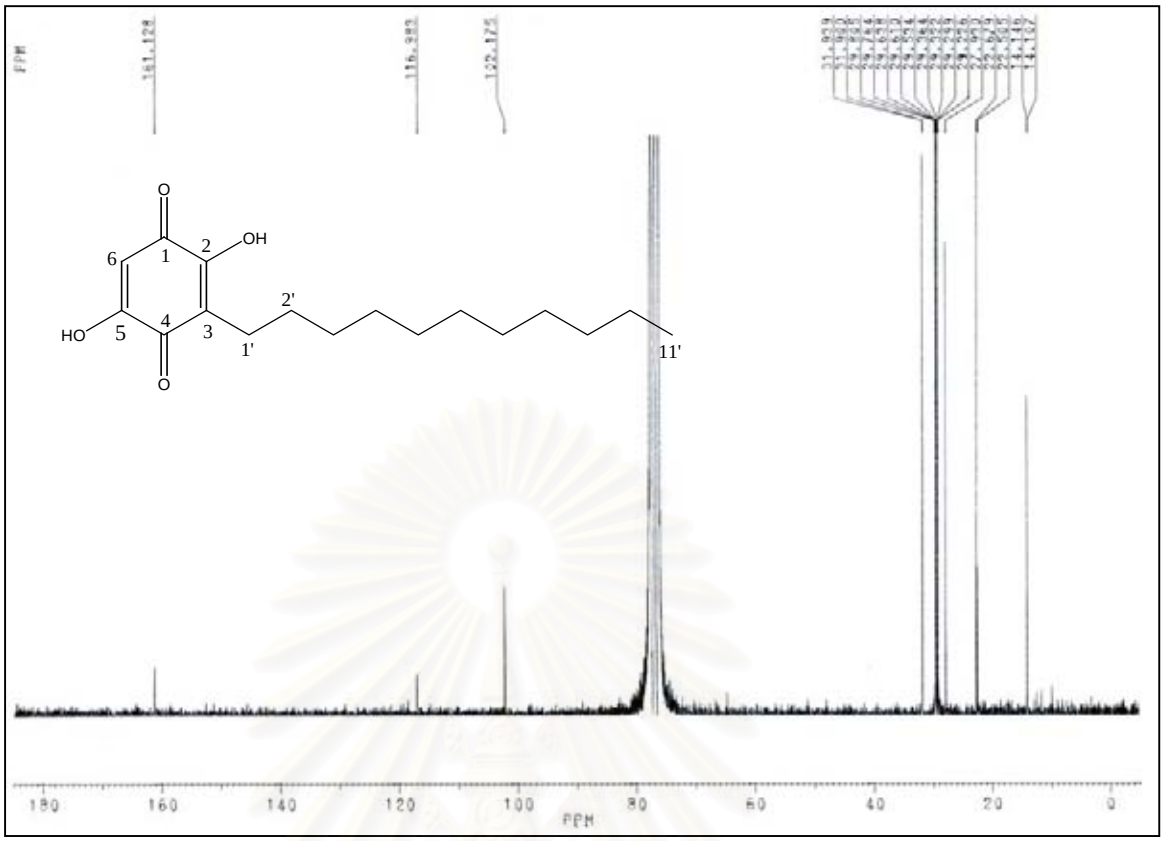
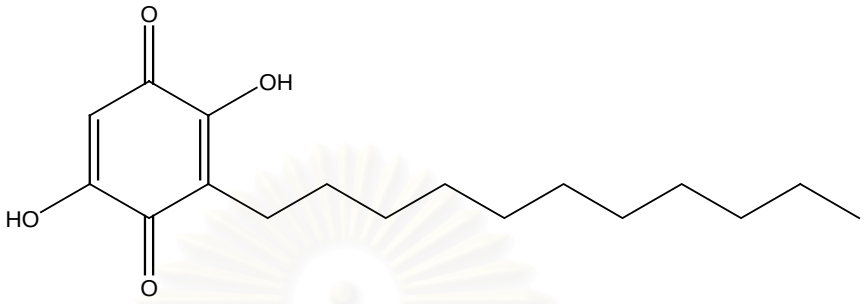


Figure 3.6 The ^{13}C NMR spectrum of Compound 1

Table 3.4 The ^{13}C NMR chemical shift assignments of Compound 1 and embelin[23]

Position	Chemical shift (ppm)	
	Compound 1	Embelin
1,2,4,5	161.1	170.0
3	102.2	103.5
6	116.1	117.5
1′	22.5	22.3
2′-10′	22.6 – 31.9	27.8 - 29.3
11′	14.1	14.0

In distinction to all spectroscopic evidences and physical properties compared with those reported in literature, Compound 1 was confident to conclude its structure as 2,5-dihydroxy-3-undecyl-1,4-benzoquinone (embelin).



Compound 1 : 2,5-dihydroxy-3-undecyl-1,4-benzoquinone

According to previous reports concerning with chemical constituents determination of *Aridisia colorata* Roxb. Luanratana *et al.* claimed that the barks and fruits of this plant contained a dihydroxybenzoquinone, 2,5-dihydroxy-3-tridecyl-1,4-benzoquinone or rapanone, as the major component.[9] Nevertheless, endorsing with various spectroscopic information this present examination manifestly demonstrated that the major content was not rapanone, but was 2,5-dihydroxy-3-undecyl-1,4-benzoquinone or embelin. The difference of these two structures: rapanone and embelin was the number of carbon atoms containing in saturated aliphatic side chain The methylene group on the side chain of embelin is less than that of rapanone by two methylene groups, 11 and 13 atoms, respectively.

As aforementioned discussion, the mass spectrum and the ^{13}C NMR spectrum of Compound 1 that revealed the molecular ion peak at m/z 294 and the 11 carbon signals of saturated side chain this molecule, respectively. These informative data strongly supported the occurrence of embelin as a major constituent in this particular plant.

3.4 Solvent Variation in Embelin Extraction Procedure

The search for appropriate solvents for embelin extraction procedure was attempted. In section 3.3, embelin was deposited upon the evaporation of solvent after soaking dry fruit material with dichloromethane. Consequently, other solvents (1000 mL) including diethyl ether, ethyl acetate and acetone were employed to examine whether it could be used in place of dichloromethane. The general protocol was as follows: 300 g of dry fruit materials was extracted with selected solvent under refluxing for 2 hours. The acquired quantity of embelin from extraction with each solvent is summarized as shown in Table 3.5.

Table 3.5 Weight and percentage yield of embelin from the extraction with selected solvents

Solvent	Weight (g) of embelin	Percentage yield
Diethyl ether	0.030	0.010
Dichloromethane	0.048	0.016
Ethyl acetate	0.015	0.005
Acetone	-	-

The extraction result showed that embelin could be extracted with non-polar solvents. The extraction of *Ardisia colorata* Roxb. fruits with dichloromethane gave the best percentage yield among all selected solvents.

3.5 Separation of Dichloromethane Extract (Fraction I)

Fraction I, the dichloromethane extract was obtained as a dark brown material 114 g (2.28% w/w of dried fruit materials). From thin-layer chromatography, it was demonstrated that at least six components (solvent system: 30% ethyl acetate in hexane) were present in this fraction. A portion of crude (100 g) was chromatographed on silica gel column chromatography to separate Fraction I into small fractions according to their polarity, using gradient elution started from *n*-hexane followed by a mixture of *n*-hexane:ethyl acetate, ethyl acetate and ethyl acetate:methanol, respectively. The eluted solution was collected approximately 500 mL. Monitoring each fraction by thin-layer chromatography technique, the fractions

that demonstrated similar features were combined. The results of separation Fraction I is presented as shown in Table 3.6.

Table 3.6 The separation of dichloromethane extract by silica gel column chromatography

Fraction	Solvent system	Remarks	Weight (g)
IA	Hexane, 20%EtOAc:Hexane	Yellow oil	6.81
IB	30%EtOAc:Hexane	Sticky brown material	17.01
IC	50%EtOAc:Hexane	Dark brown semisolid	5.22
ID	75%EtOAc:Hexane	Dark brown semisolid	4.45
IE	EtOAc	Dark brown semisolid	11.38
IF	30%MeOH:EtOAc	Dark brown semisolid	19.68
IG	50%MeOH:EtOAc	Sticky dark brown material	12.54

3.5.1 Separation of Fraction IA

Thin-layer chromatography of Fraction IA using 30% ethyl acetate-hexane as a developing solvent demonstrated that there were at least 2 components in Fraction IA. This fraction was further subjected to silica gel column chromatography. Hexane, a mixture of ethyl acetate and hexane, and ethyl acetate were used as eluents. The eluted solution was collected about 50 mL for each fraction and monitored by TLC. The results of the separation of Fraction IA are presented in Table 3.7.

Table 3.7 The results of the separation of Fraction IA

Eluents	Fraction no.	Remarks	Weight (g)
Hexane	1-5	White solid in yellow oil, Substance 2	0.98
3%EtOAc:Hexane	6-12	Yellow liquid, Mixture 3	3.24
10%EtOAc:Hexane	13-21	Sticky brown material	1.14
30%EtOAc:Hexane	22-30	Sticky brown material	1.05

From the results of separation, Fraction no. 1-5 was purified by recrystallization with hot ethyl acetate several times to afford bright white plates (21.5 mg) designated as MIxture 2. In addition, according to TLC monitoring of Fraction no. 6-12, one spot with tail was clearly detected. This fraction was then purified by flash column chromatography. The yellow semisolid (2.5 g) designated as Mixture 3 was received.

3.5.1.1 Structural Elucidation of Mixture 2

Mixture 2 (21.5 mg, 0.0245 % w/w of dichloromethane crude extract) had R_f 0.80 (40% EtOAc:Hexane), m.p. 51-53 °C soluble in dichloromethane, chloroform and methanol; slightly soluble in hexane and ethyl acetate. The IR spectrum of this mixture (Figure 3.7) displayed the characteristic absorption peak of hydroxyl group at 3446 cm^{-1}. Other signals were tentatively assigned as shown in Table 3.8.

The ^1H NMR spectrum of Mixture 2 (Figure 3.8) showed signals of terminal methyl proton at δ 0.86 ppm, methylene proton at δ 1.24 ppm and methylene protons which directly connected to oxygen atom at δ 1.55 ppm.

Table 3.8 The IR absorption band assignments of Mixture 2

Wave number (cm^{-1})	Intensity	Tentative assignment
3446	Broad	O-H stretching vibration of hydroxyl group
2909,2842	Strong	C-H stretching vibration of CH_2 and CH_3
1465	Medium	C-H bending vibration of CH_2

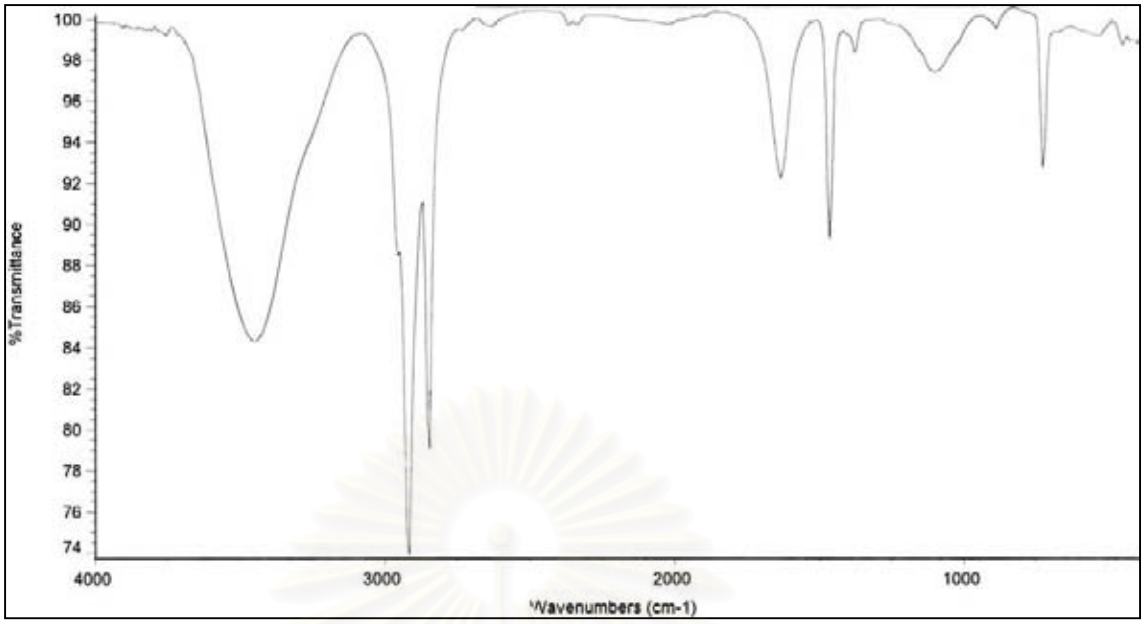


Figure 3.7 The IR spectrum of Mixture 2

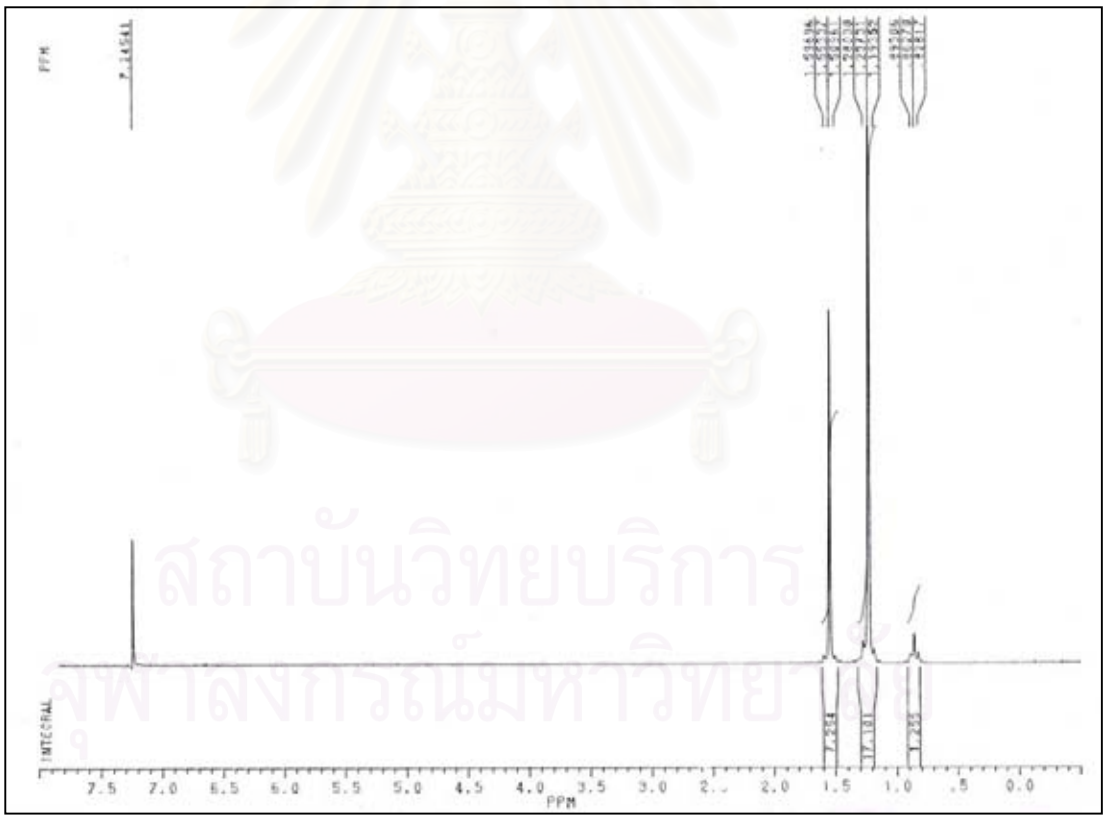


Figure 3.8 The ^{1}H NMR spectrum of Mixture 2

From physical properties and all spectroscopic data, it could be obviously concluded that this substance was a mixture of long chain saturated alcohols.

$$CH_3\text{-}(CH_2)_n\text{-}OH$$

Mixture 2

3.5.1.2 Structural Elucidation of Mixture 3

Mixture 3, the yellow semisolid (2.5 g, 2.85% w/w of crude extract) had R_f 0.62 (10% EtAOc:Hexane). It was soluble in ethyl acetate, dichloromethane, chloroform and methanol but slightly soluble in hexane. This mixture showed green-blue solution with Liebermann Burchard's reagent expressing that this mixture contained a steroidal nuclei.

The IR spectrum of this mixture (Figure 3.9) showed a major characteristic absorption band of carbonyl group belonging to an ester at 1743 cm^{-1}. Other signals were tentatively assigned as shown in Table 3.9.

Table 3.9 The IR absorption band assignments of Mixture 3

Wave number (cm^{-1})	Intensity	Tentative assignment
2921, 2846	Medium	C-H stretching vibration of CH_2 and CH_3
1743	Strong	C=O stretching vibration of ester
1461	Medium	C-H bending vibration of CH_2
1376	Weak	C-H bending vibration of CH_3
1160	Strong	C-O stretching vibration
723	Weak	CH_2 rocking vibration

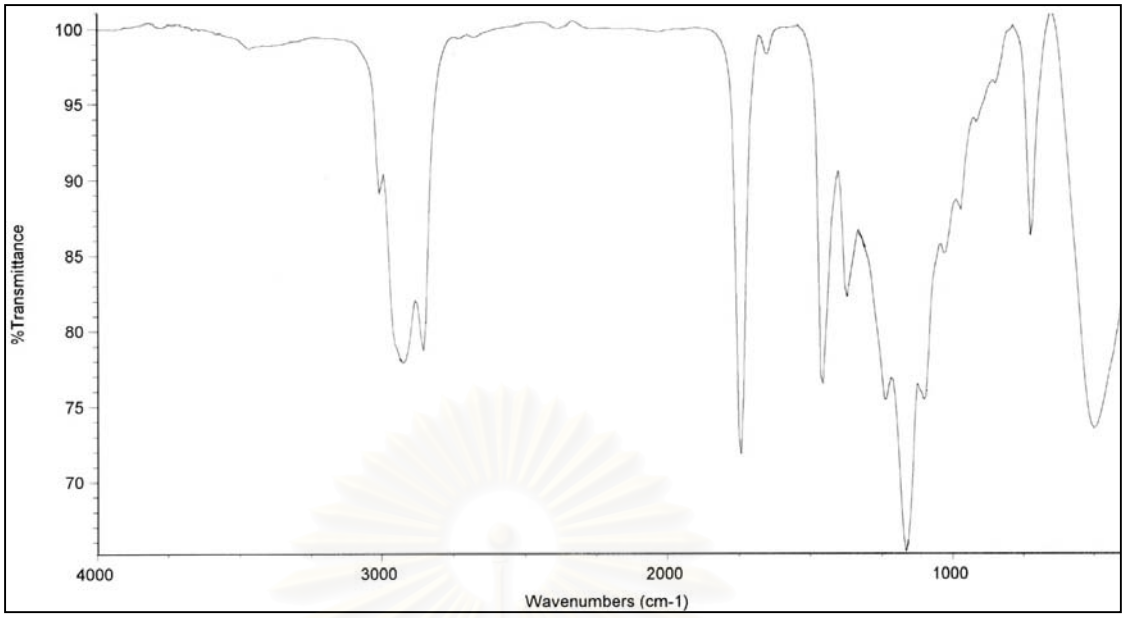


Figure 3.9 The IR spectrum of Mixture 3

From the physical properties and all spectroscopic data, this mixture could be concluded as a steroidal ester. Therefore, the hydrolysis reaction was set up for further identification of this mixture.

Hydrolysis of Mixture 3

A solution of 10% ethanolic KOH (40 mL) was added to Mixture 3 (2.0 g) and the solution was stirred under refluxing on a water bath for 4 hours. Evaporation of ethanol gave a solid, which was further extracted with diethyl ether 150 mL three times. The combined diethyl ether was dried over anhydrous sodium sulfate. Evaporation of the solvent furnished a solid with yellow oil. After recrystallization with a mixture of hexane-ethyl acetate several times, white amorphous solid designated as Mixture 3A (18.4 mg), m.p. 146-147 °C was received.

Study on Mixture 3A

Mixture 3A was thought to be a mixture of steroids which were generally detected as constituents in plants. Thus, this mixture was *co*-TLC with a mixture of authentic steroids, namely campesterol, stigmasterol and β-sitosterol, previously isolated and characterized from *Rhizophora apicalata* Bl.[18] The same R_f values on TLC plates suggested that Mixture 3A be steroidal compounds. Further study was

carried on utilizing GC compared with authentic steroids, namely cholesterol, campesterol, stigmasterol and β-sitosterol. The results of GC analysis (Figure 3.10) of this mixture showed the retention times at 28.16 and 31.91 min, respectively corresponding to those of stigmasterol and β-sitosterol, respectively. The composition of steroids in this mixture is presented in Table 3.10.

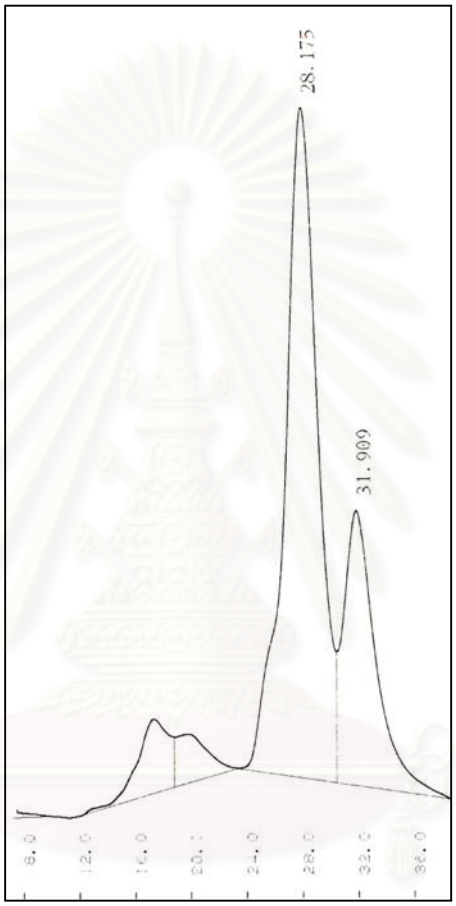
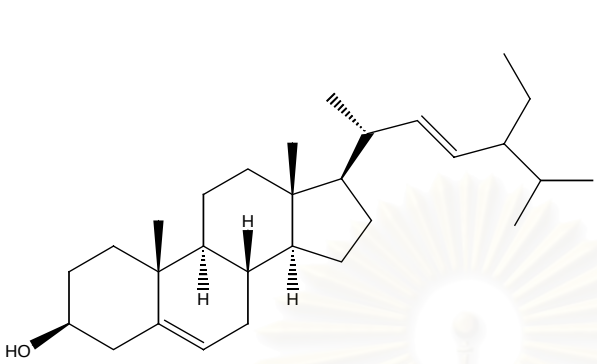


Figure 3.10 The chromatogram of Mixture 3A from GC analysis

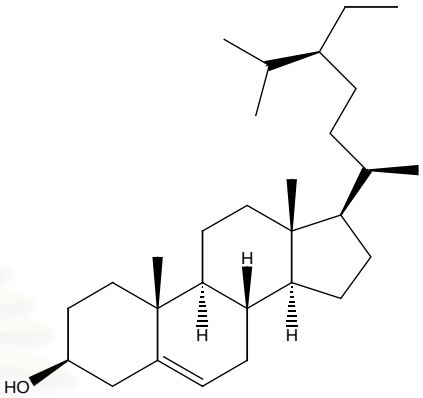
Table 3.10 The composition of steroids in Mixture 3A

Name	Retention time (min)	% Composition
Stigmasterol	28.16	71
β-sitosterol	31.91	28

Based upon the color test result, physical properties and all spectroscopic data including GC analysis, it could be concluded that Mixture 3A is a mixture of stigmasterol and β-sitosterol.



Stigmasterol



β-Sitosterol

Study on Mixture 3B

Mixture 3B is an acid part from the hydrolysis of Mixture 3. This mixture was purified with column chromatography using gradient elution of *n*-hexane and ethyl acetate as solvent system. Methylation of this mixture by tetramethylsilyl diazomethane followed the method described in section 2.6.3 led to the formation of methyl ester of Mixture 3B. The methyl ester derivative was further examined by GC-MS analysis without further purification. The gas chromatography was carried out on DB-35 column. The chromatogram of this derivative is exhibited as shown in Figure 3.11.

The component with retention time at 11.99 min was found to be a major component in this mixture. The mass spectrum of this component gave the molecular ion peak, M^+, at m/z 270. Compared with library data (NIST database), it was found that this major composition was corresponding to hexadecanoic acid methyl ester (Calcd. for $C_{17}H_{34}O_2$: MW 270.45). The mass spectrum of this component and library data of hexadecanoic acid (palmitic acid) methyl ester is shown in Figure 3.12.

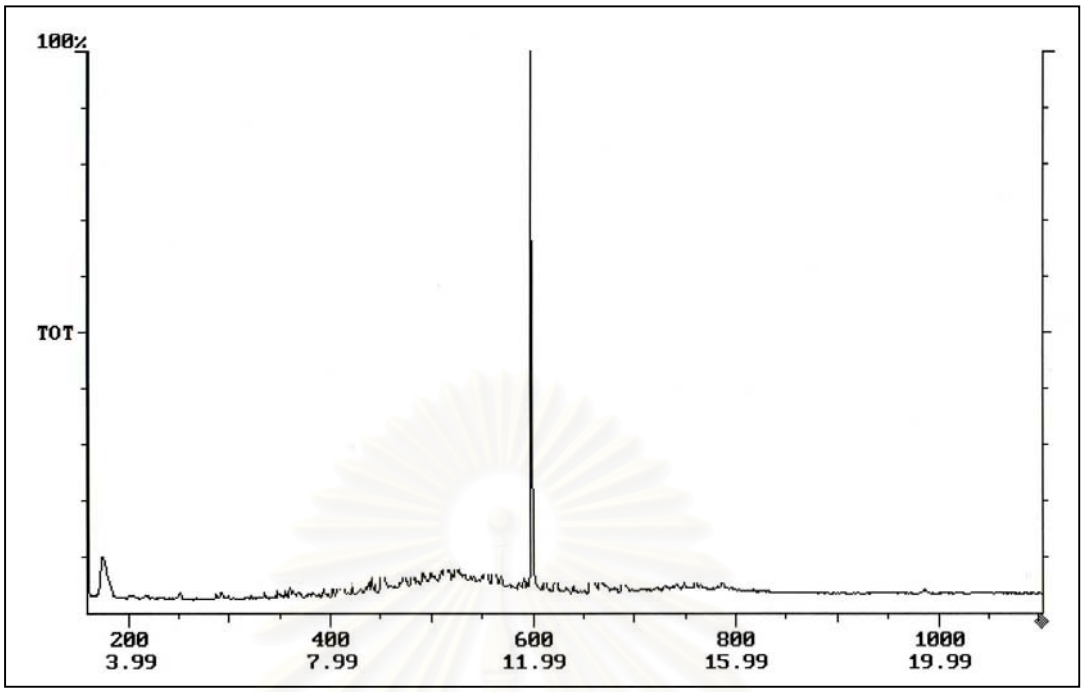
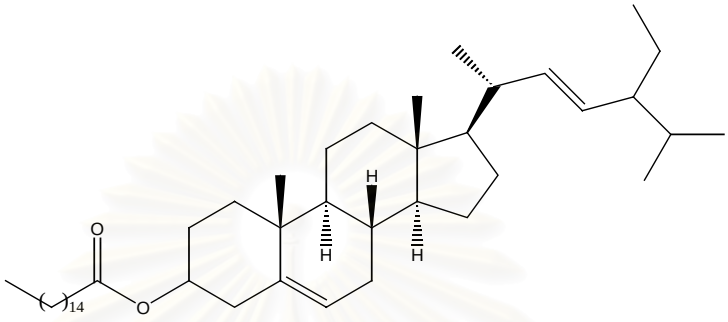


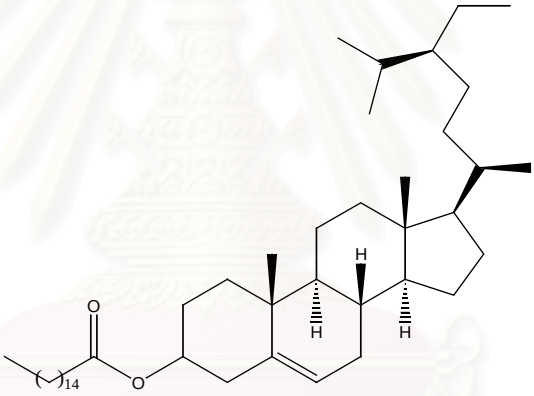
Figure 3.11 The chromatogram of Mixture 3B methyl ester

Figure 3.12 The mass spectrum of the major component (R_t = 11.99 min) of Mixture 3B

With the aids of the chemical reaction together with spectroscopic data and GC-MS analysis, it could be obviously concluded that Mixture 3 was stigmasteryl-3-*O*-palmitate and β-sitosteryl-3-*O*-palmitate.



Stigmasteryl-3-*O*-palmitate



β-Sitosteryl-3-*O*-palmitate

3.5.2 Separation of Fraction IB

The TLC of Fraction IB in 30% ethyl avetate:hexane revealed a spot identical with the authentic sample of α-amyrin or β-amyrin. Thus, Fraction IB was further separated with silica gel column, and the result of the separation of Fraction IB is presented in Table 3.11. The gradient elution of hexane and ethyl acetate was used. Each fraction was monitored by thin-layer chromatography. According to the TLC results, the fraction which exhibited the same spot as authentic sample was Fraction IB.2. Therefore, Fraction IB.2 was further purified by silica gel column. The obtained solid was further recrystallized with hot ethanol several times to yield white needle crystal (59.6 mg) specified as Mixture 4.

Table 3.11 The results of the separation of Fraction IB

Eluents	Fraction	Remarks	Weight (g)
10% EtOAc:hexane	IB.1	Yellow oil	0.52
15% EtOAc:hexane	IB.2	White crystal in yellow solution; Mixture 4	0.95
30% EtOAc:hexane	IB.3	Sticky dark yellow material	1.78
60% EtOAc:hexane	IB.4	Sticky brown material	2.05

3.5.2.1 Structural Elucidation of Mixture 4

Mixture 4, white needle crystal 59.6 mg (0.0679% w/w of dichloromethane crude extract) R_f 0.62 (40% EtOAc:hexane) melted at 189-190 °C. This compound was soluble in chloroform, dichloromethane and ethyl acetate. From the Liebermann-Burchad's test, Mixture 4 provided a red solution, suggesting the presence of triterpenoidal nuclei.

This mixture was subjected to GC-MS analysis to explore the mixture components using DB-35 column. The chromatogram of Mixture 4 revealed that there were 3 components present as shown in Figure 3.13.

The results of GC-MS analysis of this mixture revealed the retention times at 17.96, 19.56 and 20.66 min, respectively. The major component was component 3 with retention time at 20.66 min. The mass spectrum of each component is exhibited as shown in Figures 3.14 – 3.16.

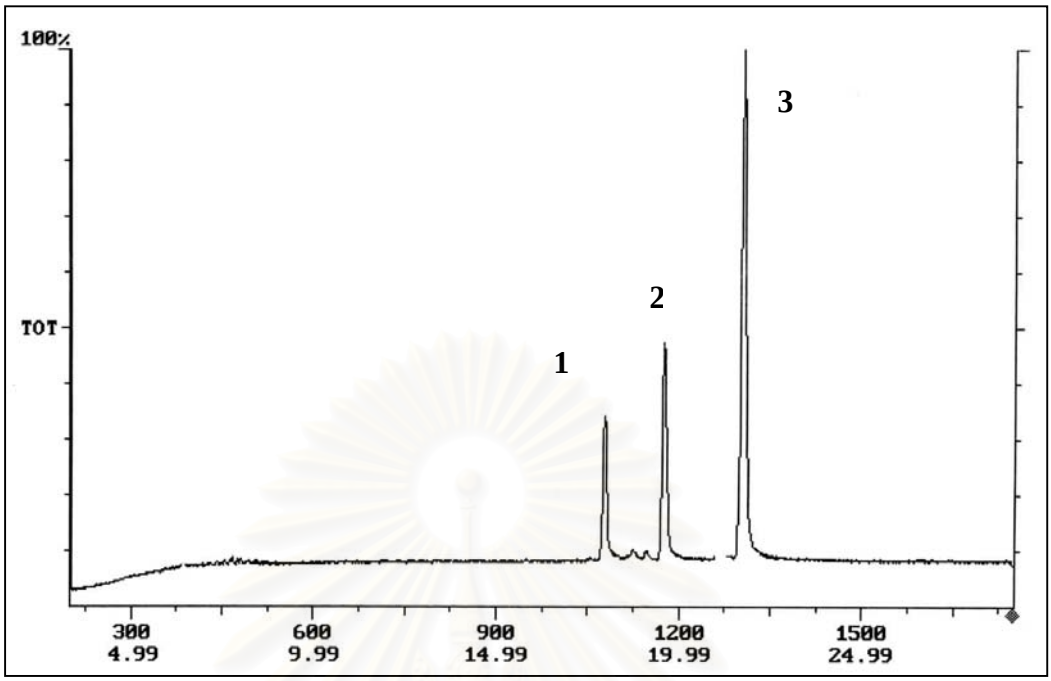


Figure 3.13 The chromatogram of Mixture 4 from GC analysis

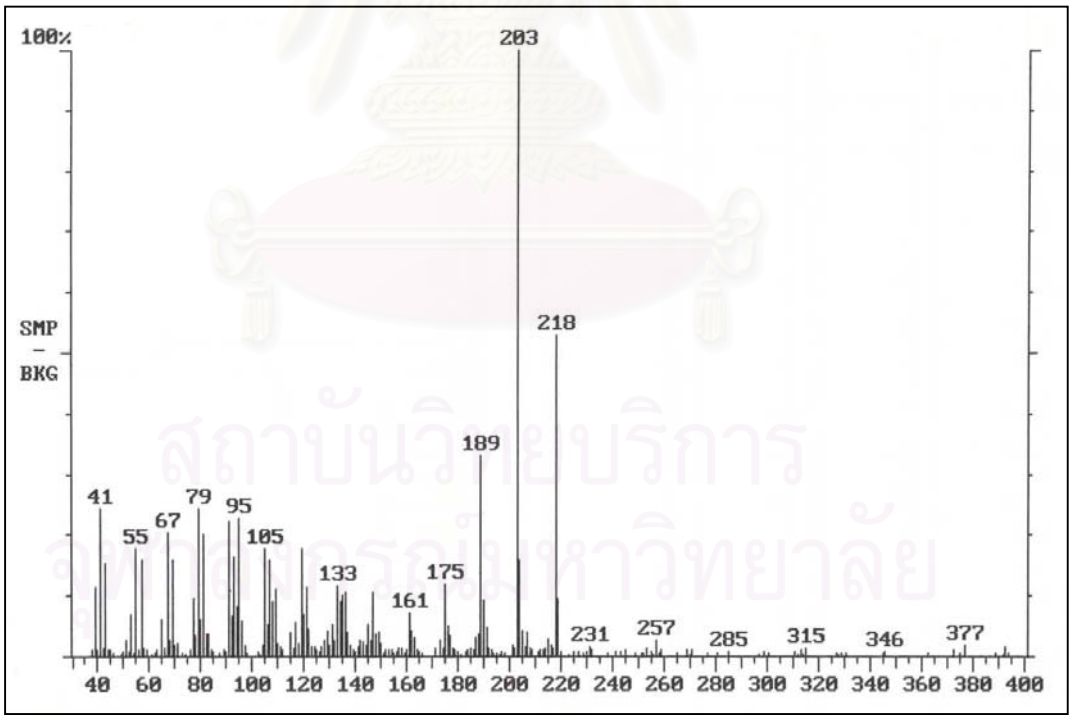


Figure 3.14 The mass spectrum of component 1 (Rt 17.96 min) of Mixture 4

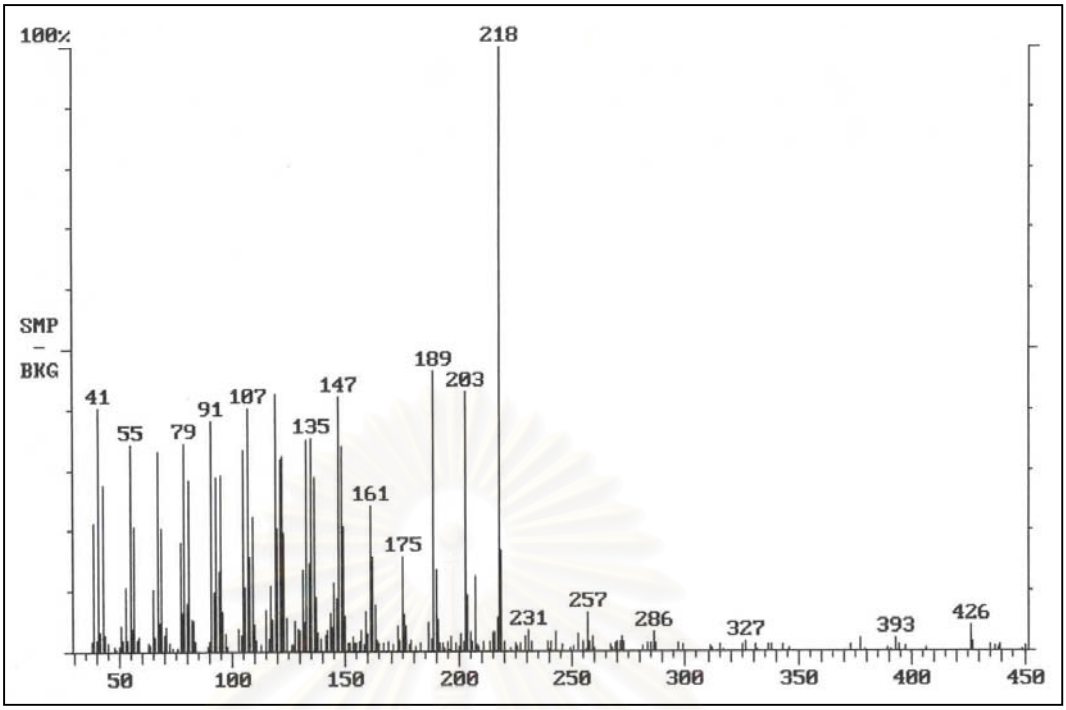


Figure 3.15 The mass spectrum of component 2 (Rt 19.56 min) of Mixture 4

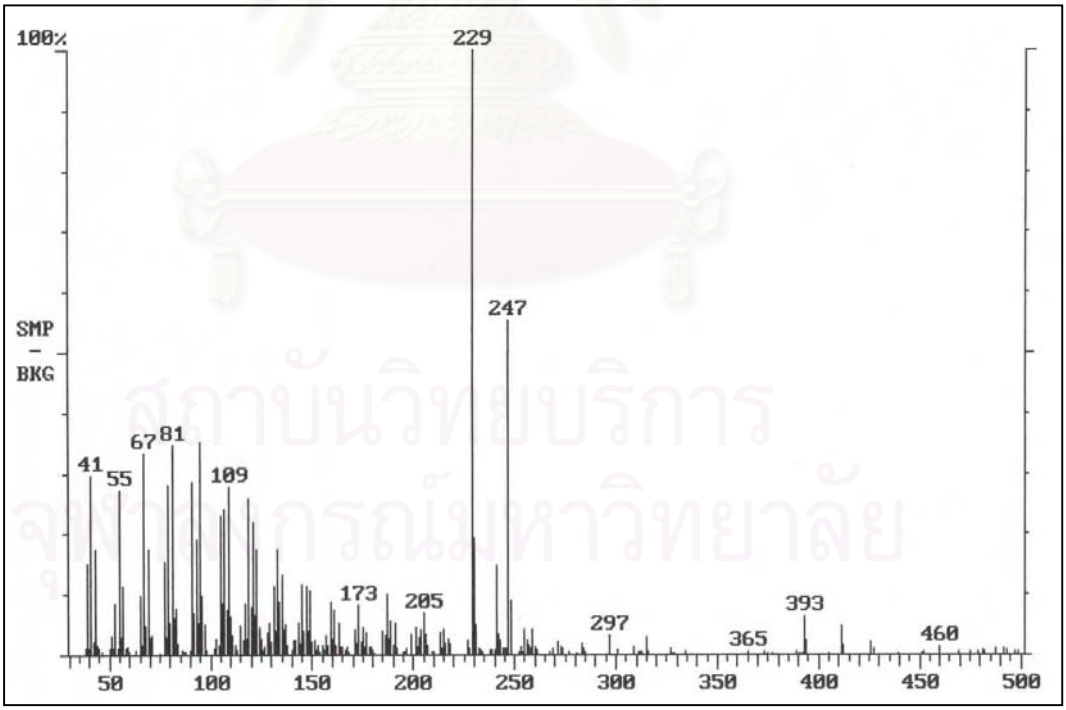


Figure 3.16 The mass spectrum of component 3 (Rt 20.66 min) of Mixture 4

Table 3.12 The composition of Mixture 4

Component	Retention time (min)	Mass	% Composition
1	17.96	346	17
2	19.56	426	21
3	21.73	460	62

From the comparison of the mass spectrum of each component with NIST data library, it was found that the first component is possibly 2(1H)Napthalenone. The second component with *m/z* at 426 was equivalent to $C_{30}H_{50}O$ (MW 426.72). The third component which showed *m/z* at 460, was perhaps an impurity that would not be considered in the meanwhile.

The spectroscopic study including IR and ^{1}H NMR for this mixture was performed as shown in Figures 3.17-3.18.

The IR spectrum of this mixture (Figure 3.17) represented the characteristic absorption band of hydroxyl group at 3248 cm^{-1}, C-H stretching at 2940 and 2856 cm^{-1} and C-O stretching at 1037 cm^{-1}. The ^{1}H NMR spectrum (Figure 3.18) exhibited the methylene proton signal at 0.72-2.16 ppm, carbinol proton (H-3) at 3.21 ppm and olefinic proton (H-12) at 5.16 ppm. The mass spectrum of this mixture was compared with that of α-amyrin from NIST data library as shown in Figure 3.19 and the possible fragmentation[18] is shown in Scheme 3.3.

Considering between α-amyrin and β-amyrin, these two triterpenoids gave closely characteristic peaks on mass spectrum. Therefore, the structure of second component from Mixture 4 was ensured by the comparison their m.p. and the ^{13}C NMR signals C-12 and C-13. The comparison data of Mixture 4, α-amyrin[24] and β-amyrin[25] are presented as shown in Table 3.13.

Table 3.13 The comparison data of α-amyrin and β-amyrin and Mixture 4

Triterpenoids	m.p. (°C)	^{13}C NMR chemical shift (ppm)	
		C-12	C-13
α-amyrin	186.0	124.3	139.3
β-amyrin	197.0-197.5	121.7	145.2
Mixture 4	189.0-190.0	124.6	138.9

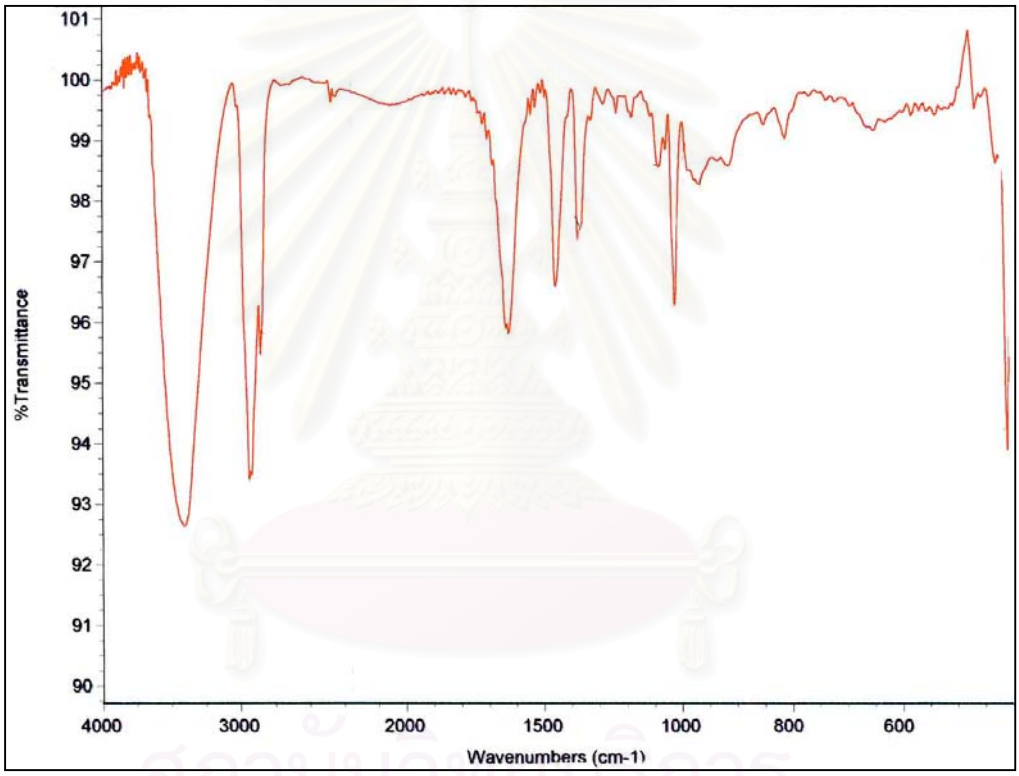


Figure 3.17 The IR spectrum of Mixture 4

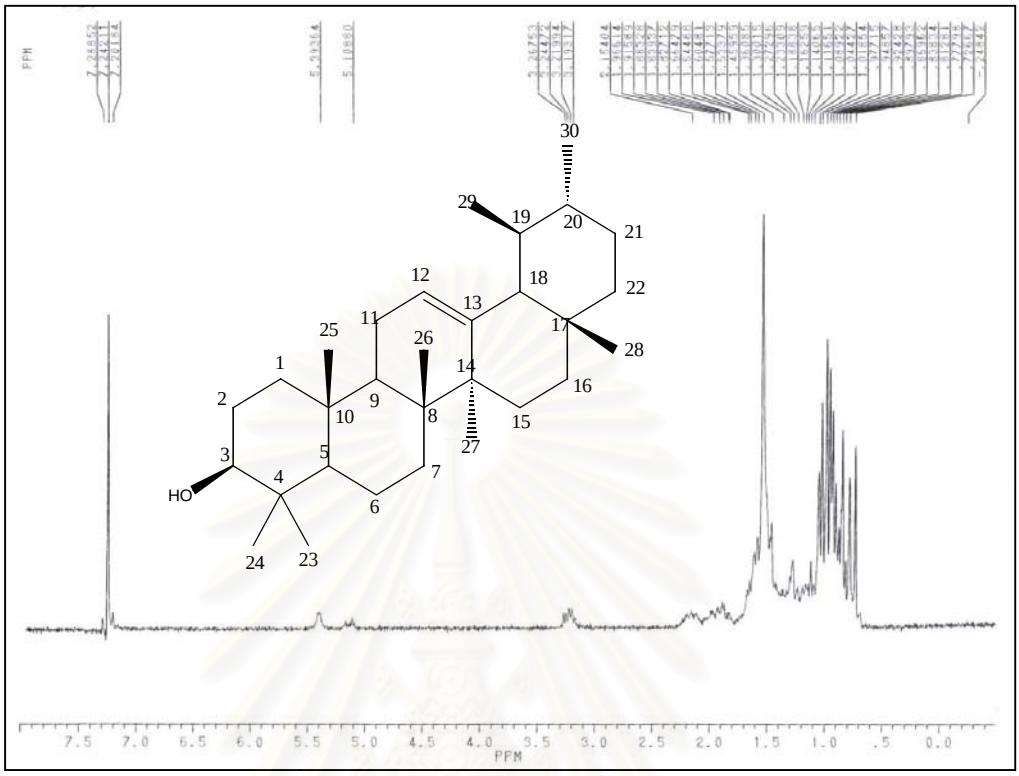


Figure 3.18 The ^{1}H NMR spectrum of Mixture 4

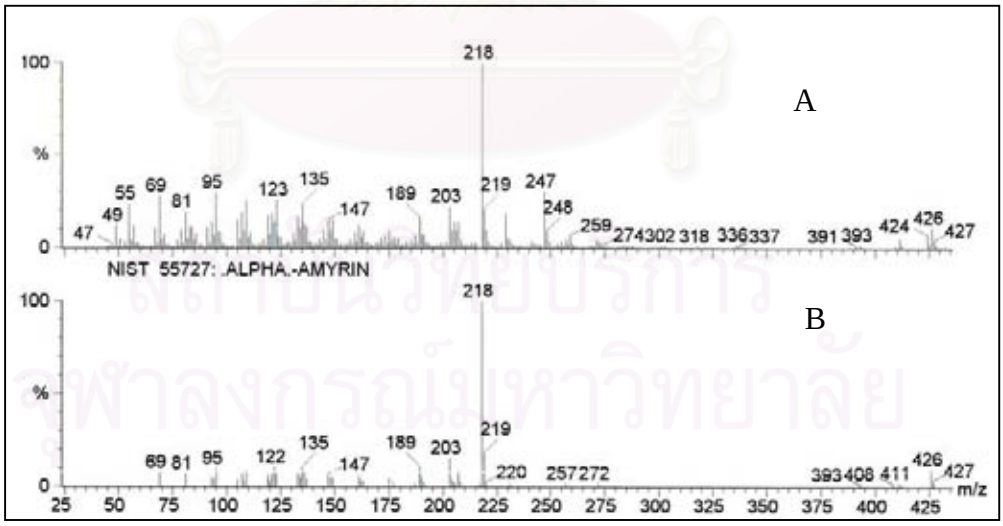
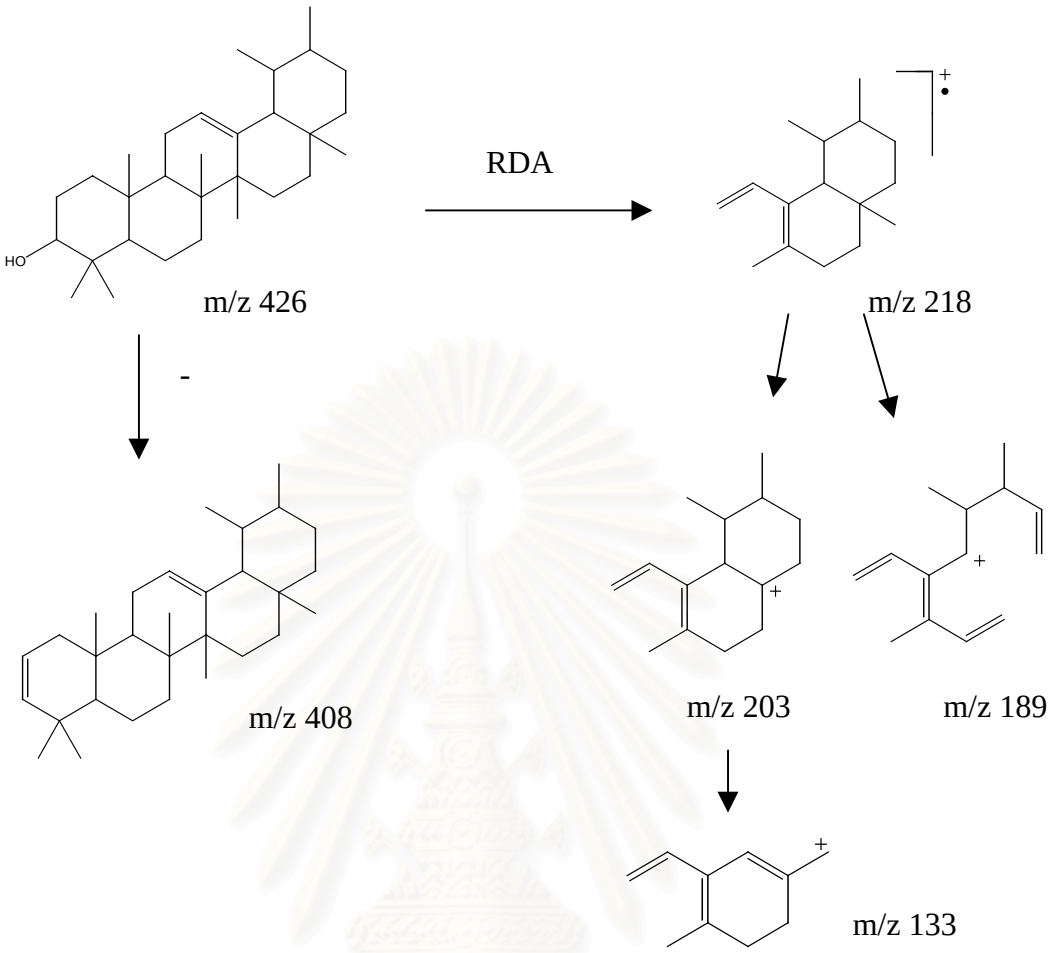
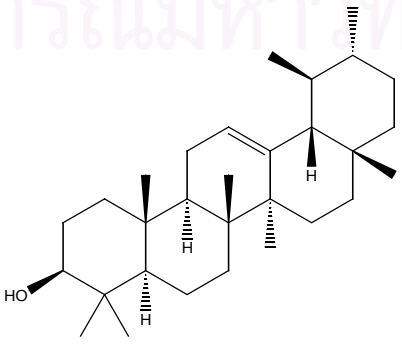


Figure 3.19 The mass spectrum of Mixture 4 (A) and α-amyrin (B)



Scheme 3.3 The possible mass fragmentation pattern of component 2 (α-amyrin) in Mixture 4[18]

From all spectroscopic evidences, physical properties and GC-MS analysis compared with literatures, it could be obviously concluded that the second component of Mixture 4 is α-amyrin.



The second component of Mixture 4; α-amyrin

3.5.3 Separation of Fractions IC and ID

According to the monitoring of Fractions IC and ID with TLC (solvent system: 30% ethyl acetate:hexane), these two fractions displayed similar pattern. Hence, Fractions IC and ID were combined and renamed as Fraction ICD. Fraction ICD was further separated with silica gel column using gradient elution system of hexane, a mixture of hexane:ethyl acetate, ethyl acetate and a mixture of ethyl acetate:methanol. Each fraction was collected approximately 50 mL, and was monitored by thin-layer chromatography technique. The fractions demonstrated similar pattern on TLC plates were combined. The results of the separation of Fraction ICD are presented as shown in Table 3.14.

Table 3.14 The results of the separation of Fraction ICD

Eluents	Fraction No.	Remarks	Weight (g)
Hexane	1-3 (ICD1)	Yellow oil	0.06
5%-10% EtOAc:hexane	4-10 (ICD2)	White solid in brown oil	1.08
20%-50% EtOAc:hexane	11-13 (ICD3)	Sticky brown material	1.54
60%-70% EtOAc:hexane	14-17 (ICD4)	Red-brown oil	1.02
70% EtOAc:hexane-EtOAc	18-25 (ICD5)	Dark brown semisolid	1.57
20% MeOH:EtOAc	26-35 (ICD6)	Dark brown semisolid	2.41

Fraction 4-10 coded as ICD2, was the white solid deposited in brown oil when it was stand at room temperature for a moment. This fraction was further purified by separation with silica gel column. Similarly, Fraction 14-17 coded as ICD4 were red-brown oil that showed two spots on TLC.

Separation of Fraction ICD2

Fraction ICD2 was chromatographed on silica gel column, using gradient elution of hexane and ethyl acetate. Each fraction was monitored with thin-layer chromatography, fraction which showed similar pattern was combined. Fraction 7-8 (see also Table 3.15) obliged white amorphous in solution. After filtration, the white amorphous was recrystallized by ethyl acetate for several times to yield off-white needle crystals (36.9 mg) designated as Compound 5.

Table 3.15 The results of the separation of Fraction ICD2

Eluents	Fraction No.	Remarks	Weight (g)
Hexane	1-2	Yellow oil	0.050
5% EtOAc:hexane	3-6	White amorphous in yellow oil	0.102
10% EtOAc:hexane	7-8	White amorphous in yellow oil; Compound 5	0.324
20% EtOAc:hexane	9-12	Sticky brown material	0.420

3.5.3.1 Structural Elucidation of Compound 5

The off-white needle crystal (36.9 mg, 0.0421 % w/w of dichloromethane crude extract) displayed a single spot on TLC at R_f 0.47 in 40% ethyl acetate:hexane. It was melted at 139-141 °C. This compound was soluble in chloroform, dichloromethane and ethyl acetate. Compound 5 showed a positive result with Liebermann-Burchard's reagent: it gave a blue-green solution. Therefore, this compound was presumed to contain a steroidal nuclei. According to the spectroscopic study, the IR spectrum (Figure 3.20) of this compound gave the absorption peaks of hydroxyl group at 3453 cm^{-1}, C=C stretching vibration at 1637 cm^{-1}. Other signals were tentatively assigned as shown in Table 3.16.

GC-MS analysis was selected to study the composition of this compound, using DB-35 column. Figure 3.21 presents the chromatogram of Compound 5. The major component with retention time at 6.56 min gave the molecular ion peak at *m/z* 412 (Figure 3.22). Compared with NIST data library, it was found that this compound was compatible with stigmasterol (Calcd for $C_{29}H_{48}O$: MW 412.69). The mass spectrum of this compound and library data of stigmasterol are shown in Figure 3.23. The possible mass fragmentation of this compound was proposed as shown in Scheme 3.4.

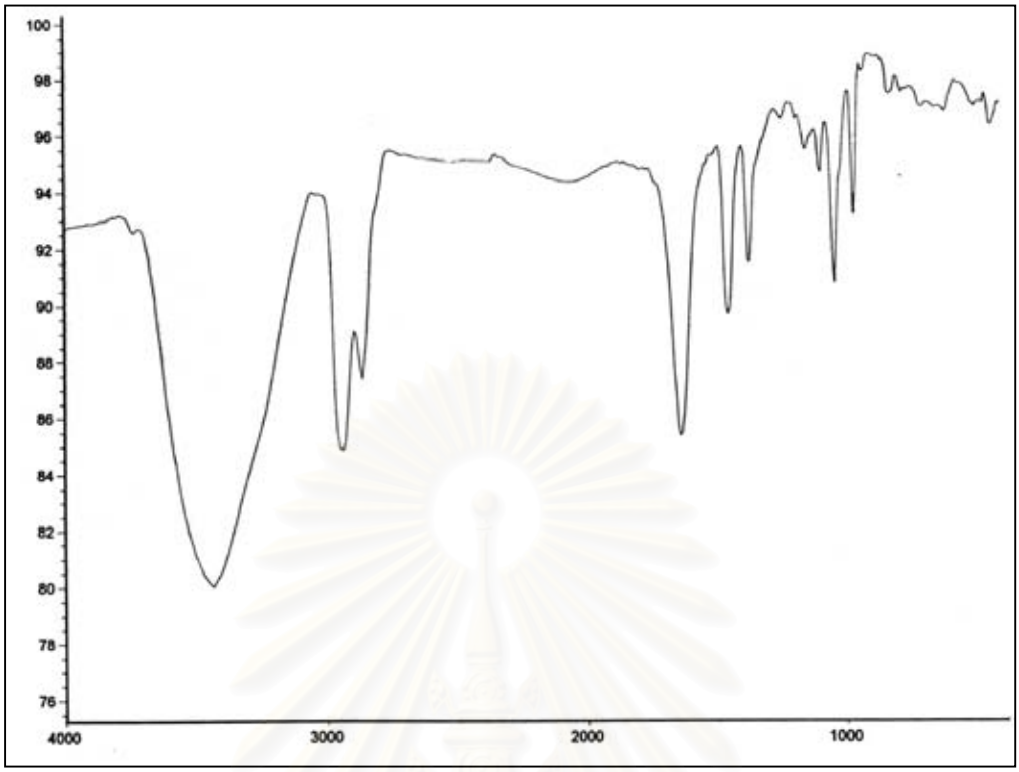


Figure 3.20 The IR spectrum of Compound 5

Table 3.16 The IR absorption band assignments of Compound 5

Wavenumber (cm^{-1})	Intensity	Tentative assignments
3435	Strong	O-H stretching vibration of hydroxyl group
2937, 2867	Medium	C-H stretching vibration of CH_2 and CH_3
1637	Medium	C=C stretching vibration
1458	Weak	C-H asymmetric bending of CH_2 and CH_3
1376	Weak	C-H symmetric bending of CH_3
1038	Weak	C-O stretching vibration

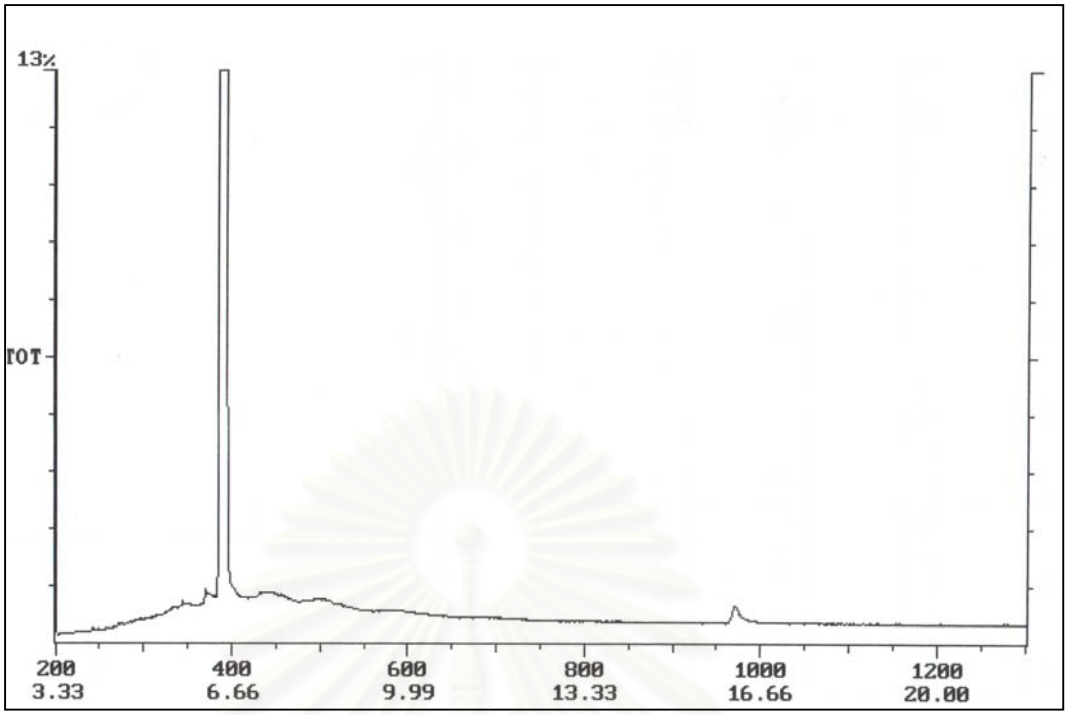


Figure 3.21 The chromatogram of Compound 5

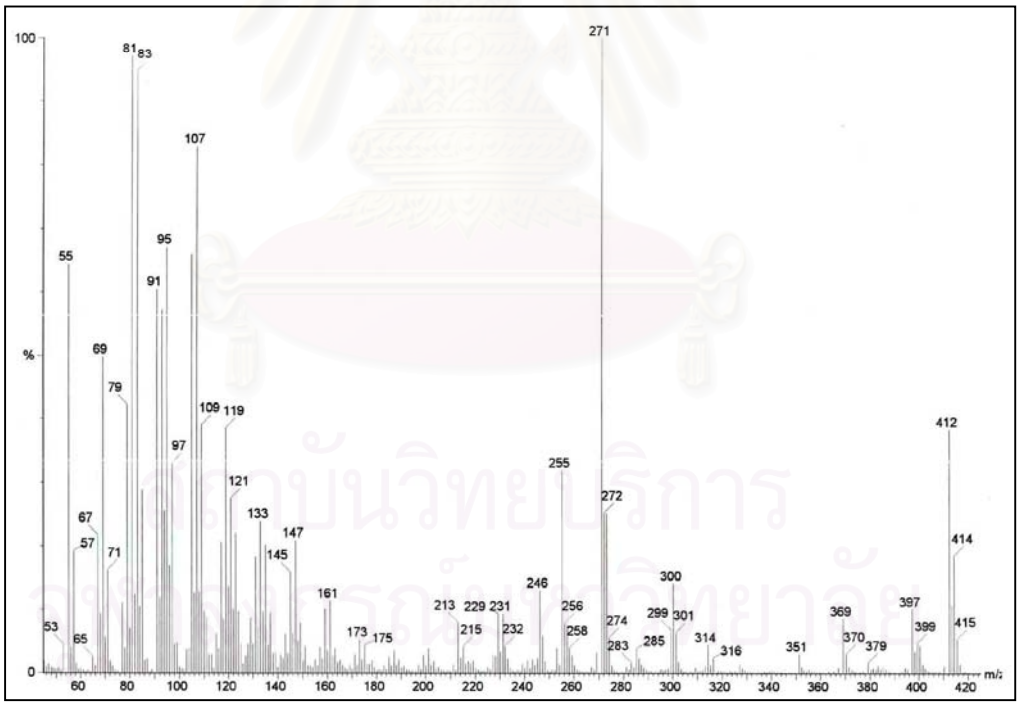


Figure 3.22 The mass spectrum of Compound 5

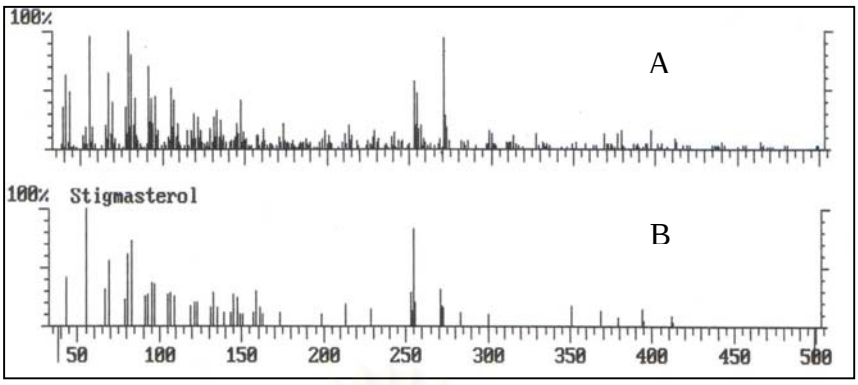
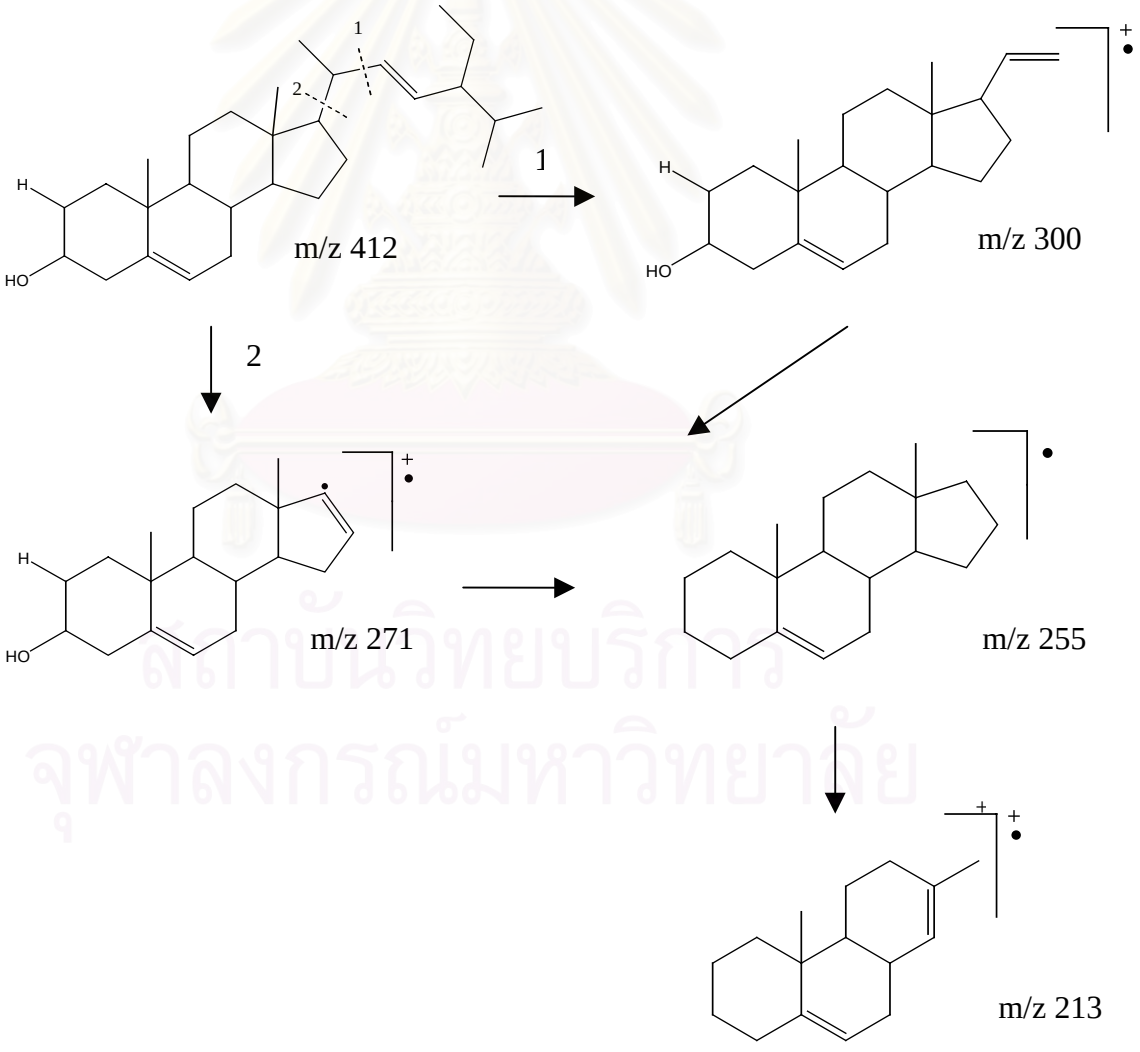
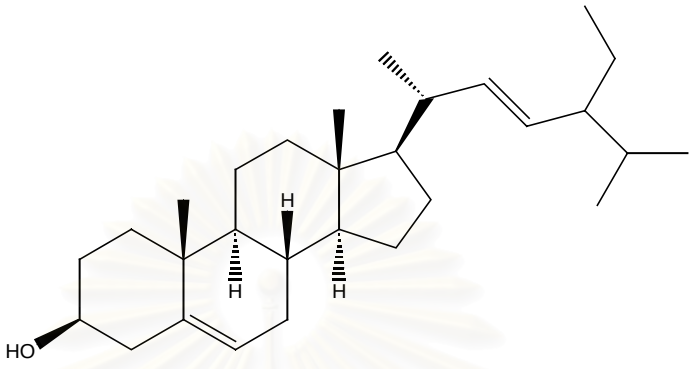


Figure 3.23 The mass spectrum of Compound 5 (A) and stigmasterol (B)



Scheme 3.4 The possible mass fragmentation pattern of Compound 5

As a result of its physical properties, GC-MS analysis and spectroscopic data, it could noticably be concluded that this compound was stigmasterol.



Compound 5: stigmasterol

Separation of Fraction ICD4

Fraction ICD4 containing red-brown oil was further separated by silica gel column. The gradient elution system of hexane, ethyl acetate and methanol was used.

In addition, from the DPPH screening for radical scavenging by TLC autographic assay, this fraction had at least 2 components that showed scavenging for DPPH assay. The results of separation are summarized as shown in Table 3.17.

Table 3.17 The results of the separation of Fraction ICD4

Eluents	Fraction No.	Remarks	Weight (g)
20% EtOAc:hexane	ICD4.1	Yellow oil	0.025
50% EtOAc:hexane	ICD4.2	White solid, Compound 5	0.084
70% EtOAc:hexane	ICD4.3	Red oil	0.898
100 % EtOAc	ICD4.4	Brown material	0.091

Fraction ICD4.2 gave a white solid which was further purified by recrystallization from hot ethyl acetate for several times to yield the off-white needle crystal. This crystal was melted at 140 °C and soluble in chloroform and dichloromethane. According to the spectroscopic study of this crystal and its physical properties, it was believed that Compound 5 was stigmasterol.

Separation of Fraction ICD4.3

According to the result of separation Fraction ICD, Fraction ICD4.3 was obtained as red oil material. The TLC of this fraction in 10% methanol in chloroform revealed 2 spots. This fraction was then rechromatographed with flash column, using gradient elution of chloroform and methanol. After separation, it still contained impurities. Therefore this portion was reseparated by chromatotron technique using gradient elution of 100% chloroform to 10 % methanol in chloroform and operated under ultraviolet light wavelength 254 nm condition. The separation of Fraction ICD4.3 by flash column chromatograph and chromatotron techniques yielded two compounds. The result of the separation of Fraction ICD4.3 is presented in Table 3.18. The first is brown oil 808.5 mg, designated as Mixture 6, and the second is pale-yellow oil 19.2 mg, specified as Compound 7. Both of them gave a positive result for TLC autographic assay for scavenging effect on DPPH radical.

Table 3.18 The results of the separation of Fraction ICD4.3

Eluents	Fraction	Remarks	Weight (g)
$CHCl_3$	1-15	Brown oil; Mixture 6	0.808
10% MeOH/$CHCl_3$	16-23	Pale-yellow oil; Compound 7	0.019

3.5.3.2 Structural Elucidation of Mixture 6

The brown oil (808.5 mg, 0.9217 % w/w of dichloromethane crude extract) revealed only one spot at R_f 0.49 in 10% methanol in chloroform. This mixture was soluble in ethyl acetate, dichloromethane and chloroform.

The IR spectrum (Figure 3.24) of this mixture exhibited absorption peaks of hydroxyl group at 3350 cm^{-1}, double bond of aromatic ring signal at 1598 cm^{-1} and 1466 cm^{-1}. Other signals were tentatively assigned as shown in Table 3.19.

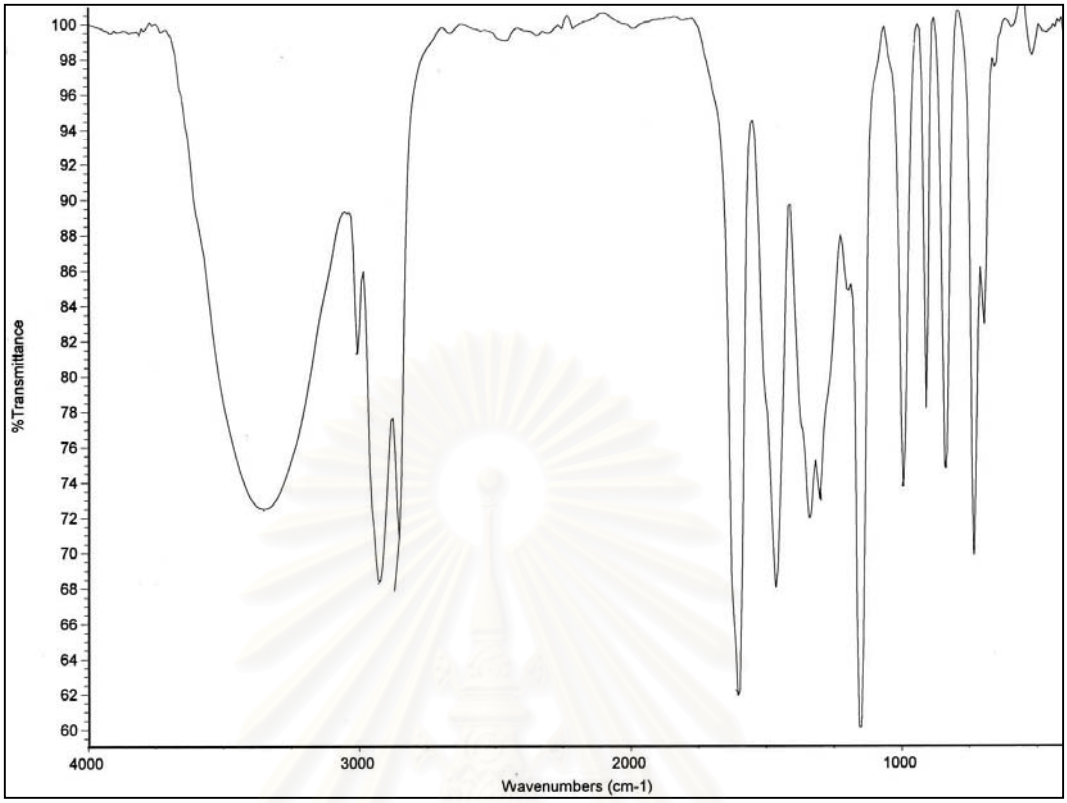


Figure 3.24 The IR spectrum of Mixture 6

Table 3.19 The IR absorption band assignments of Mixture 6

Wavenumber cm^{-1}	**Intensity**	**Tentative assignments**
3305	Strong	O-H stretching vibration of hydroxyl group
2925,2855	Strong	C-H stretching vibration of CH_2 and CH_3
1598, 1466	Strong	C-C stretching vibration of aromatic
1341,1299	Strong	=CH bending vibration in plane
1147	Strong	C-H bending in plane vibration of aromatic
991, 909, 835	Medium	C-H bending out of plane vibration of aromatic

The ^{1}H NMR spectrum ($CDCl_3$) of this mixture (Figure 3.26) demonstrated the signal of aromatic protons at δ 6.25 ppm (m, 2H) and δ 6.16 ppm (m, 1H), olefinic proton at δ 5.36 ppm (m, 3H), bisallylic methylene proton at δ 2.76 ppm (t, 1H, *J*=5.78 Hz), benzyl methylene protons at δ 2.42 ppm (t, 2H, *J*=7.36 Hz), allylic methylene protons at δ 2.02 ppm (br, 4H), homobenzyl methylene protons at δ 1.50 ppm (br, 2H), methylene protons at δ 1.27 ppm (overlappig, 16H) and terminal methyl protons at δ 0.87 ppm (t, 3H, *J*=6.28 Hz).

The ^{13}C NMR spectrum ($CDCl_3$) of this mixture (Figures 3.27-28) gave the signals for tetra-substituted benzene at δ 156.2, 146.4, 108.2 and 100.3 ppm and six olefinic carbon signals at δ 130.3, 130.2, 129.9, 129.8, 128.0 and 127.9 ppm.

According to the infrared, ^{1}H and ^{13}C NMR spectra, it was found that this mixture may contain resorcinol ring and unsaturated side chain. Thoroughly, this unsaturated side chain is consisted of 15 and 17 carbon atoms, respectively. This observation was confirmed by MS analysis (Figure 3.25). To illustrate this, the mass spectrum showed two molecular ion peaks (M^+) at m/z 344 (calcd. for $C_{23}H_{36}O_2$: MW 344.27) designated as Mixture 6A and m/z 318 (calcd. for $C_{21}H_{34}O_2$: MW 318.26) designated as Mixture 6B. Its also showed a base peak at m/z 124 corresponding to a benzylic fragmentation.

The unsaturated 17 carbon atom side chain of Mixture 6A contained two skipped double bonds. This was endorsed by the bisallylic methylene proton signal at δ 2.76 ppm and olefinic carbon signals at δ 127.9, 128.0, 130.2, and 130.3 ppm. While Mixture 6B had one double bond on 15 carbon atom side chain that showed two olefinic carbon signals at δ 129.8 and 129.9 ppm.

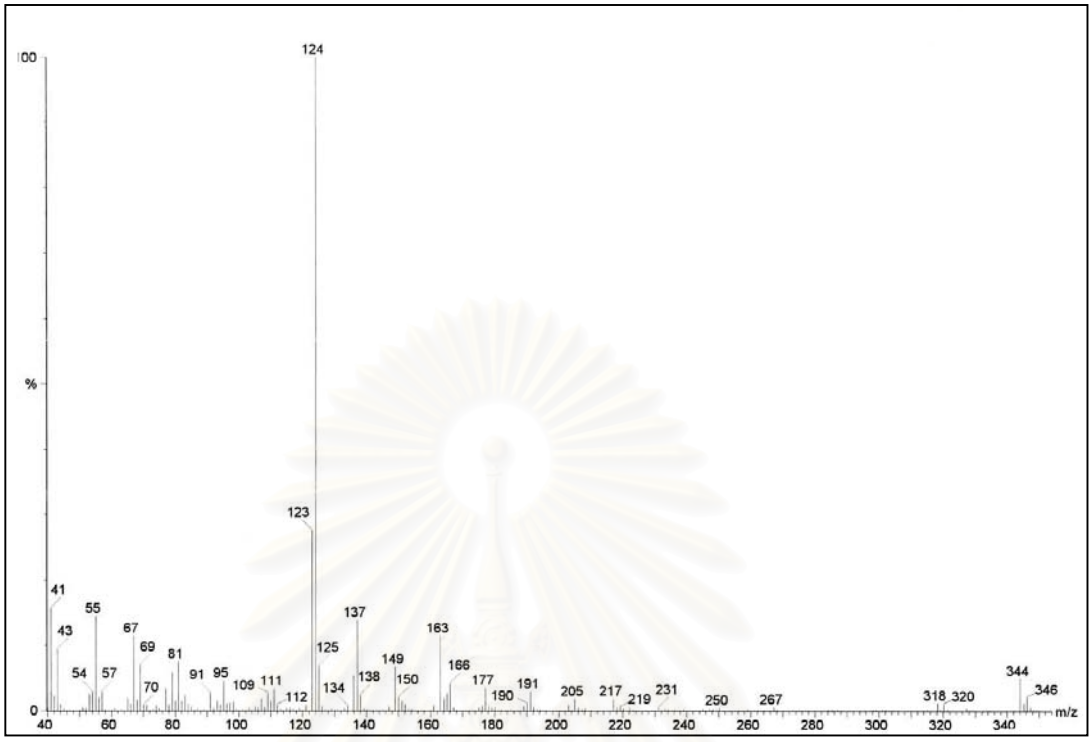


Figure 3.25 The mass spectrum of Mixture 6

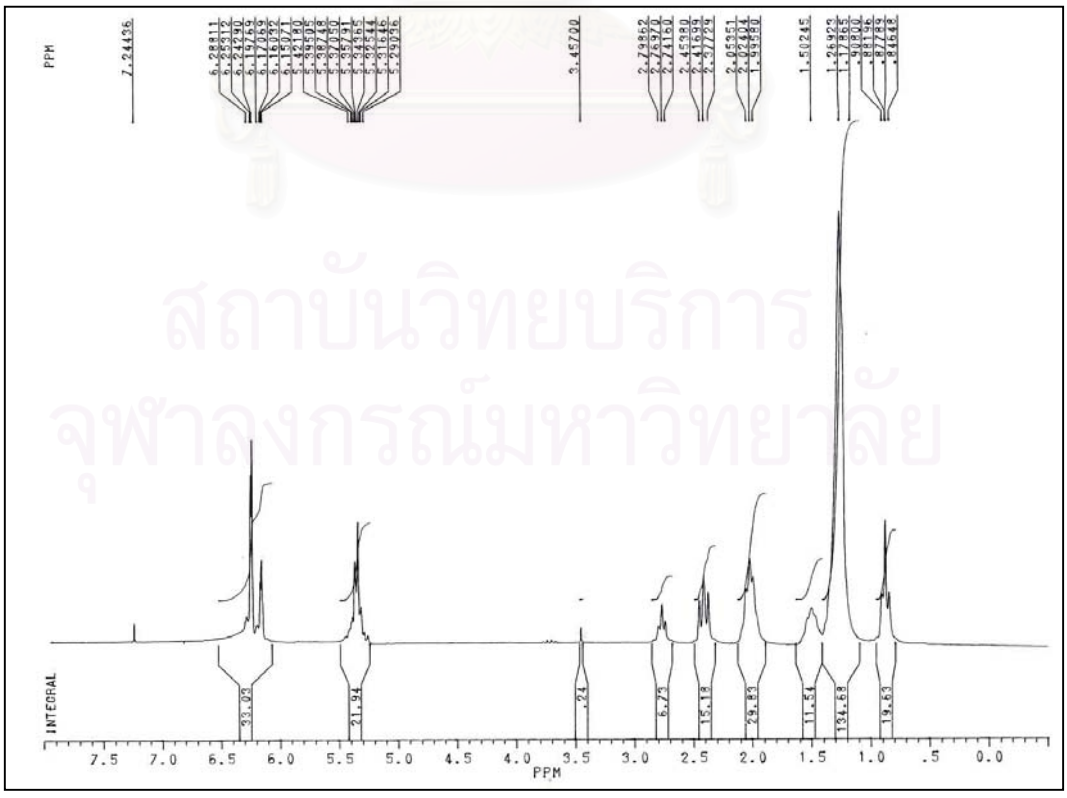


Figure 3.26 The ^{1}H NMR spectrum of Mixture 6

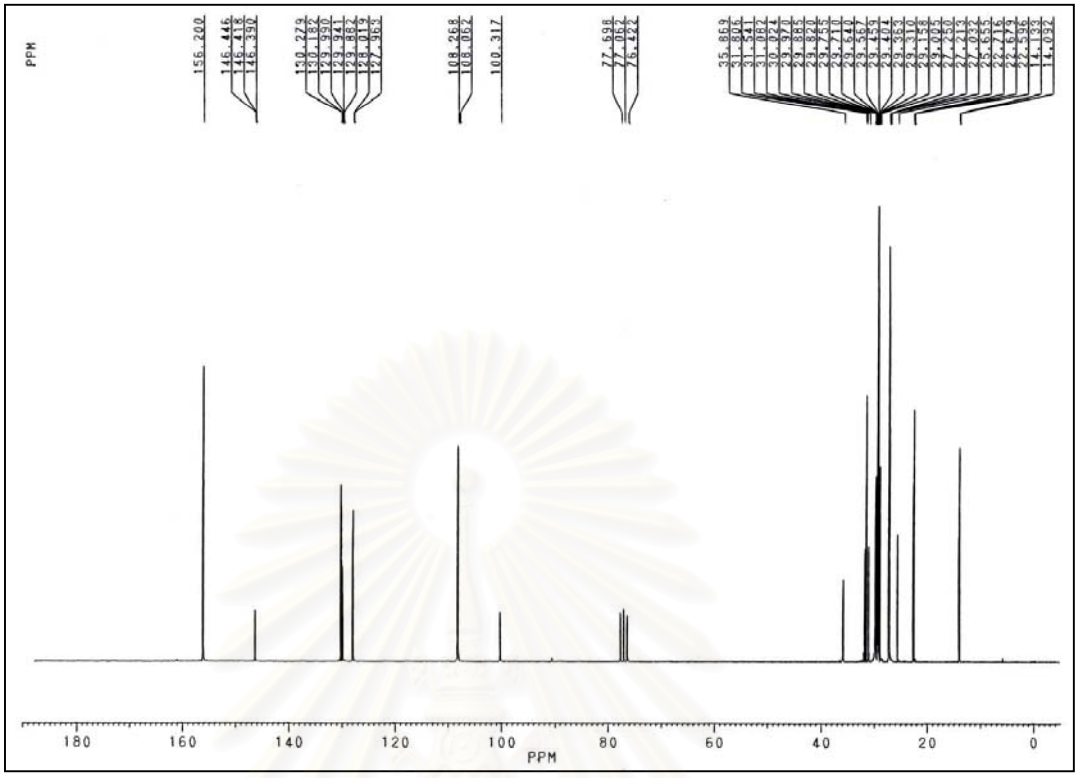


Figure 3.27 The ^{13}C NMR spectrum of Mixture 6

Figure 3.28 The ^{13}C NMR spectrum of Mixture 6 (expanded)

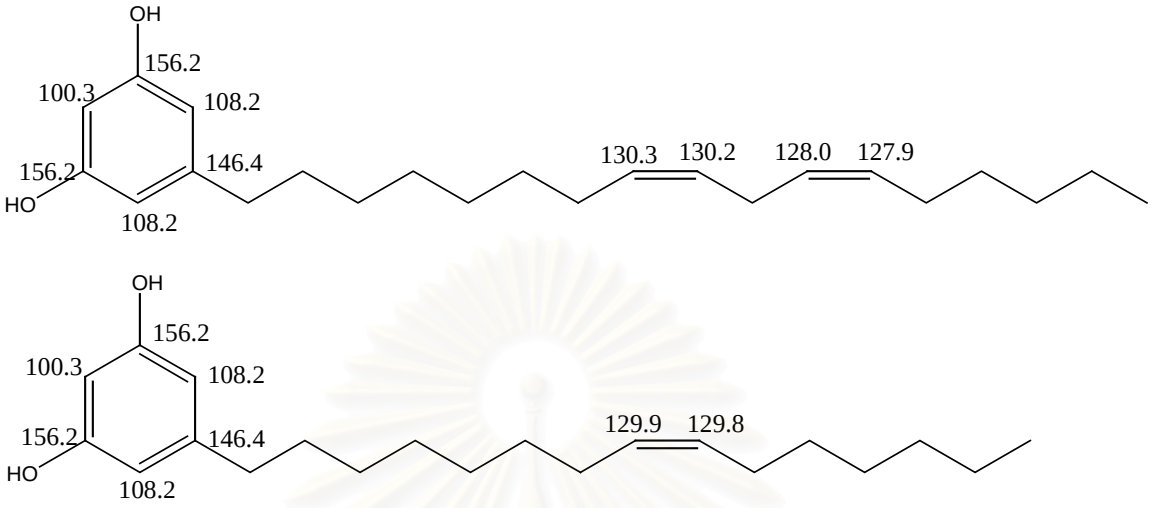
The double bond was unequivocally determined to be *Z* stereochemistry from the comparison of the ^{13}C NMR data with previous reports[26], the known 3-(heptadec-8′-*Z*-enyl)-1,2-dimethoxybenzene and its 8′-*E* isomer. The comparison of ^{13}C NMR data of Mixture 6 with both *Z*- and *E*-isomers are summarized as shown in Table 3.20.

Table 3.20 The ^{13}C NMR data of Mixture 6 compared with their references[26]

Position	**Chemical shift (ppm)**		
	Mixture 6	***Z*-isomer**	***E*-isomer**
Allylic carbon	27.21	27.18	32.58
Olefinic carbon	129.91, 128.02	129.82, 129.87	130.28,130.34

According to Table 3.20, it was found that the carbon signals of Mixture 6 were coincident with those of 8′-*Z*-isomer, but not with those of 8′-*E*-isomer. Thus, the stereochemistry of double bond in unsaturated side chain of Mixture 6 was believed to be *Z*-form. The assignments of proton and carbon signals of Mixtures 6A and 6B are summarized as shown in Figure 3.29 and Table 3.21.

^{1}H NMR assignments



^{13}C NMR assignments

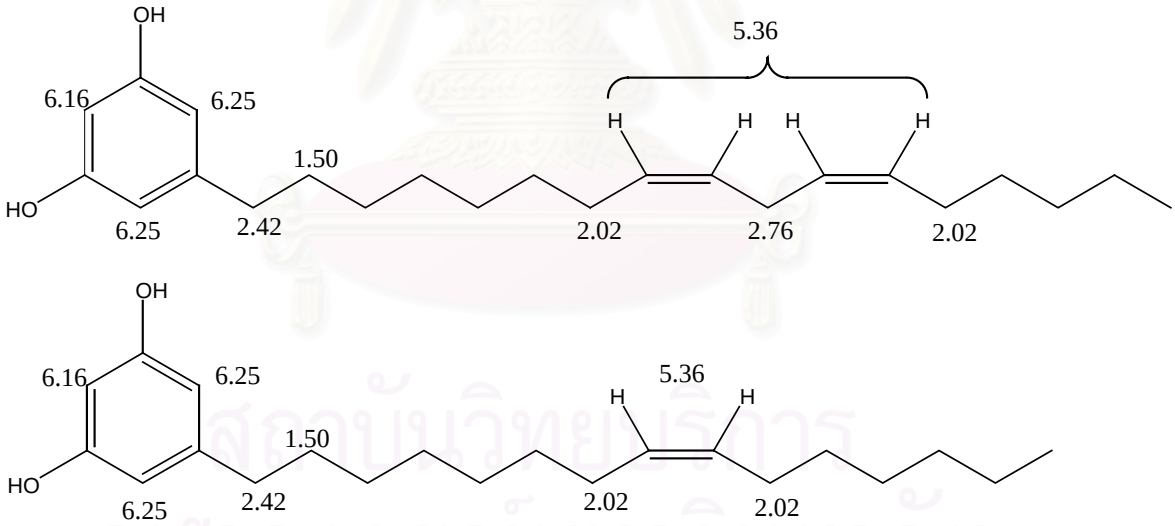
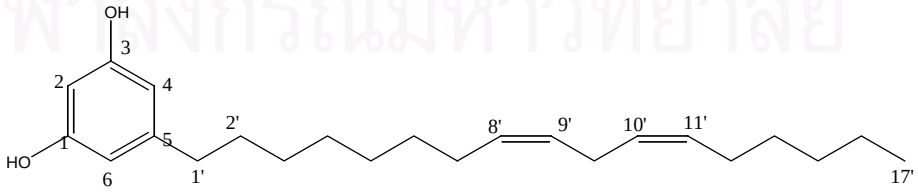
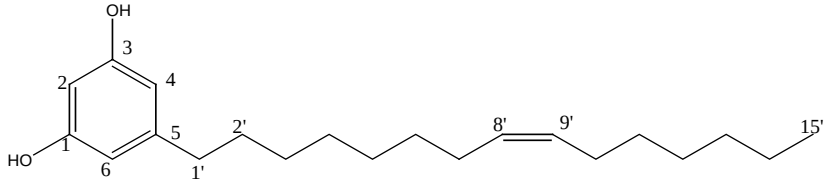


Figure 3.29 The ^{1}H NMR and ^{13}C NMR assignments of Mixture 6

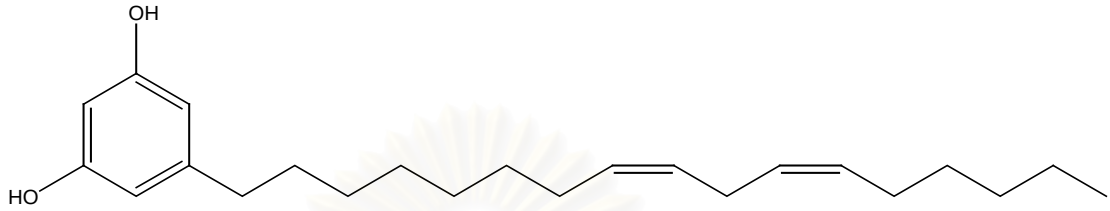
Table 3.21 The ^{1}H and ^{13}C NMR chemical shift assignments of Mixture 6

Position	Mixture 6A		Position	Mixture 6B	
	^{1}H	^{13}C		^{1}H	^{13}C
1	-	156.2	1	-	156.2
2	6.16 (m, 1H)	100.3	2	6.16 (m,1H)	100.3
3	-	156.2	3	-	156.2
4,6	6.25 (m, 2H)	108.2	4,6	6.25 (m, 2H)	108.2
5	-	146.4	5	-	146.4
1′	2.42 (t, 2H, *J*=7.36 Hz)	35.9	1′	2.42 (t, 2H, *J*=7.36 Hz)	35.9
2′	1.50 (br, 2H)	31.8	2′	1.50 (br, 2H)	31.8
3′	1.27 (overlapping)	31.5	3′	1.27 (overlapping)	31.5
4′		31.1	4′		31.1
5′		29.7	5′		29.7
6′		29.3	6′		25.3
7′,13′	2.02 (br, 4H)	27.2	7′,10′	2.02 (br, 4H)	27.2
8′	5.36 (m, 3H)	130.3	8′	5.36 (m, 3H)	129.9
9′	5.36 (m, 3H)	130.2	9′	5.36 (m, 3H)	128.0
10′	2.76 (t, 1H, *J*=5.78 Hz)	25.6	11′	1.27 (overlapping)	27.2
11′	5.36 (m, 3H)	128.0	12′		25.6
12′	5.36 (m, 3H)	127.9	14′		22.7
14′	1.27 (overlapping)	25.7	15′	0.87 (t, 3H, *J*=6.28 Hz)	14.1
15′		25.6			
16′		22.7			
17′	0.87 (t, 3H, *J*=6.28 Hz)	14.1			

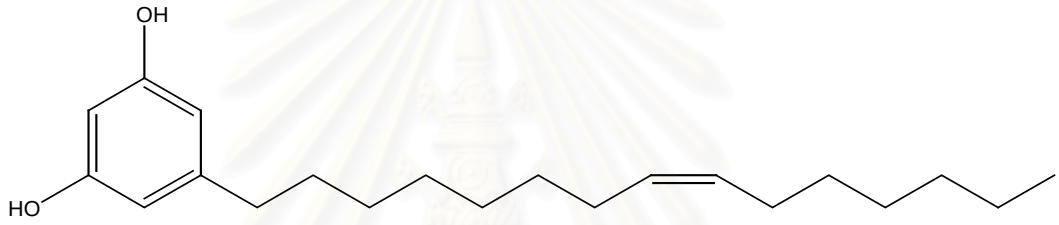




According to all spectroscopic data, the physical properties and previous study[28] of this mixture. It was believed that this mixture was composed of two components as Mixture 6A or 1,3-dihydroxy-5-(heptadec-8,11-dienyl)benzene and Mixture 6B or 1,3-dihydroxy-5-(pentadec-8-enyl)benzene.



1,3-dihydroxy-5-(heptadec-8,11-dienyl)benzene; Mixture 6A



1,3-dihydroxy-5-(pentadec-8-enyl)benzene; Mixture 6B

Mixture 6

3.5.3.3 Structural Elucidation of Compound 7

Compound 7, the pale-yellow oil 19.2 mg (0.0219 % w/w of dichloromethane crude extract) showed a single spot at R_f 0.65 in 10% methanol in chloroform was obtained from re-separation of Fraction ICD4.3. This compound was soluble in chloroform and methanol.

The IR spectrum of this compound (Figure 3.30), exhibited the absorption peaks of hydroxyl group of carboxylic acid in region of 3100-3300 cm^{-1}, carbonyl group at 1712 cm^{-1}. Other signals were tentatively assigned in Table 3.22.

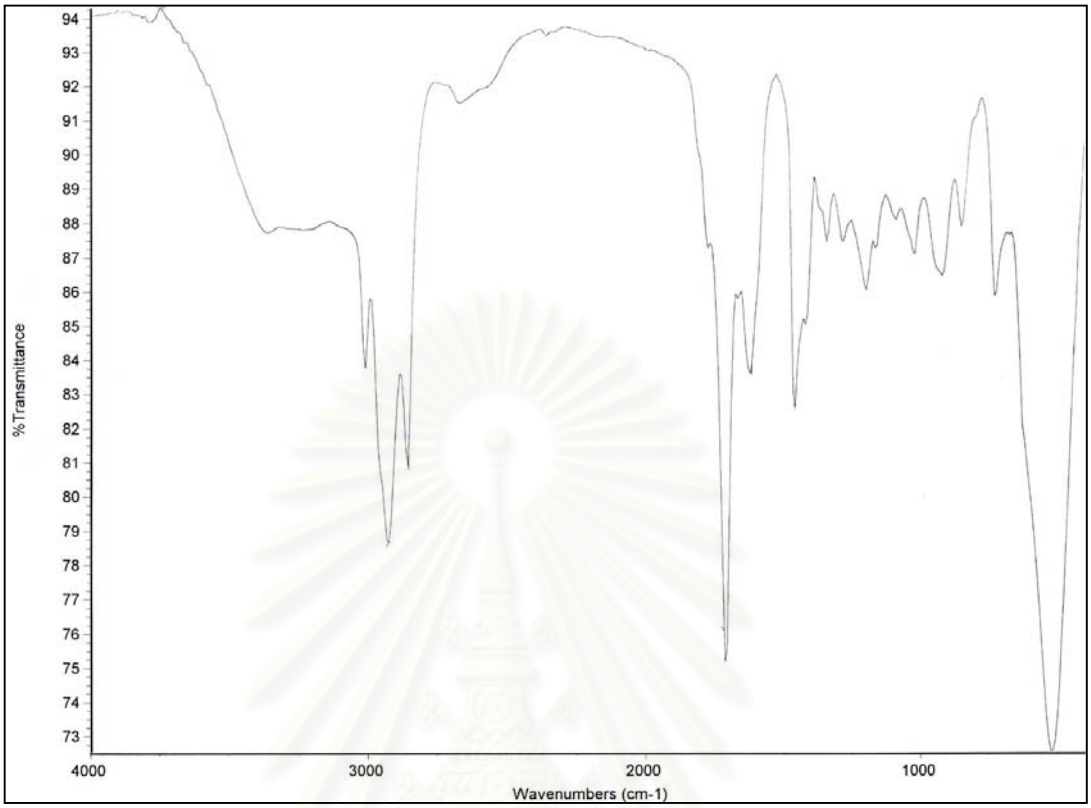


Figure 3.30 The IR spectrum of Compound 7

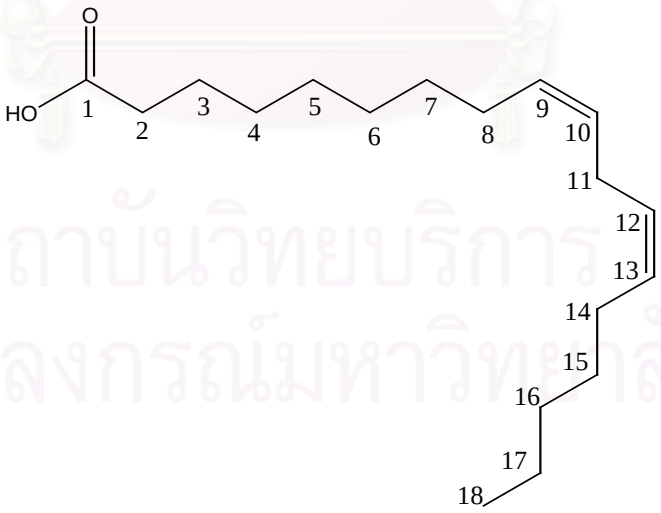
Table 3.22 The IR absorption band assignments of Compound 7

Wavenumber (cm^{-1})	**Intensity**	**Tentative assignments**
3100-3300	Broad	O-H stretching vibration of carboxylic acid
2921, 2856	Strong	C-H stretching vibration of CH_2 and CH_3
1712	Strong	C=O stretching vibration of carboxylic acid
1456	Medium	C-H bending vibration of CH_2 and CH_3

As an informative result of mass spectrometry (Figure 3.31), this compound showed the molecular ion peak at m/z 280 (Calcd. for $C_{18}H_{32}O_2$: MW 280.45) corresponding to unsaturated long chain fatty acid, linoleic acid. Furthermore, the relatively intense fragment ions at m/z (relative intensity) 60(22), 67(100), 81(87), 95 (83) and 109(43) are detected. The fragment ion at m/z 60 is formed by McLafferty (H)rearrangement. The possible mass fragmentation pattern is proposed as shown in Scheme 3.5.

The ^{1}H NMR spectrum of this compound (Figure 3.32) exhibited the chemical shift of proton signals of the olefinic signal at 5.32 ppm (m, 4H), the bisallylic signal at 2.76 ppm (t, 2H, *J*=5.83 Hz), the α-proton (H-2) at 2.33 ppm (t, 2H, *J*=7.53 Hz), the allylic signal at 2.04 ppm (br, 4H), the β-proton (H-3) at 1.61 ppm (br, 2H), the methylene signal is overlapped at 1.29 ppm (14H), and the terminal methyl proton at 0.87 ppm (t, 3H, *J*=6.97 Hz).

According to the ^{13}C NMR of this compound (Figure 3.33), it displayed 18 carbon signals. The important signals are as follows: the carbonyl signal (C-1) at 179.4 ppm and four olefinic carbons at 127.9, 128.0, 130.0, and 130.2 ppm, respectively. The other carbon signals are assigned as presented in Table 3.23 (included with the proton signals). The structure of octadeca-9,12-dienoic acid or linoleic acid, is shown below.



Octadeca-9,12-dienoic acid or linoleic acid

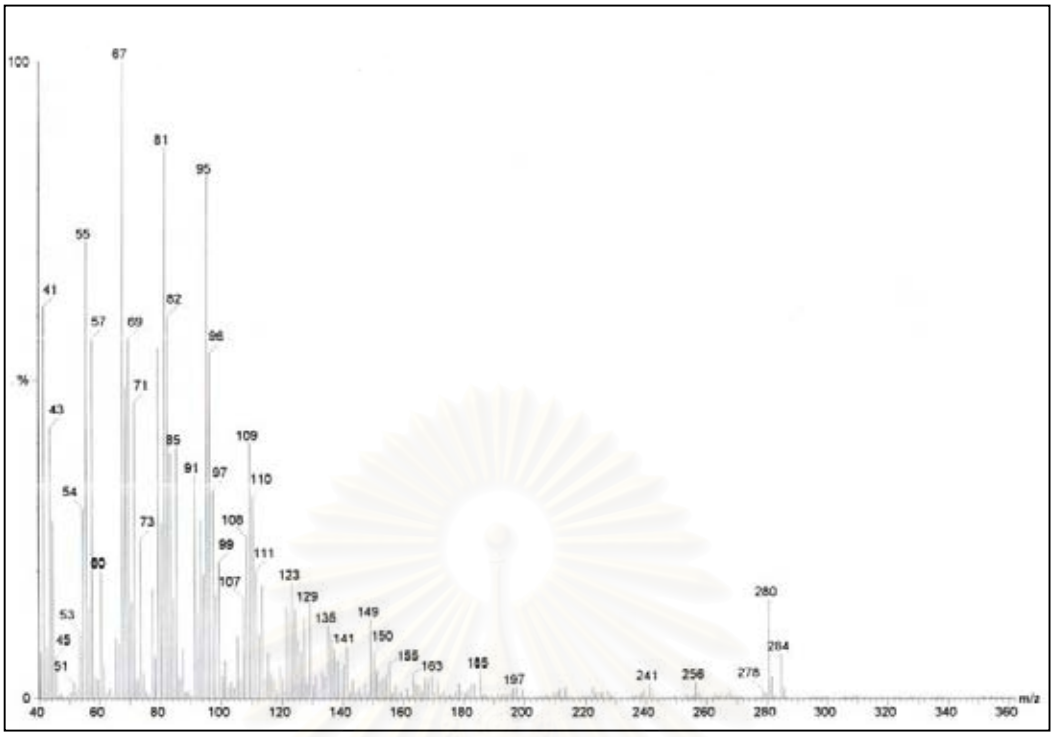
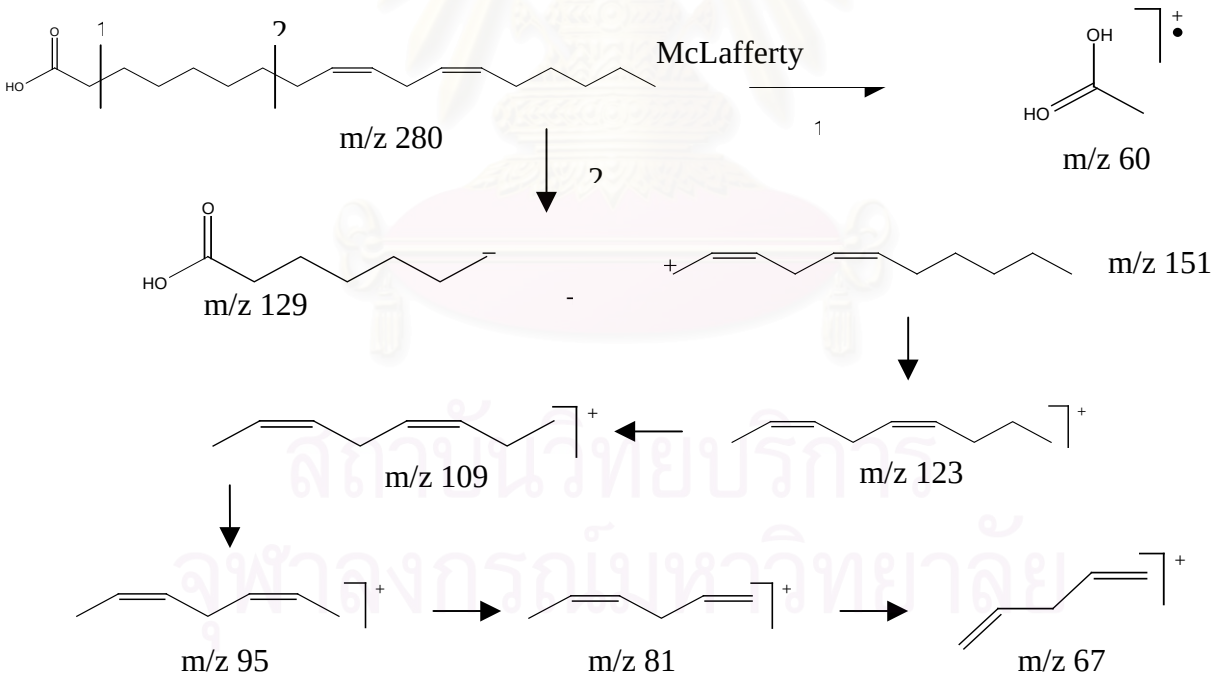


Figure 3.31 The mass spectrum of Compound 7



Scheme 3.5 The possible mass fragmentation pattern of Compound 7

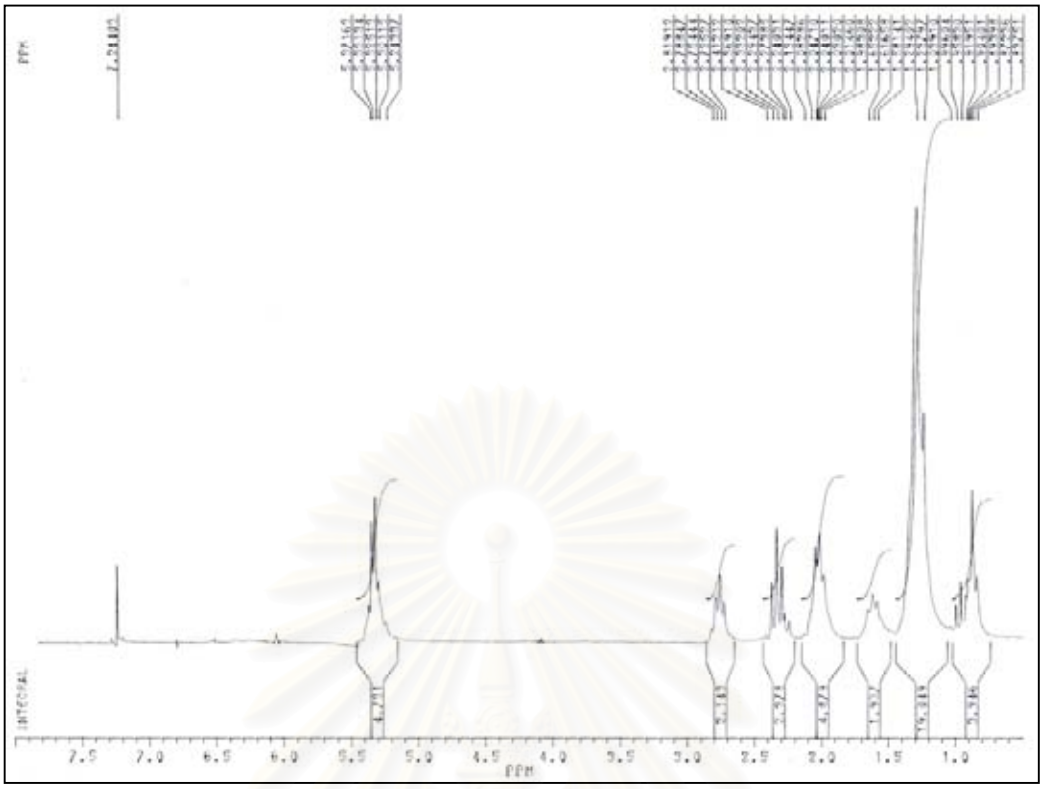


Figure 3.32 The ^{1}H NMR spectrum of Compound 7

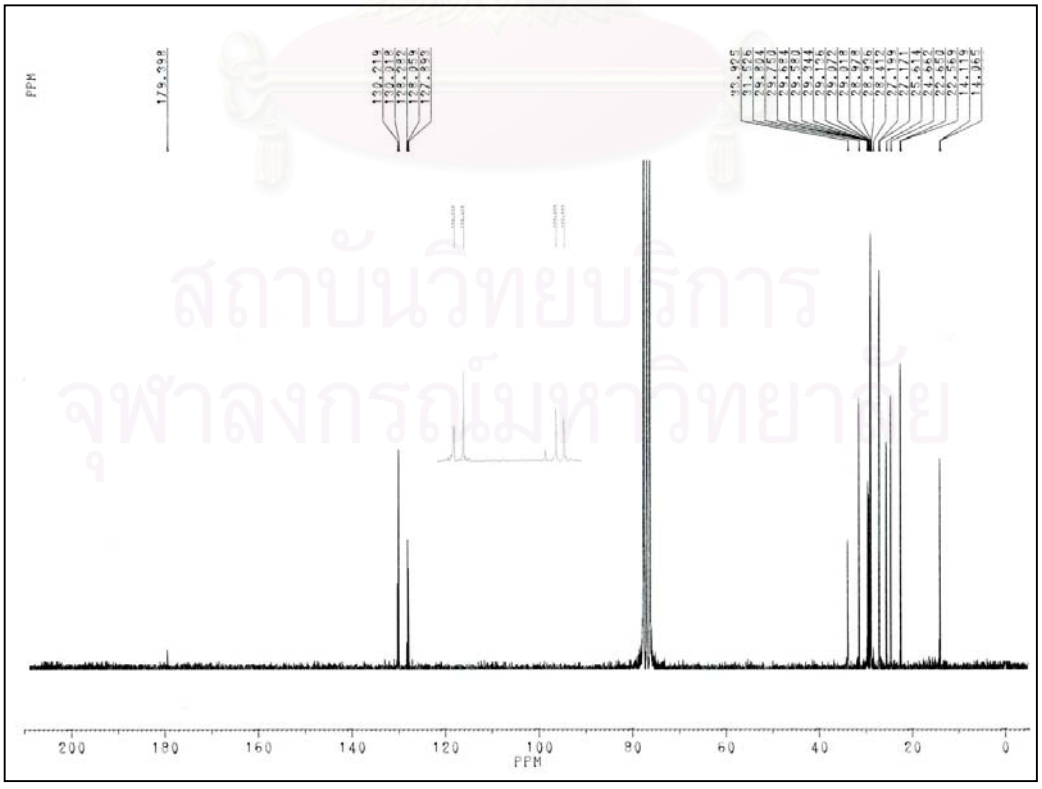


Figure 3.33 The ^{13}C NMR spectrum of Compound 7

Table 3.23 The ^{1}H and ^{13}C chemical shift assignments of Compound 7 compared with linoleic acid[28]

Position	Chemical shift (ppm)			
	Compound 7		linoleic acid	
	^{1}H	^{13}C	^{1}H	^{13}C
1	-	179.4	-	177.0
2	2.33 (t, 2H, *J*=5.83)	33.9	2.37 (t, 2H, *J*=7.1)	35.8
3	1.61 (m, 2H)	24.7	1.67 (m, 2H)	25.1
4	*	29.8	**	29.7
5	*	29.3	**	30.1
6	*	29.1	**	30.4
7	*	29.0	**	30.3
8	2.04 (m, 2H)	27.2	2.10 (m, 2H)	27.4
9	5.32 (m, 2H)	130.2	5.32 (m, 2H)	131.0
10		129.0		128.4
11	2.76 (t, 2H,*J*=7.53)	25.6	2.80 (m, 2H)	25.2
12	5.32 (m, 2H)	127.9	5.32 (m, 2H)	128.0
13		130.0		130.6
14	2.04 (m, 2H)	27.2	2.10 (m, 2H)	27.4
15	*	29.0	**	30.0
16	*	31.5	**	32.6
17	*	22.6	**	23.2
18	0.87 (t, 3H,*J*=6.97)	14.1	0.91 (t, 3H, *J*=7.4)	14

Note: The methylene signals of Compound 7* were resonated at 1.29 ppm (overlapping, 14H), and those of linoleic acid** were reported at 1.35 ppm (br, 14H).

Additionally, the structure of Compound 7 was deduced from the analysis of the two-dimensional ^{1}H correlation spectroscopy (COSY), and heteronuclear multiple bond connectivity (HMBC) spectra. The COSY spectrum of Compound 7 (Figure 3.35) reasonably exposed correlations between H-2 (δ2.33) and H-3 (δ1.61), between allylic protons H-8, H-14 (δ2.04) and olefinic protons H-9, H-13 (δ5.32), and between bisallylic proton H-11 (δ2.76) and olefinic protons H-10, H-12 (δ5.32), respectively.

According to the HMBC spectrum (Figure 3.36), it was clearly manifested the correlations between C-1 (δ179.4) and H-2, H-3, between C-8 (δ27.2) and H-10, between C-14 (δ27.2) and H-12, and C-11 (δ25.6) and H-9, H-13, respectively. The selected COSY and HMBC correlations of Compound 7 are demonstrated as depicted in Figure 3.34.

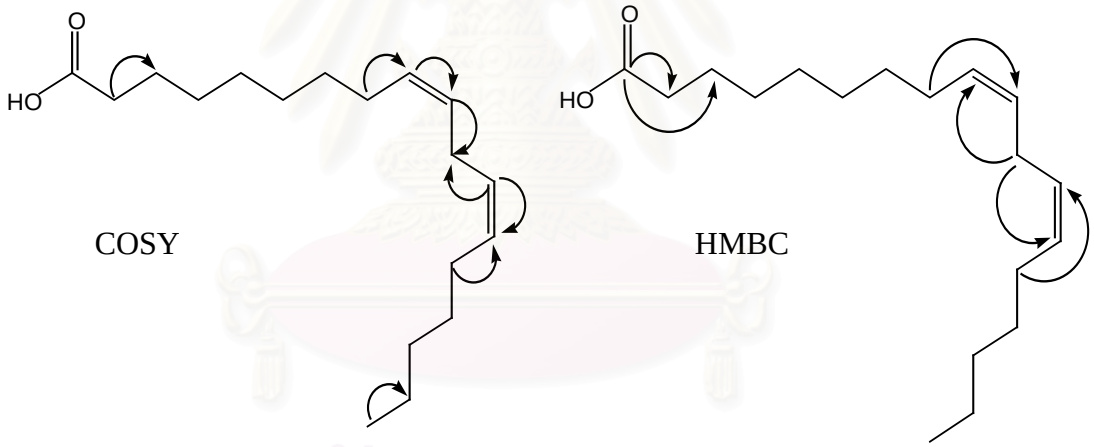


Figure 3.34 The selected COSY and HMBC correlations of Compound 7

Additional proof was carried out by allowing Compound 7 and authentic linoleic acid react with trimethylsilyl diazomethane ($TMSCHN_2$) followed the procedure described in Section 2.6.3. The methylation reaction of Compound 7 yielded the methyl ester derivative. The methyl esters of both Compound 7 and authentic linoleic acid were further subjected to GC analysis to confirm that this compound is octadeca-9,12-dienoic acid or linoleic acid. The chromatograms and retention time of both compounds were presented as shown in Figure 3.37.

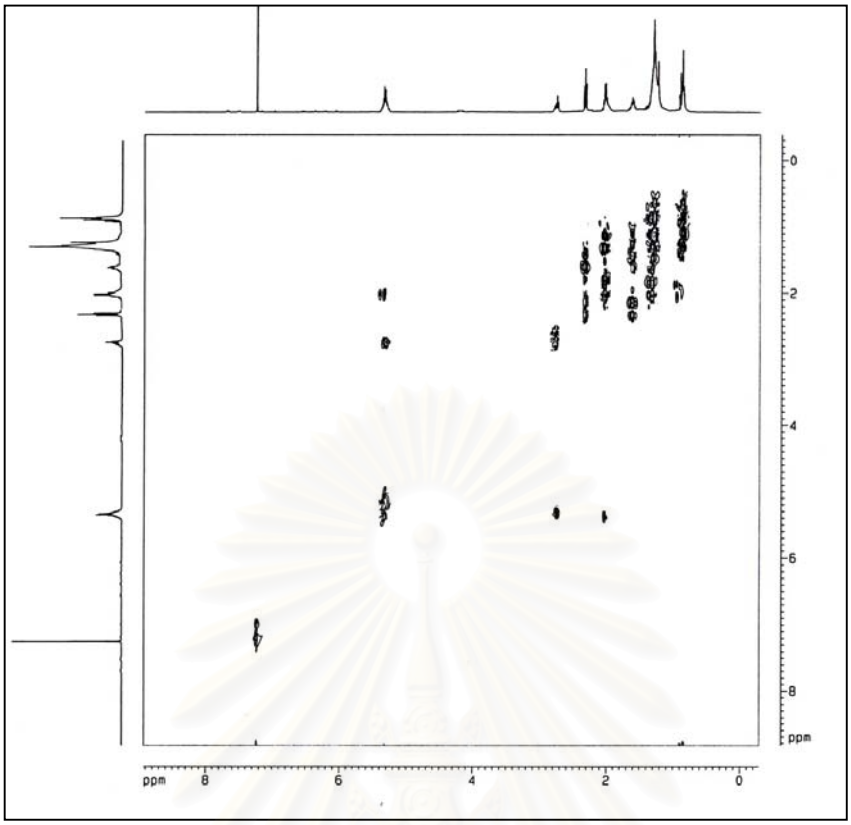


Figure 3.35 The COSY spectrum of Compound 7

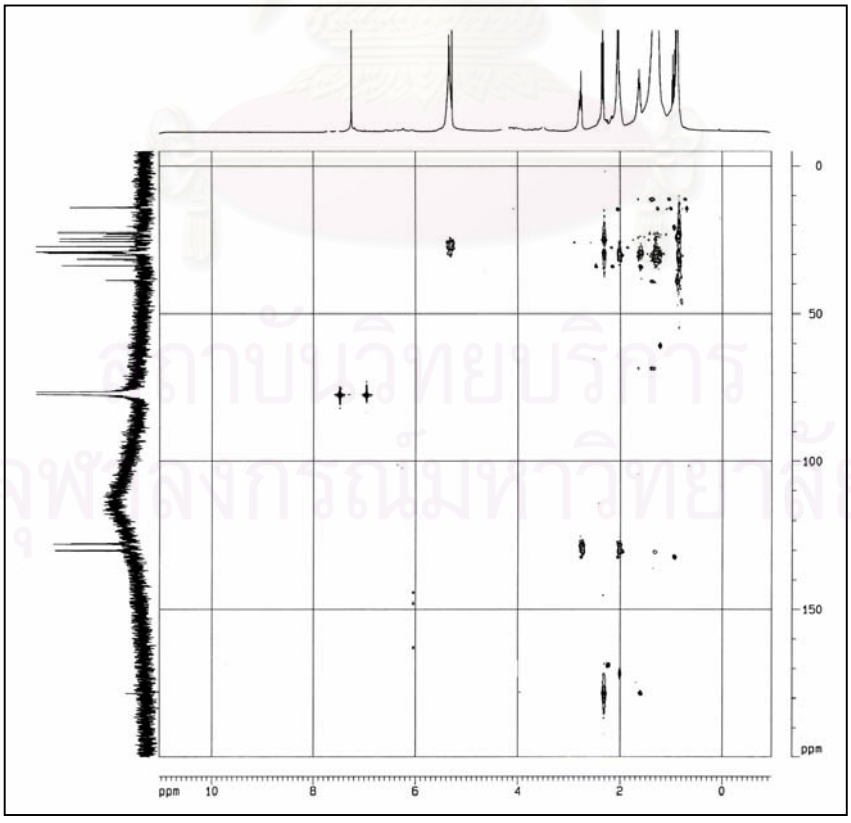


Figure 3.36 The HMBC spectrum of Compound 7

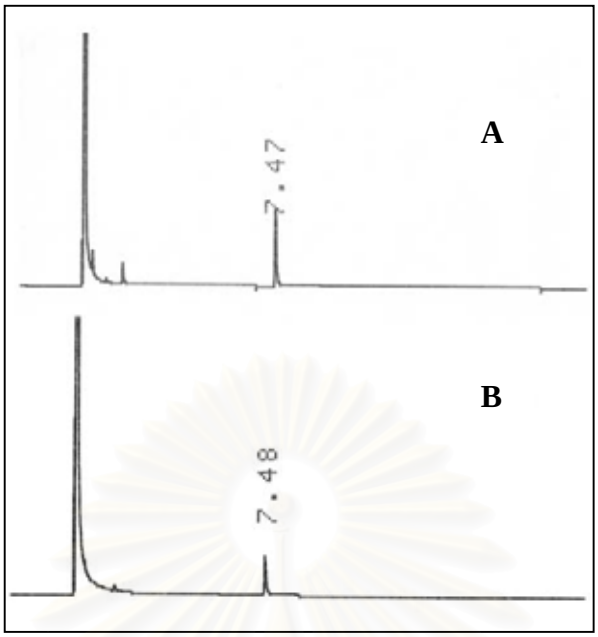
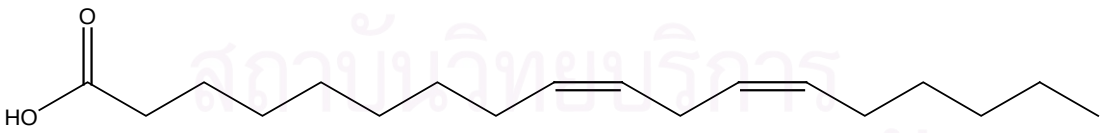


Figure 3.37 The chromatograms of methyl ester derivatives of authentic linoleic acid (A) and Compound 7 (B)

The GC analysis of methyl ester derivative of this compound revealed the retention time at 7.48 min, which was corresponded to that of authentic linoleic acid.

Therefore, as a result of physical properties, spectroscopic evidences, chemical reaction, and GC-analysis, Compound 7 was confident to conclude its structure as octadeca-9,12-dienoic acid (linoleic acid).



Compound 7; Octadeca-9,12-dienoic acid (linoleic acid)

Furthermore, according to the monitoring the EtOAc and MeOH crude extracts from *Ardisia colorata* Roxb. fruits by thin layer chromatography, these crude extracts exhibited almost similar spots as detected in CH_2Cl_2 crude extract. Moreover, these two crudes showed insignificant effect to both primary screening methods (brine shrimp cytotoxicity and scavenging effect towards DPPH radical). Therefore, there is rationalized not to separate and further explore these two fractions.

3.6 Study on Biological Activities of Isolated Substances from CH_2Cl_2 Extract of *Ardisia colorata* Fruit

As discussed above, the separation of dichloromethane extract from *Ardisia colorata* Roxb. fruits led to the isolation of seven substances. The isolated substances were further studied to search for biological activity. The cytotoxicity test, radical scavenger effect on DPPH radical, antifungal activity and insecticidal activity were selected for exploration followed the protocols described in Chapter II.

3.6.1 Brine Shrimp Lethality Cytotoxic Test

Seven isolated substances were tested for cytotoxic activity against brine shrimp, the LC_{50} values determined by probit analysis program are exhibited as shown in Table 3.24.

Table 3.24 Cytotoxicity activity against *Artemia salina* (brine shrimp)

Substance	LC_{50} (μg/mL)		Toxicity
	Acute	Chronic	
1	5.76	1.72	High
2	-	1486.31	No
3	-	650.32	Low
4	-	698.55	Low
5	-	709.22	Low
6	230.44	32.36	Moderate
7	443.96	4.99	High

Note: 0-10 μg/mL; High toxicity, 10-100 μg/mL; Moderate toxicity
100-1000 μg/mL; Low toxicity, >1000 μg/mL; No toxicity

Among all isolated substances, Compounds 1 and 7 showed high cytotoxicity against brine shrimp at LC_{50} 1.72 and 4.99 μg/mL, respectively. The Mixture 6 showed moderate cytotoxicity at LC_{50} 32.36 μg/mL. The other substances were inactive or gave low toxicity against brine shrimp.

From the result above, the cytotoxic activity against brine shrimp of Compounds 1 and 7 were found to comparatively be higher than that of dichloromethane extract (LC_{50} 13 μg/mL), whereas the other substances were lower. This attained result could clearly convince that the active components responsible for cytotoxic activity from dichloromethane extract should be Compound 1 or embelin and Compound 7 or linoleic acid.

3.6.2 Scavenging Effect on DPPH Radical

The DPPH radical scavenging activity by TLC autographic method of isolated substances revealed that Compounds 1 and 7 and Mixture 6 showed significant activity against DPPH radical.

The quantitative analysis for determination IC_{50} value of those substances were then performed using spectrophotometric method as described in Chapter II. The result of radical scavenging effect on DPPH of each compound is presented as shown in Table 3.25. The curve between the percentage of radical scavenging and the concentration of each sample was plotted. Figure 3.38 as an instance shows the curve of radical scavenging effect for Compound 1.

Table 3.25 Radical scavenging effect on DPPH radical

Sample	Concentration (μg/mL)	% radical scavenging
Compound 1	1000	74.66
	500	73.39
	250	66.17
	125	57.76
	62.5	41.26
Mixture 6	1000	62.81
	500	51.84
	250	48.83
	125	31.49
	62.5	24.88
Compound 7	500	26.06
	250	16.80
	125	13.54
	62.5	7.40
BHA	1000	68.38
	500	81.67
	250	79.77
	125	75.26
	62.5	66.39

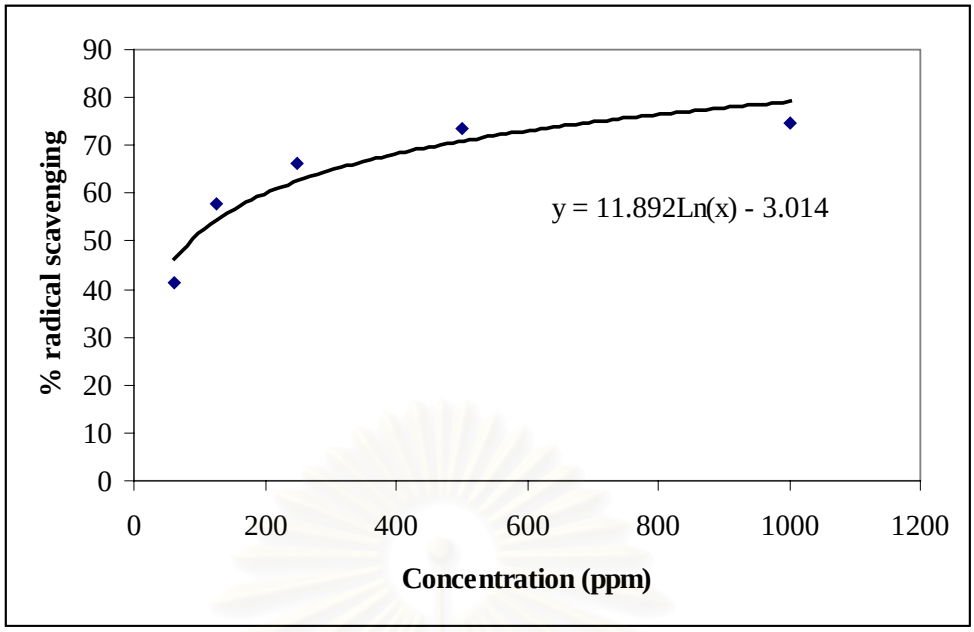
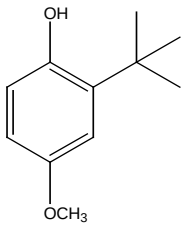


Figure 3.38 DPPH radical scavenging effect of Compound 1

The IC_{50} values of each substance were calculated from the logarithmic equation of each curve and were tabulated in Table 3.26.

Table 3.26 IC_{50} values of radical scavenging effect on DPPH radical

Substance	IC_{50} (μg/mL)
1	86.34
6	386.03
7	9505.40
BHA*	16.88



Butylated hydroxyanisole (BHA)

From the result of radical scavenging effect on DPPH radical assay, it was found that Compound 1 (embelin) showed the highest activity at IC_{50} 86 ppm among all tested substances. From the comparison with BHA (butylated hydroxyanisole), a commercial antioxidant, the activity of Compound 1 was lower than that of BHA.

As the comparison with preliminary result of dichloromethane crude extract, it was detected that the active component which scavenged the DPPH radical on TLC (Section 3.2.2) should be Compound 1 or Compound 7 or Mixture 6. Therefore, this inference was ensured by spectrophotometric method. The IC_{50} values of each substance pointed out that Compound 1 or embelin gave the lowest IC_{50} among those substances tested, although it gave higher value than that of BHA. By this reason, it could obviously conclude that embelin was the active antioxidant agent from dichloromethane crude extract from *A. colorata* fruits.

3.6.3 Preliminary Antifungal Activity on Fusarium oxysporum and Alternaria sp. by Bioautographic Assay

Seven isolated substances from the dichloromethane extract from the fruits of *Ardisia colorata* Roxb. were investigated for antifungal activity. Two phytophatogenic fungi *Fusarium oxysporum* and *Alternaria* sp. were employed. The TLC plate containing spot of each substance was sprayed with spore suspension of those two mentioned fungi and incubated in a water agar for 3 days. The inhibition was observed by clear zone of active component against blue background.

When the TLC plate was sprayed with spore suspension of *Fusarium oxysporum*, all of isolated substances did not show any inhibition effect. That was noticed by no clear zone on the TLC plate as displayed in Figure 3.39.

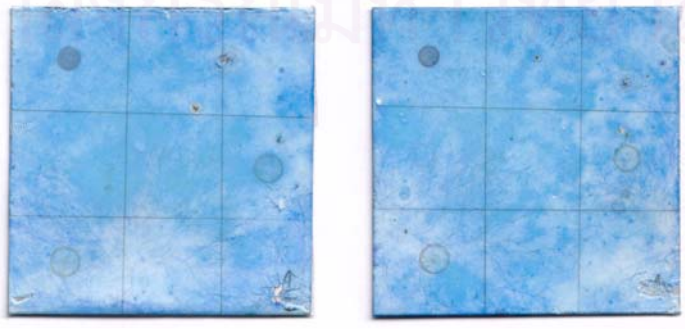


Figure 3.39 Bioautographic pattern of isolated substances against *Fusarium oxysporum* 43-68

Furthermore, when spore suspension of *Alternaria* sp. was sprayed on TLC plate, the spot of Mixture 6 displayed a clear zone which indicated the inhibition zone. The TLC plates are shown in Figure 3.40

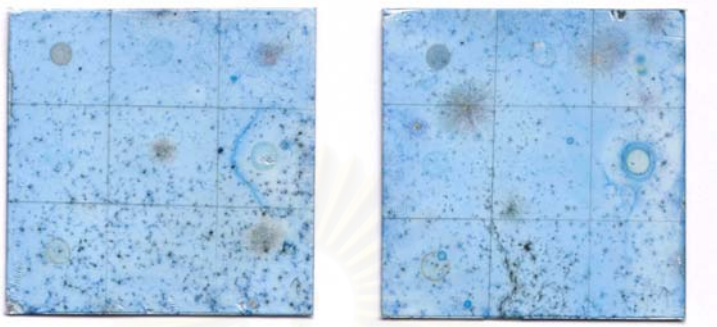


Figure 3.40 Bioautographic pattern of isolated substances against *Alternaria* sp.43-89

According to preliminary result of antifungal activity, Mixture 6 (a mixture of alkenylresorcinol) exhibited inhibition zone on TLC plate when sprayed with spore suspension of *Alternaria* sp., while the other substances did not show inhibition zone. Therefore, it could be elementary proposed that the resorcinol moiety of Mixture 6 displayed as important portion responsible for this activity.

3.6.4 Insect Contact Toxicity Test

The contact toxicity against the larvae of *S. litura* by the vial test method was performed followed the procedure that described in Section 2.7.4. Two potential substances, Compound 1 and Mixture 6 containing phenolic lipid derivatives were investigated. The dead larvae amount after 5 days is reported as shown in Table 3.27.

The correlation curve between the concentration and the percentage of died larvae were plotted, and the results are exhibited in Figure 3.41. The LD_{50} of each sample was calculated by probit analysis program, and the LD_{50} results are demonstrated in Table 3.28.

Table 3.27 The died larvae (*S. litura*) after treating with Compound 1 and Mixture 6

Concentration (μg/mL)	Compound 1		Mixture 6	
	Died larvae	Percentage	Died larvae	Percentage
1000	24	53	-	-
500	23	51	45	100
100	15	33	43	96
50	11	24	26	58
10	14	31	15	33

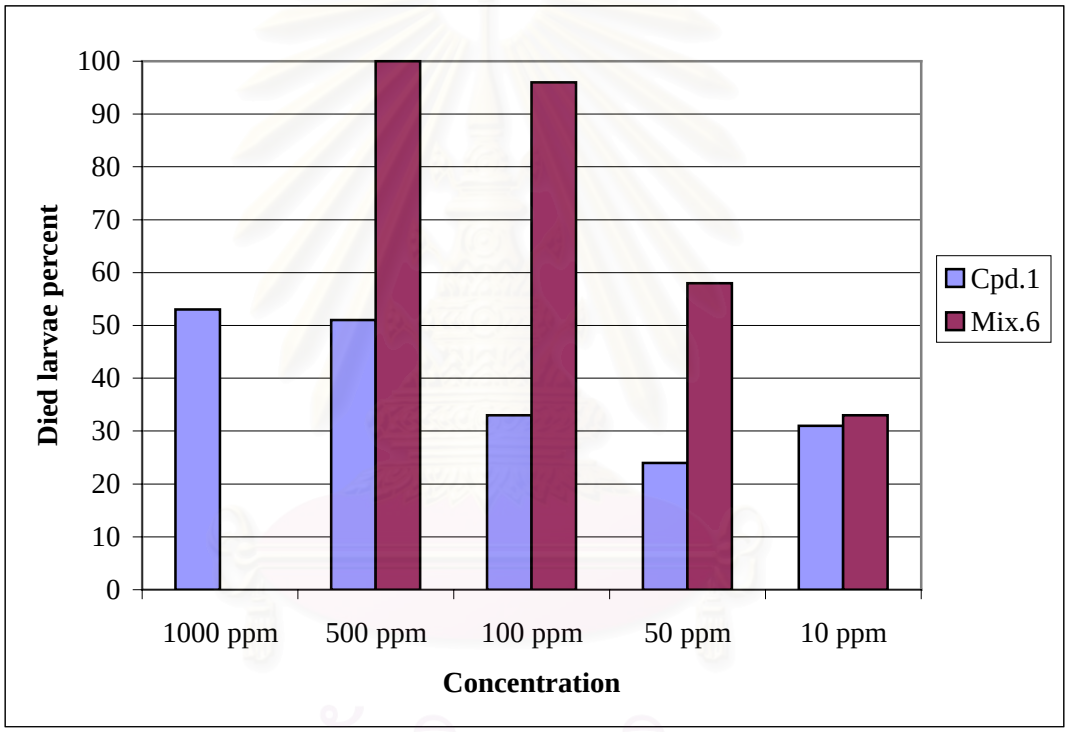


Figure 3.41 Insecticidal activity (contact toxicity vial test) against *S. litura*

Table 3.28 The LD_{50} of Compound 1 and Mixture 6 against *S. litura* by contact toxicity vial test

Substance	LD_{50} (μg/mL)
Compound 1	746.73
Mixture 6	33.6

The LD_{50} of Mixture 6 is significantly less than that of Compound 1 about 80 times. Mixture 6 displayed higher insect toxicity than Compound 1. Considering the structure of both substances, it could be uncomplicatedly recommended that the resorcinol moiety or the unsaturated aliphatic side chain may be the active part of Mixture 6.

3.6.5 Anticancer Activity

As aforementioned, phenolic lipid derivatives (Compound 1 and Mixture 6) are known to possessed cytotoxic activity against brine shrimp, the anticancer activity was selected for further investigation. The anticancer activity against human mouth carcinoma (KB), breast cancer (BC), and small cell lung cancer (NCI-H187) was performed by Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology using microculture tatrazolium assay. This assay utilized the maximum concentration at 20 μg/mL. The cytotoxicity results are deduced as shown in Table 3.29.

Table 3.29 The results of anticancer activity

Substances	Cancer Cell					
	KB		BC		NCI-H187	
	IC_{50} μg/mL	Acitvity	IC_{50} μg/mL	Activity	IC_{50} μg/mL	Activity
Compound 1	-	Inactive	2.03	Strong	0.30	Strong
Mixture 6	-	Inactive	1.95	Strong	3.30	Strong

Note: >20 μg/mL; Inactive, 10-20 μg/mL; Weak, 5-10 μg/mL; Moderate, <5 μg/mL; Strong

As the results presented in Table 3.29, it was found that both Compound 1 and Mixture 6 demonstrated potent cytotoxic against the breast cancer (BC) and the small cell lung cancer (NCI-H187), whereas they were inactive for the human mouth carcinoma (KB). The presence of *o*-hydroxybenzoquinone nucleus of Compound 1 and resorcinol moiety of Mixture 6 might be important portion for expressing this activity.

The antifungal and insecticidal activities result indicated that Mixture 6 or a mixture of alkenylresorcinol showed positive result for both assays. The other biological activity of resorcinolic lipid derivative was additionally literated from previous reports. In 1999 Kozubek and Tyman reviewed the biological activity of resorcinolic lipid.[29] Antibacterial activity is one of activities that have been reported. Recent experiment indicated that the extracts from *Gingko biloba* fruit, *Ardisia japonica* plant and seed cover of *Myristica fragrans* or cashew nut shell liquid (CNSL) which contained resorcinolic lipid derivative as a major component exhibited high activity toward phatogenic Gram-positive bacteria, *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* as well as phytophatogenic bacteria. Similarly to their antibacterial activity, resorcinolic lipid displayed antifungal activity that inhibited the growth of *Trichophyton mentagrophytes* and *Saccharomyces cerevisiae*. The resorcinolic lipids are nontoxic to higher animals, it was found by the toleration by rats with an oral intake of 5 g/kg. This result was accordingly led to other applications such as the treatment of mouth and gingival infections, antifungal fluids and also in hair restoration lotion preparations. Furthermore, the biological activity of this substance as the growth regulators and in host-parasite relationship, effect of resorcinolic lipids on nucleic acid, interaction with proteins and effects on enzymatic activity, contact dermatitis, interaction of resorcinolic lipids with phospholipids bilayer biological membranes and the modulators of lipid oxidation have been addressed.

Moreover, as a collaboration work with Dr. Masanori Morimoto from Department of Agricultural Chemistry, Kinki University, Nakamachi, Nara, Japan, in some biological activity tests on Compound 1 or embelin, a major component of *Ardisia colorata* Roxb. fruits were conducted. The bioassay studied at Kinki University included antifeedant activity, mitochondria respiration inhibitor, antimicrobial activity, and the inhibitory enzymatic activity.

The insect antifeedant activity of Compound 1 using leaf disk bioassay[30] against *Spodoptera litura* gave the ED_{50} of 0.585 mg/disk (moderate activity).

For the antimicrobial activity against *Bacillus subtilis*, the gram-positive bacteria that normally considered to be non-pathogenic, Compound 1 showed complete inhibition at 31.5 ppm.

In the case of the inhibitory enzymatic activity on *p*-hydroxypyruvate dioxygenase or HPPD enzyme, the inhibition of HPPD enzyme will prevent the

accumulation of toxic fumarylacetoacetate and succinylacetone in the liver, Compound 1 exhibited IC_{50} value of 24.5 μg/mg protein.

For the mitochondria respiration inhibitor test, mitochondria are essential to the creation of ATP. In general, the mitochondria is an important organelle in respiration system. In this experiment, Compound 1 displayed as electron transport inhibitory on succinic acid to H_2O was tested. Compound 1 demonstrated the weak mitochondria inhibition, and showed IC_{50} at 3.56×10^{-4} M.

The biological activity result of isolated substances indicated that the active components of the dichloromethane extract from *A. colorata* fruit are Compound 1 or embelin and Mixture 6. These two substances could be classified as phenolic lipid type derivative. Compound 1 (embelin) exhibited the highly cytotoxic activity against brine shrimp at LC_{50} 1.72 μg/mL, and the extremely antioxidant against DPPH radical. Mixture 6 revealed the moderately cytotoxic activity against brine shrimp at LC_{50} 32.36 μg/mL, but it showed high activity on the growth inhibitory effect of fungi *Alternaria* sp. and the insecticidal activity against *S. litura*. Furthermore, Compound 1 and Mixture 6 exhibited strongly cytotoxic activity against the breast cancer cell at IC_{50} 2.03 μg/mL and 1.95 μg/mL, and also showed powerfully cytotoxic activity against the small cell lung cancer at IC_{50} 0.30 μg/mL and 3.30 μg/mL, respectively.

Compound 7 or linoleic acid belonged to the unsaturated long chain fatty acid demonstrated the highly cytotoxic activity against brine shrimp at LC_{50} 4.99 μg/mL. However, no other activity was observed. The other substances, Mixtures 3 and 4 and Compound 5 that categorized in triterpenoid and steroid type illustrated low cytotoxicity against brine shrimp.

CHAPTER IV

CONCLUSION

During the course of this research, with the aim to study chemical constituents and their bioactivities from Thai medicinal plants, it was found that the dichloromethane extract of *Ardisia colorata* Roxb. fruits, belonging to Mysinaceae family, showed highly cytotoxic activity against *Artemia salina* (brine shrimp) and exhibited significant radical scavenging effect on DPPH radical. After fractionation and purification, three pure compounds and four mixtures were obtained. All isolated substances were further elucidated by means of their physical properties, chemical reactions and spectroscopic evidences. The structures of all isolated substances are summarized as shown in Table 4.1.

Table 4.1 Isolated substances of dichloromethane extract from *Ardisia colorata* Roxb. fruits

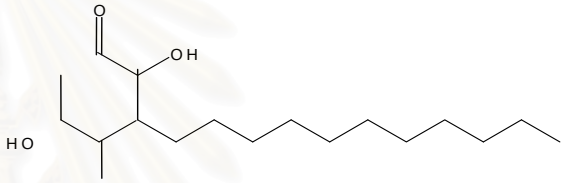
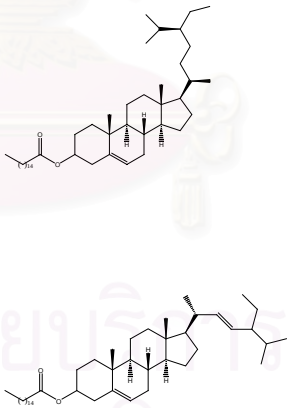
Substance	Weight	% yield*	Structure	Remarks
Compound 1: 2,5-dihydroxy-3-undecyl-1,4-benzoquinone (embelin)	9.33 g	0.19		Orange plate solid
Mixture 2: Mixture of long chain alcohols	21.5 mg	0.0245	CH_3-$(CH_2)_n$-OH	Bright white plate solid
Mixture 3: Stigmasteryl-3-*O*-palmitate and β-Sitosteryl-3-*O*-plamitate	2.5 g	2.85		Yellow semisolid

Table 4.1 (Continued)

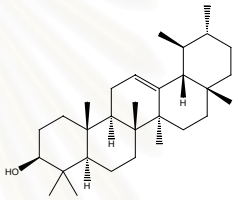
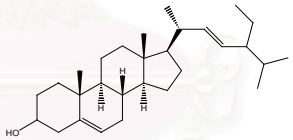
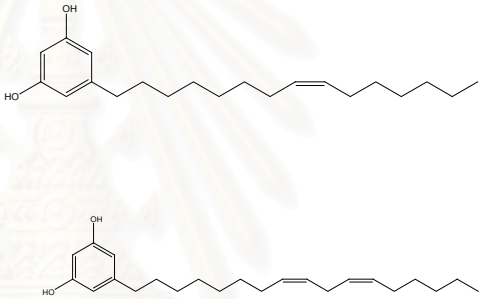
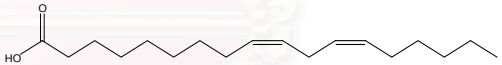
Substance	Weight	% yield*	Structure	Remarks
Mixture 4:				
α-amyrin	56.9 mg	0.0679		White needle crystal
Compound 5:				
Stigmasterol	36.9 mg	0.0421		Off-white needle crystal

Table 4.1 (Continued)

Substance	Weight	% yield*	Structure	Remarks
Mixture 6 1,3-dihydroxy-5-(heptadec-8,11-dienyl)benzene and 1,3-dihydroxy-5-(pentadec-8-enyl) benzene	808.5 mg	0.9217		Brown oil
Compound 7: Octadeca-9,12-dienoic acid	19.20 mg	0.0219		Pale-yellow oil

*The percentage yield of all isolated substances was calculated based on crude CH_2Cl_2 extract (114.20 g), while Compound 1 was calculated based on dried fruit materials (5 kg).

All isolated substances were further bioassayed. The bioactivity assay that selected for determination in this research are as follows; cytotoxicity test against brine shrimp, scavenging effect on DPPH radical, antifungal (plant phatogenic) activity, insecticidal activity (contact toxicity, vial test against *S. litura*) and anticancer activity. Compound 1 (embelin), the orange dihydroxybenzoquinone pigment obtained as a major component, exhibited high cytotoxic activity against brine shrimp, the breast cancer and the small cell lung cancer as well as the highest scavenging activity toward DPPH radical, and moderate toxicity against the common cutworm *S. litura*. While this compound was subjected to antifungal activity, it displayed inactive for growth inhibitor of *Fusarium oxysporum* and *Alternaria* sp.

Previous report on the chemical constituent determination of *A. colorata* fruit resulted that the major orange pigment was rapanone. Nevertheless, in this course of research endorsed by various apectroscopic evidences, the major orange pigment was obviously identified as embelin.

Concerning with bioactivities of other substances, Mixture 6 or a mixture of alkenylresorcinol exhibited moderate toxicity and strong cytotoxic activity against the breast cancer and the small cell lung cancer. It also showed significant activity on antifungal against *Alternaria* sp. Compound 7 or linoleic acid displayed high toxicity against brine shrimp while it showed inactive for other assays tested. The surplus substances revealed insignificant bioactivities result.

The occurrence of linoleic acid clearly showed the possible biosynthesis pathway for Mixture 6A[30] or 1,3-dihydroxy-5-(heptadec-8,11-dienyl)benzene. The unsaturated side chain moiety of Mixture 6A which had non-conjugated two double bonds should be arose by the decarboxylation of linoleic acid and then attached to position 3 of resorcinol moiety.

The antifungal activity and insecticidal activity of chemical constituents from the dichloromethane extract from *A. colorata* fruit was addressed for the first time in chemical literature.

In conclusion, it could be clearly seen that the fruits of *Ardisia colorata* Roxb. could be used as a good source of embelin, a bioactive benzoquinone pigment. Moreover, this plant produced resorcinol substance in good yield.

Proposal for The Future Work

The incidence of embelin as a major constituent, one of an active ingredient and the outcome from this research opened many possibilities to carry on for further exploration. For example, the utilization of embelin as a cytotoxic agent against various cell lines. The possible future work on structure activity relationship of embelin and other substances may provide an opportunity to understand what parts of the molecule have an influence for those interest activity. For instance, the most importantly active part of embelin was *o*-hydroxybenzoquinone moiety, the exploration of structure activity relationship of this compound should be studied on the variation of number of carbon atom and degree of unsaturation in side chain and substituted group on *o*-hydroxybenzoquinone moiety. Another aspect that would make this research fulfill is the chemical constituents and biological activity investigations of ethyl acetate and methanol crude extracts from the dried fruits, and other parts of *Ardisia colorata* Roxb. This would provide informative data for the chemotaxonomy of this plant. This present investigation is one of excellent examples to endorse the concept of the necessity on studying chemical constituents in conjunction with biological activity which will certainly be a gateway for disclosing lead bioactive compounds for specific proposes.

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APPENDIX

Probit analysis (brine shrimp) of dichloromethane extract from *A.colorata* fruit

* * * * * * * * * * * * P R O B I T A N A L Y S I S * * * * * * * * * * * *

Parameter estimates converged after 19 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.03594	.01687	2.12985

Intercept	Standard Error	Intercept/S.E.
-.47802	.19253	-2.48284

Pearson Goodness-of-Fit Chi Square = .114 DF = 2 P = .945

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

- -

* * * * * * * * * * * * P R O B I T A N A L Y S I S * * * * * * * * * * * *

Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	30.0	30.0	30.000	.000	1.00000
100.00	30.0	30.0	29.973	.027	.99908
10.00	30.0	13.0	13.584	-.584	.45279
.00	30.0	10.0	9.490	.510	.31632

* * * * * * * * * * * * PROBIT ANALYSIS * * * * * * * * * * * *

Confidence Limits for Effective VAR00001

		95% Confidence Limits	
Prob	VAR00001	Lower	Upper
.01	-51.42761	-714.02422	-23.18061
.02	-43.84287	-619.02806	-19.11494
.03	-39.03059	-558.77621	-16.51524
.04	-35.41050	-513.46537	-14.54530
.05	-32.46584	-476.62021	-12.93120
.06	-29.95947	-445.26953	-11.54700
.07	-27.76187	-417.79070	-10.32375
.08	-25.79418	-393.19586	-9.21930
.09	-24.00465	-370.83684	-8.20584
.10	-22.35738	-350.26433	-7.26395
.15	-15.53726	-265.22331	-3.22959
.20	-10.11684	-197.91892	.26030
.25	-5.46660	-140.65421	3.73078
.30	-1.29055	-90.23857	7.85723
.35	2.57919	-46.41435	14.57437
.40	6.25119	-14.95507	31.07391
.45	9.80390	-1.44194	63.96149
.50	13.30028	4.18560	103.99899
.55	16.79666	7.67931	146.17032
.60	20.34936	10.45487	189.79546
.65	24.02137	12.95749	235.25166
.70	27.89111	15.38708	283.36344
.75	32.06716	17.87396	335.41864
.80	36.71740	20.54494	393.48285
.85	42.13782	23.57827	461.24381
.90	48.95794	27.32006	546.57740
.91	50.60521	28.21516	567.19670
.92	52.39474	29.18459	589.59974
.93	54.36243	30.24735	614.23627
.94	56.56002	31.43080	641.75490
.95	59.06639	32.77661	673.14397
.96	62.01106	34.35315	710.02669
.97	65.63115	36.28550	755.37512
.98	70.44342	38.84606	815.66612
.99	78.02817	42.86708	910.70693

Probit analysis (brine shrimp) of ethyl acetate extract from *A.colorata* fruit

* * * * * * * * * * * * P R O B I T A N A L Y S I S * * * * * * * * * * * *

Parameter estimates converged after 12 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.00689	.00299	2.30258

Intercept	Standard Error	Intercept/S.E.
-.42568	.17554	-2.42494

Pearson Goodness-of-Fit Chi Square = .370 DF = 2 P = .831

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

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Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	30.0	30.0	30.000	1.4954E-09	1.00000
100.00	30.0	18.0	18.119	-.119	.60397
10.00	30.0	12.0	10.819	1.181	.36064
.00	30.0	9.0	10.055	-1.055	.33517

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Confidence Limits for Effective VAR00001

Prob	VAR00001	95% Confidence Limits Lower	Upper
.01	-275.73480	-2070.13544	-128.97750
.02	-236.18814	-1804.72630	-107.21648
.03	-211.09704	-1636.39808	-93.34444
.04	-192.22199	-1509.81681	-82.86355
.05	-176.86860	-1406.88942	-74.30144
.06	-163.80044	-1319.31401	-66.98179
.07	-152.34223	-1242.55670	-60.53468
.08	-142.08277	-1173.85725	-54.73451
.09	-132.75219	-1111.40451	-49.43282
.10	-124.16339	-1053.94295	-44.52631
.15	-88.60343	-816.41558	-23.83323
.20	-60.34151	-628.38063	-6.64275
.25	-36.09530	-468.17573	9.21749
.30	-14.32145	-326.28751	25.44121
.35	5.85527	-199.13301	44.80099
.40	25.00100	-90.32605	75.02174
.45	43.52472	-16.02898	135.23560
.50	61.75475	20.80164	230.78329
.55	79.98478	41.40057	342.56267
.60	98.50849	56.71072	461.76345
.65	117.65423	70.17032	587.33157
.70	137.83094	83.13328	720.88289
.75	159.60479	96.38341	865.74469
.80	183.85101	110.62935	1027.56390
.85	212.11293	126.83892	1216.57976
.90	247.67288	146.87823	1454.76089
.91	256.26169	151.67826	1512.32893
.92	265.59226	156.87926	1574.88236
.93	275.85173	162.58362	1643.67762
.94	287.30994	168.93886	1720.52680
.95	300.37809	176.16952	1808.19120
.96	315.73149	184.64417	1911.20605
.97	334.60654	195.03717	2037.87521
.98	359.69763	208.81729	2206.29535
.99	399.24430	230.47297	2471.80983

Probit analysis (brine shrimp) of methanol extract from *A.colorata* fruit

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Parameter estimates converged after 18 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.00459	.00153	3.01209

Intercept	Standard Error	Intercept/S.E.
-1.60776	.21486	-7.48277

Pearson Goodness-of-Fit Chi Square = .345 DF = 2 P = .842

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

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Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	30.0	30.0	29.958	.042	.99859
100.00	30.0	3.0	3.762	-.762	.12541
10.00	30.0	2.0	1.775	.225	.05917
.00	30.0	2.0	1.618	.382	.05394

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Confidence Limits for Effective VAR00001

		95% Confidence Limits	
Prob	VAR00001	Lower	Upper
.01	-156.41324	-584.14924	-47.69094
.02	-97.07766	-421.81899	-4.20030
.03	-59.43114	-321.69132	26.25878
.04	-31.11113	-248.98521	51.78800
.05	-8.07498	-192.43352	75.14318
.06	11.53240	-146.90687	97.62982
.07	28.72423	-109.57749	119.93478
.08	44.11747	-78.63855	142.39129
.09	58.11700	-52.78684	165.10061
.10	71.00360	-31.00374	188.01801
.15	124.35754	40.63195	301.45434
.20	166.76156	82.83411	406.34156
.25	203.14043	113.59304	501.77222
.30	235.80979	138.78415	589.90325
.35	266.08281	160.83745	672.85981
.40	294.80895	180.98608	752.35527
.45	322.60182	199.96427	829.78389
.50	349.95405	218.27318	906.35330
.55	377.30628	236.30260	983.20219
.60	405.09915	254.39912	1061.51248
.65	433.82529	272.91613	1142.63955
.70	464.09832	292.26599	1228.29956
.75	496.76767	312.99607	1320.89163
.80	533.14654	335.93213	1424.14516
.85	575.55057	362.51119	1544.65547
.90	628.90451	395.76919	1696.46949
.91	641.79110	403.77677	1733.16241
.92	655.79063	412.46626	1773.03395
.93	671.18387	422.00997	1816.88568
.94	688.37571	432.65636	1865.87365
.95	707.98308	444.78386	1921.75944
.96	731.01923	459.01376	1987.43640
.97	759.33924	476.48316	2068.20232
.98	796.98577	499.66888	2175.60336
.99	856.32135	536.14039	2344.95275

Probit analysis (brine shrimp) of Compound 1

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>Warning # 13527
>Parameter estimates did not converge in maximum number of iterations.

Number of iterations = 20
Optimal solution not found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.49095	.89505	.54852

Intercept	Standard Error	Intercept/S.E.
-.84316	.26094	-3.23122

Pearson Goodness-of-Fit Chi Square = .001 DF = 2 P = 1.000

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

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Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	30.0	30.0	30.000	.000	1.00000
100.00	30.0	30.0	30.000	.000	1.00000
10.00	30.0	30.0	29.999	.001	.99998
.00	30.0	6.0	5.987	.013	.19957

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Confidence Limits for Effective VAR00001

		95% Confidence Limits	
Prob	VAR00001	Lower	Upper
.01	-3.02105	.	.
.02	-2.46580	.	.
.03	-2.11352	.	.
.04	-1.84851	.	.
.05	-1.63294	.	.
.06	-1.44946	.	.
.07	-1.28858	.	.
.08	-1.14454	.	.
.09	-1.01354	.	.
.10	-.89295	.	.
.15	-.39367	.	.
.20	.00313	.	.
.25	.34355	.	.
.30	.64927	.	.
.35	.93255	.	.
.40	1.20136	.	.
.45	1.46144	.	.
.50	1.71740	.	.
.55	1.97335	.	.
.60	2.23343	.	.
.65	2.50224	.	.
.70	2.78553	.	.
.75	3.09124	.	.
.80	3.43166	.	.
.85	3.82847	.	.
.90	4.32774	.	.
.91	4.44833	.	.
.92	4.57933	.	.
.93	4.72338	.	.
.94	4.88425	.	.
.95	5.06774	.	.
.96	5.28330	.	.
.97	5.54831	.	.
.98	5.90060	.	.
.99	6.45584	.	.

Probit analysis (brine shrimp) of Mixture 2

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Parameter estimates converged after 12 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.00070	.00029	2.38385

Intercept	Standard Error	Intercept/S.E.
-1.03937	.16634	-6.24859

Pearson Goodness-of-Fit Chi Square = .118 DF = 2 P = .943

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

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Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	30.0	11.0	11.007	-.007	.36690
100.00	30.0	5.0	4.985	.015	.16616
10.00	30.0	5.0	4.528	.472	.15095
.00	30.0	4.0	4.479	-.479	.14932

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Confidence Limits for Effective VAR00001

Prob	VAR00001	95% Confidence Limits Lower	Upper
.01	-1840.38413	-11983.85760	-816.37638
.02	-1450.56560	-9799.55977	-594.27935
.03	-1203.23824	-8415.78934	-451.26940
.04	-1017.18351	-7376.53566	-341.98567
.05	-865.84233	-6532.76338	-251.51137
.06	-737.02722	-5816.15712	-172.92704
.07	-624.08157	-5189.48319	-102.37440
.08	-522.95221	-4630.16026	-37.41410
.09	-430.97910	-4123.47557	23.66185
.10	-346.31771	-3659.35826	82.16913
.15	4.20309	-1788.46557	375.08187
.20	282.78591	-515.79263	822.13289
.25	521.78516	100.82163	1680.88866
.30	736.41387	365.96120	2740.67699
.35	935.29937	533.93725	3800.44407
.40	1124.02225	668.33479	4831.05472
.45	1306.61382	787.57282	5838.97558
.50	1486.31047	899.23264	6836.60398
.55	1666.00712	1007.45678	7837.66806
.60	1848.59869	1115.12917	8857.15456
.65	2037.32158	1224.75578	9912.53614
.70	2236.20707	1338.99718	11026.03788
.75	2450.83578	1461.21506	12228.74789
.80	2689.83503	1596.36507	13568.96792
.85	2968.41785	1752.98857	15132.06838
.90	3318.93865	1949.06727	17099.79511
.91	3403.60005	1996.29918	17575.18779
.92	3495.57315	2047.56353	18091.68407
.93	3596.70251	2103.88004	18659.65080
.94	3709.64816	2166.71889	19294.03852
.95	3838.46327	2238.31968	20017.62831
.96	3989.80445	2322.35964	20867.83493
.97	4175.85919	2425.56971	21913.16227
.98	4423.18654	2562.61382	23302.89855
.99	4813.00507	2778.31773	25493.58949

Probit analysis (brine shrimp) of Mixture 3

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Parameter estimates converged after 10 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.00138	.00031	4.42221

Intercept	Standard Error	Intercept/S.E.
-1.46642	.20124	-7.28677

Pearson Goodness-of-Fit Chi Square = .559 DF = 2 P = .756

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

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Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	30.0	14.0	13.917	.083	.46388
100.00	30.0	2.0	2.759	-.759	.09195
10.00	30.0	3.0	2.195	.805	.07316
.00	30.0	2.0	2.138	-.138	.07127

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Confidence Limits for Effective VAR00001

		95% Confidence Limits	
Prob	VAR00001	Lower	Upper
.01	-625.05993	-1517.23675	-253.03731
.02	-426.91570	-1170.75000	-106.35727
.03	-301.19953	-953.08202	-11.12673
.04	-206.62816	-790.98050	62.15311
.05	-129.70164	-660.53497	123.17216
.06	-64.22510	-550.79749	176.40112
.07	-6.81497	-455.80566	224.29897
.08	44.58895	-371.94205	268.37602
.09	91.33875	-296.84236	309.63325
.10	134.37203	-228.87389	348.77151
.15	312.54128	36.57611	526.77200
.20	454.14455	222.74675	693.04174
.25	575.62755	362.25991	855.89106
.30	684.72304	473.50914	1016.17268
.35	785.81627	567.56933	1173.72628
.40	881.74386	651.04218	1329.01032
.45	974.55491	727.97626	1483.07629
.50	1065.89448	801.03618	1637.35400
.55	1157.23405	872.16226	1793.56556
.60	1250.04510	942.95608	1953.77178
.65	1345.97269	1014.94349	2120.54126
.70	1447.06592	1089.81417	2297.28437
.75	1556.16141	1169.73547	2488.89393
.80	1677.64441	1257.91315	2703.07872
.85	1819.24768	1359.87035	2953.56191
.90	1997.41693	1487.22243	3269.66032
.91	2040.45021	1517.85860	3346.13088
.92	2087.20001	1551.09456	3429.25184
.93	2138.60393	1587.58819	3520.69887
.94	2196.01406	1628.28789	3622.88885
.95	2261.49060	1674.63819	3739.50499
.96	2338.41712	1729.01059	3876.59716
.97	2432.98849	1795.74528	4045.24384
.98	2558.70466	1884.29591	4269.59173
.99	2756.84889	2023.55350	4623.50093

Probit analysis (brine shrimp) of Compound 5

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Parameter estimates converged after 10 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.00113	.00029	3.95835

Intercept	Standard Error	Intercept/S.E.
-.80130	.15271	-5.24718

Pearson Goodness-of-Fit Chi Square = .487 DF = 2 P = .784

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

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Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	30.0	19.0	18.862	.138	.62875
100.00	30.0	6.0	7.369	-1.369	.24563
10.00	30.0	7.0	6.443	.557	.21476
.00	30.0	7.0	6.344	.656	.21148

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Confidence Limits for Effective VAR00001

		95% Confidence Limits	
Prob	VAR00001	Lower	Upper
.01	-1349.79433	-3006.06564	-772.02852
.02	-1108.52104	-2532.75401	-606.05341
.03	-955.44087	-2233.31279	-499.88769
.04	-840.28462	-2008.67315	-419.40503
.05	-746.61390	-1826.45825	-353.42648
.06	-666.88540	-1671.82101	-296.81210
.07	-596.97911	-1536.65976	-246.74701
.08	-534.38636	-1416.04800	-201.51068
.09	-477.46076	-1306.75874	-159.96773
.10	-425.06065	-1206.56046	-121.32472
.15	-208.11019	-797.67528	44.63014
.20	-35.68485	-484.57464	188.39415
.25	112.24074	-232.43855	328.20725
.30	245.08250	-28.16119	475.91236
.35	368.18018	135.70813	638.20727
.40	484.98784	268.19373	815.21962
.45	598.00061	379.51553	1003.34068
.50	709.22160	478.01922	1199.53229
.55	820.44259	569.41313	1402.83368
.60	933.45535	657.56096	1614.12870
.65	1050.26302	745.37278	1835.81484
.70	1173.36070	835.47367	2071.87817
.75	1306.20245	930.78662	2328.54770
.80	1454.12804	1035.30504	2615.97846
.85	1626.55339	1155.65469	2952.49346
.90	1843.50384	1305.55598	3377.43221
.91	1895.90395	1341.57338	3480.25611
.92	1952.82955	1380.63394	3592.02776
.93	2015.42230	1423.50982	3714.99997
.94	2085.32860	1471.31394	3852.42219
.95	2165.05710	1525.74130	4009.24643
.96	2258.72782	1589.57444	4193.60675
.97	2373.88407	1667.90571	4420.39778
.98	2526.96424	1771.82728	4722.08316

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Probit analysis (brine shrimp) of Mixture 6

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Parameter estimates converged after 13 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.00805	.00306	2.63309

Intercept	Standard Error	Intercept/S.E.
-.26040	.17233	-1.51099

Pearson Goodness-of-Fit Chi Square = 1.144 DF = 2 P = .565

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

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Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	30.0	30.0	30.000	1.0303E-13	1.00000
100.00	30.0	21.0	21.207	-.207	.70689
10.00	30.0	15.0	12.858	2.142	.42861
.00	30.0	10.0	11.918	-1.918	.39728

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Confidence Limits for Effective VAR00001

		95% Confidence Limits	
Prob	VAR00001	Lower	Upper
.01	-256.72814	-1108.52756	-129.77412
.02	-222.85329	-976.33625	-110.02892
.03	-201.36079	-892.51302	-97.45332
.04	-185.19281	-829.48842	-87.96082
.05	-172.04141	-778.24821	-80.21398
.06	-160.84750	-734.65635	-73.59858
.07	-151.03263	-696.45411	-67.77888
.08	-142.24459	-662.26636	-62.55023
.09	-134.25222	-631.19081	-57.77815
.10	-126.89523	-602.60194	-53.36921
.15	-96.43531	-484.45730	-34.89412
.20	-72.22673	-390.95220	-19.81818
.25	-51.45793	-311.23268	-6.38484
.30	-32.80690	-240.35955	6.39617
.35	-15.52395	-175.84143	19.39609
.40	.87589	-116.72356	33.83525
.45	16.74292	-63.79820	52.07717
.50	32.35838	-20.37055	78.68848
.55	47.97384	10.24693	118.10995
.60	63.84087	30.74445	168.77971
.65	80.24071	46.23451	226.84668
.70	97.52366	59.74947	290.84976
.75	116.17469	72.80932	361.44406
.80	136.94349	86.40817	440.99806
.85	161.15207	101.59042	534.39685
.90	191.61198	120.13902	652.46798
.91	198.96898	124.56014	681.04467
.92	206.96135	129.34381	712.10864
.93	215.74939	134.58353	746.28531
.94	225.56426	140.41389	784.47689
.95	236.75817	147.03966	828.05837
.96	249.90957	154.79674	879.28835
.97	266.07754	164.29957	942.30262
.98	287.57005	176.88603	1026.11500
.99	321.44490	196.64372	1158.29380

Probit analysis (brine shrimp) of Compound 7

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Parameter estimates converged after 13 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.11353	.03565	3.18500

Intercept	Standard Error	Intercept/S.E.
-.16789	.23000	-.72997

Pearson Goodness-of-Fit Chi Square = 3.334E-10 DF = 2 P = 1.000

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

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Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	30.0	30.0	30.000	.000	1.00000
100.00	30.0	30.0	30.000	.000	1.00000
10.00	30.0	25.0	25.000	.000	.83333
.00	30.0	13.0	13.000	.000	.43333

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Confidence Limits for Effective VAR00001

		95% Confidence Limits	
Prob	VAR00001	Lower	Upper
.01	-19.01191	-56.41258	-9.86288
.02	-16.61083	-50.20620	-8.34007
.03	-15.08742	-46.27304	-7.36931
.04	-13.94142	-43.31725	-6.63607
.05	-13.00924	-40.91521	-6.03737
.06	-12.21580	-38.87256	-5.52592
.07	-11.52012	-37.08317	-5.07585
.08	-10.89721	-35.48244	-4.67143
.09	-10.33071	-34.02797	-4.30228
.10	-9.80924	-32.69039	-3.96123
.15	-7.65021	-27.16843	-2.53317
.20	-5.93428	-22.80527	-1.37268
.25	-4.46217	-19.08970	-.34946
.30	-3.14017	-15.78571	.60214
.35	-1.91514	-12.76555	1.52542
.40	-.75270	-9.95554	2.45735
.45	.37197	-7.31598	3.43815
.50	1.47881	-4.83515	4.52029
.55	2.58565	-2.52963	5.77773
.60	3.71032	-.44038	7.30885
.65	4.87275	1.39302	9.21738
.70	6.09779	2.97776	11.57607
.75	7.41979	4.38324	14.42619
.80	8.89190	5.71215	17.83607
.85	10.60783	7.08233	21.98954
.90	12.76685	8.66055	27.36132
.91	13.28832	9.02685	28.67367
.92	13.85483	9.42006	30.10407
.93	14.47774	9.84756	31.68172
.94	15.17342	10.31991	33.44883
.95	15.96686	10.85310	35.46974
.96	16.89904	11.47330	37.85028
.97	18.04504	12.22829	40.78432
.98	19.56845	13.22195	44.69459
.99	21.96953	14.77118	50.87456

Probit analysis (insecticidal activity) of Compound 1

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Parameter estimates converged after 8 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.00071	.00023	3.16759

Intercept	Standard Error	Intercept/S.E.
-.53391	.11637	-4.58810

Pearson Goodness-of-Fit Chi Square = 2.422 DF = 3 P = .490

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

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Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1000.00	45.0	24.0	25.733	-1.733	.57185
500.00	45.0	23.0	19.349	3.651	.42999
100.00	45.0	15.0	14.485	.515	.32189
50.00	45.0	11.0	13.913	-2.913	.30919
10.00	45.0	14.0	13.463	.537	.29918

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Confidence Limits for Effective VAR00001

		95% Confidence Limits	
Prob	VAR00001	Lower	Upper
.01	-2506.93112	-7155.18332	-1400.23227
.02	-2125.67060	-6157.51347	-1162.30375
.03	-1883.77302	-5524.94477	-1010.92490
.04	-1701.80290	-5049.38638	-896.75009
.05	-1553.78424	-4662.80092	-803.63308
.06	-1427.79712	-4333.97214	-724.15972
.07	-1317.33105	-4045.85389	-654.27686
.08	-1218.42187	-3788.07006	-591.51341
.09	-1128.46793	-3553.81397	-534.24456
.10	-1045.66517	-3338.36877	-481.34058
.15	-702.83965	-2449.16968	-259.50237
.20	-430.37279	-1748.29830	-77.35653
.25	-196.62048	-1156.51441	88.40974
.30	13.29631	-643.57006	255.76885
.35	207.81551	-209.79494	452.39627
.40	392.39520	116.87816	723.91352
.45	570.97819	335.08711	1084.46067
.50	746.72981	494.06568	1495.06223
.55	922.48143	629.19020	1929.51785
.60	1101.06442	755.51817	2381.94597
.65	1285.64411	880.24325	2855.41125
.70	1480.16331	1008.13660	3357.92044
.75	1690.08010	1143.74837	3902.61212
.80	1923.83240	1292.95612	4510.95453
.85	2196.29926	1465.37682	5221.55106
.90	2539.12479	1680.89581	6117.06937
.91	2621.92754	1732.78362	6333.53073
.92	2711.88149	1789.09499	6568.74431
.93	2810.79067	1850.95051	6827.43607
.94	2921.25673	1919.96567	7116.42202
.95	3047.24386	1998.60121	7446.08861
.96	3195.26252	2090.89748	7833.49481
.97	3377.23264	2204.25014	8309.87536
.98	3619.13021	2354.77196	8943.30108
.99	4000.39073	2591.72193	9941.94948

Probit analysis (insecticidal activity) of Mixture 6

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Parameter estimates converged after 14 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
VAR00001	.02252	.00385	5.84637

Intercept	Standard Error	Intercept/S.E.
-.75734	.20607	-3.67518

Pearson Goodness-of-Fit Chi Square = 1.515 DF = 2 P = .469

Since Goodness-of-Fit Chi square is NOT significant, no heterogeneity factor is used in the calculation of confidence limits.

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Observed and Expected Frequencies

VAR00001	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
500.00	45.0	45.0	45.000	.000	1.00000
100.00	45.0	43.0	41.964	1.036	.93254
50.00	45.0	26.0	28.974	-2.974	.64387
10.00	45.0	15.0	13.380	1.620	.29733

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Confidence Limits for Effective VAR00001

		95% Confidence Limits	
Prob	VAR00001	Lower	Upper
.01	-69.66227	-127.88879	-39.91774
.02	-57.55919	-109.85618	-30.67907
.03	-49.88017	-98.43710	-24.79540
.04	-44.10354	-89.86124	-20.35508
.05	-39.40470	-82.89624	-16.73242
.06	-35.40525	-76.97682	-13.64007
.07	-31.89851	-71.79432	-10.92102
.08	-28.75865	-67.16086	-8.47958
.09	-25.90307	-62.95321	-6.25290
.10	-23.27450	-59.08593	-4.19736
.15	-12.39154	-43.14836	4.38712
.20	-3.74210	-30.59700	11.32510
.25	3.67835	-19.94873	17.39696
.30	10.34214	-10.52004	22.98346
.35	16.51714	-1.93981	28.31703
.40	22.37660	6.01248	33.56759
.45	28.04571	13.47402	38.87995
.50	33.62493	20.53315	44.39220
.55	39.20415	27.25301	50.24372
.60	44.87325	33.69391	56.57673
.65	50.73272	39.93573	63.53776
.70	56.90771	46.09708	71.29021
.75	63.57151	52.35117	80.05131
.80	70.99195	58.95204	90.17057
.85	79.64139	66.30914	102.30281
.90	90.52436	75.23234	117.90166
.91	93.15293	77.34862	121.70819
.92	96.00851	79.63437	125.85679
.93	99.14837	82.13352	130.43253
.94	102.65511	84.90935	135.55825
.95	106.65456	88.05810	141.42127
.96	111.35340	91.73759	148.32944
.97	117.13002	96.23646	156.84675
.98	124.80904	102.18298	168.20299
.99	136.91213	111.49578	186.16146

VITA

Pichaya Prasertsang was born on April 5, 1979 at Ramathibodi Hospital in Bangkok, Thailand. He obtained a Bachelor Degree of Science, majoring in Chemistry from Srinakarinwirote University, Bangkok, Thailand in 1999. Since 2000, he has been a graduate student studying Organic Chemistry at Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. During the course study, he obtained financial support from Graduate School Chulalongkorn University.