PHYTOCHEMICAL STUDY OF *ZANTHOXYLUM LIMONELLA* (DENNST.) ALSTON PERICARP

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Thesis
entitled

PHYTOCHEMICAL STUDY OF
***ZANTHOXYLUM LIMONELLA* (DENNST.) ALSTON PERICARP**

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PHYTOCHEMICAL STUDY OF *ZANTHOXYLUM LIMONELLA* (DENNST.) ALSTON PERICARP

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ABSTRACT

The phytochemical study of *Zanthoxylum limonella* pericarp ethanol extract, which inhibited *Candida albicans* ATCC 13802, resulted in the isolation of compounds 1, 2 and 3. They were identified using spectroscopic methods (UV, IR, MS, ^{1}H-NMR and ^{13}C-NMR). The compounds were as follows: **1** was β-sitosterol, **2** was comprised of a mixture of 3-*O*-β-D-glucopyranosyl-(24S)-24α-ethylcholesta-5-ene (β-sitosteryl glucoside, **2.1**) and 3-*O*-β-D-glucopyranosyl-22E,(24S)-24α-ethyl cholest-5,22-diene(stigmasteryl glucoside, **2.2**) in a proportion of 58:42, and **3** was lupeol. The isolated compounds 2 and 3 did not inhibit *C. albicans* ATCC 13802. Compound 3 was selected as a marker compound in the quality assessment (standardization) of the pericarp extract because it could be isolated in sufficient amount without difficulty and had a reported antiinflammatory effect. A quantitative analysis of **3** using high-performance liquid chromatography (HPLC) was performed. An appropriate isocratic condition analysis was performed on a Hypersil® Gold C18 column (150×4.6 mm i.d.) with a solvent system comprised of a mixture of methanol and water (with 1% acetic acid) in the ratio of 95:5 which include UV detection at 214 nm. The peak of **3** appeared at the retention time of 10 min. The test of system suitability resulted in a precision (%RSD) of 0.68-1.26, number of theoretical plates (N) of 7,584, a tailing factor (TF) of 0.8 and a resolution factor (R_s) of 2.46. A test of method validation was performed. A calibration curve of **3** was linear in the range of 10-100 μg/ml, with a regression coefficient (r^2) of 0.9994 (n = 15). The recovery of **3** spiked into the extract was 98.24%. The limits of detection and quantitation were 1.47 μg/m and 4.45 μg/ml, respectively. The results of the system suitability test and method validation conformed to the USP 26 requirement. The content of **3** in the pericarp extract quantitated by the HPLC method was 0.195% w/w.

KEY WORDS: *ZANTHOXYLUM LIMONELLA* / β-SITOSTEROL / β-SITOSTERYL GLUCOSIDE / STIGMASTERYL GLUCOSIDE / LUPEOL / HPLC QUANTITATIVE ANALYSIS

121 pages

การศึกษาพฤกษเคมีของสมุนไพรพริกพราน
PHYTOCHEMICAL STUDY OF *ZANTHOXYLUM LIMONELLA* (DENNST.) ALSTON PERICARP

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บทคัดย่อ

จากการศึกษาพฤกษเคมีของสารสกัดอัลกอฮอล์จากเปลือกผลพริกพราน *Zanthoxylum limonella* ซึ่งมีฤทธิ์ยับยั้ง *Candida albicans* ATCC 13802 สามารถแยกสาร 1, 2 และ 3 และพิสูจน์สูตรโครงสร้างทางเคมีโดยอาศัยเทคนิคสเปคโตรสโคปี (UV, IR, MS, ^{1}H-NMR and ^{13}C-NMR) พบว่าสาร **1** คือ β-sitosterol, **2** คือ สารผสมของ 3-*O*-β-D-glucopyranosyl-(24S)-24α-ethylcholesta-5-ene (β-sitosteryl glucoside, **2.1**) และ 3-*O*-β-D-glucopyranosyl-22E,(24S)-24α-ethylcholest-5,22-diene (stigma steryl glucoside, **2.2**) ในสัดส่วน 58:42 และสาร **3** คือ lupeol สาร **2** และ **3** ไม่มีฤทธิ์ยับยั้งเชื้อ *C. albicans* ATCC 13802 การทดลองได้เลือกสาร **3** เป็น marker compound ในการควบคุมคุณภาพสารสกัดจากเปลือกผลเนื่องจากแยกได้ไม่ยากในปริมาณเพียงพอและมีรายงานฤทธิ์ต้านอักเสบ การวิเคราะห์ปริมาณของสาร **3** ในสารสกัดเปลือกผลพริกพรานใช้เทคนิค high-pressure liquid chromatography (HPLC) โดยสภาวะที่เหมาะสมสำหรับการวิเคราะห์ประกอบด้วยคอลัมน์ Hypersil® Gold C18 (150×4.6 มม., i.d.) ส่วนผสมของเมทานอล-น้ำ (95:5) ซึ่งในน้ำมีกรดน้ำส้ม 1% เป็นตัวทำละลายเคลื่อนที่ ในอัตราส่วนคงที่ UV detection ที่ความยาวคลื่น 214 นาโนเมตร พีคของสาร **3** ปรากฏที่นาทีที่ 10 ผลการทดสอบความเหมาะสมของระบบให้ค่าความแม่นยำของวิธีวิเคราะห์คิดเป็น % RSD เท่ากับ 0.68-1.26 ค่าประสิทธิภาพของคอลัมน์เท่ากับ 7,584 ค่าความสมมาตรของพีคเท่ากับ 0.8 และประสิทธิภาพการแยกของคอลัมน์เท่ากับ 2.46 ผลการทดสอบวิธีวิเคราะห์ให้กราฟมาตรฐานของสาร **3** ในช่วงความเข้มข้น 10-100 ไมโครกรัมต่อมิลลิลิตร มีค่าสัมประสิทธิ์การถดถอย (regression coefficient, r^2) 0.9994 ค่าความถูกต้องของวิธีซึ่งคิดเป็นค่า % recovery เท่ากับ 98.24% ขีดจำกัดของการวัดและขีดจำกัดการหาปริมาณคือ 1.47 และ 4.45 ไมโครกรัมต่อมิลลิลิตรตามลำดับ ผลการทดสอบความเหมาะสมของระบบและวิธีวิเคราะห์เป็นไปตามข้อกำหนดของ USP 26 จากการวิเคราะห์หาปริมาณโดยวิธี HPLC พบว่าสารสกัดเปลือกผลมีปริมาณสาร **3** เท่ากับ 0.195 % w/w

121 หน้า

CONTENTS

	Page
ACKNOWLEDGEMENTS	**iii**
ABSTRACT (ENGLISH)	**iv**
ABSTRACT (THAI)	**v**
LIST OF TABLES	**viii**
LIST OF FIGURES	**ix**
LIST OF ABREVIATIONS	**xii**
CHAPTER I INTRODUCTION	**1**
CHAPTER II OBJECTIVE	**3**
CHAPTER III LITERATURE REVIEW	**4**
3.1 *Zanthoxylum limonella*	4
3.2 The chemical studies of the genus *Zanthoxylum*	13
3.3 Analytical procedure	23
CHAPTER IV MATERIALS AND METHODS	**39**
Part I: Isolation and identification the phytochemical from *Z. limonella* (Dennst.) Alston pericarp.	39
Part II: HPLC Quantitative Analysis	47
CHAPTER V RESULTS AND DISCUSSION	**53**
Part I : Isolation and identification	53
5.1 Extraction	53
5.2 The isolation	55
5.3 Structure elucidation	57
Part II : High-Performance Liquid Chromatography (HPLC) Quantitative Analysis	103
5.1 System suitability test	103
5.2 Method validation	106

CONTENTS (cont.)

	Page
5.3 Determination of the compound ZL3 in *Z.limonella* pericarp extract	110
CHAPTER VI CONCLUSION	**113**
REFERENCES	**116**
BIOGRAPHY	**121**

LIST OF TABLES

Table **Page**

3.1 Ethnomedical uses of *Z. limonella* 8

3.2 Ethnomedical use and phytochemicals of *Zanthoxylum* plants 20

3.3 The phytochemicals of *Z. limonella* 21

3.4 Common names of *Zanthoxylum* Species in Thailand 22

3.5 System suitability parameters and recommendation 24

3.6 HPLC analysis method of lupeol from natural product 38

5.1 The Percent extractives of *Z. limonella* 53

5.2 ^{13}C-NMR chemical shifts of compound 1 in $CDCl_3$ together with their literature values 60

5.3 ^{1}H- and ^{13}C-NMR data of compound 1 ($CDCl_3$) 61

5.4 ^{13}C-chemical shifts of compound 2.1 and compound 2.2 (Pyridine-d_5) 73

5.5 ^{1}H- and ^{13}C-NMR data of compound 2.1 and compound 2.2 (Pyridine-d_5) 74

5.6 ^{13}C-chemical shifts of compound 3 ($CDCl_3$) 83

5.7 NMR data of compound 3 ($CDCl_3$) 84

5.8 Data of compounds isolated from *Z. limonella* pericarp ethanolic extract 101

5.9 Retention time data of compound 3 104

5.10 The results of system suitability test 106

5.11 Linearity of analysis of compound 3 107

5.12 Percentage recoveries obtained from spiking method of compound 108

5.13 Intra-day precision of analysis of compound 3 108

5.14 Inter-day precision of analysis of compound 3 109

5.15 The results of method validation test 110

5.16 The linearity data of lupeol 111

5.17 Peak area of *Z. limonella* pericarp extract 111

LIST OF FIGURES

Figure		Page
3.1	*Zanthoxylum limonella* (Dennst.) Alston	7
3.2	Various streptococci in a biofilm in the oral cavity.	8
3.3	The dominant bacterial species in dental plaque	8
3.4	The USP eight steps of method validation	29
4.1	The extraction method	42
4.2	The isolation scheme of the extraction and isolation	44
5.1	Thin-layer chromatogram of the extracts from the pericarp	54
5.2	Thin-layer chromatogram of the isolated compounds	56
5.3	The UV spectrum of compound 1	58
5.4	FABMS spectrum of compound 1	58
5.5	FTIR spectrum of compound 1	59
5.6	125.7 MHz ^{13}C-NMR spectrum of compound 1 in $CDCl_3$	62
5.7	125.7 MHz ^{13}C-NMR expanded spectrum of compound 1 mixture in $CDCl_3$ ($\Delta\delta$ = 11-57 ppm)	63
5.8	DEPT 90 spectrum of compound 1 in $CDCl_3$	64
5.9	DEPT 135 spectrum of compound 1 in $CDCl_3$	65
5.10	DEPT 135 expanded spectrum of compound 1 in $CDCl_3$ ($\Delta\delta$ = 11-57 ppm)	66
5.11	500 MHz ^{1}H-NMR spectrum of compound 1 in $CDCl_3$	67
5.12	500 MHz ^{1}H-NMR expanded spectrum of compound 1 in $CDCl_3$ ($\Delta\delta$ = 0-5.5 ppm)	68
5.13	The UV spectrum of compound 2	71
5.14	FABMS spectrum of compound 2 mixture	71
5.15	FTIR spectrum of compound 2 mixture	72
5.16	125.7 MHz ^{13}C-NMR spectrum of the compound 2 mixture in Pyridine-d_5	75
5.17	500 MHz ^{1}H-NMR spectrum of compound 2 mixture in Pyridine-d_5	76

LIST OF FIGURES (cont.)

Figure		Page
5.18	500 MHz ^{1}H-NMR expanded spectrum of compound 2 mixture in Pyridine-d_5 ($\Delta\delta$ = 0.56 - 2.24 ppm)	77
5.19	500 MHz ^{1}H-NMR expanded spectrum of compound 2 mixture in Pyridine-d_5 ($\Delta\delta$ = 2.4 - 7.3 ppm)	78
5.20	The UV spectrum of compound 3	81
5.21	FABMS spectrum of compound 3 in $CDCl_3$	81
5.22	FTIR spectrum of compound 3	82
5.23	125.7 MHz ^{13}C-NMR spectrum of ompound3 in $CDCl_3$	85
5.24	125.7 MHz ^{13}C-NMR expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta$ = 14 - 56 ppm)	86
5.25	DEPT 90 and 135 spectrum of compound 3 in $CDCl_3$	87
5.26	DEPT 135 spectrum of compound 3 in $CDCl_3$	88
5.27	DEPT 135 expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta$ = 13 - 57 ppm)	89
5.28	500 MHz ^{1}H-NMR spectrum of compound 3 in $CDCl_3$	90
5.29	500 MHz ^{1}H NMR expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta$ = 0.6 - 2.5 ppm)	91
5.30	500 MHz ^{1}H NMR expanded spectrum of compound 3 in $CDCl_3$	92
5.31	HMQC spectrum of compound 3 in $CDCl_3$	93
5.32	HMQC expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta$ = 0.7 - 2.0 ppm)	94
5.33	HMQC expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta$ = 0.7 - 2.5 ppm)	95
5.34	HMBC spectrum of compound 3 in $CDCl_3$	96
5.35	HMBC expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta$ = 0.65 - 2.5 ppm)	97

LIST OF FIGURES (cont.)

Figure **Page**

5.36 HMBC expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta$ = 0.7 - 2.5 ppm) 98

5.37 COSY spectrum of compound 3 in $CDCl_3$ 99

5.38 COSY expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta$ = 0.6 - 2.5 ppm) 100

5.39 HPLC chromatogram of the ethanol extract 103

5.40 The calibration curve of compound 3 107

5.41 The calibration curve of lupeol 111

LIST OF ABBREVIATIONS

cm	=	centimeter(s)
DEPT	=	distorsionless enhancement by polarization transfer
EI-MS	=	electron impact mass spectrometry
Fig.	=	figure
FT-IR	=	Fourier transformed infrared spectrometer
g	=	gram
HPLC	=	high-pressure liquid chromatography
IC_{50}	=	concentration giving 50% inhibition
i.d.	=	inner diameter
IR	=	infrared
J(Hz)	=	coupling constant (hertz)
kg	=	kilogram(s)
LPLC	=	low-pressure liquid chromatrography
M	=	multiplicity
mg	=	milligram
μg	=	microgram
min	=	minute(s)
ml	=	milliliter(s)
mm	=	millimeter(s)
m.p.	=	melting point
m/z	=	mass to charge ratio
NMR	=	nuclear magnetic resonance spectroscopy
No.	=	number
ppm	=	part per million
Rf	=	mobility relative to front
TLC	=	thin-layer chromatography
UV	=	ultraviolet
°C	=	degree Celcius

LIST OF ABBREVIATIONS (cont.)

δ	=	chemical shift
S, *s*	=	singlet
D, *d*	=	doublet
T, *t*	=	triplet
Q, *q*	=	quartet
M, *m*	=	multiplet
br, *s*	=	broad signal

CHAPTER I
INTRODUCTION

Zanthoxylum limonella (Dennst.) Alston (syn. *Z. rhetsa* (Roxb.) D.C. and *Z. budrunga* Wall. ex Hook.f.) (1, 2) belonging to the Rutaceae family which is a large genus of aromatic, prickly deciduous (or rarely monoecious) trees or shrubs. They are distributed mainly in the pantropics but also in the subtropics (2, 3) and can grow to a height of 35 m. The major constituent of the essential oil has been reported (4, 5, 6) as sabinene 35.7–67.7%. The essential oil from the fruits is reported to have anti-inflammatory, anesthetic and hypotensive activities (7). The bark is used to treat toothache (8). The phytochemical studies of this genus are wellknown to contain several types of compounds, including alkaloids, terpenoids (9, 10), triterpenes and lignans (21). The fruits and seeds are reported to contain fatty acids, carbohydrates and proteins (9), phytosterols, flavanones, volatile oils (10), monoterpenes (11), amides, coumarins and flavones (12).

Many plant products have been successfully incorporated into dentifrice or mouthwash in many countries (13). The commission of higher education supported the joint project between Mahidol University and the private sector to develop *Z. limonella* products for oral health. Essential oil of *Z. limonella* is widely used for dental caries or flavoring of food in Thailand and other countries. (14). It also produces local anesthesia at the tongue after biting the pericarp, which has a good scent.

Dental caries is one of the most important problems in public health because of its ubiquitousness in civilized populations (15). Dental caries is known to be associated with specific gram-positive oral bacteria, of which the most prominent member is *Streptococcus mutans*, whereas periodontitis is associated with specific gram-negative oral bacteria, such as *Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis* (16). Mild periodontal disease is called gingivitis and is not usually serious. More severe periodontal disease, called periodontitis, can lead to teeth falling out. The herbs can help in the alleviation of problems dental caries and

periodontal disease. The herbs act as oral antiseptic or astringent, local anesthetic, and antiinflammation.

The present study aims at the isolation and identification of the phytochemicals from *Z. limonella* pericarp using chromatographic methods. Then the analysis of the isolated compound will be developed. The result of the study will be applied for the quality assessment of the plant products.

CHAPTER II
OBJECTIVE

This study aimed at the extraction, isolation and identification of the compounds from *Z. limonella* indigenous to Thailand, and the HPLC quantitative analysis of the isolated compound. The development of the oral cavity products of the extract was determined from the cytotoxicity to normal cell line L-929 (tested by Faculty of Dentistry, Mahidol University), so the ethanol extract that has no or less toxicity than hexane extract was selected for continuing study. The result of the study will be applied to standardize *Z. limonella* pericarp extract, which is used as herbal ingredient in the oral products. The Committee of Higher Education, Ministry of Education has supported the joint project between the university and the private sector to develop the oral products from *Z. limonella*.

CHAPTER III
LITERATURE REVIEW

3.1 *Zanthoxylum limonella*

3.1.1 Botany

Zanthoxylum limonella (Dennst.) Alston (syn. *Z. rhetsa* (Roxb.) D.C. and *Z. budrunga* Wall. ex Hook.f.) (1, 2) belonging to the Rutaceae family is a large genus of aromatic, prickly deciduous (or rarely monoecious) trees or shrubs, which is distributed mainly in the pantropics but also in the subtropics (3), can grow to a height of 35 m, **branches** sparsely armed with straight or ascending prickles, generally swollen and hollow; **leaves** alternate, paripinate or imparipinate, 30-40 cm long, **leaflets** opposite to subopposite, 10-17, ovate to elliptical, 7-13 cm × 3-5 cm, occasionally pellucid dotted, margin entire to glandular crenate; panicle terminal or axillary, 8-14 cm long; **flowers** up to 2.5 mm long, 4-merous, sepal 4, petal 4, white or pale yellow; male flowers with 4 stamens, rudimentary carpel 1; females flowers with ovary 1-carpellate; **fruits** are globose, single-seeded capsules 5 to 6 mm in diameter. During ripening, they turn from green to reddish brown. At maturity, the round, black, shiny seeds hang from the capsules. *Z. limonella* occurs in rather dry, often monsoonal forest and thickets from sea-level up to 500 m altitude (2, 17)

a. a. a.

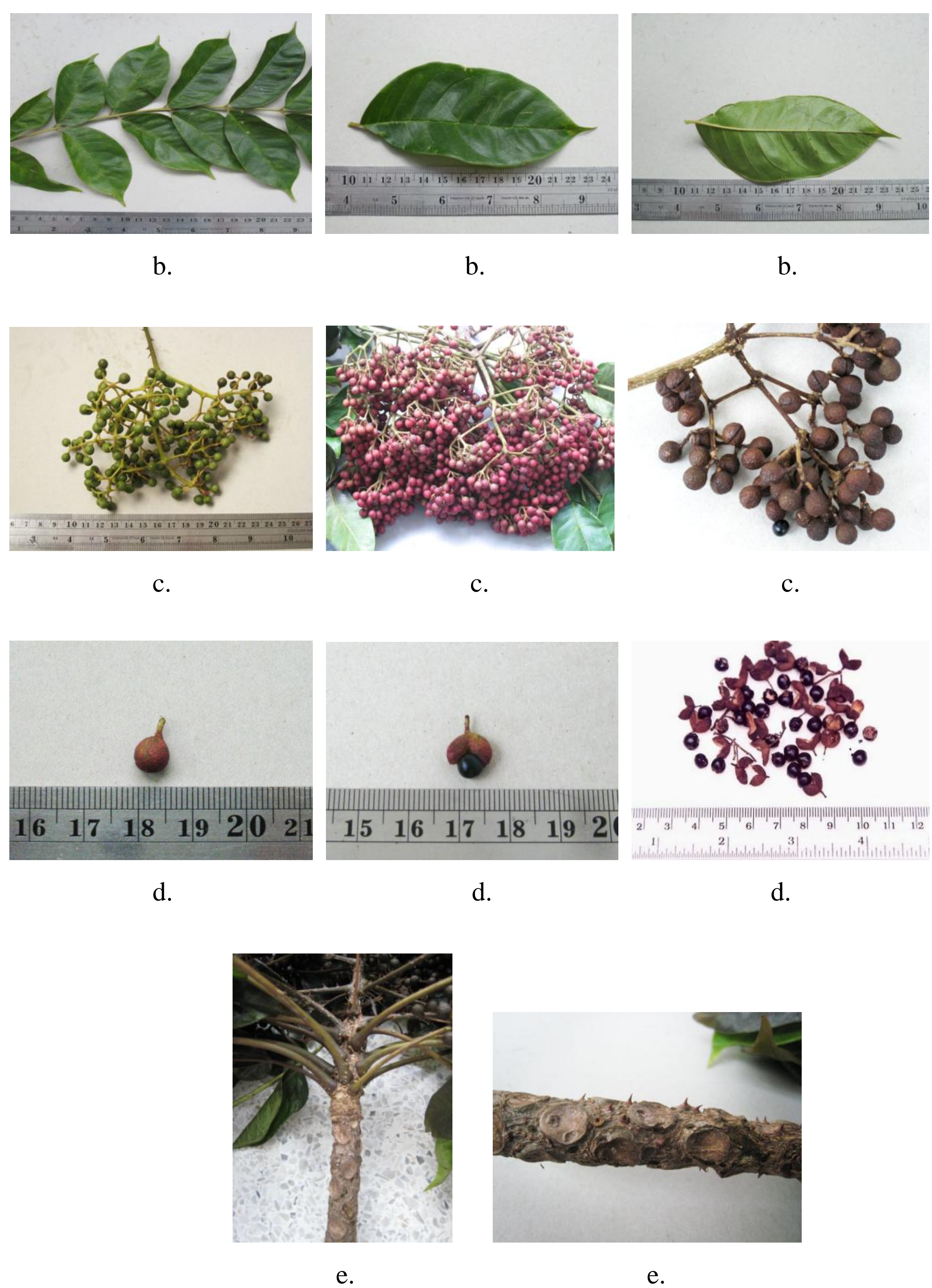


Fig. 3.1 *Zanthoxylum limonella* (Dennst.) Alston

a. *Z. limonella* tree b. *Z.limonella* leaves

c. *Z. limonella* fruits d. *Z.limonella* pericarp and seed

e. *Z. limonella* stem

3.1.2 Distribution

The plants are distributed from India and Sri Lanka to Burma (Myanmar), Indo-China, Malaysia (2, 17), Thailand, Peninsular, Java, the Lesser Sunda Islands, Moluccas (Wetar), Sulawesi, the Philippines and southern Papua New Guinea (17). In Thailand is found mainly in ChaingMai, Lumpang and Petchaburi provinces.

3.1.3 Ethnomedical uses of *Z. limonella*

In Philippines, the bark pounded and mixed with oil is used externally as a remedy for stomach pains. A decoction of the bark is taken internally as a cure for pains in the chest. When chewed it is applied to snakebites. In India, the fruits are used in dyspepsia, asthma and bronchitis, heart troubles, toothache and rheumatism. The pericarps are credited with astringent, stimulant and digestive properties. The oil obtained by steam distillation is used as a traditional remedy for cholera. It is further applied as an antiseptic and disinfectant. In Java, the young fruits are eaten as a spice. In Burma (Myanmar), the young leaves are used as a seasoning. (17). The aqueous extract of the leaves has a folkloric reputation for treating dyspepsia and some form of diarrhea. The bark juice is used in dysentery, cough, a headache and vomiting (11). The fruits of the plants are used for the treatment of asthma, bronchitis, piles and other diseases. The fruits are also claimed to possess stimulant properties. The roots and fruits of the plants have weak antifungal and anti-inflammatory activity, respectively (9). The bark is used to treat toothache (8).

Natural products have been used for thousands of years in folk medicine for several purposes. As most of the oral diseases are due to bacterial infections and it has been well documented that medicinal plants confer considerable antibacterial activity against various microorganisms including bacterial responsible for dental caries (35). Essential oil of *Z. limonella* is widely used for dental caries (14).

Bacterial biofilms in the oral cavity are considered to be composed of complex microbial communities with more than 500 species and phenotypes identified. Dental plaque is an example of a biofilm; its presence is natural and it supports the host in its defense against invading microbes. Oral microbial flora is responsible for two major human diseases, dental caries and periodontitis (16).

Dental caries, otherwise known as tooth decay, is one of the most prevalent chronic diseases of people worldwide; individuals are susceptible to this disease throughout their lifetime. Dental caries forms through a complex interaction over time between acid-producing bacteria and fermentable carbohydrate, and many host factors including teeth and saliva (36). Tooth decay is mainly caused by a group of cariogenic Gram-positive bacteria such as *Streptococcus mutans* (16, 36). Given a suitable carbohydrate nutrient (such as simple dimmer sugars like sucrose), these bacteria produce insoluble glucans and acids in dental plaque. The glucans produced by *S. mutans* are very sticky, enabling it to adhere to the tooth's surface while the acids attach the tooth's mineral structure causing demineralization that may lead to cavitation (36).

Streptococcus mutans appears to be important in the initiation of dental caries because its activities lead to colonization of the tooth surfaces, plaque formation, and localized demineralization of tooth enamel. It is not however, the only cause of dental decay (15).

Periodontal disease is caused by bacteria found in dental plaque, and about 10 species have been identified as putative pathogens in periodontal disease, mainly gram negative rods. *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Bacteroides forsythus* are the gram-negative bacteria most commonly associated with periodontitis (16, 36, 37).

Oral yeast infection is a condition which occurs due to the infection of mucous membranes of the throat and mouth. The real cause for oral yeast infection is a certain kind of yeast that is known as *Candida albicans* (*C. albicans*) that is in fact also found in fifty percent of the world’s population. *C. albicans* will not cause any problems as long as it is well balanced with the good bacteria present in the system though when this balance is disturbed the same *C. albicans* causes unwanted and additional fungal growth that in turn will become an infection (38).

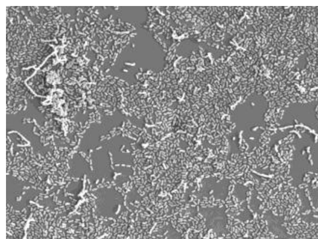
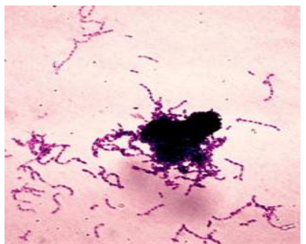
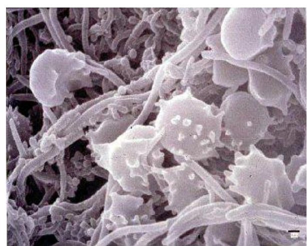


Fig. 3.2 Various streptococci in a biofilm in the oral cavity



Streptococcus mutans.
Gram stain. CDC.



Dental plaque,
scanning electron micrograph illustrating
the diversity of microbes in plaque.

Fig. 3.3 The dominant bacterial species in dental plaque

Table 3.1 Ethnomedical uses of *Z. limonella*

Part used	Traditional used	Ref.
Fruits	condiment, spice, digestive, appetizing, treatment of asthma, bronchitis, piles, stimulant properties and other diseases, anti-inflammatory	17, 18, 19
Leaves	condiments, treating dyspepsia and some forms of diarrhea	17
Bark juice	dysentery, cough, headache and vomiting	11
Bark	toothache	20
Roots	weak antifungal	11
Pericarps	astringent, stimulant and digestive properties	17

3.1.4 Chemical studies of the *Z. limonella*

The stem bark has also been reported to contain alkaloids, triterpenoids and coumarins (6), furoquinolone, indolequinazoline alkaloids and terpenoids (7). The barks, seeds and fruits have been shown to be a good source of alkaloid (9, 11). The barks have also been reported to contain terpenoids. The fruits and seeds are reported to contain fatty acids, carbohydrates and protein (9). Quinoline and indopyrido-quinazoline alkaloids, phytosterol and flavanone, volatile oils (10, 11) and terpenoids have previously been reported from this plant (10). The leaves and barks have also been reported to contain monoterpenes (11).

3.1.4.1 Alkaloids

Rutaecarpine was isolated from the stem bark by Somanabandhu, A., Ruangrungsi, N., Lange, L.G. and Organ, G.M. 1992 (6), pseudophrynamine, lunacridine, 2-(2′, 4′, 6′-trimethyl-heptenyl)-4-quinozolone were isolated from the leaves by Ahmad, M.U., Rahman, M.A., Huq, E., Chowdhury, R., 2003 (9), N-methylflndersine, zanthobungeanine, γ-fagarine and canthine-6-one were isolated from the stem bark by Rahmana, M.M., Islamb, A.M., Proma, K., Alexander, I.G. 2005 (10). Dictamine, rutaecarpine, (+)-evodiamine and skimiammine were previously reported from the stem bark of *Z. limonella* (10).

rutaecarpine

N-methylflndersine

zanthobungeanine

γ-fagarine

canthine-6-one

pseudophrynamine

3.1.4.2 Essential oils

Z. limonella oils from fruits and seeds have also been reported to contain sabinene, α-terpineol, terpinen-4-ol, β-pinene, limonene, α-pinene, γ-terpinene, α-terpinene and para-cymene, β-phellandrene, linalool, myrcene, terpinolene, camphene, α-phellandrene, trans-β -ocimene, sabinene hydrate, linalyl acetate and delta-3-carene, β-elemene, β-caryophyllene, γ-cadinene, α-farnesene, delta-cadinene, farnesol, γ-cadinol, delta-cadinol, β-cubebene, germacrene D, β-bisabolene, spathulenol, α-selinene, γ-crene B and α-humulene (18, 19).

farnesol

β-cubebene

myrcene camphene para-cymene

germacrene D β-bisabolene α-humulene

3.1.4.3 Lignans

(+)-seasamine has been described in a number of rutaceous plants including *Z. limonella*, while another lignan, (+)-syringaresinol was isolated from the stem bark by Rahmana, M.M., Islamb, A.M., Proma, K., Alexander, I.G. 2005 (10).

(+)-seasamine (+)-syringaresinol

3.1.4.4 Coumarins

Previously work on *Z. limonella* has disclosed the presence of xanthoxyletin, ostrol and scopoletin which were isolated from the stem bark by Somanabandhu, A., Ruangrungsi, N., Lange, L.G. and Organ, G.M. 1992 (6).

Xanthoxyletin

ostrol

scopoletin

3.1.4.5 Terpenoids

Previously work on *Z. limonella* has disclosed the presence of lupeol which was isolated from the stem bark by Somanabandhu, A., Ruangrungsi, N., Lange, L.G. and Organ, G.M. 1992 (6).

lupeol

3.2 The chemical studies of the genus *Zanthoxylum*

The plants of this genus are well known to contain several types of compounds, including alkaloids. The barks, seeds and fruits have been shown to be a good source of alkaloids (9, 11) like quinoline and indopyridoquinazoline alkaloids (8). The barks have also been reported to contain terpenoids (9, 10), triterpenes and lignans (21). The fruits and seeds are reported to contain fatty acids, carbohydrates and proteins (9). Phytosterols, flavanones, volatile oils (10), monoterpenes (11), coumarins, and lignans (12) have previously been reported from this plant.

3.2.1 Alkaloids

The presence of several types of alkaloids, especially benzophenanthridines, which are typical of the Rutaceae, have previously been reported in these plants (12).

Rutaecarpine was isolated from the stem bark of *Z. limonella* by Somanabandhu, A., Ruangrungsi, N., Lange, L.G. and Organ, G.M. 1992 (6)

Pseudophrynamine, lunacridine and 2-(2′,4′,6′-trimethyl-heptenyl)-4-quinozolone were isolated from the leaves of *Z. budrunga* by Ahmad, M.U., Rahman, M.A., Huq, E., Chowdhury, R. 2003 (9).

Chelerythrine, rhoifoline A, dihydrochelerythrinyl-8-acetaldehyde, oxyavicine, isoarnottianamide, integriamide, dictamnine, 8-hydroxydihydrochelerythrine, 8-methoxydihydrochelerythrine, sanguinarine, decarine, aronttianamide, liriodenine, nitidine and dihydrochelerythrine were isolated from roots of *Z. nitidum* by Jiang, H., Wei-Dong, Z., Yun-Heng, S., Chuan, Z., Lei, X., Run-Hui, L., Bin, W., Xi-Ke, X. 2007 (12).

Two benzophenanthrene alkaloids, 8-acetonyldihydronitidine and 8-acetonyldihydroavicine were isolated from stem bark of *Z.tetraspermum* by Nissanka, A.P.K., Karunaratne, V., Bandara, B.M.R., Kumar, V., Nakanishi, T., et al. 2001 (28).

Zanthoxyline, dihydronitidine, 6-oxynitidine and skimmianine were isolated from the bark of *Z. rhoifolium* by Moura, F.D.N, Ribeiro, B.H., Machado, C.S.E., Ethur, M.D., Zanatta N. et al. 1997 (29).

Benzophenanthridine alkaloid, 6-[2′-ethoxy-2′-(2″,4″,5″-trimethoxy phenyl)] ethyl-7,8-dimethoxy-5-methyl-2,3-methylenedioxy-5,6-dihydro-benzo [*c*]

phenanthridine named buesgeniine as well as the known decarine, was isolated from the stem bark of *Z. buesgenii* by Pierre, T., Hippolyte, K.W., Joseph, D.C. 2005. (33).

buesgeniine

oxynitidine

zanthoxyline

skimmianine

[2-(2′, 4′, 6′-trimethyl heptenyl)-4-quinozolone]

lunacridine

8-acetonyldihydronitridine

quinoline

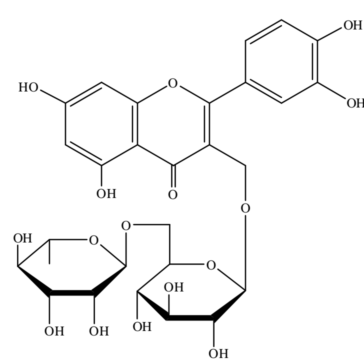
3.2.2 Flavonoids

Previous work on *Z. bungeanum* pericarp has disclosed the presence of several types of flavonol glycosides such as 3, 5, 6-trihydroxy-7, 4'-dimethoxyflavone, quercitrin (quercetin-3-rhamnoside), hyperin (quercetin-3-galactoside), quercetin, foeniculin (quercetin-3-arabinoside), rutin, isorhamnetin-7-glucoside were isolated by Quanbo, X., Dawen, S., Mizuo, M. 1995 (22).

3,5,6-trihydroxy-7,4'-dimethoxyflavone

hyperin (quercetin-3-O-galactoside)

quercitrin (quercetin-3-O-rhamnoside)



rutin

3.2.3 Esseential oil

Previous work on *Z. limonella* oils from leaves, fruits and seeds has disclosed the presence of α-pinenes, β-pinene, sabinene, limonene, α-terpinene, β-phellandrene, terpinen-4-ol, cuminic aldehyde, phlorophenone dimethyl ether, 1, 4-cineol, octanal, decanal, cryptone, linalool, β-caryophyllene (19, 23).

α-terpinene α-pinene β-pinene

sabinene limonene β-phellandrene

terpinen-4-ol linalool β-caryophyllene

3.2.4 Steroids and triterpenes

Sterols are common components of many plants and have been isolated from virtually all plants. β-sitosterol appears ubiquitous in nature. Lupeol, β-sitosterol, usually associated with stigmasterol and campesterol and β-amyrin have been isolated from the various morphological parts of all the *Zanthoxylum* species investigated (24). Previously work on *Z. limonella* has disclosed the presence of lupeol in the stem bark by Somanabandhu, A., Ruangrungsi, N., Lange, L.G. and Organ, G.M. 1992 (6). β-amyrin and friedelin were isolated from bark of *Z. schinifolium* by Ih-Sheng, C., Yuh-Chwen, L., Ian-Lih, T., Che-Ming, T., Feng-Nien, K., Tsutomu, I., Hisashi, I. 1995 (25).

β - Sitosterol (R = H)
β - Sitosteryl glucoside
(R = β-D-glucose)

Stigmasterol (R = H)
Stigmasteryl glucoside
(R = β-D-glucose)

lupeol

3.2.5 Coumarins

The presence of several types of coumarin such as schinicoumarin, acetoxyaurapten, epoxycollinin, schininallylol, schinilenol, schinindiol, aurapten, collinin, epoxyaurapten, hydrangetin, umbelliferone, acetoxycollinin and aesculetin dimethyl ether have previously been reported in genus *zanthoxylum* were isolated from bark of *Z. schinifolium* by Ih-Sheng, C., Yuh-Chwen, L., Ian-Lih, T., Che-Ming, T., Feng-Nien, K., Tsutomu, I., Hisashi, I. 1995 (25). Previously work on *Z. limonella* has disclosed the presence of xanthoxyletin, ostrol and scopoletin in the stem bark by Somanabandhu, A., Ruangrungsi, N., Lange, L.G. and Organ, G.M. 1992 (6).

hydrangetin

umbelliferone

aurapten

epoxyaurapten

3.2.6 Amides

The hot, peppery taste of *the Zanthoxylum* roots is a common feature of all the roots known. The benumbing effect on the pallate when chewed is characteristic and has been used to advantage in the treatment of mouth infections particularly in the elderly. The genus *Zanthoxylum* is characterized chemically by the frequent accumulation of olefinic alkamides (unsaturated aliphatic acid amides), a biogenetic capacity derived from the condensation of fatty acids such as linolenic and linoleic acids with isobutyl amines. Pellitorine and many other structurally-related components, for example, N-isobutyl-*trans*-2-*trans*-4-octadienamide, N-isobutyl-t*rans*-2-*trans*-4-tetradecadienamide, N-isobutyl-*trans*-2-*trans*-4-eicosadienamide, gamma-sanshool (N-isobutyl-2,4,8,10,12-tetradecapentaenamide) are pungent (24).

The presence of several types of amides such as hydroxy-α-sanshool, hydroxy-β-sanshool, hydroxy-γ-sanshool, hydroxy-γ-isosanshool, bungeanool, isobungeanool, γ-sanshool, tetrahydrobungeanool, dihydrobungeanool and dehydro-γ-sanshool were isolated from the pericarps of *Z. bungeanum* by Quanbo, X., Dawen, S., Hirofumi, Y., Mizuo, M. 1997 (26).

hydroxy-α-sanshool

O
N
H
OH

hydroxy-β sanshool

O
N
H
OH

hydroxy-γ-sanshool,

O
N
H
OH

hydroxy-γ-isosanshool

O
N
H
OH

bungeanool

O
N
H
OH

isobungeanool

O
N
H

γ-sanshool

O
N
H
OH

tetrahydrobungeanool

Table 3.2 Ethnomedical use and phytochemicals of *Zanthoxylum* plants

Species	Part used	Chemistry	Ref.
Z. limonella (*Z. budrunga*, *Z. rhetsa*)	- stem bark - bark, seeds, fruits - bark - fruits and seeds - fruits and seeds	- alkaloids, triterpenoids, coumarins - alkaloids - terpenoids - fatty acid, carbohydrate and protein - essential oil	6 9 9 9 18, 19
Z. nitidum	root	benzophenanthridines, lignans, coumarins	12
Z. bungeanum	pericarps	alkylamides, flavonol glucosides	22, 26
Z. fagara	leaves and fruits	alkaloids, lignans, coumarins	27
Z. elephantiasis	bark	alkaloids and coumarins	27
Z. martinicense	bark	alkaloids, triterpenes, lignans and carbohydrates	27
Z. tetraspermum	stem bark	benzophenanthrene alkaloids	28
Z. rhoifolium	bark, leaves	benzophenanthidine alkaloids, volatile oil and terpenes	23, 29
Z. piperitum	leaves and fruits	terpenoids, aliphatic amides, flavonoids	30, 31
Z. armatum	bark	amides	32
Z. buesgenii	stem bark	benzophenanthidine alkaloids, lignan	33
Z. schinifolium	bark	coumarins, alkaloids and triterpenoids	34

Table 3.3 The phytochemicals of *Z. limonella*

1. Alkaloid

Compounds	Sources	Ref
Rutaecarpine	stem bark	6
Pseudophrynamine, lunacridine, 2-(2′, 4′, 6′-trimethyl-heptenyl)-4-quinozolone	leaves	9
N-methylflndersine, zanthobungeanine, γ-fagarine, canthine-6-one, dictamine, rutaecarpine, (+)-evodiamine, skimiammine	stem bark	10

2. Essential oils

Compounds	Sources	Ref
sabinene, α-terpineol, terpinen-4-ol, β-pinene, limonene, α-pinene, γ-terpinene, α-terpinene and para-cymene, β-phellandrene, linalool, myrcene, terpinolene, camphene, α-phellandrene, trans-β -ocimene, sabinene hydrate, linalyl acetate and delta-3-carene, β-elemene, β-caryophyllene, γ-cadinene, α-farnesene, delta-cadinene, farnesol, γ-cadinol, delta-cadinol, β-cubebene, germacrene D, β-bisabolene, spathulenol, α-selinene, γ-crene B and α-humulene	fruits, leaves and seeds	4, 5, 18, 19

3. Lignans

Compounds	Sources	Ref
(+)-seasamine, (+)-syringaresinol	stem bark	10

4. Coumarins

Compounds	Sources	Ref
xanthoxyletin, ostrol and scopoletin	stem bark	6

5. Terpenoids

Compounds	Sources	Ref
lupeol	stem bark	6

The genus of *Zanthoxylum* consists of ca. 200 species, of which are 8 or 9 species are found in Thailand (3). Species in Southeast Asia, including Thailand are *Z. nitidum*, *Z. rhetsa* (synonym *Z. limonella*), *Z. armatum* (synonyms *Z. alatum, Z. planispinum*), *Z. avicennae* and *Z. acanthopodium* (34). Some of the common names of various *Zanthoxylum* species in Thailand are listed in Table 3.4.

Table 3.4 Common Names of *Zanthoxylum* Species in Thailand (1)

Scientific names	Common names	Thai names
Z. acanthopodium DC.	Mak kak doi suthep (Northern)	หมักก้ากดอยสุเทพ (ภาคเหนือ)
Z. armatum DC. (syn. *Z. alatum* Roxb.)	Mak kak (Northern)	หมักก้าก (ภาคเหนือ)

Table 3.4 Common Names of *Zanthoxylum* Species in Thailand (1) (cont.)

Scientific names	Common names	Thai names
Z. limonella (Dennst.) Alston (syn. *Z. budrunga* Wall. ex Hook.f., *Z. rhetsa* (Roxb.) DC.)	Kamchat ton, Phrik hom, Mak mat (Bangkok), Ma khuang, Ma khwaen (Northern), Ma khaen (Laos), Luk re mat (Central), Mak khuang (Mae Hong Son)	กำจัดต้น, พริกหอม, หมากมาศ (กรุงเทพฯ), มะข่วง, มะแขว่น (ภาคเหนือ), มะแข่น (ลาว), ลูกระมาศ (ภาคกลาง), หมักข่วง (แม่ฮ่องสอน)
Z. nitidum (Roxb.) DC. (Syn. *Z. collinsae Craib*, *Z. hamiltonianum* Wall.)	Kamchat nuai (Peninsular), Ngu hao (Udon Thani)	กำจัดหน่วย (เพนิซูลา), งูเห่า (อุดรธานี)

3.3 Analytical procedure

Chromatographic methods are commonly used for the quantitative and qualitative analysis of environmental and pharmaceutical samples. High-performance liquid chromatography (HPLC) analysis has become the requirement in the development of drug substances because of its potential, speed and convenient for use at routine work (39).

3.3.1 System Suitability Testing

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. (40)

Table 3.5 System suitability parameters and recommendations (41)

Parameter	Recommendation
Column capacity (*k'*)	The peak of interest should be well resolved from other peaks and the void volume; generally k should be greater than 2.0
Repeatability	An RSD of less than or equal to 2% for N greater than or equal to 5 is desirable
Relative retention	Not essential if the resolution is stated
Resolution (R_s)	R_s should be greater than 2 between the peak of interest and the closest eluted potential interferent (impurity, excipient, degradation product, or internal standard)
Tailing factor (*T*)	T should be less than or equal to 2
Theoretical plate (*N*)	In general should be greater than 2,000

Definition of Terms for the System Suitability Parameters. (42)

Where

W_X = width of the peak determined at either 5% (0.05) or 10% (0.10) from the baseline of x the peak height

f = distance between peak maximum and peak front at W_X

t_O = elution time of the void volume or non-retained components

t_R = retention time of the analyte

t_W = peak width measured at baseline of the extrapolated straight sides to baseline

3.3.1.1 Column Capacity (*k'*)

$$k' = (t_R - t_O) / t_O$$

The capacity factor is a measure of where the peak of interest is located with respect to the void volume, i.e., elution time of the non-retained components.

Recommendations:

The peak should be well-resolved from other peaks and the void volume. Generally the value of k' is > 2.

3.3.1.2 Determination of System Precision

Injection precision expressed as RSD (relative standard deviation) indicates the performance of the HPL chromatograph which includes the plumbing, column, and environmental conditions, at the time the samples are analyzed. It should be noted that sample preparation and manufacturing variations are not considered.

Recommendations:

RSD of 1% for n 5 is desirable.

3.3.1.3 Relative retention (α)

$$\alpha = k'1 / k'2$$

Relative retention is a measure of the relative location of two peaks. This is not an essential parameter as long as the resolution (Rs) is stated.

3.3.1.4 System Resolution (R_s)

$$R_s = (t_{R2} - t_{R1}) / (1/2)(t_{W1} + t_{W2})$$

R_s is a measure of how well two peaks are separated. For reliable quantitation, well-separated peaks are essential for quantitation. This is a very useful parameter if potential interference peak(s) may be of concern. The closest potential eluting peak to the analyte should be selected.

R_s is minimally influenced by the ratio of the two compounds being measured.

Recommendations:

R_s of > 2 between the peak of interest and the closest potential interfering peak (impurity, excipient, degradation product, internal standard, etc.) is desirable.

3.3.1.5 Asymmetry Factor (Tailing Factor)

$$T = Wx / 2f$$

The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculationof the area under the peak. Integrator variables are preset by the analyst for optimum calculation of the area for the peak of interest.

Recommendations:

T of ≤ 2

3.3.1.6 Theoretical plate number (N)

$$N = 16\,(t_R / t_W)^2 = L / H$$

Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram. N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions. H, or HETP, the height equivalent of a theoretical plate, measures the column efficiency per unit length (L) of the column. Parameters which can affect N or H include peak position, particle size in column, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the analyte.

Recommendations:

The theoretical plate number depends on elution time but in general should be > 2000.

General Recommendation:

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. The amount of testing required will depend on the purpose of the test method. For dissolution or release profile test methods using an external standard method, k', T and RSD are minimum recommended system suitability tests. For acceptance, release, stability, or impurities/degradation methods using external or internal standards, k', T, Rs and RSD are recommended as minimum system suitability testing parameters. In practice, each

method submitted for validation should include an appropriate number of system suitability tests defining the necessary characteristics of that system. Additional tests may be selected at the discretion of the applicant or the reviewer.

3.3.2 Method validation

Validation is the process of demonstrating or confirming the performance characteristics of a method of analysis.

This process of validation is separate from the question of acceptability or the magnitude of the limits of the characteristics examined, which are determined by the purpose of the application. Validation applies to a specific operator, laboratory, and equipment utilizing the method over a reasonable concentration range and period of time.

Typically the validation of a chemical method of analysis results in the specification of various aspects of reliability and applicability. Validation is a time-consuming process and should be performed only after the method has been optimized and stabilized because subsequent changes will require revalidation. The stability of the validation must also be verified by periodic examination of a stable reference material.

The method of analysis is the detailed set of directions, from the preparation of the test sample to the reporting of the results that must be followed exactly for the results to be accepted for the stated purpose.

The term “method of analysis” is sometimes assigned to the technique, e.g., liquid chromatography or atomic absorption spectrometry, in which case the set of specific directions is referred to as the “protocol.”

The performance characteristics of a method of analysis are the functional qualities and the statistical measures of the degree of reliability exhibited by the method under specified operating conditions.

The functional qualities are the selectivity (specificity), as the ability to distinguish the analyte from other substances; applicability, as the matrices and concentration range of acceptable operation; and degree of reliability, usually expressed in terms of bias as recovery, and variability as the standard deviation or equivalent terms (relative standard deviation and variance).

Measurements are never exact and the "performance characteristics of a method of analysis" usually reflect the degree to which replicate measurements made under the same or different conditions can be expected or required to approach the "true" or assigned values of the items or parameters being measured. For analytical chemistry, the item being measured is usually the concentration, with a statement of its uncertainty, and sometimes the identity of an analyte (53).

Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications (43).

Method validation is completed to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range that analyte will be analyzed. Method validation provides an assurance of reliability during normal use, and is sometime referred to as "the process of providing documented evidence that the method does what it is intended to do". Regulated laboratories must perform method validation in order to be in compliance with US Food and Drug Administration (FDA) regulations (44). The guideline for submitting samples and analytical data for method validation, the FDA designated the specifications in the current edition of the USP as those legally recognized when determining compliance with the Federal Food, Drug, and Cosmetic Act.

For method validation, these specifications are listed in USP Chapter <1225> (43), and can be referred to as the "Eight Steps of Method Validation", as shown in Fig. 3.4.

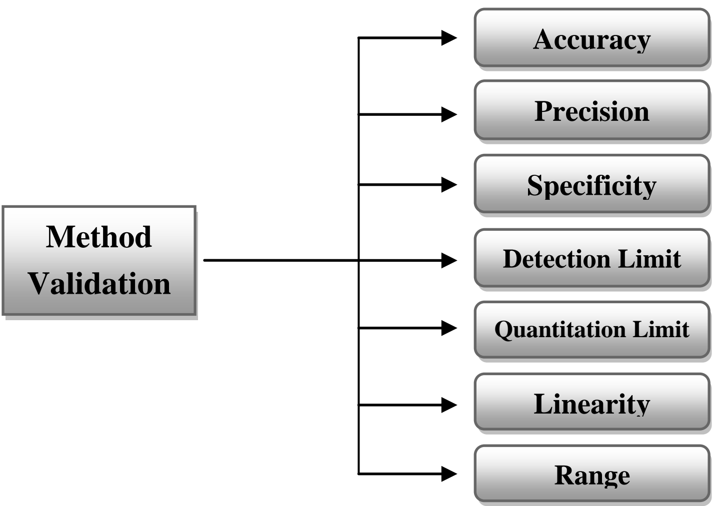


Fig. 3.4 The USP eight steps of method validation (43)

3.3.2.1 Accuracy (40)

Accuracy should be established across the specified range of the analytical procedure.

3.3.2.1.1 Assay

I. Drug Substance

Several methods of determining accuracy are available:

a) application of an analytical procedure to an analyte of known purity (e.g. reference material);

b) comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined (independent procedure, see 1.2.);

c) accuracy may be inferred once precision, linearity and specificity have been established.

II. Drug Product

Several methods for determining accuracy are available:

a) application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analysed have been added;

b) in cases where it is impossible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated and/or defined.

c) accuracy may be inferred once precision, linearity and specificity have been established.

3.3.2.1.2 Impurities (Quantitation)

Accuracy should be assessed on samples (drug substance/drug product) spiked with known amounts of impurities.

In cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure. The response factor of the drug substance can be used.

It should be clear how the individual or total impurities are to be determined e.g., weight/weight or area percent, in all cases with respect to the major analyte.

3.3.2.1.3 Recommended Data

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure).

Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

3.3.2.2 Precision (43)

Definition: The precision of analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of a homogeneous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions. Reproducibility refers to the use of the analytical procedure in different laboratory. Repeatability refers to the use of the analytical procedure equipment.

Determination: The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation. Assays are independent analyses of samples that have been carried through the complete analytical procedure from sample preparation to final test result.

3.3.2.3 Specificity (45)

Specificity and selectivity both give an idea of the reliability of the analytical method. Some authors give different definitions for both terms while, for others, they are identical. The term ‘specific’ generally refers to a method that produces a response for a single analyte only, while the term ‘selective’ is used for a method producing responses for different chemical entities or analytes which can be distinguished from each other. A method is called ‘selective’ if the response is distinguished from all other responses. The method is perfectly able to measure accurately an analyte in the presence of interferences. According to Eurachem, specificity and selectivity essentially reflect the same characteristic and are related very closely to each other in such a way that specificity means 100% selectivity. In other words, a method can only be specific if it is for 100% selective. Another related term is ‘confirmation of identity’, which is the proof that ‘the measurement signal, which has been attributed to the analyte, is only due to the analyte and not to the presence of something chemically or physically similar or arising as coincidence’. A method must first show high specificity before true quantification can be performed. There is no single expression for specificity. It is rather something that must be

demonstrated. The way that this is done depends on the objective and the type of analytical method (see also below). For identification tests, the goal is to ensure the identity of an analyte. Specificity is here the ability to discriminate between compounds of closely related structures that can be present.

3.3.2.4 Linearity (40)

A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighings of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample.

For the establishment of linearity, a minimum of 5 concentrations is recommended. Other approaches should be justified.

3.3.2.5 Range (40)

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

The following minimum specified ranges should be considered:

- for the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration;
- for content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified;
- for dissolution testing: +/-20 % over the specified range;

e.g., if the specifications for a controlled released product cover a region from 20%, after 1 hour, up to 90%, after 24 hours, the validated range would be 0-110% of the label claim.

- for the determination of an impurity: from the reporting level of an impurity1 to 120% of the specification;
- for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the detection/quantitation limit should be commensurate with the level at which the impurities must be controlled;

Note: for validation of impurity test procedures carried out during development, it may be necessary to consider the range around a suggested (probable) limit.

- if assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities1 to 120% of the assay specification.

3.3.2.6 Detection Limits

In chapter 1225, the U.S. Pharmacopeia (USP) defines limit of detection as the lowest concentration of an analyte in a sample that can be detected but not necessarily quantitated. A limit of detection test specifies whether or not an analyte

is above or below a certain value. The USP defines the limit of quantitation as the lowest concentration of an analyte in a sample that can be determined (quantitated) with acceptable precision and accuracy under the stated operational conditions of the method. Like the limit of detection, the limit of quantitation also is expressed as a concentration (43).

A new convention is coming into common usage as a result of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q2 (R1) Methodology guideline on analytical method validation (40).

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

I. Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

II. Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

III. Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$$DL = \frac{3.3\,\sigma}{S}$$

where σ = the standarddeviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example:

I. Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

II. Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended Data

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

3.3.2.7 Quantitation Limit

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

I. Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

II. Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

III. Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

$$QL = \frac{10\,\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways for example:

I. Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

II. Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended Data

The quantitation limit and the method used for determining the quantitation limit should be presented.

The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit.

3.3.2.8 Ruggedness and robustness (45)

Although the terms ruggedness and robustness are often treated as the same and used interchangeably, separate definitions exist for each. To have an idea about the ruggedness, Eurachem recommends introducing deliberate variations to the method, such as different days, analysts, instruments, reagents, variations in sample preparation or sample material used. Changes should be made separately and the effect evaluated of each set of experimental conditions on the precision and trueness. To examine the effects of different factors, a ‘‘factorial design’’ methodology can be applied, as described by Holst et al. (46). By combining changes in conditions and performing a set of experiments, one can determine which factors have a significant or even critical influence on the analytical results. In ICH/USP guidelines, ruggedness is not defined separately but treated under the same denominator as reproducibility precision: it is ‘the degree of reproducibility of the results obtained under a variety of conditions, expressed as %RSD’. Robustness is a term introduced by USP/ICH. Although Eurachem has included the term robustness in its official list of definitions, the term is not used by official organizations other than USP/ICH. According to Eurachem, both parameters do present the same and are thus synonyms.

3.3.3 Quantitative Determination of Lupeol in Plant Materials

In most HPLC methods, acetonitrile/water was used as a mobile phase but acetonitrile/water with 0.01% phosphoric acid or acetic acid/methanol/water or *n*-hexane/isopropanol has also been used. Separation of lupeol was achieved by using a Super ODS column with isocratic elution by acetonitrile/water (95:5, *v*/*v*) at 40°C in 32 min. In a longer procedure, with run time 90 min and gradient elution by water and acetonitrile (both containing 0.01% phosphoric acid), separation of triterpenoids on a C18 column was also successful (51).

Table 3.6 HPLC analysis methods of lupeol from natural products

Plant	Column	Detection	Mobile phase/ Flow rate/ Retention time	Ref
Brassica oleracea L.	Hypersil BDS C18 (250 × 3 mm i.d., 3 µm)	UV 200 nm	acetonitrile : water (95:5 *v/v*), 0.8 ml/min, 15.1 min	51
Vernonia cinerea L.	C18 column (150 × 4.6 mm i.d., 5 µm)	UV 210nm	methanol : acetonitrile (30:70 *v/v*), 1.0 ml/min, 4.45 min	52

CHAPTER IV
MATERIALS AND METHODS

Part I : Isolation and identification the phytochemical from *Z. limonella* (Dennst.) Alston pericarp

A. Materials

4.1 Plant materials

Z. limonella was collected from Petchaburi Province, Thailand. The plant was identified by Professor Wongsatit Chuakul. The herbarium specimens were readied for systematic-identification. Voucher herbarium specimens (PBM 04888) were deposited at Pharmaceutical Botany Mahidol Herbarium (PBM), Pharmaceutical Botany Department, Faculty of Pharmacy, Mahidol University. The pericarps were obtained from the fruits (2 kg). They were dried and ground to a coarse power (800 g).

4.2 Chemicals

4.2.1 Solvents

- Hexane (AR grade) J.T. Baker
- Dichloromethane (AR grade) J.T. Baker
- Methanol (AR grade) J.T. Baker
- Ethyl acetate (AR grade) J.T. Baker
- Hexane (Commercial grade)
- Dichloromethane (Commercial grade)
- Methanol (Commercial grade)
- 95% Ethanol (commercial grade)

4.2.2 Spray reagent

- 10% Sulfuric acid in ethanol

4.2.3 Solvent systems for thin-layer chromatography

- Chloroform-methanol, 9:1 for phytosteryl glucosides and their aglycones

4.2.4 Reference compounds

- The reference compounds, a mixture of β-sitosterol and stigmasterol (isolated from *Murdannia loriformis* by Dr. Weena Jiratchariyakul)
- The reference compounds, a mixture of β-sitosteryl and stigmasteryl glucosides (isolated from *Murdannia loriformis* by Dr. Weena Jiratchariyakul)

4.3 Chromatographic materials

4.3.1 Silica gel 60 for column chromatography, 63-200 μm, Merck, Germany

4.3.2 Thin-layer chromatography (TLC), Silica gel GF_{254}, pre-coated on TLC aluminium sheets 20×20 cm, layer thickness 0.25 cm, Merck, Germany

4.3.3 Glass columns with inner diameter (i.d.) 5 cm × 60 cm long, i.d. 3 cm × 60 cm long and i.d. 2 cm × 60 cm long.

4.4 Instruments for physical and structural identification

4.4.1 Melting points

The melting points were determined on an Electrothermal 9100.

4.4.2 Ultraviolet spectra (UV)

Ultraviolet spectra were recorded by UV spectrophotometry, Perkin-Elmer, Lambda 35™, USA.

4.4.3 Fourier Transform Infrared spectra (FTIR)

Fourier Transform Infrared spectra (FTIR, Magna-IR™ spectrometer 550 Nicolet) spectra were recorded at Faculty of Pharmacy, Mahidol University.

4.4.4 Nuclear Magnetic Resonance spectra (NMR)

^{1}H-NMR, ^{13}C-NMR, DEPT 90 and DEPT 135 spectra were recorded on an INOVA 500 (500 Hz) for compound 1 at the Scientific and Technological Research Equipment Center, Chulalongkorn University. ^{1}H-NMR, ^{13}C-NMR were recorded on an INOVA 500 (500 Hz) for compound 2 at the Scientific and Technological Research Equipment Center, Chulalongkorn University. ^{1}H-NMR, ^{13}C-NMR, DEPT 90, DEPT 135, HMQC and HMBC spectra were recorded on an AVANCE 500 (500 Hz) for compound 3 at Faculty of Science, Mahidol University.

4.4.5 Mass spectra

Fast Atom Bombardment Mass Spectra (FABMS) was recorded at Faculty of Science, Mahidol University

4.5 Other instruments

Rotary evaporator	Buchi,
Ultrasonic bath	Sonorex, Germany
TLC tank size 13×13×5 cm	CAMAG, Germany
TLC tank size 20×20×5 cm	CAMAG, Germany
Spraying bottle	
Hot plate	

B. Methods

4.1 The extraction

The coarse powder (2×400 g) of *Z. limonella* pericarp was macerated with hexane and 95% ethanol (3×1.2 L), each for 7 days. The solvents were removed under reduced pressure at 40-50°C by rotary evaporator. The yields of each extracts were recorded. The procedures of the extraction were shown in Fig. 4.1.

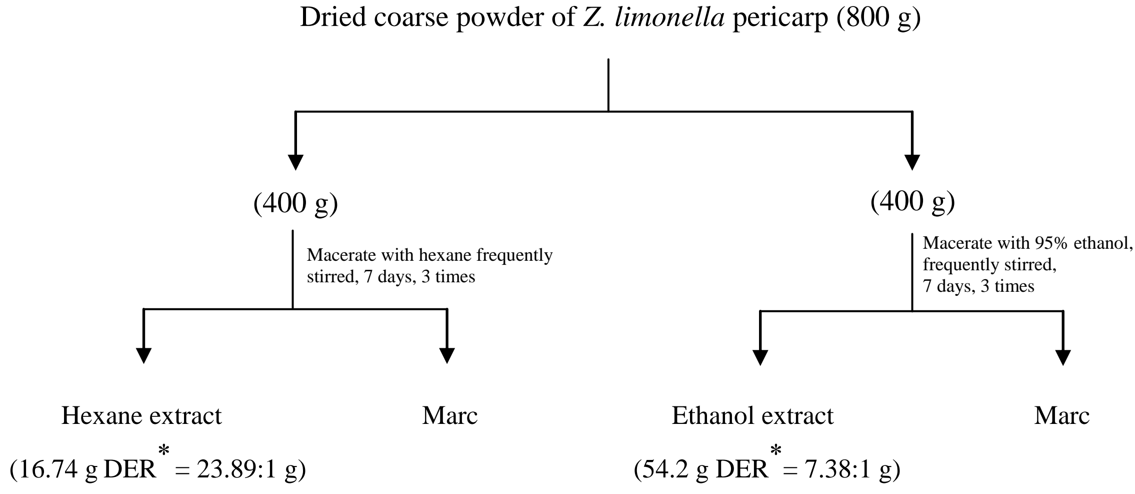


Fig. 4.1 The extraction method

4.2 The Isolation of compounds from ethanol extract of *Z.limonella*

The ethanol extract (20 g) was prepared as extract granules and subjected to conventional column chromatography eluted with hexane, hexane/ethyl acetate (as a gradient from 100:0→90:10), dichloromethane, dichloromethane/methanol (as a gradient from 100:0→90:10) and methanol. The collected fractions were be monitored by TLC (silica gel GF_{245}, dichloromethane/methanol 90:10). The compounds obtained were further purified by recrystallization.

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* DER = Drug Extract Ratio is the weight (g) of crude extract that produces 1 g of extract. e.g. DER of hexane extract is 23.89 : 1 means that 23.89 g of crude extract produces 1 g of hexane extract.

4.2.1 Isolation of compound 1

The ethanol extract (20 g) was chromatographed on the silica gel column (5 cm, i.d. × 60 cm, length) eluted with hexane/ethyl acetate (as a gradient from 100:0→99:1→95:5→90:10). Fractions of 50 ml were collected and combined. The compound 1 enriched fraction (fraction 4, 160.0 mg). This fraction was washed and recrystallized in hot methanol affording 11.7 mg of compound 1 (yield = 0.058%).

4.2.2 Isolation of compound 2

The ethanol extract (20 g) was chromatographed on the silica gel column (5 cm, i.d. × 60 cm, length) eluted with dichloromethane/methanol (as a gradient from 100:0→99:1→97:3→95:5→90:10). Fractions of 50 ml were collected and combined. The compound 2 enriched fraction (fraction 12, 627.0 mg). This fraction was washed and recrystallized in hot methanol affording 26.3 mg of compound 2 (yield = 0.13%).

4.2.3 Isolation of compound 3

The ethanol extract (20 g) was chromatographed on the silica gel column (5 cm, i.d. × 60 cm, length) eluted with hexane/ethyl acetate(as a gradient from 100:0→99:1→95:5→90:10). Fractions of 50 ml were collected and combined. The compound 3 enriched fraction (fraction 3, 421.3 mg).

Fraction 3 was chromatographed on the silica gel column (1 cm, i.d. × 60 cm, length) eluted with hexane/ethyl acetate, (as a gradient from 100:0→99:1→97:3→95:5). Fractions of 10 ml were collected and combined. The compound 3 not enriched fraction (fraction 4, 13.2 mg). This fraction was washed and recrystallized in hot methanol affording 10.4 mg of compound 3 (yield = 0.052%).

The procedures of the extraction and isolation were summarized in the flow chart (Fig 4.2)

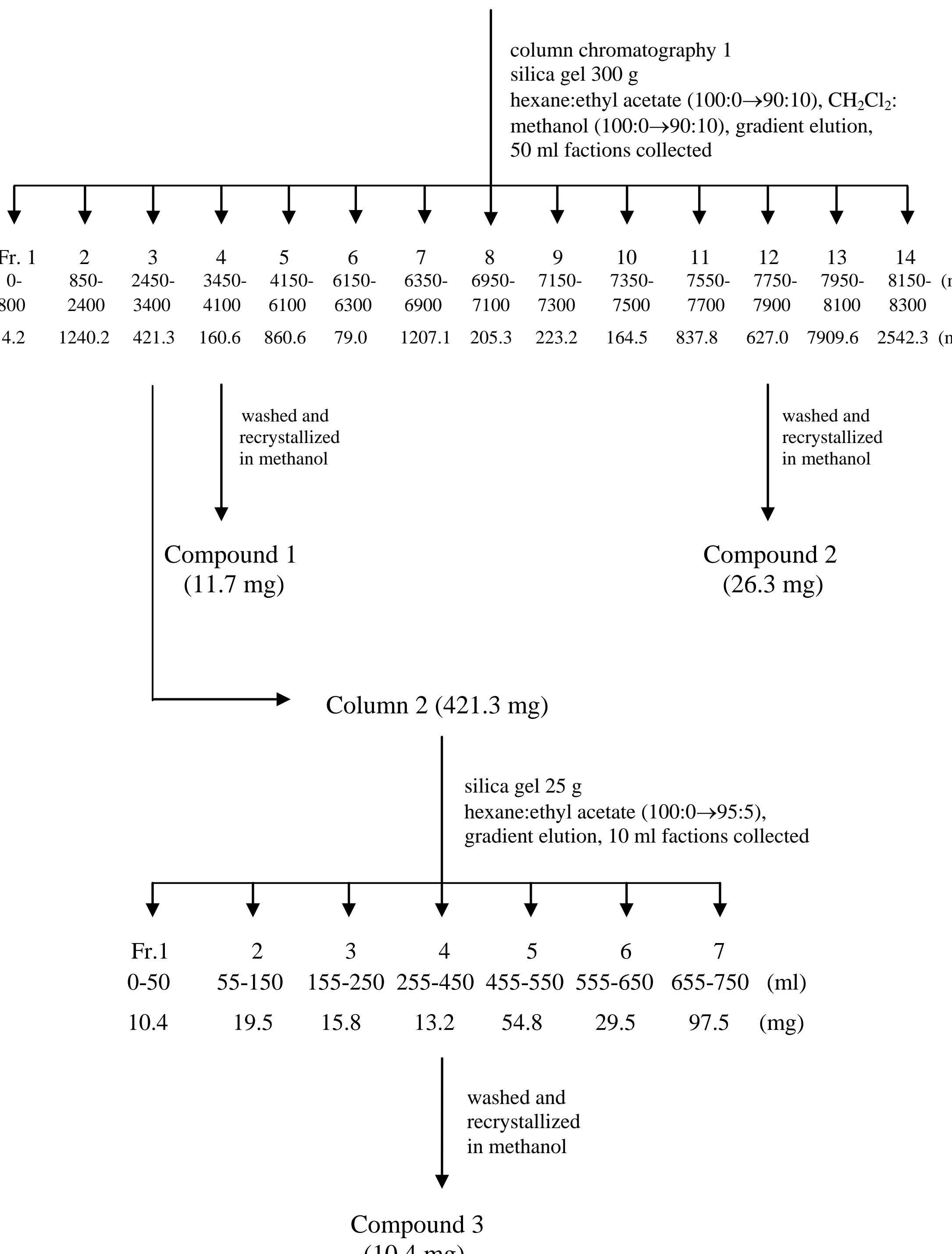


Fig. 4.2 The isolation scheme of the extraction and isolation of compounds from *Z. limonella*

4.3 Identification

4.3.1 Melting points

The melting points of the isolated compounds were recorded on the Electrothermal 9100, Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University.

4.3.2 Ultraviolet spectra

The ultraviolet spectra of compound 1, 2 and 3 in chloroform were recorded on the UV spectrophotometer, Perkin-Elmer, Lambda 35™, USA at the Central Laboratory, Faculty of Pharmacy, Mahidol University.

4.3.3 Infrared spectra

The IR-spectra in KBr disc of the isolated compounds were recorded on the FT-IR spectrometer (Magna-IR™ spectrometer 550 Nicolet) at the Central Laboratory, Faculty of Pharmacy, Mahidol University.

4.3.4 Nuclear magnetic resonance spectra

The 1D- and 2D-NMR spectra were recorded on NMR spectrometer (Avance 500) at the Scientific and Technological Research Equipment Center, Chulalongkorn University and Faculty of Science, Mahidol University.

4.3.5 Mass spectra (FABMS)

Fast Atom Bombardment Mass Spectrometry was recorded at the Faculty of Science, Mahidol University, Thailand.

C. Antimicrobial Tests (Tested by Faculty of Dentistry, Mahidol University)

4.1 Tested materials

The hexane extract and the ethanol extract of *Z. limonella* pericarp, compound 2 and 3

4.2 Studied activity

Antimicrobial activity of *Z. limonella* extracts, compound 2 and 3 were screening using standard disk diffusion technique.

Minmum Inbibitory Concentration (MIC) and Minimum Cidal Concentration (MCC) were determined using Millipore membrane technique (Tantaoui-Elaraki, et al; 1992)

4.3 Microorganisms

Streptococcus mutans KPSK2, *Lactobacilli* spp., *Candida albicans* (ATCC13802 and clinical strain)

Part II : HPLC Quantitative Analysis

4.1 Materials and instruments

4.1.1 Plant materials

Z. limonella was collected from Petchaburi Province, Thailand. The pericarps were separated and ground into coarse powder. The pericarps were collected from the fruits (2 kg). They were ground to a coarse powder (800 g).

4.1.2 Chemicals

Methanol HPLC grade	E.Merck
Methanol AR grade	J.T.Baker
Water HPLC grade	E.Merck
Water, distilled	

4.1.3 Equipment

Ultrasonic bath

Centrifuge

Filter membrane

4.1.4 Chromatographic materials

HPLC column, 5µm Hypersil® Gold C18, 150×4.6 mm, Thermo electron corperation, United Kingdom.

Javelin® BDS C18 guards column, ThermoHypersil, United Kingdom.

4.1.5 Chromatograpic instruments

High-pressure liquid chromatography (HPLC), Water Model 717 plus autosampler

High pump, Water Model 717

Water 600 controller

Water 2996 Photodiode array detector

Water Data Module

4.1.6 Marker compound

The compound 3 (lupeol) was isolated from the pericarp in our laboratory and identified by NMR, MS, and IR spectra.

4.2 Methods

4.2.1 Preparation of compound 3 standard solution

4.2.1.1 Stock solution

A compound 3 was accurately weight, 5 mg into 10 ml volumetric flask. One ml of dichloromethane was added and the mixture sonicated until the clear solution was obtained. Adjusted to volume with methanol.

4.2.1.2 Standard solutions

The stock solution was quantitatively diluted with methanol to concentrations of 10, 20, 50, 80, and 100 ppm immediately before used.

4.2.2 HPLC procedure

The standard solution and sample solutions were injected into HPLC column using the following chromatographic conditions

Column :	5µm HYPERSIL® Gold C18
Solvent system :	methanol : water (95:5)
Flow rate :	1 ml/ min
Detection :	214 nm
Volume :	20 µl

The chromatograms and retention times were recorded.

4.2.3 Validation of HPLC quantitative method

4.2.3.1 System suitability test

The suitability test for isocratic system was determined from four parameters: precision (%CV), number of theoretical plates (N), tailing factor (TF), and resolution factor (R_s).

4.2.3.1.1 Precision

The precision was determined in term of percentage of coefficient of variation (%CV) or relative standard deviation (RSD), which was calculated by using the following equation:

$$\%CV = [SD/\overline{X}] \times 100 \qquad (1)$$

where

$\overline{X}$ = mean value

SD = standard deviation

4.2.3.1.2 The column coefficiency (number of theoretical plates, N)

The number of theoretical plate (N) was calculated from the following equation;

$$N = 5.54 \times [t_R/W_{1/2h}]^2 \qquad (2)$$

where

t_R = the retention time of peak

$W_{1/2h}$ = the peak width at the half height.

4.2.3.1.3 Tailing factor (TF)

Tailing factor (TF) was calculated by using the following equation;

$$TF = W_{0.05} / [2 \times A_{0.05}] \qquad (3)$$

where

$W_{0.05}$ = the width of peak at 5% of the peak height

$A_{0.05}$ = the distance from peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from baseline.

4.2.3.1.4 The resolution factor (R_s)

The resolution factor (R_s) was calculated by using the following eqution :

$$R_s = 2 \times (t_{RB} - t_{RA}) / (W_B + W_A)$$

where

t_{R1} = the retention time of the peak A

t_{R2} = the retention time of the peak B

W_1 = the peak width of the peak A

W_2 = the peak width of the peak B

4.2.3.2 Method validation

In this study, typical parameters to be considered in the method validation study were linearity (r^2), accuracy (recoveries), and precision (%CV).

4.2.3.2.1 Linearity

Five final concentrations of standard solution were used for evaluation of linearity. The standard calibration plot was constructed by least-square linear regression of the peak area of the compound 3 versus concentration.

4.2.3.2.2 Accuracy

The accuracy of the proposed method was evaluated as the percentage of recovery by using the standard addition technique. The percentage of recovery was calculated as follows:

$$\% \text{ Recovery} = \left[\frac{C_S - C_A}{C_A^*} \right] \times 100$$

where

C_S = measured concentration of spiked sample

C_A = measured concentration of unspiked sample

C_A^* = concentration of spiking solution

4.2.3.2.3 Precision

Precision was evaluated by intra-day and inter-day precision. The sample solutions of the homogeneous sample were prepared by the consecutive injections of five sample solutions on the same day. Inter-day precision was performed on the same way of intra-day test, but on three different days. The relative standard deviation (RSD) or percentage of coefficient of variation (%CV) was calculated.

4.2.3.2.4 Limit of detection and quantitation

The calculation was based on the standard deviation of the response (σ) and the slope of the calibration curve (S) at levels approaching the limit according to equations 4 and 5:

$$LOD = 3.3\ (\sigma/S) \qquad (4)$$

$$LOQ = 10\ (\sigma/S) \qquad (5)$$

The standard deviation of the response could be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y intercepts of regression

lines. The ICH calculation method could reduce the bias that sometimes occurs when determining the S/N. This bias can result because of differences in opinion about how to determine and measure the noise.

4.2.4 HPLC analysis of compound 3 (Lupeol) in *Z. limonella* extract

4.2.4.1 Preparation of the crude extract

Accurately weighed 100 mg of crude ethanol extract and transferred into 25 ml volumetric flask. Five ml of methanol were added and the mixture sonicated for 15 min in an ultrasonic bath, the volume was adjusted with methanol. Filtered through 0.45 μm filter membrane before HPLC analysis.

CHAPTER V
RESULTS AND DISCUSSIONS

Part I : Isolation and identification

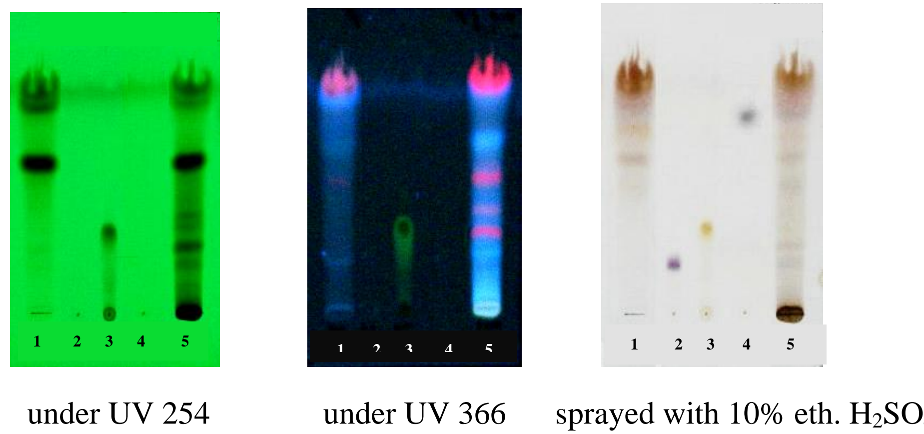
5.1 Extraction (Fig. 4.1, Chapter IV)

The coarse powder (pericarp, 2×400 g) was macerated with hexane (1.2 L) and 95% ethanol (1.2 L) for 7 days 3 times. The solvent was removed under reduced pressure at 40-50°C by rotary evaporator. The yield of each extract was recorded (Table 5.1) and thin-layer chromatogram of the extracts were shown in Figure 5.1.

Table 5.1 The Percent extractives of *Z. limonella*

Sample extract	Wt.crude extract (g)	% Yields (w/w)
Hexane	16.74	4.18
95% Ethanol	54.2	13.55

For further development of the oral cavity products of the extract was determined from the cytotoxicity to normal continuous cell line L-929 (tests by Faculty of Dentistry, Mahidol University), so the ethanol extract that has no or less toxicity than hexane extract was selected for continuing study.



under UV 254 under UV 366 sprayed with 10% eth. H_2SO_4

Adsorbent	silica gel GF_{254}
Solvent system	dichloromethane : methanol (90 : 10)
Detection	The plate was sprayed with 10% sulfuric acid in ethanol and heated at 110 °C for 1-2 minutes, the purple, bluish-grey, yellow and brown colors appeared.

Samples :	Track 1	=	hexane extract, 15 μl
	Track 5	=	ethanol extract, 15 μl
Reference compounds :	Track 2	=	a mixture of β-sitosteryl glucoside and stigmasteryl glucoside, 5μl
	Track 3	=	quercetin, 5μl
	Track 4	=	β-sitosterol, 5μl

Fig. 5.1 Thin-layer chromatogram of the extracts from the pericarp

TLC chromatograms of the hexane and ethanol extracts from *Z. limonella* pericarp (Fig 5.1) were developed using dichloromethane : methanol (90:10). The extract contained quenching bands under UV 254, 2 fluorescence bands blue and red under UV 366. After spraying the plate with 10% sulfuric acid in ethanol and heated at 110 °C for 1-2 minutes, purple and bluish-grey colored spots appeared. The major compounds shown the similar hRf values and corresponded with the mixture of β-sitosteryl glucoside and stigmasteryl glucoside (hRf = 13) and β-sitosterol (hRf = 64) as reference standards.

5.2 The isolation (Fig 4.2, Chapter IV)

5.2.1 Compound 1

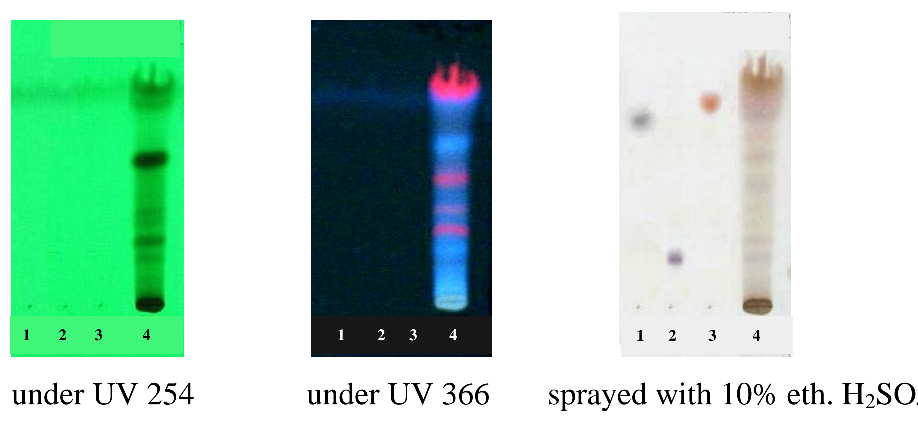
The ethanol extract (20 g) was separated using silica gel 60 column and eluted with solvents of increasing polarity, i.e. hexane (300 ml), hexane : ethyl acetate (4,000 ml) , dichloromethane (700 ml), dichloromethane : methanol (2,700 ml) and methanol (600 ml). Compound 1 came up at the retention volume of 4,100 ml (160.6 mg). It was then washed with hot methanol and recrytallized in methanol. Eleven milligrams of compound 1 could be obtained.

5.2.2 Compound 2

The ethanol extract (20 g) was separated using silica gel 60 column and eluted with solvents of increasing polarity, i.e. hexane (300 ml), hexane : ethyl acetate (4,000 ml) , dichloromethane (700 ml), dichloromethane : methanol (2,700 ml) and methanol (600 ml). Compound 2 came up at the retention volume of 7,900 ml (627.0 mg). It was then washed with hot methanol and recrytallized in methanol. Twenty-six milligrams of compound 2 could be obtained.

5.2.3 Compound 3

The ethanol extract (20 g) was separated using silica gel 60 column and eluted with solvents of increasing polarity, i.e. hexane (300 ml), hexane : ethyl acetate (4,000 ml) , dichloromethane (700 ml), dichloromethane : methanol (2,700 ml) and methanol (600 ml). Compound 3 came up at the retention volume of 3,400 ml (421.3 mg) which was further purified by conventional column chromatography. Compound 3 (421.3 mg) was separated using silica gel 60 column and eluted with solvents of hexane (50 ml), hexane : ethyl acetate (500 ml) and methanol (100 ml). Compound 3 came up at the retention volume of 350 ml (13.2 mg). It was then washed with hot methanol and recrytallized in methanol. Ten milligrams of compound 3 could be obtained.



Adsorbent	silica gel GF_{254}
Solvent system	dichloromethane : methanol (90 : 10)
Detection	The plate was sprayed with 10% sulfuric acid in ethanol and heated at 110 °C for 1-2 minutes, the purple, bluish-grey, orange and brown colors appeared.
Compound	Track 1 = compound 1, 5 µl
	Track 2 = compound 2, 5 µl
	Track 3 = compound 3, 5 µl
Sample	Track 4 = ethanol extract, 15 µl

Fig. 5.2 Thin-layer chromatogram of the isolated compounds

5.3 Structure elucidation

5.3.1 Compound 1

Compound 1

β-sitosterol $C_{29}H_{50}O$ $M_R = 414$

Compound 1 appeared as white needle-shape crystals with a melting point of 132°C (with decomposition). It was isolated using silica gel column and hexane:ethyl acetate (85:15) as the eluting solvent system. The identification of compound 1 was performed using NMR and the spectroscopic data was compared with the literature values.

The UV spectrum in $CHCl_3$ of compound 1 exhibited maximum absorption at 210 nm (Fig.5.3). The molecular formula of compound 1 was deduced as $C_{29}H_{50}O$ (M_R = 414) from the FABMS (Fig. 5.4), which showed the molecular ion $[M+Na]^+$ at 437.37. The FTIR spectrum (Fig 5.5) showed O-H stretching (ν) at 3434 cm^{-1}, νC-H at 2931 and 2861 cm^{-1}, and the CH_3 bending (δ) at 1463 and 1379 cm^{-1}.

The ^{13}C spectral data of compound 1 was compared with literature (47) (Table 5.2). It showed olefinic carbon signals at δ 140.748 and δ 121.709 which were caused by the olefinic carbons C-5 and C-6 (Fig 5.6). The carbon types were determined by DEPT 90 and 135 experiments (Fig. 5.8-5.10) which showed signals of 11 methylene. The carbon signals in the region of δ 10-20 which were signals of 6 methyl.

The ^1H-NMR data of ZL1 (Table 5.3) showed the characteristic signals of a Δ^5-3β-OH sterol at δ 5.33 (*br.d*, J = 5 Hz) and a multiplet for 3-H at δ3.50. The absorptions in the high field region were rather complex, which was typical for steroidal compounds.

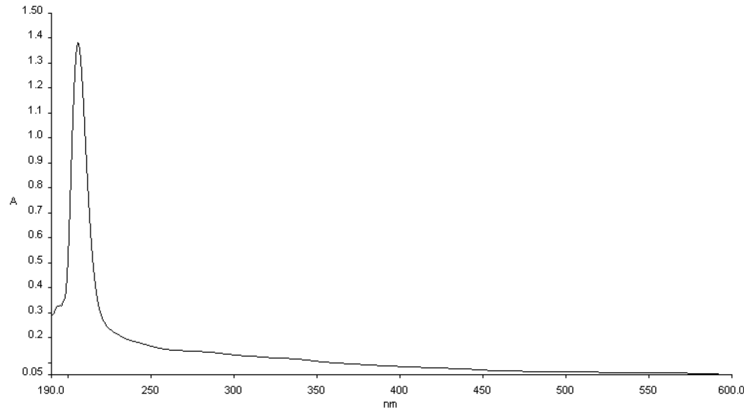


Fig. 5.3 The UV spectrum of compound 1

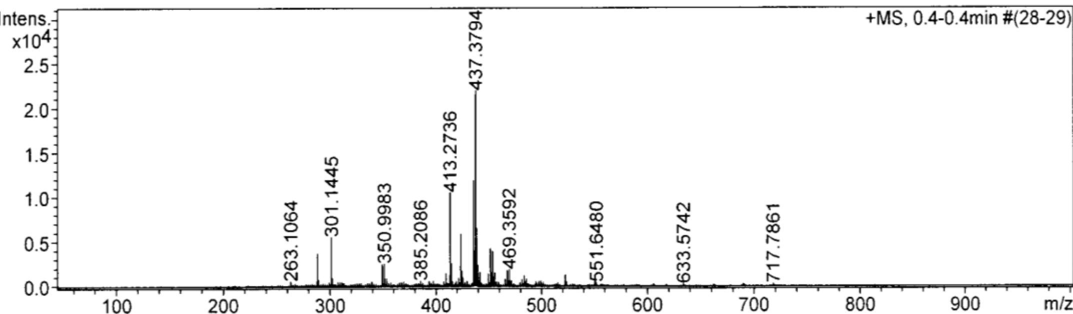


Fig. 5.4 FABMS spectrum of compound 1

Fig. 5.5 FTIR spectrum of compound 1

Table 5.2 ^{13}C-NMR chemical shifts of compound 1 in $CDCl_3$ together with the literature values

C	Compound 1 δ (ppm)	Lit. (47)	Δδ
1	37.239 T	37.243	0.004
2	31.649 T	31.65	0.001
3	71.806 D	71.8	-0.006
4	42.291 T	42.2	-0.091
5	140.748 S	140.74	-0.008
6	121.709 D	121.5	-0.209
7	31.895 T	31.89	-0.005
8	31.895 D	31.89	-0.005
9	50.118 D	50.14	0.022
10	36.494 D	36.5	0.006
11	39.761 T	39.76	-0.001
12	42.291 T	42.29	-0.001
13	28.231 S	28.24	0.009
14	56.755 D	56.76	0.005
15	24.290 T	24.29	0
16	28.231 T	28.24	0.009
17	56.041 D	56.04	-0.001
18	11.848 Q	11.85	0.002
19	19.381 Q	19.39	0.009
20	36.129 D	36.12	-0.009
21	18.763 Q	18.77	0.007
22	33.933 T	33.93	-0.003
23	23.053 T	22.06	-0.993
24	45.820 D	45.82	0
25	29.135 D	29.14	0.005
26	19.810 Q	19.81	0
27	19.017 Q	19.02	0.003
28	23.053 T	23.05	-0.003
29	11.967 Q	12.04	0.073

Table 5.3 ^{1}H- and ^{13}C-NMR data of compound 1 ($CDCl_3$)

C	Compound 1	
	^{13}C	^{1}H
1	37.239	
2	31.649	
3	71.806	3.50 *dddd* (J = 15.5, 11, 9.5, 4.5)
4	42.291	2.23 *m*
5	140.748	
6	121.709	5.33 *br.d* (J = 5)
7	31.895	
8	31.895	
9	50.118	
10	36.494	
11	39.761	
12	42.291	2.23 *m*
13	28.231	
14	56.755	
15	24.290	
16	28.231	
17	56.041	
18	11.848	0.65 *s*
19	19.381	0.91 *s*
20	36.129	
21	18.763	0.84 *d* (J = 1.5)
22	33.933	
23	20.190	
24	45.820	
25	29.135	
26	19.810	0.77 *d* (J = 3)
27	19.017	0.75 *d* (J = 1.5)
28	23.053	
29	11.967	0.76 *t* (J = 1.5, .3)

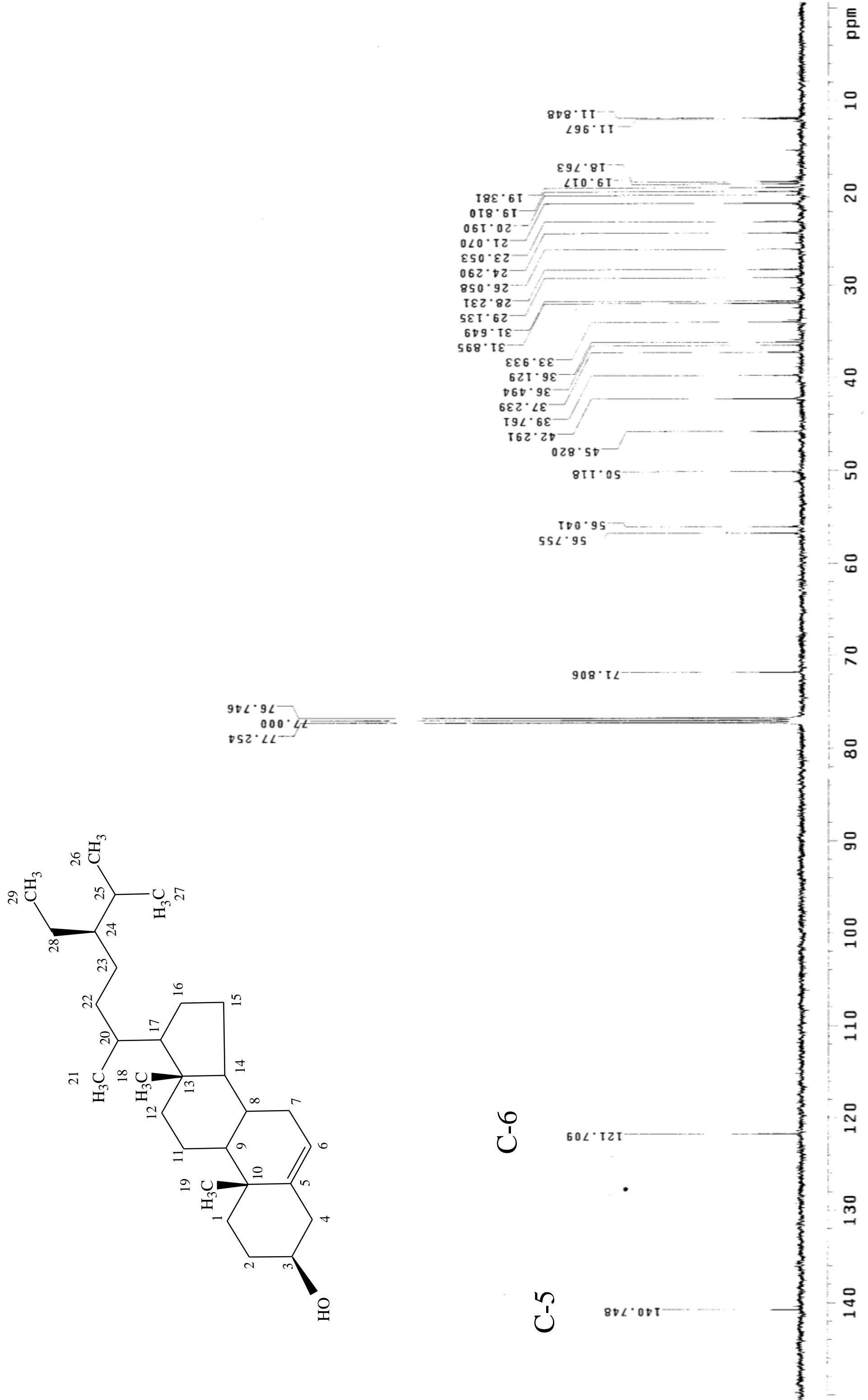


Fig. 5.6 125.7 MHz ^{13}C-NMR spectrum of compound 1 in $CDCl_3$

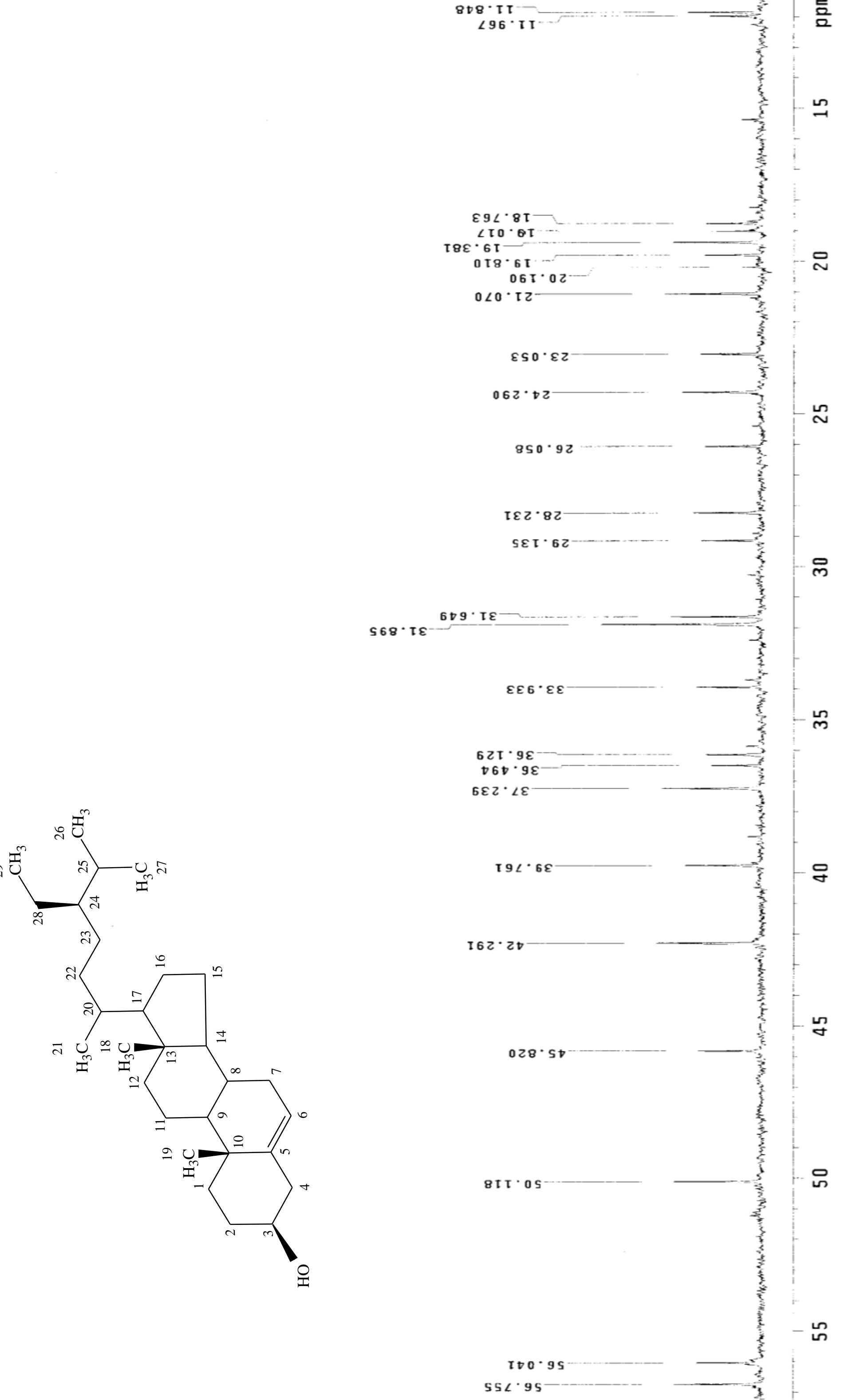
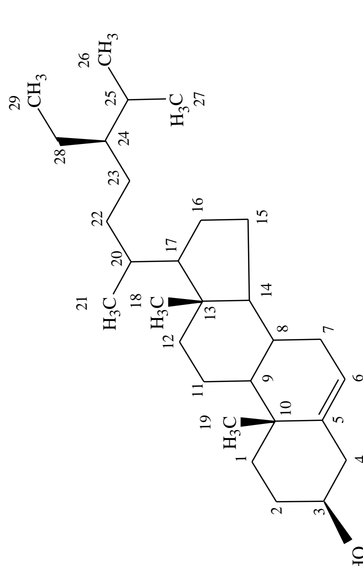


Fig. 5.7 125.7 MHz ^{13}C-NMR expanded spectrum of compound 1 mixture in $CDCl_3$ ($\Delta\delta$ = 11-57 ppm)



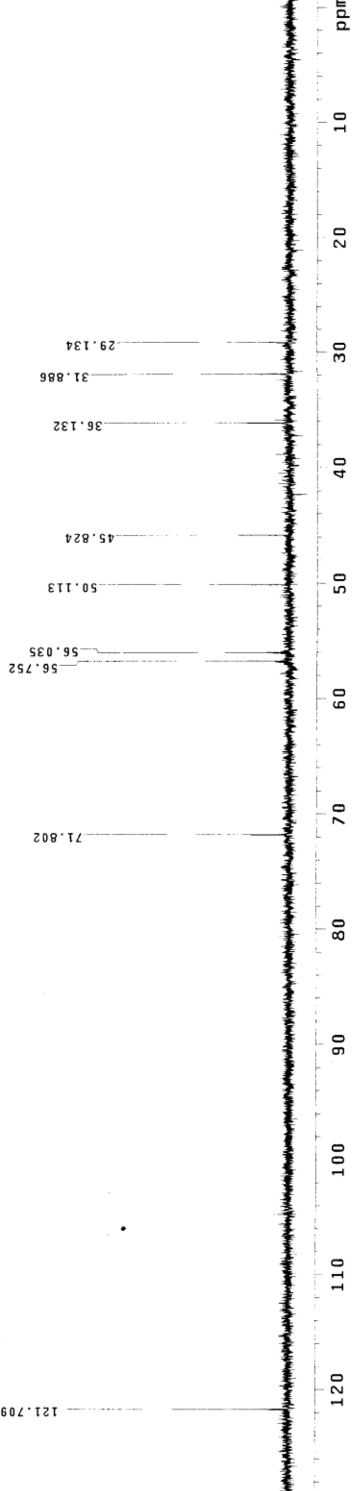


Fig. 5.8 DEPT 90 spectrum of compound 1 in $CDCl_3$

Fig. 5.9 DEPT 135 spectrum of compound 1 in $CDCl_3$

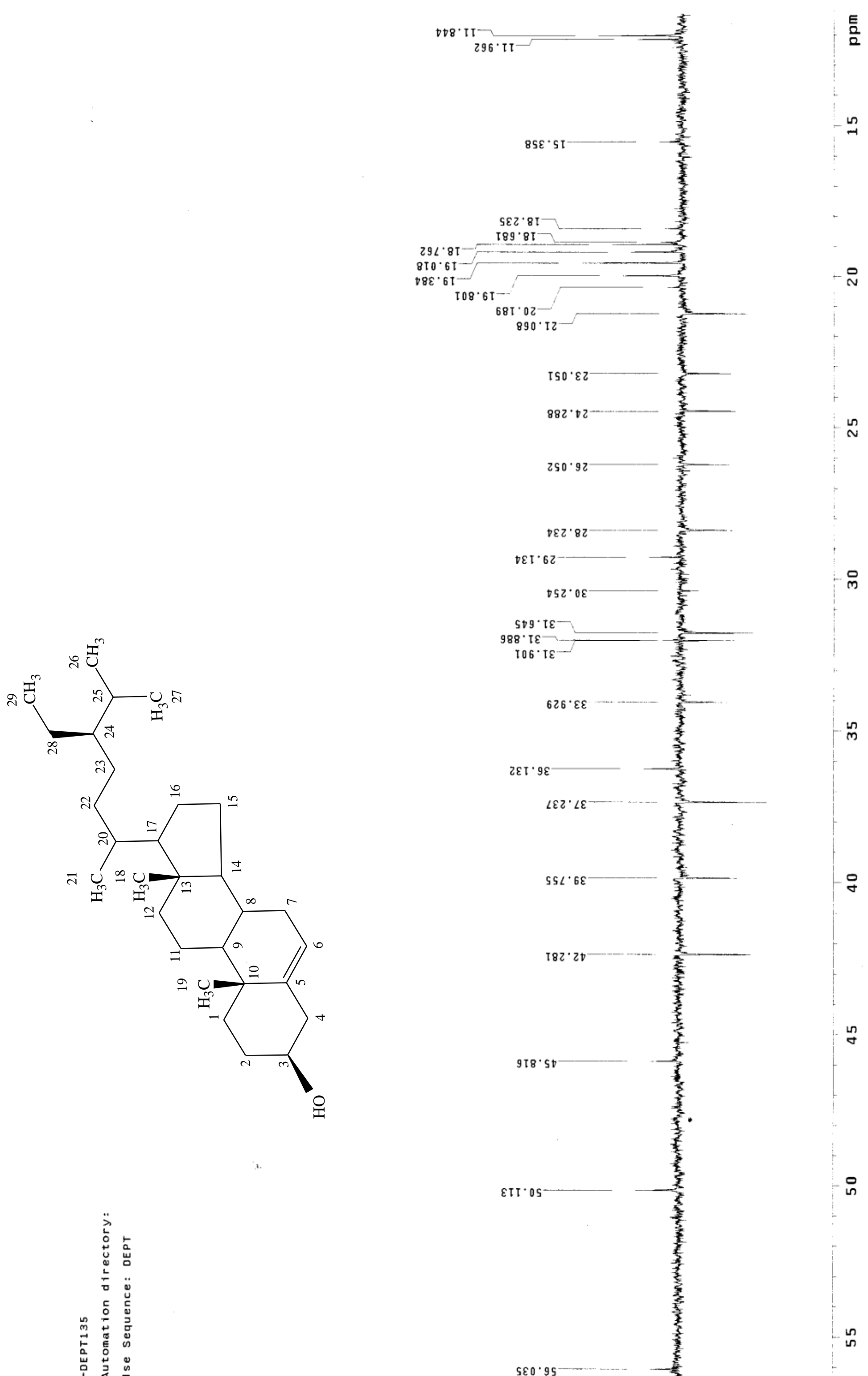


Fig. 5.10 DEPT 135 expanded spectrum of compound 1 in $CDCl_3$ ($\Delta\delta$ = 11-57 ppm)

Fig. 5.11 500 MHz ^{1}H-NMR spectrum of compound 1 in $CDCl_3$

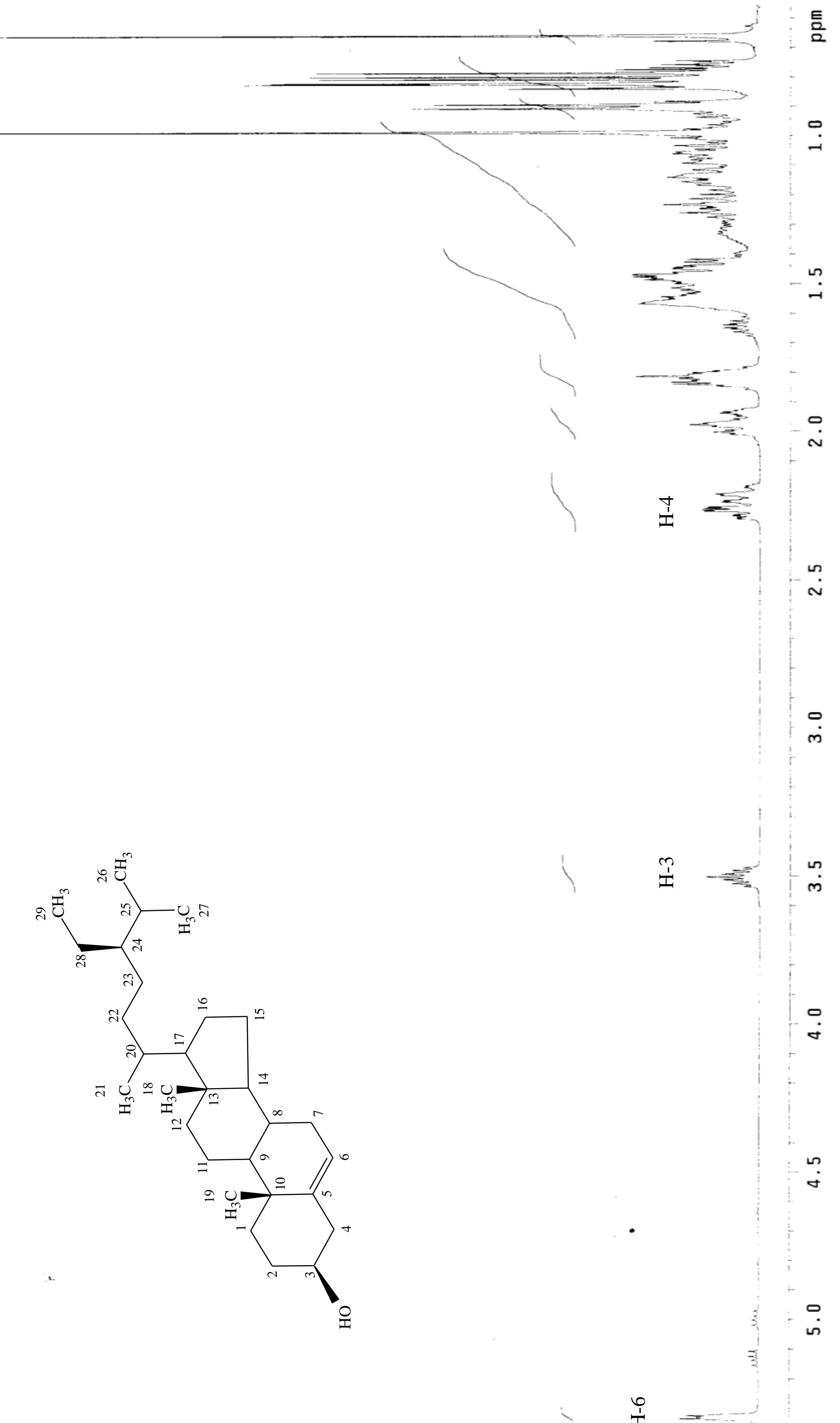


Fig. 5.12 500 MHz ^{1}H-NMR expanded spectrum of compound 1 in $CDCl_3$ ($\Delta\delta$ = 0-5.5 ppm)

5.3.2 Compound 2

Compound 2.1

β-sitosteryl glucoside $C_{35}H_{60}O_6$ $M_R = 576$

Compound 2.2

Stigmasteryl glucoside $C_{35}H_{58}O_6$ $M_R = 574$

Compound 2 appeared as a white solid with a melting point of 267 °C (with decomposition). It was isolated using silica gel column and dichloromethane : methanol (90:10) as the eluting solvent system. It consisted of 3-*O*-β-D-glucopyranosyl-(24S)-24α-ethylcholesta-5-ene (β-sitosteryl glucoside), compound 2.1 and 3-*O*-β-D-glucopyranosyl-22E,(24S)-24α-ethylcholest-5,22-diene (stigmasteryl glucoside), compound 2.2. The identification of compound 2 was performed using NMR and the spectroscopic data was compared with the literature values.

The UV spectrum in $CHCl_3$ of compound 2 exhibited maximum absorption at 210 nm (Fig. 5.13). The molecular formula of compound 2 was deduced as $C_{35}H_{60}O_6$ (M_R = 576) from the FABMS (Fig. 5.14), which showed the molecular ion $[M+Na]^+$ at 599.42. The FTIR spectrum (Fig. 5.15) showed O-H stretching (ν) at

3403 cm^{-1}, νC-H at 2931 and 2864 cm^{-1}, and CH_3 bending (δ) at 1460 and 1382 cm^{-1}. An intensive peak was also seen at 1018 due to the C-O stretching of the sugar moiety.

The ^{13}C chemical shifts of the compound 2.1 and compound 2.2 were assigned by comparing with literature data (48) as shown in Table 5.5. The chemical shifts of C-19 to C-29 of compound 2.1 and compound 2.2 were different. The signals of the olefinic carbons (C-5, C-6) appeared at δ 140.644 and δ 121.629, respectively. The two remaining olefinic carbons signals at δ 138.440 (C-22) and 129.170 (C-23) belonged to the side chain double bond of compound 2.2. The presence of the sugar moiety was identified by anomeric carbon signal at δ 102.058 together with those of the other sugar carbons in the region of δ 62-78 (Fig. 5.16).

The ^{1}H-NMR whole spectrum shown in Fig 5.17. The characteristic olefinic 6-H proton signal of the Δ^5-3β-OH sterol was found at δ 5.34 (*d*, J = 3.5 Hz). The trans olefinic proton signal for 22-H and 23-H of compound 2.2 were observed at δ 5.21 (*dd*, J = 17.5, 9 Hz) and at 5.05 (*dd*, J = 11, 7.5 Hz), respectively. The isolated two multiplets at δ 2.73 and δ 2.47 belonged to the two 4-H methylene protons. The two most upfield shifted singlets at δ 0.65 and δ 0.66 belonged to the C-18 methyl signals of compound 2.1 and compound 2.2, respectively. The ratio of 70:50 for compound 2.1 and compound 2.2 was derived from the ^{1}H-integration of these two peaks. The remaining signals in the region δ 0.8-2.2 (Fig. 5.18) were complex multiplets due to the methine and methylene protons of the skeleton and side chain.

The ^{1}H-NMR spectrum (Fig. 5.19) showed the doublet derived form the anomeric proton (1′-H) at δ 5.08 with the coupling constant of 11 Hz suggesting the β-glycosidic linkage. The signals of 2′-H, 5′-H and 3′-H were seen as multiplets overlapping in the region of δ 3.9-4.36. The signal groups of two 6′-proton were obserbed at δ 4.41 (*dd*, J = 11, 5 Hz, 6′b-H) and δ 4.56 (*br.d*, J = 11 Hz, 6′a-H)

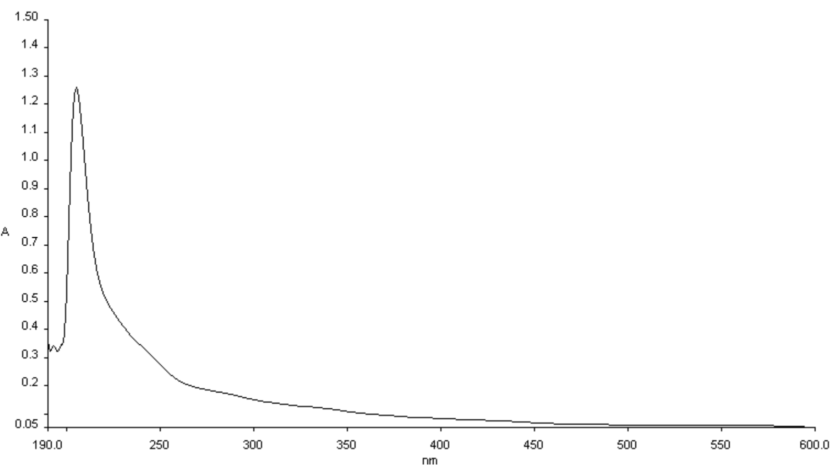


Fig. 5.13 The UV spectrum of compound 2

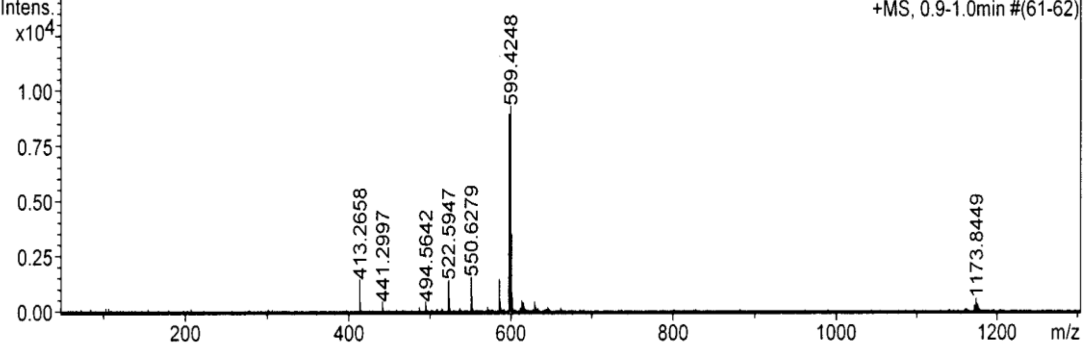


Fig. 5.14 FABMS spectrum of compound 2 mixture

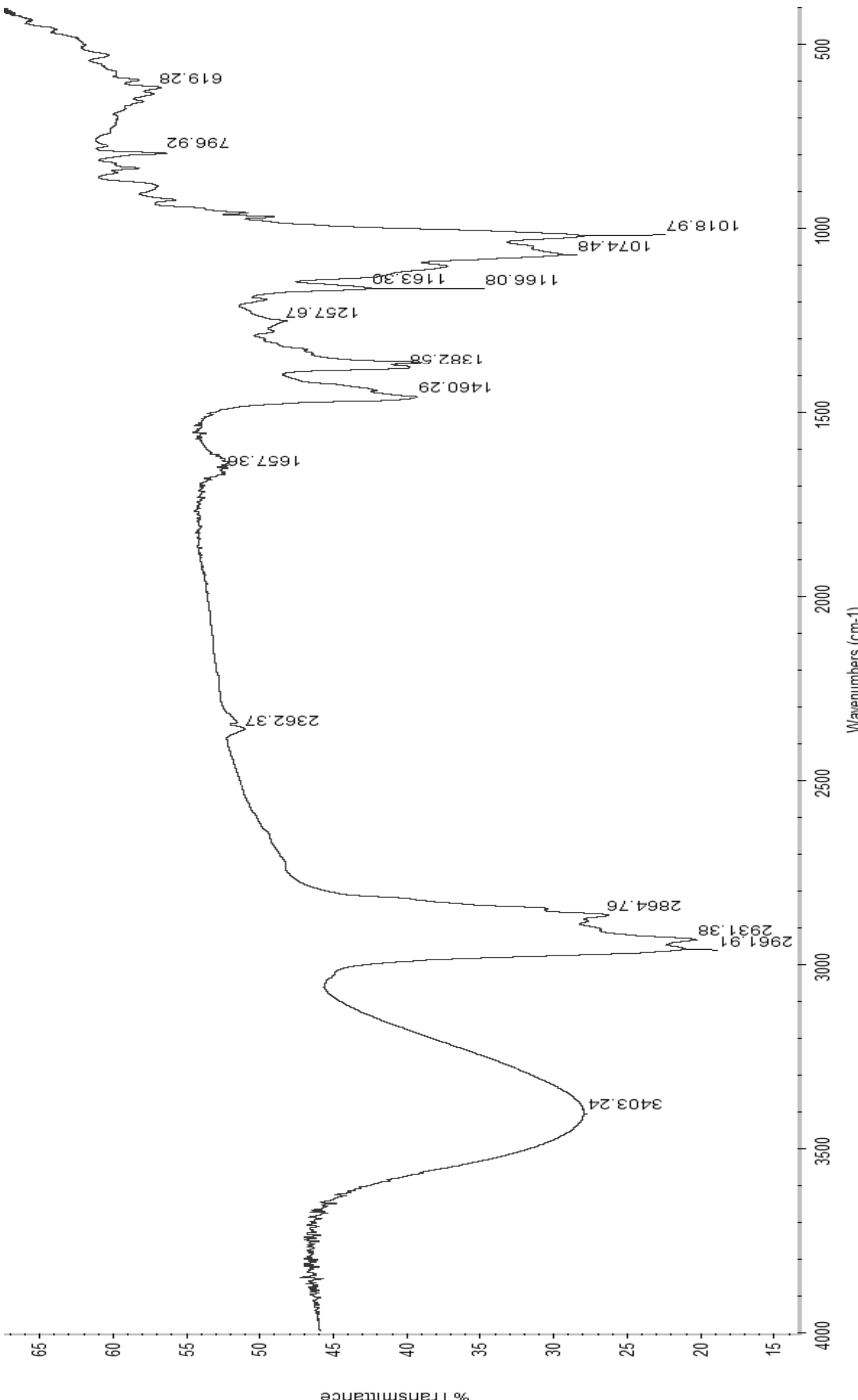


Figure 5.15 FTIR spectrum of compound 2 mixture

Table 5.4 ^{13}C-chemical shifts of compound 2.1 and compound 2.2 (pyridine-d_5)

C	Compound 2.1			Compound 2.2		
	δ (ppm)	Lit. (48)	Δδ	δ (ppm)	Lit. (48)	Δδ
1	37.159	37.5	0.341	37.159	37.6	0.441
2	29.562	30.2	0.638	29.832	30.3	0.468
3	78.157	78.5	0.343	78.157	78.3	0.143
4	39.641 T	40.0	0.359	38.927	39.4	0.473
5	140.644	140.9	0.256	140.644	141.0	0.356
6	121.629	121.8	0.171	121.629	122.0	0.371
7	31.830	32.1	0.27	31.83	32.2	0.37
8	31.727	32.1	0.373	31.727	32.1	0.373
9	50.021	50.3	0.279	50.021	50.4	0.379
10	37.159	36.9	-0.259	37.159	37.0	0.159
11	20.950	21.3	0.35	20.950	21.4	0.450
12	38.927	39.3	0.373	39.641	39.9	0.259
13	42.155	42.5	0.345	42.028	42.4	0.372
14	56.540	56.9	0.36	56.627	57.1	0.473
15	24.194	24.5	0.306	24.194	24.7	0.506
16	28.222	28.6	0.378	29.134	29.4	0.266
17	55.913	56.3	0.387	55.913	56.2	0.287
18	11.672	12.0	0.328	11.847	12.3	0.453
19	19.134	19.4	0.266	18.873	19.3	0.427
20	36.017	36.4	0.383	40.442	40.9	0.458
21	18.873	19.1	0.227	21.157	21.7	0.543
22	33.852	34.3	0.448	138.440	138.9	0.46
23	26.002	26.5	0.498	129.170	129.5	0.33
24	45.684	41.6	0.416	51.068	51.5	0.432
25	29.134	29.4	0.266	31.830	32.2	0.37
26	19.134	19.2	0.066	21.157	21.4	0.243
27	19.697	20.0	0.303	19.697	20.1	0.403
28	23.068	23.4	0.332	25.367	25.8	0.433
29	11.847	12.2	0.353	12.259	12.6	0.341
1′	102.058	102.5	0.442	102.058	102.6	0.542
2′	74.794	75.2	0.406	74.794	75.4	0.606
3′	77.951	78.3	0.349	78.157	78.7	0.543
4′	71.218	71.7	0.482	71.218	71.7	0.482
5′	77.824	78.2	0.376	77.951	78.5	0.549
6′	62.273	62.8	0.527	62.273	62.9	0.627

Table 5.5 ^{1}H- and ^{13}C-NMR data of compound 2.1 and compound 2.2 (pyridine-d_5)

C	Compound 2.1		Compound 2.2	
	^{13}C	^{1}H	^{13}C	^{1}H
1	37.159	1.85 *m*	37.159	1.85 *m*
2	29.562	1.71 *m*, 2.13 *m*	29.832	1.71 *m*, 2.13 *m*
3	78.157	3.94 *m*	78.157	3.94 *m*
4	39.641 T	2.73 *dd* (J = 2, 13), 2.47 *dd* (J = 2, 13)	38.927	2.73 *dd* (J = 2, 13), 2.47 *dd* (J = 2, 13)
5	140.644		140.644	
6	121.629	5.34 *d* (J = 3.5)	121.629	5.34 *d* (J = 3.5)
7	31.830		31.83	
8	31.727		31.727	
9	50.021		50.021	
10	37.159		37.159	
11	20.950		20.950	
12	38.927		39.641	
13	42.155		42.028	
14	56.540		56.627	
15	24.194		24.194	
16	28.222		29.134	
17	55.913		55.913	
18	11.672	0.65 *s*	11.847	0.66 *s*
19	19.134	0.92 *s*	18.873	0.92 *s*
20	36.017		40.442	
21	18.873	1.06 *d* (J = 6.5)	21.157	1.09 *d* (J = 6.5)
22	33.852		138.440	5.21 *dd* (J = 15, 3)
23	26.002		129.170	5.05 *dd* (J = 15, 9)
24	45.684		51.068	
25	29.134		31.830	
26	19.134		21.157	
27	19.697		19.697	
28	23.068		25.367	
29	11.847		12.259	
1′	102.058	5.05 *d* (J = 7.5)	102.058	5.05 *d* (J = 7.5)
2′	74.794	4.05 *t* (J = 8, 7.5)	74.794	4.05 *t* (J = 8, 7.5)
3′	77.951	4.29 *m*	78.157	4.29 *m*
4′	71.218	4.27 *m*	71.218	4.27 *m*
5′	77.824	3.99 *m*	77.951	3.99 *m*
6′a	62.273	4.56 *br.d* (J = 11)	62.273	4.56 *br.d* (J = 11)
6′b	62.273	4.41 *dd* (J = 11, 5)	62.273	4.41 *dd* (J = 11, 5)

Fig. 5.16 127.5 MHz ^{13}C-NMR spectrum of compound 2 mixture in pyridine-d_5

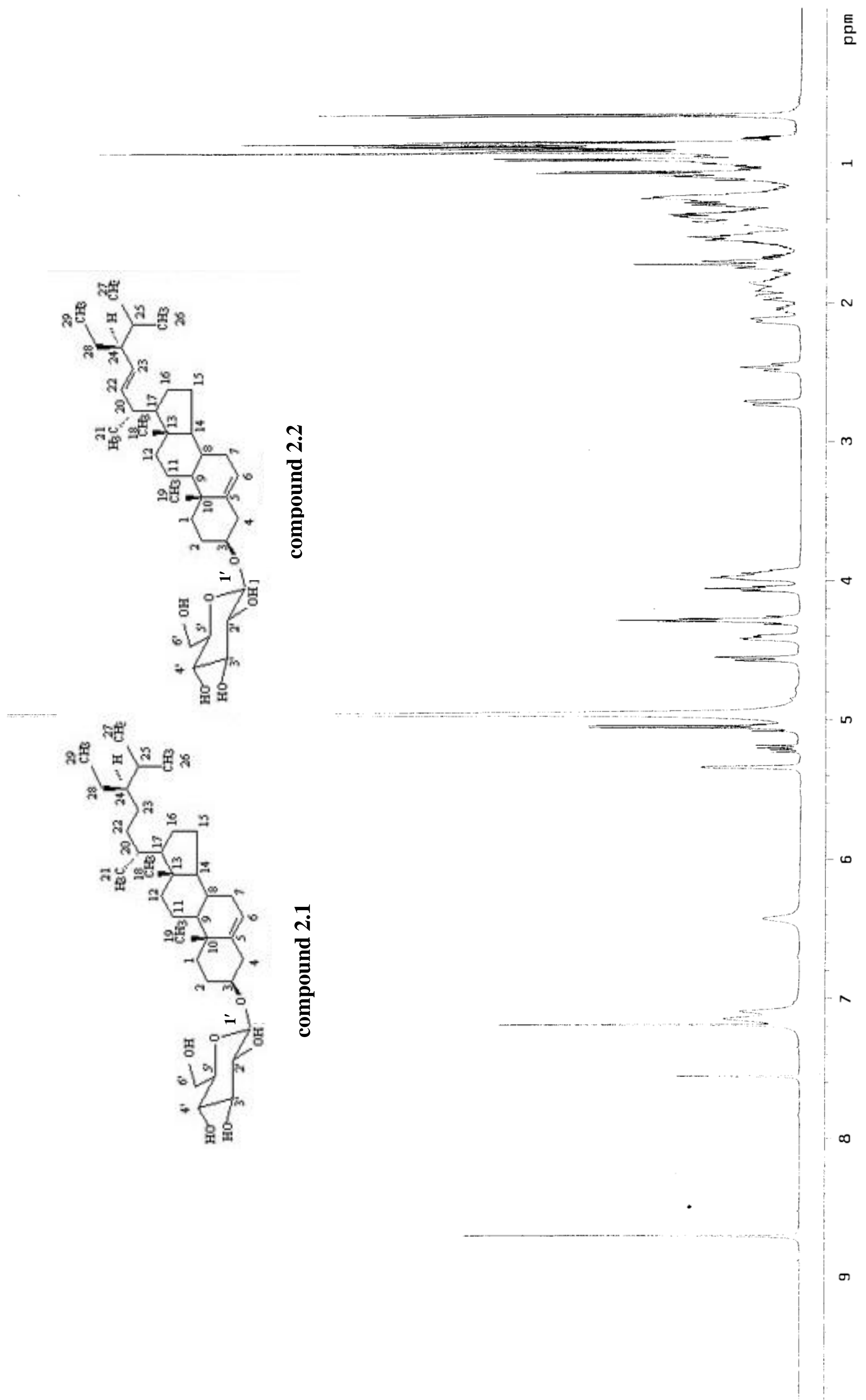


Fig. 5.17 500 MHz ^{1}H-NMR spectrum of compound 2 mixture in pyridine-d_5

Fig. 5.18 500 MHz ^{1}H-NMR expanded spectrum of compound 2 mixture in pyridine-d_5 ($\Delta\delta = 0.56 - 2.24$ ppm)

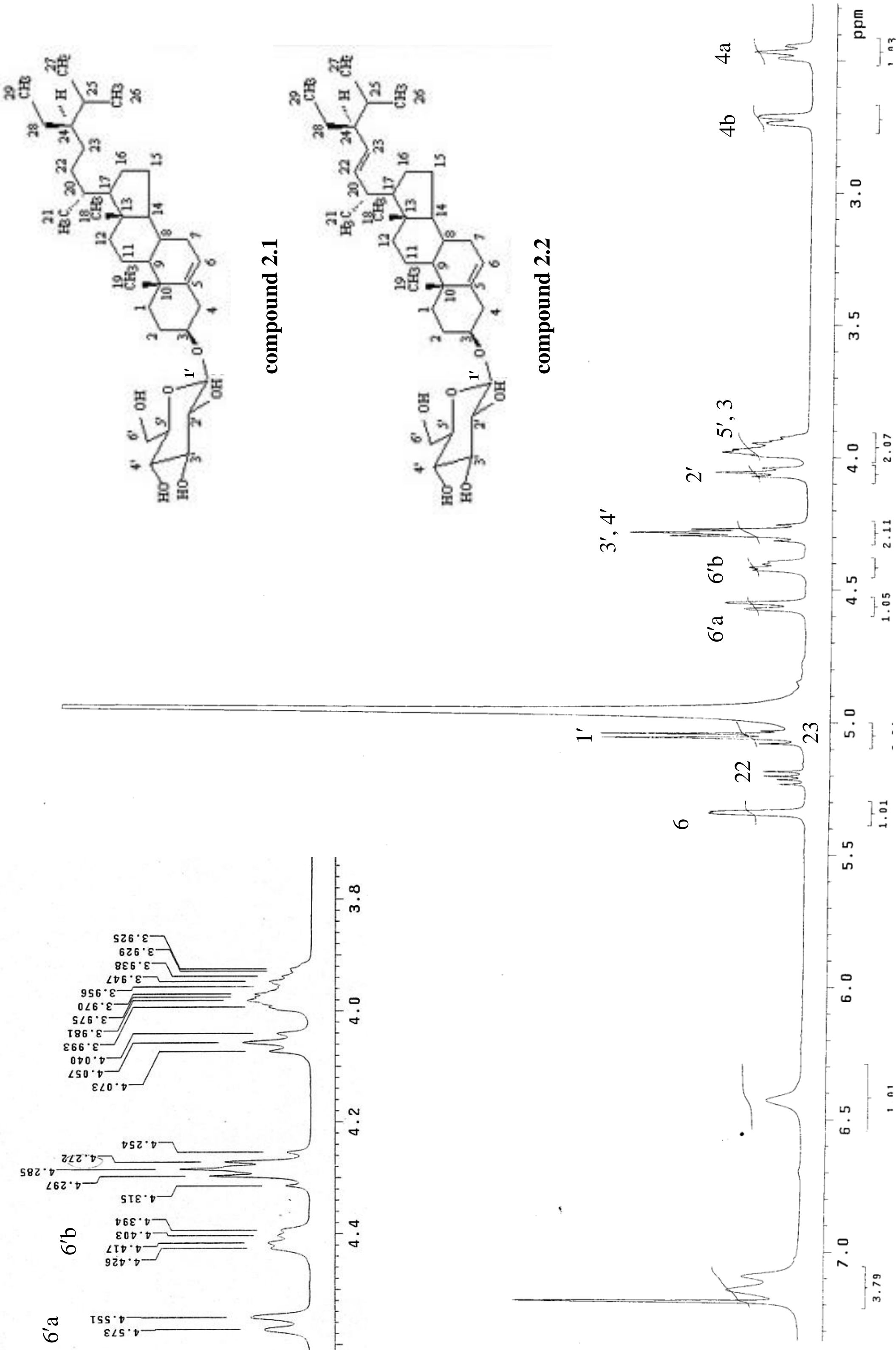


Fig. 5.19 500 MHz ^{1}H-NMR expanded spectrum of compound 2 mixture in pyridine-d_5 ($\Delta\delta$ = 2.4 - 7.3 ppm)

5.3.3 Compound 3

Compound 3

Lupeol $C_{30}H_{50}O$, $M_R = 426$

Compound 3 appeared as a white solid with a melting point of 209°C (with decomposition). It was isolated using silica gel column and hexane : ethyl acetate (85:15) as the eluting solvent system. The identification of compound 3 was performed using NMR and the spectroscopic data was compared with the literature values.

The UV spectrum in $CHCl_3$ of compound 3 exhibited maximum absorption at 214 nm (Fig. 5.20). The molecular formula of compound 3 was deduced as $C_{30}H_{50}O$ ($M_R = 426$) from the FABMS (Fig. 5.21), which showed the molecular ion $[M+Na]^+$ at 449.37. The FTIR spectrum (Fig. 5.22) showed O-H stretching (ν) at 3344 cm^{-1}, νC-H at 2942 and 2848 cm^{-1}, and CH_3 bending (δ) at 1454 and 1382 cm^{-1}. The vibration of νC=C appeared at 1641 cm^{-1} as a weak band and δC-C at 1044 cm^{-1}.

The ^{13}C-NMR spectral (Fig. 5.23-5.24) compound 3 showed 30 carbon signals suggesting that this compound was a triterpene. The carbon types were determined by DEPT 90 and 135 experiments (Fig. 5.25-5.27) which showed signals of 7 methyl, 11 methylene, 6 methane, 5 quaternary and 2 olefinic carbons. The ^{13}C chemical shifts of compound 3 were assigned by comparing with the literature values (49) as shown in Table 5.6. It could be concluded that the structure of compound 3 was lupeol.

The ^{1}H-NMR spectral (Fig.5.28-5.29) showed the signals for seven methyl protons at δ0.78 (H_3-24), δ0.81 (H_3-28), δ0.85 (H_3-25), δ0.96 (H_3-27), δ0.99 (H_3-23), δ1.05 (H_3-26) and δ1.70 (H_3-30) ppm. The signal of H-3 proton appeared as a doublet

of doublet (*dd*) at δ3.21 (*J*= 5, 11 Hz) and H-29 olefinic protons (Ha, Hb) as doublets at Ha-29 δ4.59 (*J* = 1, 2, 5 Hz) and Hb-29 δ4.71 (*J* = 2 Hz), respectively (Fig. 5.30).

The heteronuclear single quantum coherence (HSQC) spectra of compound 3 revealed the presence of C-H correlation (Fig. 5.31-5.33).

The heteronuclear multiple bond correlation (HMBC) spectra of compound 3 showed the C-H long-range correlation (Fig. 5.34-5.36), which supported the assignment.

The assignment of ^{1}H was also confirmed by COSY spectra (Fig. 5.37-5.38).

The complete NMR data of compound 3 was shown in Table. 5.7.

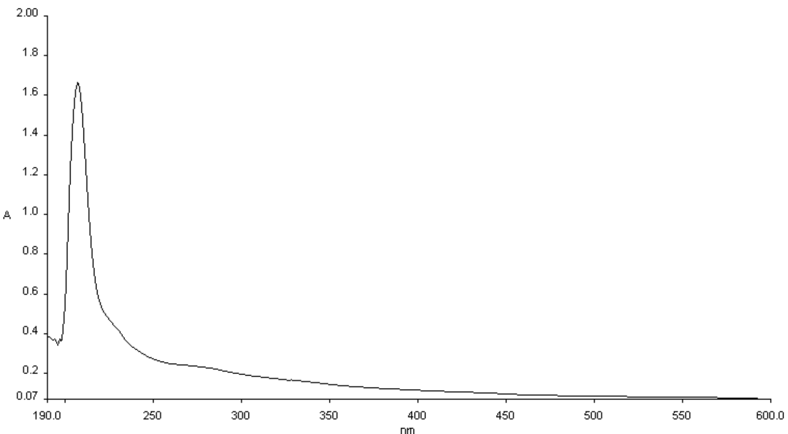


Fig. 5.20 The UV spectrum of compound 3

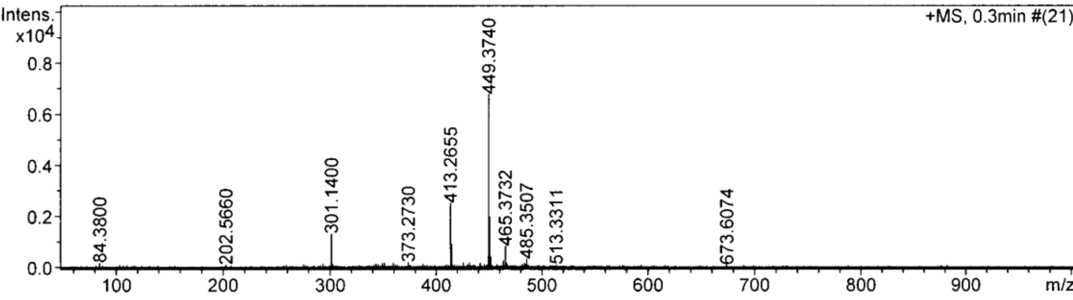


Fig. 5.21 FABMS spectrum of compound 3 in $CDCl_3$

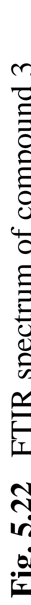


Fig. 5.22 FTIR spectrum of compound 3

Table 5.6 ^{13}C-chemical shifts of compound 3 ($CDCl_3$)

C	Compound 3 δ (ppm)	Lit. (49)
1	38.7 T	38.728
2	27.4 T	27.462
3	78.9 D	79.023
4	38.8 S	38.867
5	55.3 D	55.322
6	18.3 T	18.33
7	34.2 T	34.304
8	40.8 S	40.854
9	50.4 D	50.462
10	37.1 S	37.187
11	20.9 T	20.945
12	25.1 T	25.17
13	38.0 D	38.077
14	42.8 S	42.845
15	27.4 T	27.429
16	35.5 T	35.598
17	43.0 S	43.009
18	48.2 D	48.328
19	47.9 D	47.994
20	150.9 S	150.97
21	29.8 T	29.868
22	40.0 T	40.013
23	28.0 Q	27.993
24	15.4 Q	15.364
25	16.1 Q	16.116
26	15.9 Q	15.986
27	14.5 Q	14.555
28	18.0 Q	18.006
29	109.3 T	109.313
30	19.3 Q	19.313

Table 5.7 NMR data of compound 3 ($CDCl_3$)

C	Compound 3		
	^{13}C	HMQC (^1H)	HMBC
1	38.7 T	0.92 (CH_2-1)	0.85 (H_3-25)
2	27.4 T		3.21 (OH-3)
3	78.9 D	3.21 *dd* (J = 5, 11 Hz) (OH-3)	0.99 (H_3-23), 0.78 (H_3-24)
4	38.8 S		0.99 (H_3-23), 0.85 (H_3-25), 0.78 (H_3-24)
5	55.3 D		0.99 (H_3-23), 0.85 (H_3-25), 0.78 (H_3-24)
6	18.3 T		
7	34.2 T	1.41 (CH_2-7)	1.05 (H_3-26)
8	40.8 S		1.05 (H_3-26), 0.96 (H_3-27)
9	50.4 D	1.27 (CH-9)	0.85 (H_3-25), 1.05 (H_3-26)
10	37.1 S		0.85 (H_3-25)
11	20.9 T		
12	25.1 T		
13	38.0 D	1.68 (CH-13)	0.96 (H_3-27), 2.40 (CH-19)
14	42.8 S		1.05 (H_3-26), 0.96 (H_3-27)
15	27.4 T		0.96 (H_3-27)
16	35.5 T	1.49 (CH_2-16)	0.81 (H_3-28)
17	43.0 S		0.81 (H_3-28)
18	48.2 D	1.38 (CH-18)	0.81 (H_3-28)
19	47.9 D	2.40 *ddd* (J = 6, 11, 17 Hz) (CH-19)	1.70 (H_3-30), 0.81 (H_3-28), 4.71 (CH_2-29), 4.59 (CH_2-29)
20	150.9 S		2.40 (CH-19), 1.70 (H_3-30), 1.38 (CH-18)
21	29.8 T	1.94 (CH_2-21)	2.40 (CH-19)
22	40.0 T	1.20 (CH_2-22)	0.81 (H_3-28)
23	28.0 Q	0.99 (H_3-23)	0.78 (H_3-24), 3.21 (OH-3)
24	15.4 Q	0.78 (H_3-24)	0.99 (H_3-23)
25	16.1 Q	0.85 (H_3-25)	
26	15.9 Q	1.05 (H_3-26)	
27	14.5 Q	0.96 (H_3-27)	
28	18.0 Q	0.81 (H_3-28)	
29a	109.3 T	4.59 *br* (J = 1, 2, 5 Hz) (CH-29)	2.40 (CH-19), 1.70 (H_3-30)
29b		4.71 *br* (J = 2 Hz) (CH-29)	4.71 (CH_2-29), 4.59 (CH_2-29), 2.40 (CH-19)
30	19.3 Q	1.70 (H_3-30)	

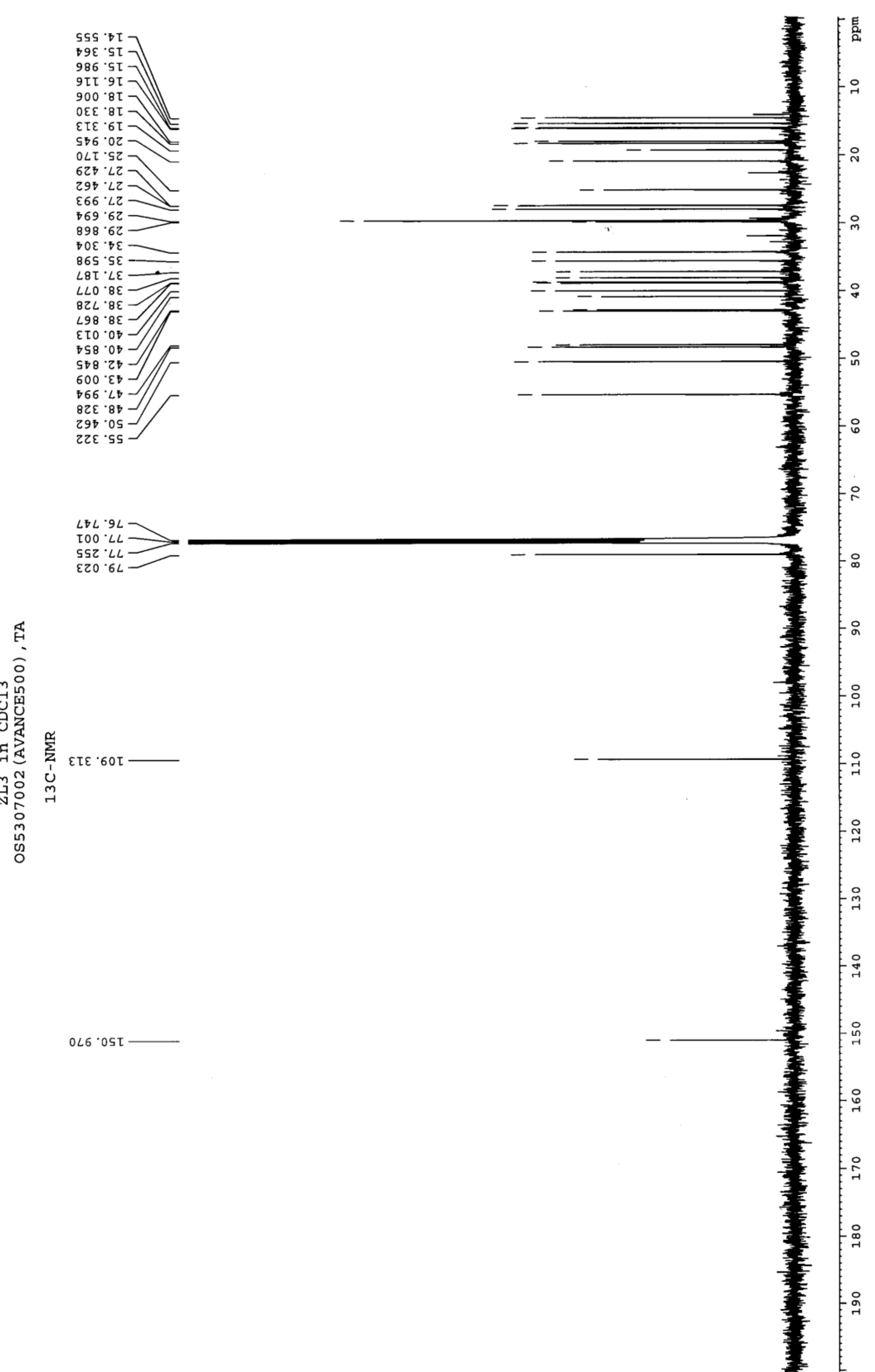


Fig. 5.23 125.7 MHz ^{13}C-NMR spectrum of compound 3 in $CDCl_3$

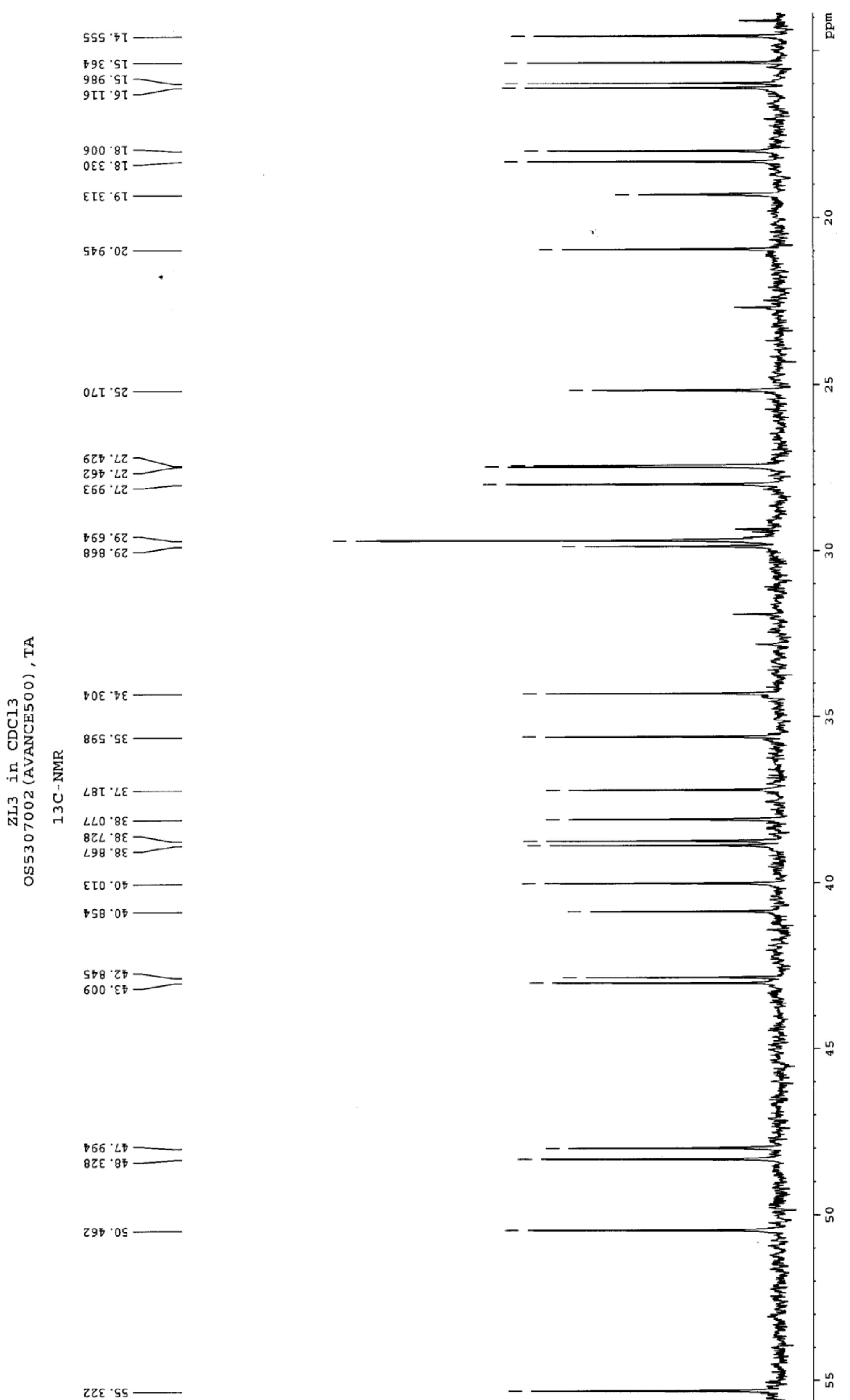


Fig. 5.24 125.7 MHz ^{13}C-NMR expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta$ = 14 – 56 ppm)

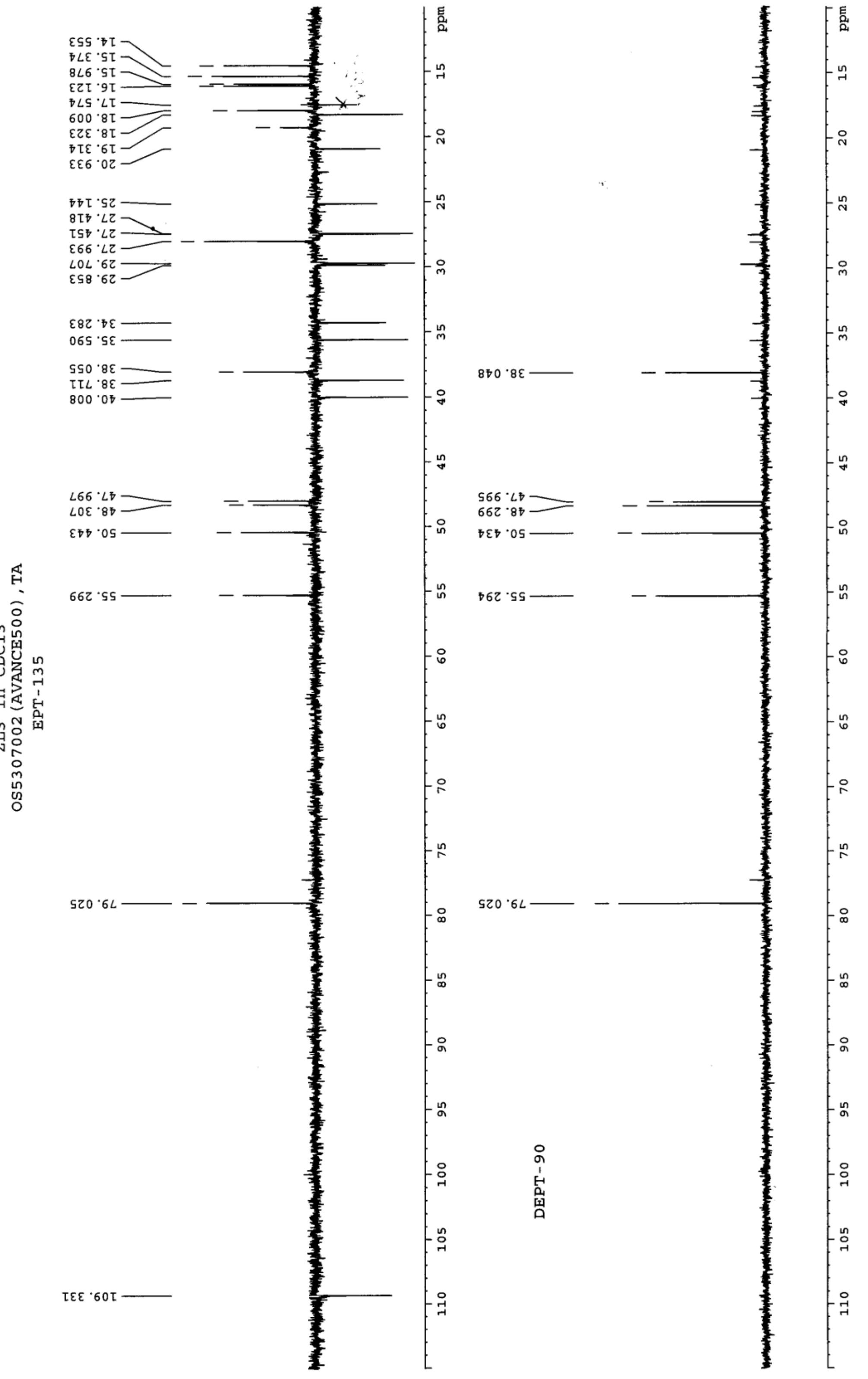


Fig. 5.25 DEPT 90 and 135 spectrum of compound 3 in $CDCl_3$

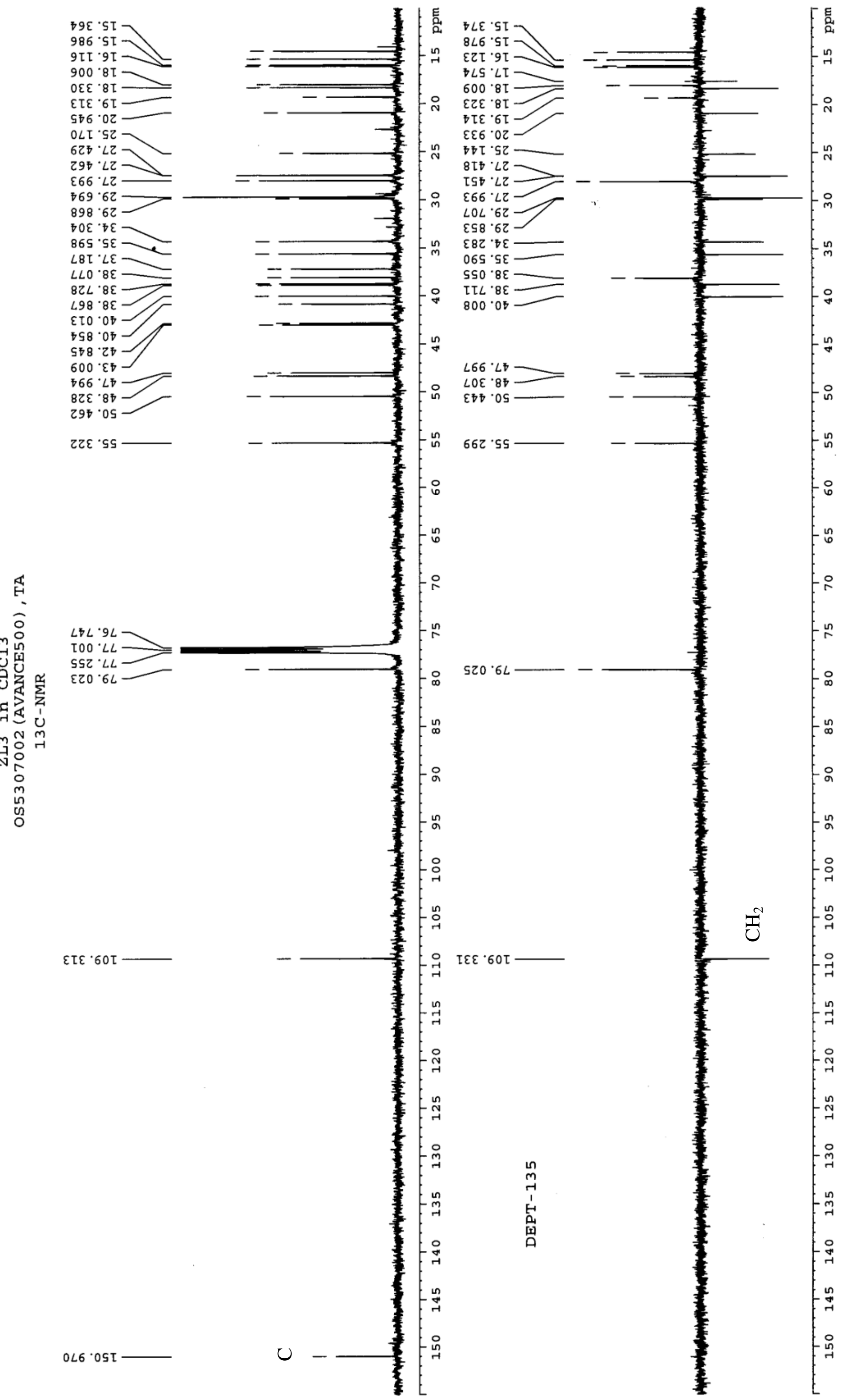


Fig. 5.26 DEPT 135 spectrum of compound 3 in $CDCl_3$

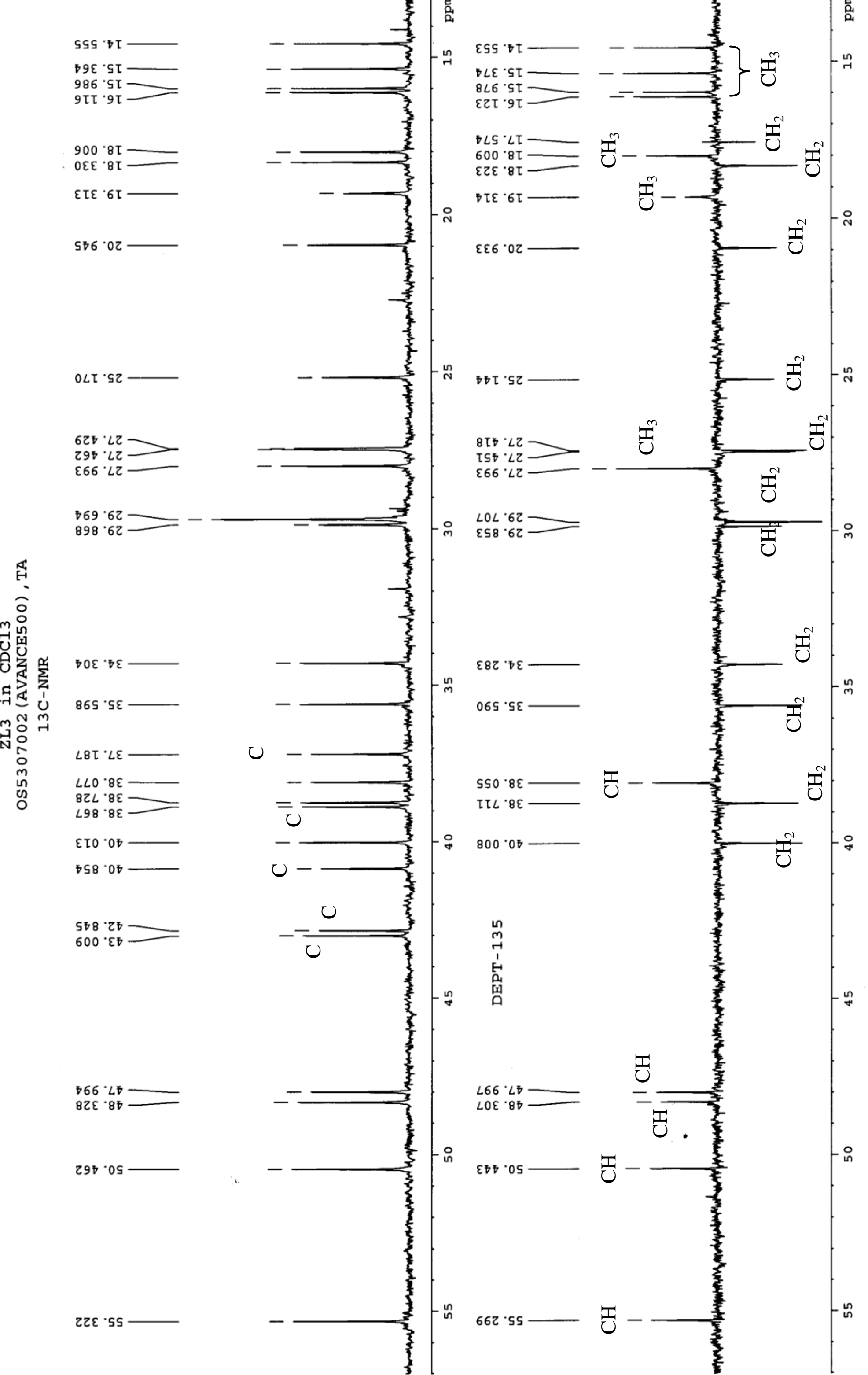


Fig. 5.27 DEPT 135 expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta = 13 - 57$ ppm)

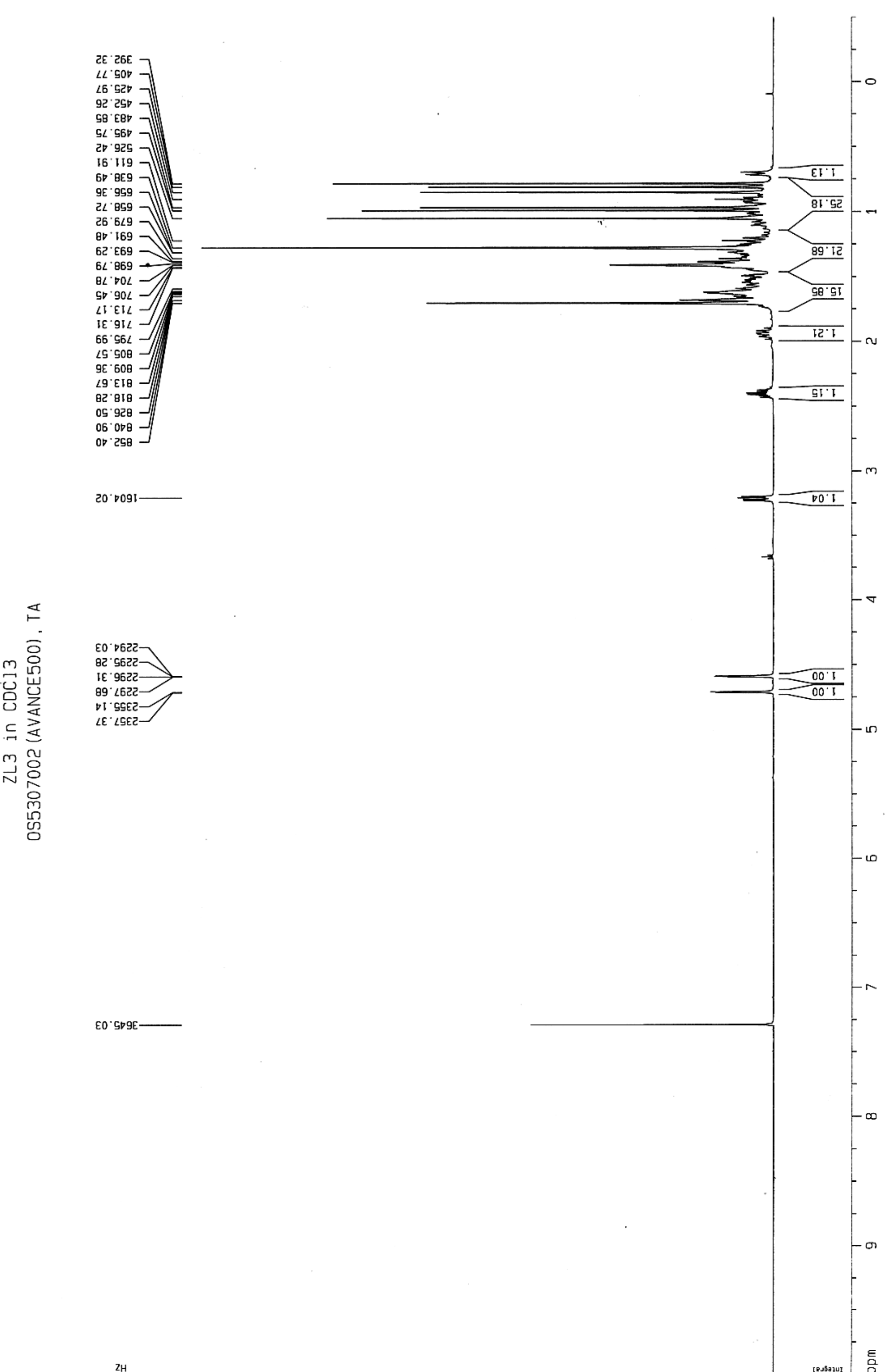


Fig. 5.28 500 MHz ^{1}H-NMR spectrum of compound 3 in $CDCl_3$

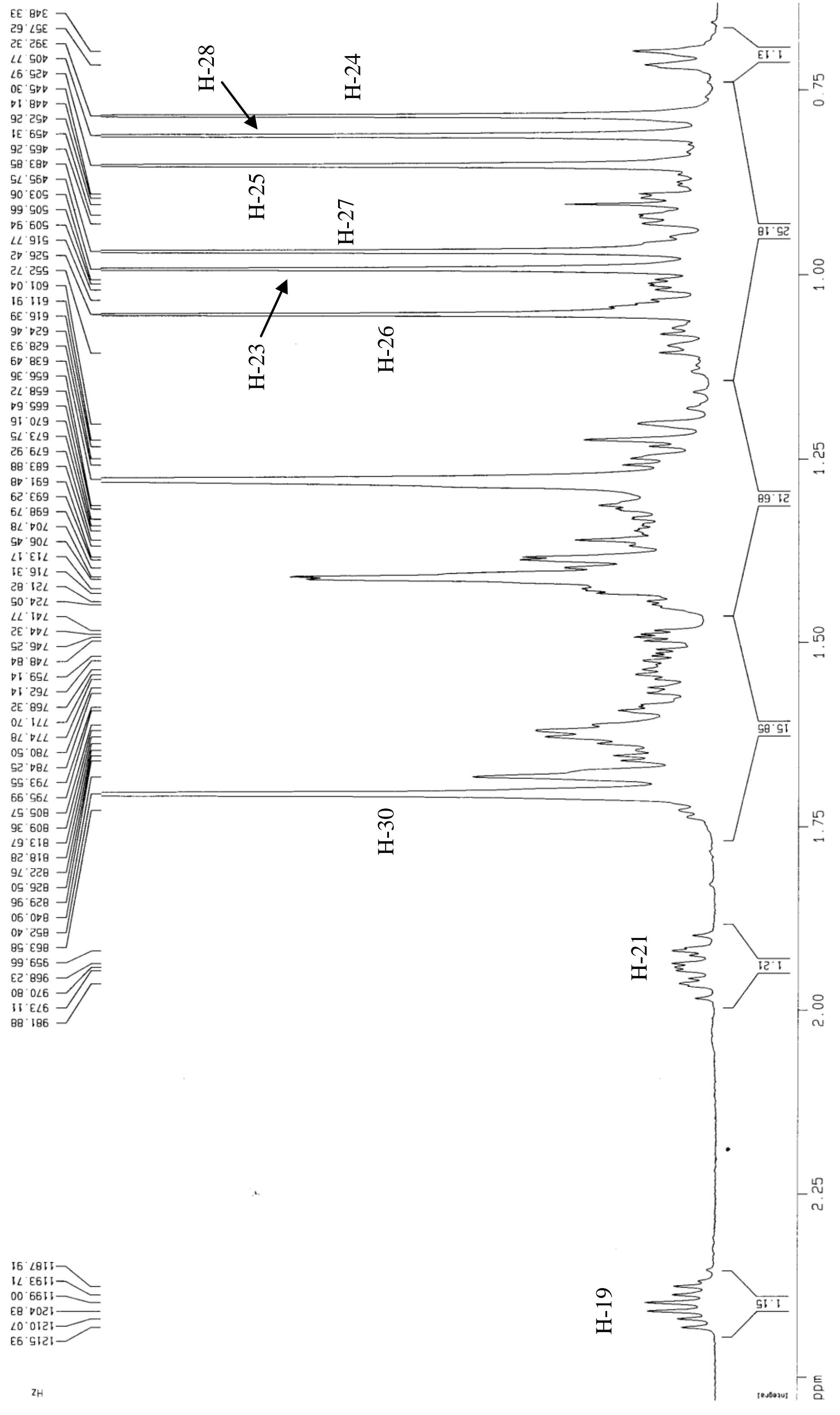


Fig. 5.29 500 MHz ^{1}H-NMR expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta = 0.6 - 2.5$ ppm)

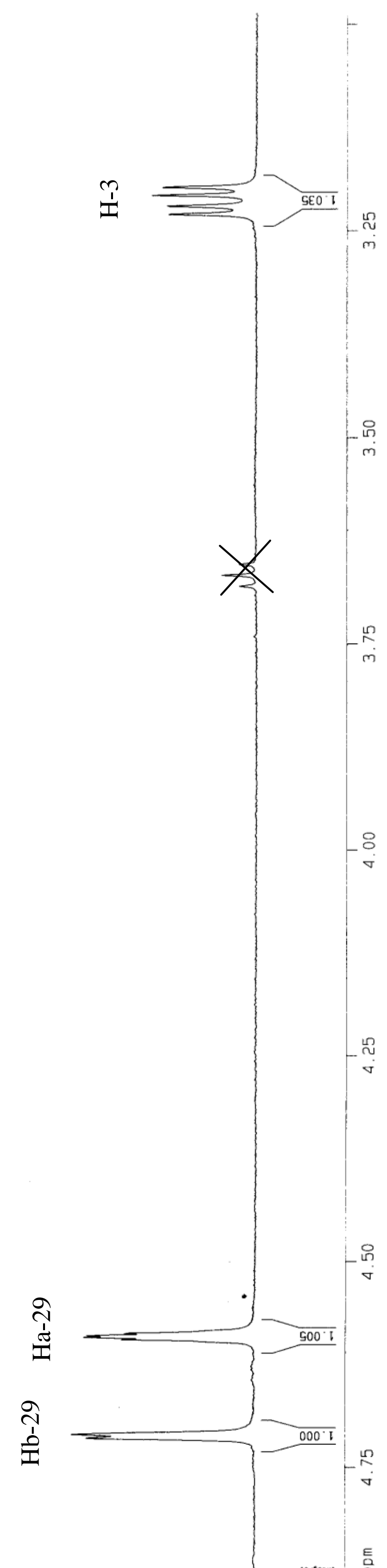
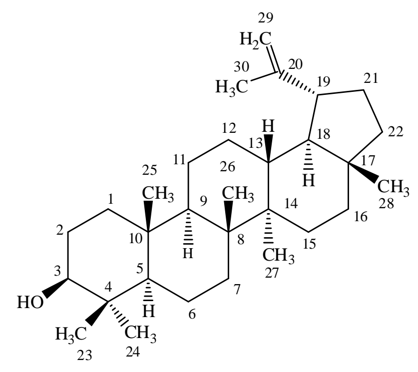


Fig. 5.30 500 MHz ^{1}H-NMR expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta = 3.00 - 4.80$ ppm)



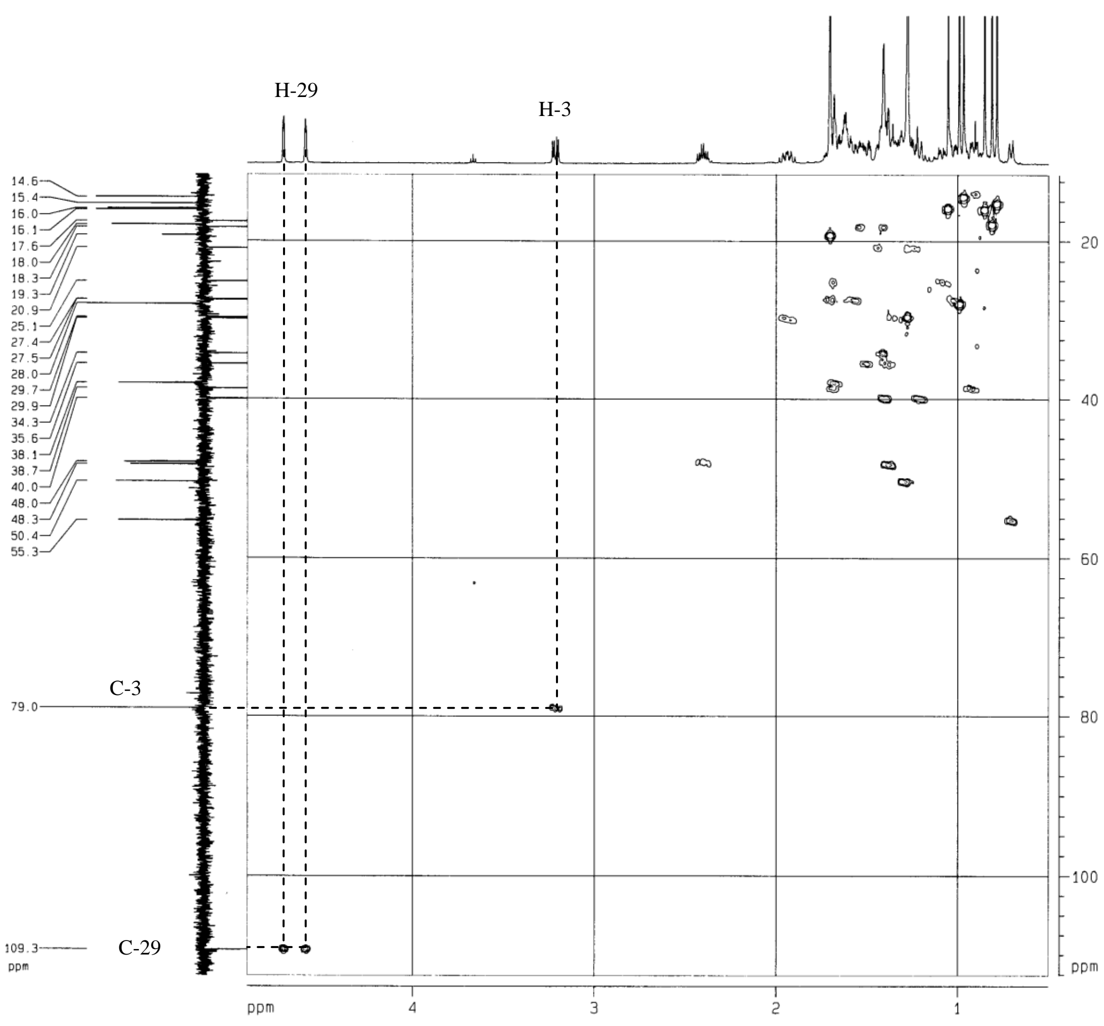


Fig. 5.31 HMQC spectrum of compound 3 in $CDCl_3$

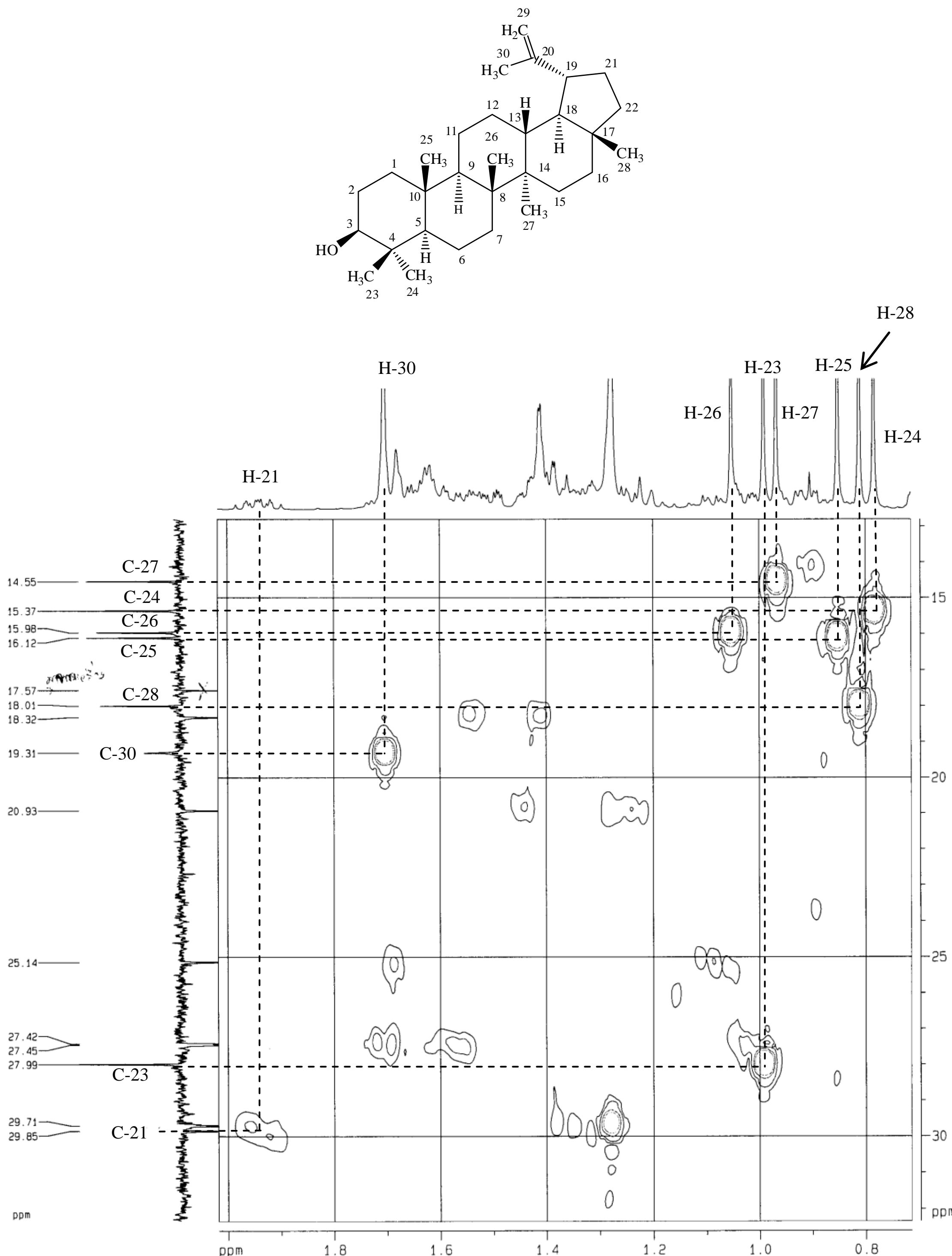
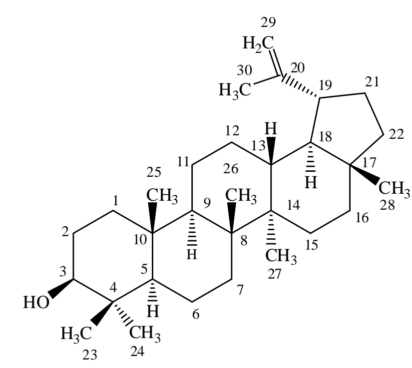


Fig. 5.32 HMQC expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta = 0.7 - 2.0$ ppm)



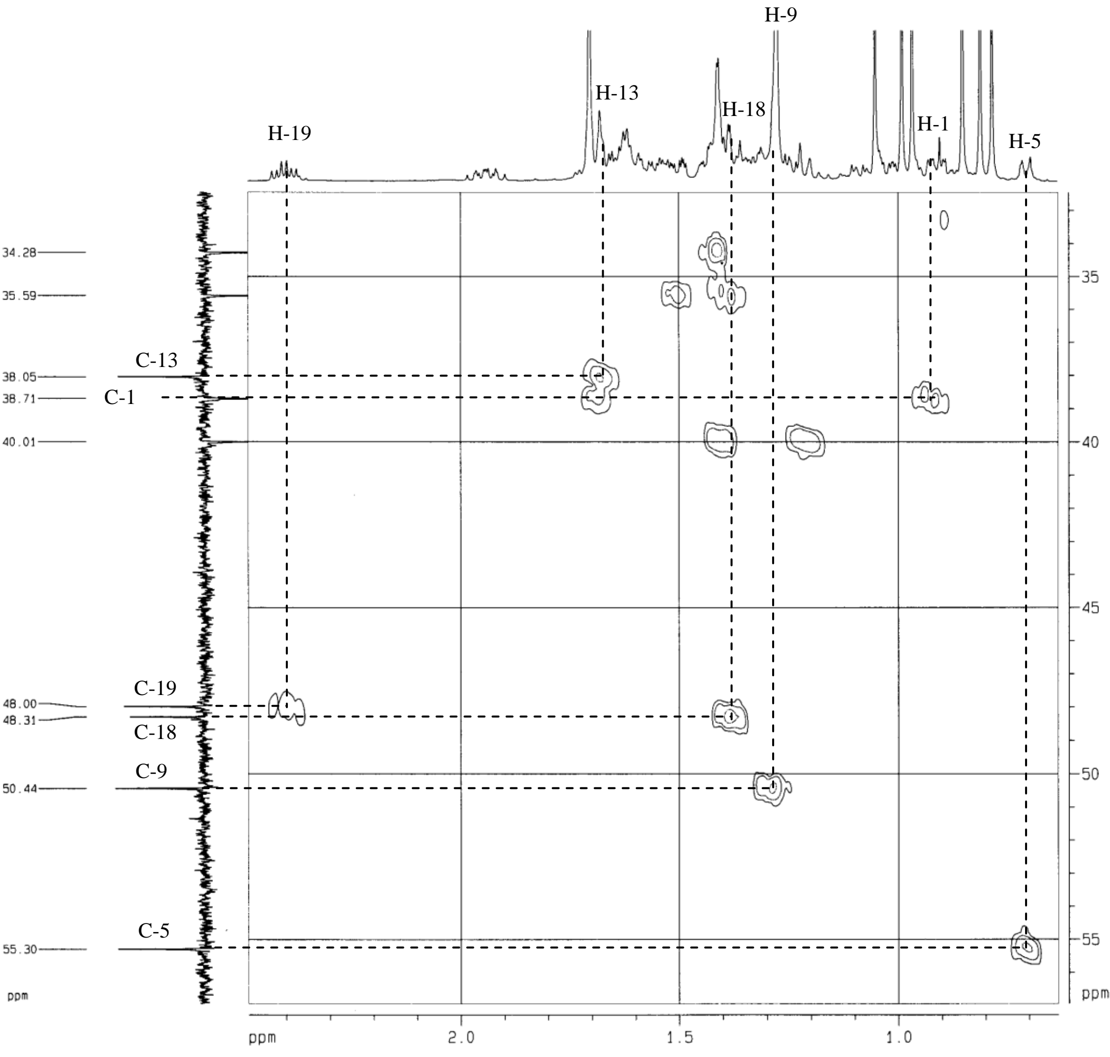


Fig. 5.33 HMQC expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta = 0.7 - 2.5$ ppm)

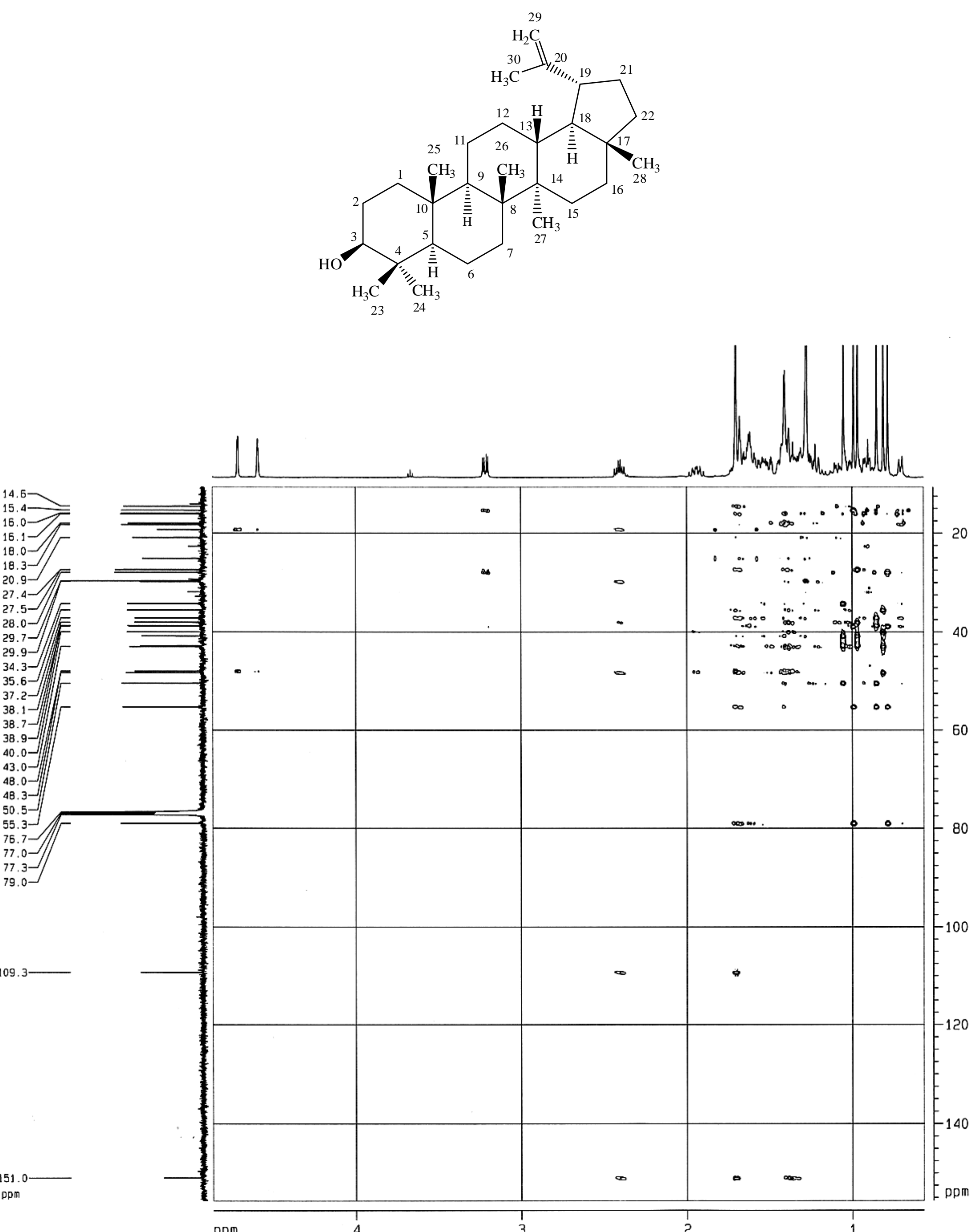
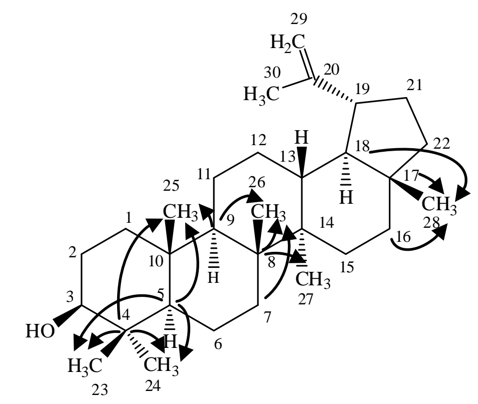


Fig. 5.34 HMBC spectrum of compound 3 in $CDCl_3$



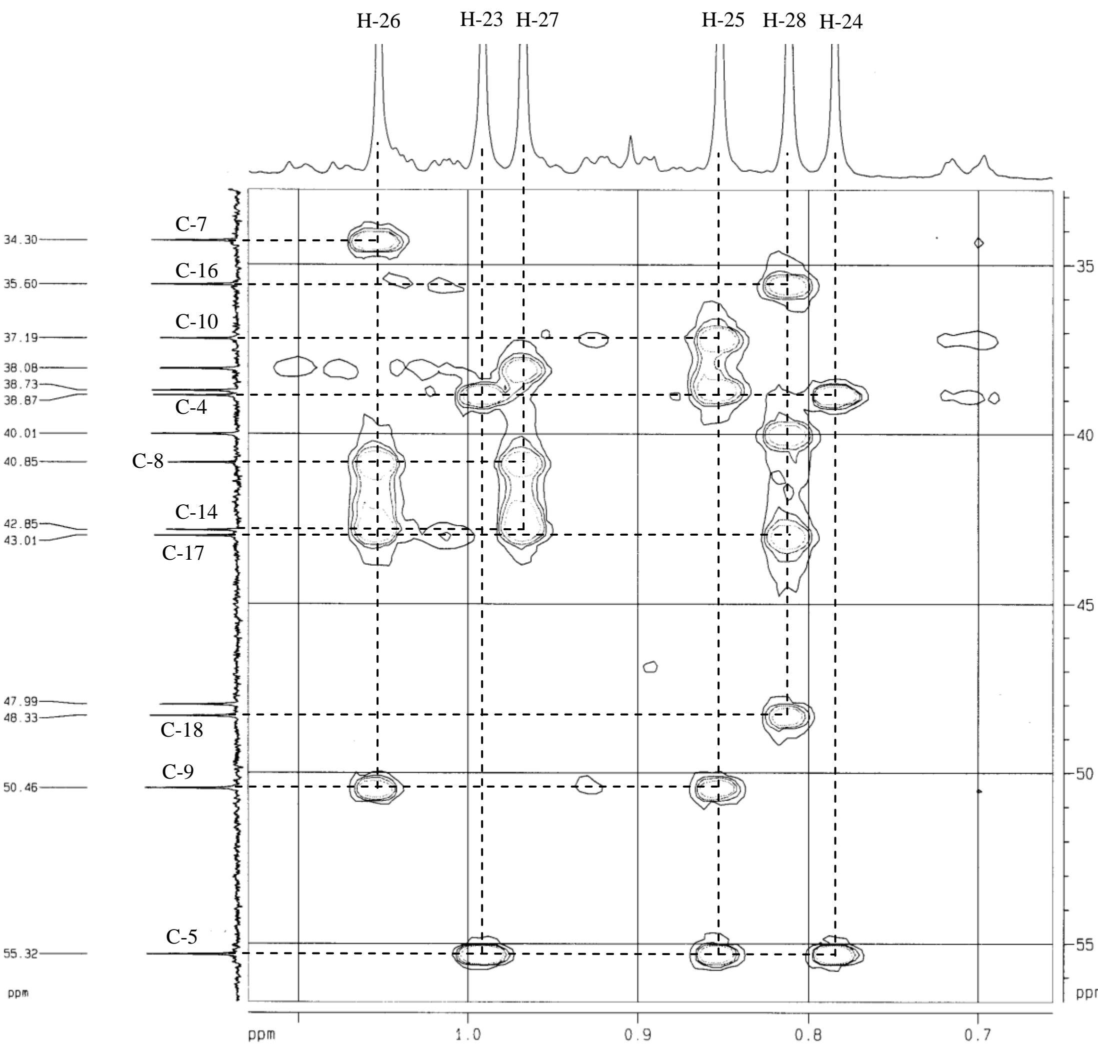
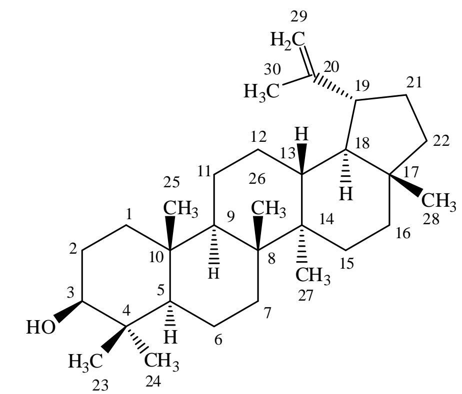


Fig. 5.35 HMBC expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta = 0.65 - 2.5$ ppm)



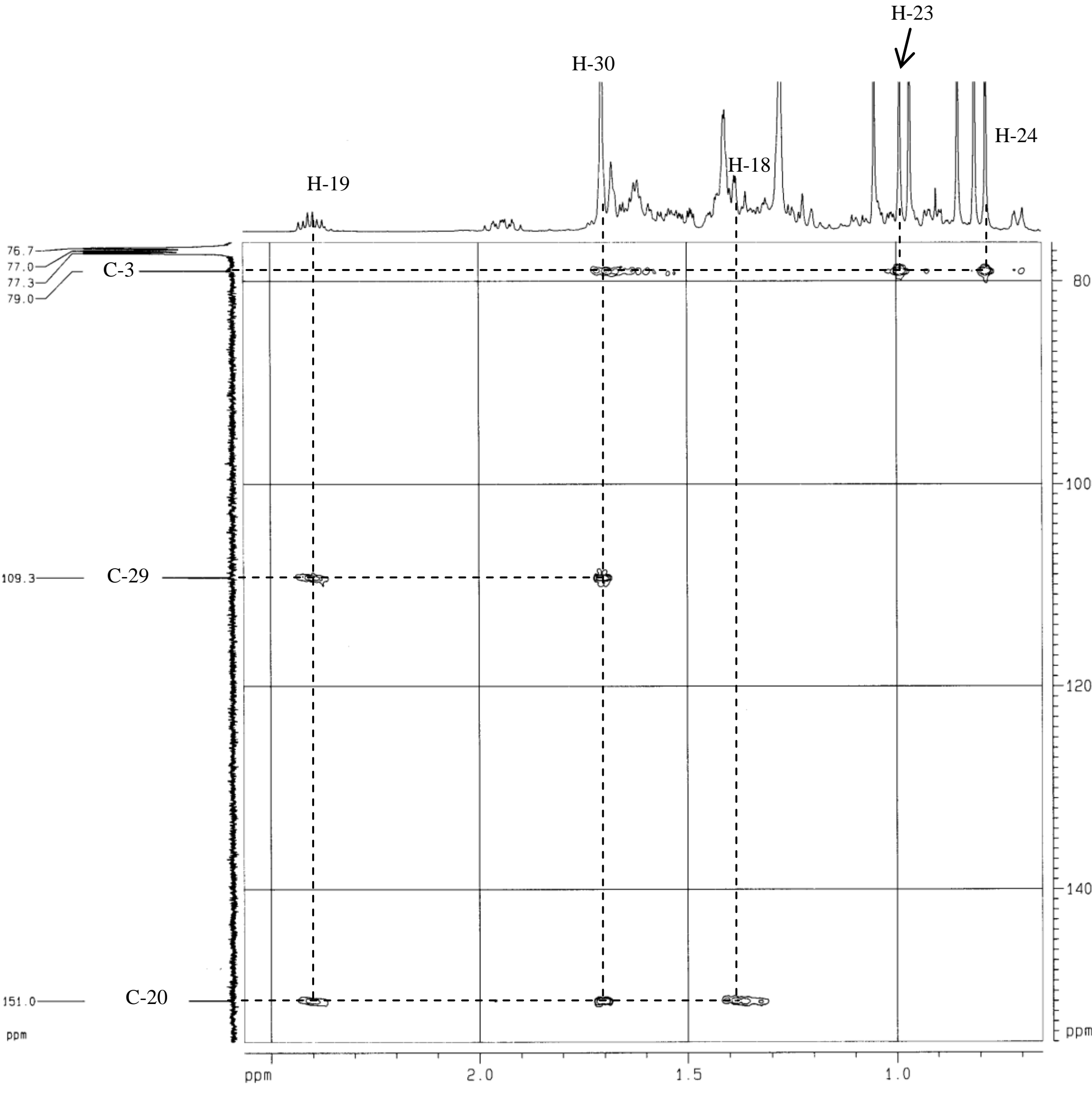
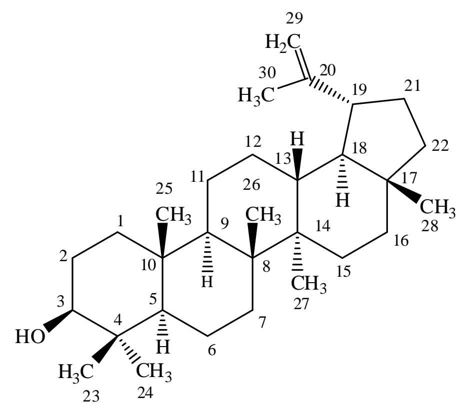


Fig. 5.36 HMBC expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta = 0.7 – 2.5$ ppm)



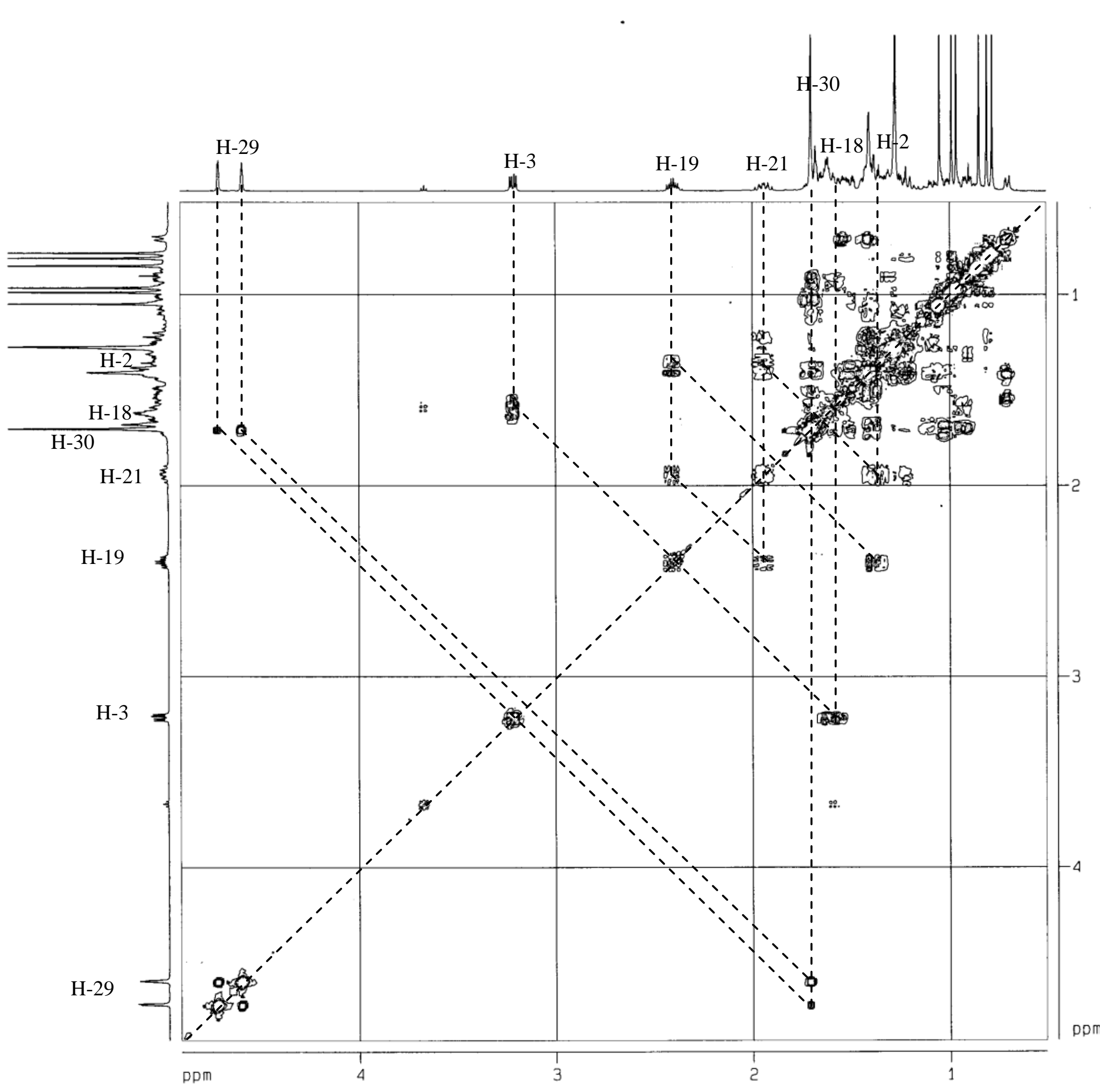


Fig. 5.37 COSY spectrum of compound 3 in $CDCl_3$

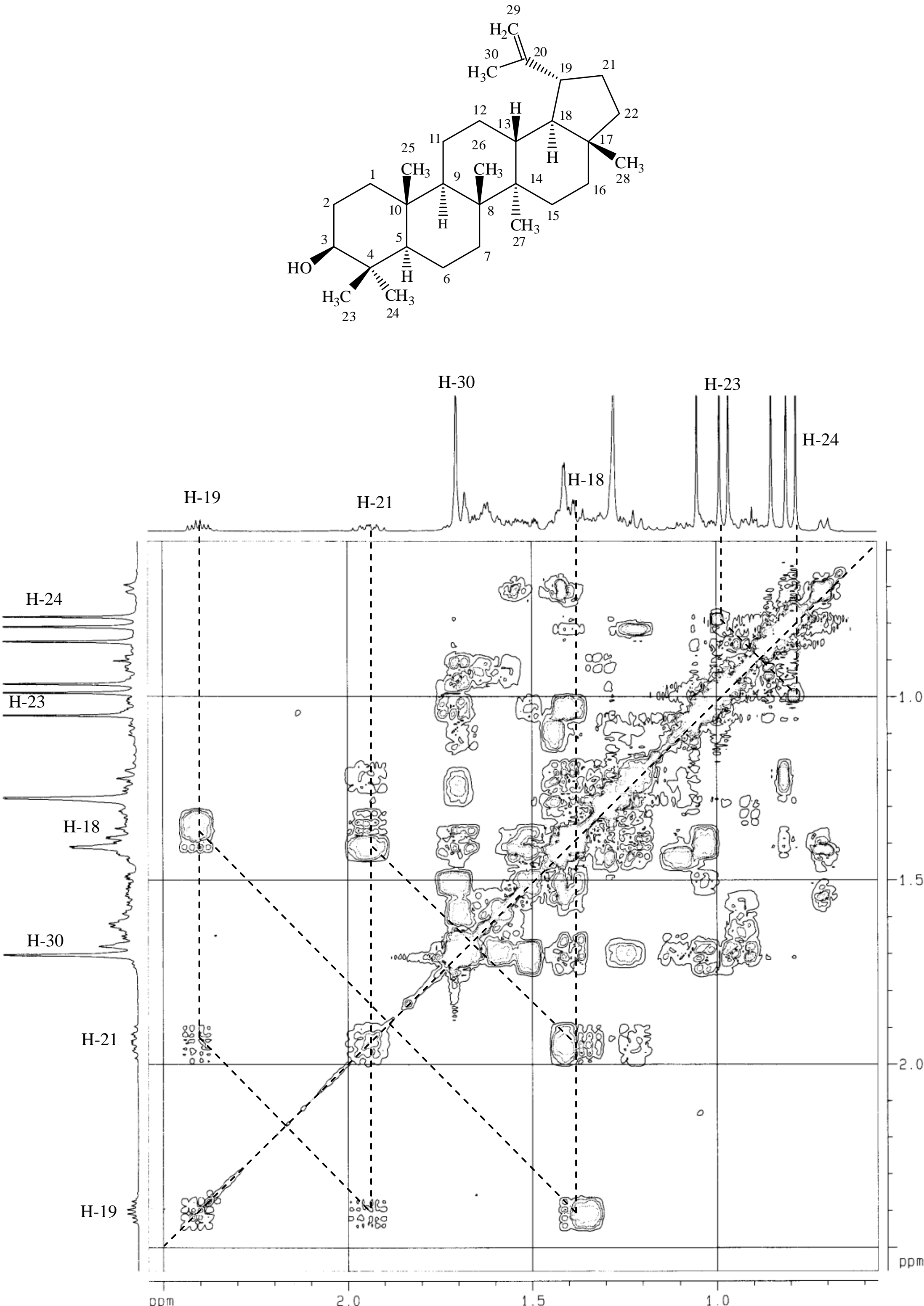


Fig. 5.38 COSY expanded spectrum of compound 3 in $CDCl_3$ ($\Delta\delta = 0.6 - 2.5$ ppm)

Table 5.8 Data of compounds isolated from *Z. limonella* pericarp ethanol extract

Compounds	**Weight (mg)**	**% Yield (w/w)**	**TLC (solvent system)**	**hRf**	**Color after spray with 10% sulfuric acid in ethanol**	**m.p.**	**Structure**
1 (β-sitosterol)	11.7	0.058	hexane: ethyl acetate (85:15)	22	Gray	132°C	
2 (The mixture of β-sitosteryl glucoside and Stigma steryl glucoside)	26.3	0.13	dichloro methane: methanol (90:10)	13	Violet	267°C	ZL 2.1 ZL 2.2
3 (lupeol)	10.4	0.052	hexane: ethyl acetate (85:15)	37	Orange	209°C	

5.4 Antimicrobial Tests (Tested by Faculty of Dentistry, Mahidol University)

Samples	Toxicity tests	Antimicrobial tests
Hexane extract	0.1%, 0.2% and 0.5% (< 50% cell viability)	Inhibit the growth of *Candida albicans* ATCC 13802 (1 mg, 0.5 mg and 0.25 mg)
Ethanol extract	No toxicity 0.1% (96.46% cell viability)	Inhibit the growth of *C. albicans* ATCC 13802 (1 mg)
Compound 2 and 3	-	Not inhibit the growth of *C. albicans* ATCC 13802

Part II High-Performance Liquid Chromatography (HPLC) Quantitative Analysis

5.1 System suitability test

In this study, the selective marker compound was compound 3 (lupeol) because it could be isolated sufficiently in pure form and was reported in the literature as an antiinflammatory agent (50).

The appropriate method of the separation of lupeol was reversed-phase chromatography (C_{18}). Various mobile phase compositions were tested on TLC in order to improve the resolution of the extract components. It started with 100% methanol and carefully increased amount of water until the separation was obtained. The solvent system of methanol : water 95 : 5 (water with 1% acetic acid) produced the hRf value of compound , which was a selected a marker compound at 30. The solvent was transferred to column chromatography. From the following parameters: precision, number of theoretical plates, tailing factor and resolution factor.

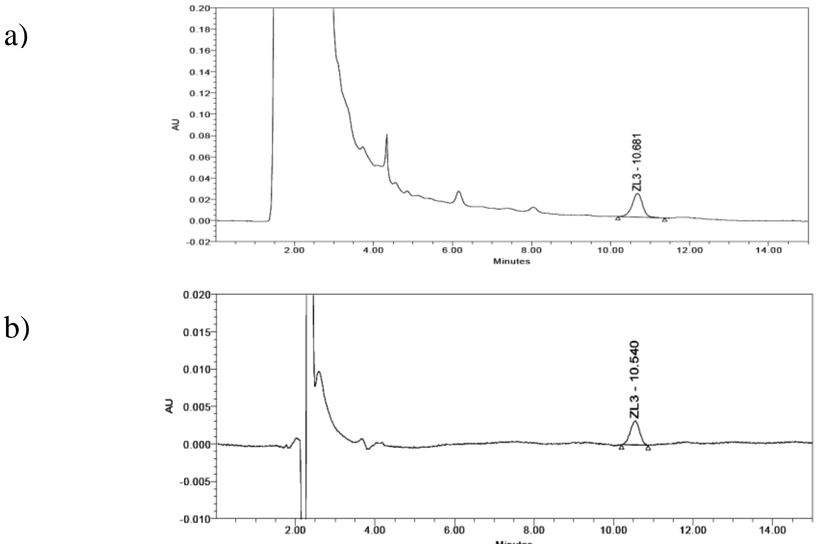


Fig. 5.39 HPLC chromatogram of the ethanol extract (5µm Hypersil® Gold C18 column, 150×4.6 mm i.d.)

a) *Z. limonella* pericarp ethanol extract b) lupeol (compound 3)

The system suitability of the chosen isocratic system was evaluated.

5.1.1 Precision

The replicated injections were performed using isocratic system. The precision was determined in term of percentage of coefficient variation (%CV) or relative standard deviation (RSD) which was calculated by using the following equation:

$$\%CV = [SD/\overline{X}] \times 100$$

where

$\overline{X}$ = mean value

SD = standard deviation

The data for precision was shown in Table 5.9.

Table 5.9 Retention time data of compound 3

Inject No.		Day I	Day II	Day III
1		10.329	10.314	10.743
2		10.259	10.545	10.715
3		10.453	10.452	10.769
4		10.440	10.389	10.763
5		10.297	10.540	10.748
6		10.461	10.476	10.701
7		10.437	10.429	10.673
8		10.334	10.536	10.621
9		10.462	10.488	10.498
10		10.386	10.424	10.532
11		10.329	10.583	10.516
12		10.446	10.506	10.534
13		10.326	10.469	10.564
14		10.359	10.551	10.621
15		10.508	10.432	10.624
Intraday	Mean	10.388	10.476	10.641
	SD	0.074	0.071	0.096
	%RSD	0.715	0.684	0.904
Interday	Mean	10.501		
	SD	0.133		
	%RSD	1.263		

5.1.2 The column efficiency (number of theoretical plates, N)

The number of theoretical plates (N) was calculated from the following equation:

$$N = 5.54 \times [t_R / W_{1/2h}]^2$$

where t_R = the retention time of peak

$W_{1/2h}$ = the peak width at half height

The number of theoretical plates for this study was 7,584.

5.1.3 Tailing factor (TF)

Tailing factor (TF) was calculated by using the following equation

$$TF = W_{0.05} / [2 \times A_{0.05}]$$

Where $W_{0.05}$ = the width of peak at 5% of the peak height

$A_{0.05}$ = the distance from the peak maximum to leading edge of the peak, the distance being measured at a point 5% of the peak height from base line.

The tailing factor value of this study was 0.8.

5.1.4 Resolution factor (R_s)

The resolution factor (R_s) was calculated by using the following equation

$$R_s = 2 \times (t_{RB} - t_{RA}) / (W_B + W_A)$$

where t_{R1} = the retention time of the peak A

t_{R2} = the retention time of the peak B

W_1 = the peak width of the peak A

W_2 = the peak width of the peak B

The resolution factor for this study was 2.46

The results of system suitability test were summarized in Table 5.10.

Table 5.10 The results of system suitability test

Parameter	compound 3	Requirement (USP 26)
Precision (%RSD)		≤ 2
- Interday	1.26	
- Intraday	0.68-0.90	
Theoretical plate (N)	7,584	> 2,000
Tailing factor (TF)	0.8	≤ 2
Resolution factor (R_s)	2.46	> 2
Retention time (min)	10	-

5.2 Method validation

Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application. Validation requirement are accuracy, precision, detection limit, quantitation and linearity.

5.2.1 Linearity

The concentration of standard solution for linearity was prepared in the range 10-100 μg/ml (Table 5.11). The result of the regression coefficient (r^2) was 0.9994. The linear regression equation was $y = 3.26\times10^3x - 3.71\times10^3$. The calibration curve of compound 3 was shown in Fig 5.40.

Table 5.11 Linearity of analysis of compound 3

Concentration (μg/ml)	Peak area			Average	SD	%RSD
10 ppm	29438	28750	28771	28986	391.29	1.35
20 ppm	59474	61869	60503	60615	1201.44	1.98
50 ppm	161517	161439	161961	161639	281.57	0.17
80 ppm	251285	252512	255573	253123	2208.4	0.87
100 ppm	325389	320964	324246	323533	2297.04	0.71

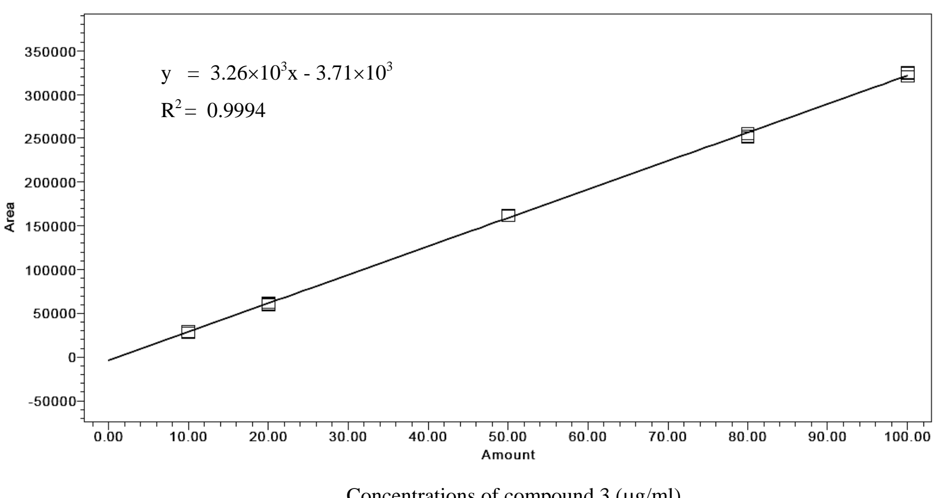


Fig 5.40 The calibration curve of compound 3

5.2.2 Accuracy

The accuracy was determined using spiking method. Three different concentrations were 10, 15 and 20 ppm of the compound 3 were added into the ethanolic extract. Each concentration was injected three times under the same conditions to evaluate the percentage of recoveries. The results were shown in Table 5.12.

Table 5.12 Percentage of recoveries obtained from spiking method of compound (n=3)

sample	HPLC measurement (ppm) / recovery (Percent)				% recovery
	No compound 3 added (0 ppm)	compound 3 10 ppm added	compound 3 15 ppm added	compound 3 20 ppm added	
Day 1	18.31	28.49 / (101.80%)	32.75 / (96.26%)	38.25 / (99.70%)	99.25
Day 2	18.47	28.19 / (97.20%)	33.27 / (98.66%)	38.49 / (100.10%)	98.65
Day 3	18.12	28.30 / (101.80%)	31.30 / (87.86%)	38.28 / (100.80%)	96.82
				Average	**98.24**
				SD	**1.26**
				%RSD	**1.29**

5.2.3 Precision

The precision of this method was expressed as the percentage of relative standard deviation (%RSD), on the basis of the peak area ratio for three replicate injections of the compound 3 ranging from 10-100 µg/ml. The %RSD was shown in Table 5.13 and Table 5.14.

Table 5.13 Intra-day precision of analysis of compound 3

Concentration (µg/ml)	Peak area			Average	SD	%RSD
10	28773	28559	27821	28384.33	499.46	**1.76**
20	57995	58776	59573	58781.33	789.01	**1.34**
50	158424	155071	156697	156730.70	1676.75	**1.07**
80	257383	257742	249686	254937.00	4551.04	**1.78**
100	324023	317964	323306	321764.30	3310.65	**1.03**

Table 5.14 Inter-day precision of analysis of compound 3

Day I	**Concentration (µg/ml)**	**Peak area**			**Average**	**SD**	**%RSD**
	10	28773	28559	27821	28384.33	499.45	**1.76**
	20	57995	58776	59573	58781.33	789.01	**1.34**
	50	158424	155071	156697	156730.70	1676.75	**1.07**
	80	257383	257742	249686	254937.00	4551.04	**1.78**
	100	324023	317964	323306	321764.30	3310.65	**1.03**

Day II	**Concentration (µg/ml)**	**Peak area**			**Average**	**SD**	**%RSD**
	10	28189	28952	28146	28429	453.44	**1.59**
	20	58995	57726	58473	58398	637.81	**1.09**
	50	158390	161030	157194	158871.3	1962.77	**1.23**
	80	261457	255347	255344	257382.7	3528.47	**1.37**
	100	322173	316334	321757	320088	3257.70	**1.01**

Day III	**Concentration (µg/ml)**	**Peak area**			**Average**	**SD**	**%RSD**
	10	29438	28750	28771	28986.33	391.29	**1.35**
	20	59474	61869	60503	60615.33	1201.44	**1.98**
	50	161517	161439	161961	161639	281.57	**0.17**
	80	251285	252512	255573	253123.3	2208.40	**0.87**
	100	325389	320964	324246	323533	2297.04	**0.71**

Day I-III	**Concentration (µg/ml)**	**Average peak area**			**Average**	**SD**	**%RSD**
		Day I	**Day II**	**Day III**			
	10	28384	28429	28986	28599	335.33	**1.17**
	20	58781	58398	60615	59264	1184.99	**2.00**
	50	156730	158871	161639	159080	2461.16	**1.55**
	80	254937	257382	253123	255147	2137.27	**0.84**
	100	321764	320088	323533	321795	1722.70	**0.53**

5.2.4 Limit of Measurement

Detection limit (LOD) was the lowest concentration of analyte in a sample that could be detected, but not necessarily quantitated, under the stated experimental conditions. Quantitation limit (LOQ) was the lowest concentration of analyte in a sample that could be determined with acceptable precision and accuracy under the stated experimental conditions.

The calculation was based on the standard deviation of y-intercepts of regression line (σ) and the slope of the calibration curve (S):

$$LOD = 3.3 (\sigma/S)$$

$$LOQ = 10 (\sigma/S)$$

which σ = 1465.65 and S = 3292.5

LOD and LOQ for this method were 1.47 μg/ml and 4.45 μg/ml, respectively.

Table 5.15 The results of method validation test

Parameter	**ZL3**	**Requirement (USP 26)**
Linearity regression coefficient (r^2)	0.9994	≥ 0.999
Accuracy (% recovery)	98.24%	80-120%
Precision (%RSD) - Interday - Intraday	 0.53-2.00 1.03-1.78	≤ 2
LOD (ppm)	1.47	-
LOQ (ppm)	4.45	-

5.3 Determination of the marker compound in *Z.limonella* pericarp extract

The determination of the marker compound in the pericarp extract was conducted using the established and validated method. Compound 3, which was identified as lupeol, was used as a marker compound. The HPLC condition comprised methanol : water (95:5) (water with 1% acetic acid) as a mobile phase, and a C18 column as a stationary phase. The chromatogram (Fig. 5.40) showed lupeol with the

retention time of 10 min. The content of lupeol in the ethanol extract was 0.195 % w/w (based on the pericarp weight).

Table 5.16 The linearity data of lupeol

Concentration (µg/ml)	Peak area			Average	SD	%RSD
10	28189	28952	28146	28429	453.44	1.59
20	58995	57726	58473	58398	637.81	1.09
50	158390	161030	157194	158871	1962.77	1.23
80	261457	255347	255344	257382	3528.47	1.37
100	322173	316334	321757	320088	3257.70	1.01

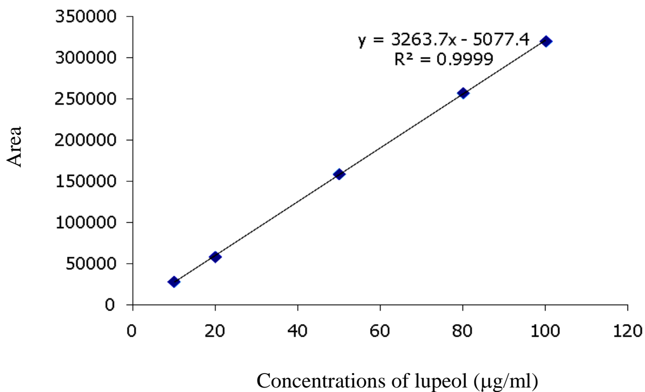


Fig. 5.41 The calibration curve of lupeol

Table 5.17 Peak area of *Z. limonella* pericarp extract

Concentration (mg/ml)	Inject No.	Peak area
25.59	1	155194
	2	155935
	3	160801
	4	161120
	Average	**158262.50**
	SD	**3132.74**
	%RSD	**1.97**

Calculation;

$$y = 3263.7x - 5077.4$$

$$y = 158262.5 \text{ (average peak area of lupeol)}$$

$$\therefore x = 50 \text{ ppm } (\mu g/ml)$$

Concentration of lupeol was 50 ppm in the pericarp extract 0.6398 g

$$\frac{50 \times 25 \text{ ml}}{1,000,000} = 0.00125 \text{ g}$$

The pericarp extract 0.6398 g contained lupeol $= 0.00125$ g

The pericarp extract 100 g contained lupeol $= \frac{0.00125 \times 100}{0.6398}$

$= 0.195\%$ (w/w)

The quantitative determination of lupeol using HPLC method mostly used acetonitrile/water as a mobile phase. But in this study we selected methanol instead of acetonitrile because it was cheaper, not harmful and produced the reasonable retention time. The content of lupeol deduced from the gravimetric method (extraction and isolation) was 0.052% w/w (based on the pericarp weight) due to the loss of lupeol during the chromatographic separation. Hence the HPLC quantitative method is suitable for the determination of lupeol in pericarp extract.

CHAPTER VI
CONCLUSION

Zanthoxylum limonella belongs to the family Rutaceae, which is the family of the citrus fruits. The roots and fruits of *Z. limonella* have weak antifungal and antiinflammatory activities, respectively (9). The bark is used to treat toothache (8). The previous chemical study of *Z. limonella* plant indicated several types of compounds, including alkaloids, triterpenoids and coumarins (6), furoquinolone and indolequinazoline alkaloids, terpenoids (7), fatty acids, carbohydrates, proteins (9), quinoline and indopyridoquinazoline alkaloids, phytosterol, flavanones, volatile oils (10, 11, 18,19) and lignans (10). This study aimed at the phytochemical investigation of *Z. limonella* indigenous to Thailand and the HPLC quantitative analysis of the isolated compound. The result of the study has been applied to standardize *Z. limonella* pericarp extract, which is incorporated in oral products, which included dentifrice, mouth wash, mouth spray and local anesthetic gel. The Committee of Higher Education, Ministry of Education has supported the joint project between the university and the private sector to develop the oral products from *Z. limonella*.

The yields of hexane and ethanol extracts were 4.18% and 13.55%, respectively. The ethanol extract, which inhibited *Candida albicans* ATCC 13802 was chromatographed on silica gel column (Fig. 4.2) and gradiently eluted with hexane-ethyl acetate, (0, 1, 5, 10% of ethyl acetate, 300, 200, 200, 3,500 ml, respectively) followed by dichloromethane (700 ml), 1, 3, 5 and 10% of methanol in dichloromethane (600, 800, 600, 700 ml, respectively) and washed with methanol.

The combined fraction of 3,450-4,100 ml was concentrated under reduced pressure and left for crystallization overnight. Compound 1 appeared as needles and was recrytallized in methanol, 11.7 mg of **1** was obtained.

The combined fraction of 7,750-7,900 ml was concentrated under reduced pressure and left for crystallization overnight. Compound 2 appeared as white powder and was recrytallized in methanol, 26.3 mg of **2** was obtained.

The combined fraction of 2,450-3,400 ml was concentrated and further chromatographed on silica gel column and gradiently eluted with hexane-ethyl acetate (0, 1, 3, 5% of ethyl acetate, 50, 100, 200, and 300 ml, respectively). The combined fraction of 255-450 ml was concentrated under reduced pressure and left for crystallization overnight. Compound 3 appeared as white powder and was recrytallized in methanol, 10.4 mg of **3** was obtained.

From physical, chemical and spectral evidences 1, 2 and 3 were identified as β-sitosterol (**1**), a mixture of sitosteryl (**2.1**) and stigmasteryl glucosides (**2.2**) and lupeol (**3**), respectively.

The quality assessment (standardization) of *Z. limonella* pericarp extract requires a marker compound, which is the chemical constituent in the pericarp. Our study could isolated **3** in a sufficient amount and without difficulty. **3** possessed antiinflammatory property (50). **3** was thus selected as a marker compound in the HPLC quantitative analysis of the pericarp extract.

The quantitative determination of lupeol using HPLC method mostly used acetonitrile/water as a mobile phase. But in this study we selected methanol instead of acetonitrile because it was cheaper, not harmful and produced the reasonable retention time.

The HPLC method using 5µm Hypersil® Gold C18 column, 150×4.6 mm as stationary phase and the methanol : water 95 : 5 (water with 1% acetic acid) as mobile phase with flow rate of 1 ml/min and detection at 214 nm was developed and validated for the quantitative determination of **3**. The peak of **3** appeared at the retention time of 10 min. The test of system suitability resulted the precision (%RSD) of 0.68-1.26, number of theoretical plates (N) of 7,584, a tailing factor (TF) of 0.8 and a resolution factor (R_s) of 2.46. A test of method validation resulted the linearity of **3** in the range of 10-100 µg/ml with the regression coefficient (r^2) 0.9994 (n = 15). The recovery of **3** in the pericarp extract was 98.24%, the limit of detection 1.47 and limit of quantitation 4.45 µg/ml. The results of the tests were conformed to the USP 26 requirement (Table 5.10 and 5.15). These results indicated that the established HPLC method was suitable for the determination of **3** in the pericarp extract. The calculated content of **3** in the pericarp extract was 0.195% w/w (based on the pericarp weight).

The content of **3** deduced from the gravimetric method (extraction and isolation) was 0.052% w/w (based on the pericarp weight) due to the loss of **3** during the chromatographic separation. Hence the HPLC quantitative method is suitable for the determination of **3** as a marker compound in the pericarp extract.

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