ISOLATION AND STRUCTURE ELUCIDATION OF SOME COMPOUNDS FROM CRUDE ETHYL ACETATE AND CRUDE METHANOL EXTRACTS OF *SPILANTHES ACMELLA* MURR.

SAOWAPA SUPHAPONG

Presented in partial fulfillment of the requirements
for the Master of Science Degree in Biological Chemistry
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การแยกและการวิเคราะห์โครงสร้างสารประกอบบางชนิดจากสารสกัดเอทิลแอซิเตดและเมทานอลของผักคราดหัวแหวน (*Spilanthes acmella* Murr.)

บทคัดย่อ
ของ
เสาวภา ศุภพงษ์

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
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ผักคราดหัวแหวนเป็นพืชสมุนไพรไทย ใช้แก้ปวดฟัน มีฤทธิ์เป็นยาชาเฉพาะที่ และเป็นยาระงับปวด จากการนำสารสกัดหยาบเอทิลแอซิเตตและเมทานอลของผักคราดหัวแหวนมาแยกและวิเคราะห์พบสารประกอบ 7 ชนิด สารประกอบที่แยกได้จากสารสกัดหยาบเอทิลแอซิเตต มี 3 ชนิด ได้แก่ 3-acetylaleuritolic acid (3-*β*-*O*-acetyltaraxer-14-en-28-oic), vanillic acid (4-hydroxy-3-methoxybenzoic acid), *β*-sitostenone (stigmast-4-en-3-one, 24*α*-ethylcholest-4-en-3-one) และสารประกอบที่แยกได้จากสารสกัดหยาบเมทานอลมี 4 ชนิด ได้แก่ scopoletin (7-hydroxy-6-methoxycoumarin), *trans*-ferulic acid (*trans*-4-hydroxy-3-methoxy cinnamic acid), *trans*-isoferulic acid (*trans*-3-hydroxy-4-methoxycinnamic acid) และสารผสม stigmasteryl-3-O-*β*-D-glucopyranoside และ *β*-sitosteryl-3-*O*-*β*-D-glucopyranoside การศึกษาโครงสร้างของสารเหล่านี้ใช้วิธีทางสเปกโตรสโกปี (COSY, HMQC และ HMBC)

ISOLATION AND STRUCTURE ELUCIDATION OF SOME COMPOUNDS FROM CRUDE ETHYL ACETATE AND CRUDE METHANOL EXTRACTS OF *SPILANTHES ACMELLA* MURR.

AN ABSTRACT
BY
SAOWAPA SUPHAPONG

Presented in partial fulfillment of the requirements
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Saowapa Suphapong. (2002). *Isolation and Structure Elucidation of Some Compounds from Crude Ethyl Acetate and Crude Methanol Extracts of Spilanthes acmella* Murr.. Master Thesis, M.Sc. (Biological Chemistry). Bangkok: Graduate school; Srinakharinwirot University. Advisor Committee: Assoc. Prof. Dr. Supaluk Prachayasittikul, Assoc. Prof. Dr. Somsak Ruchirawat

Spilanthes acmella Murr., a Thai medicinal plant has long been used as traditional medicine for toothache treatment, local anesthetic and analgesic actions. Our investigation on its crude ethyl acetate and crude methanol extracts led to the isolation of seven compounds. Three isolates from crude ethyl acetate extract are 3-acetylaleuritolic acid (3-β-*O*-acetyltaraxer-14-en-28-oic), vanillic acid (4-hydroxy-3-methoxy benzoic acid) and β-sitostenone (stigmast-4-en-3-one, 24α-ethyl-cholest-4-en-3-one). In addition, four isolated compounds from crude methanol extract are scopoletin (7-hydroxy-6-methoxycoumarin), *trans*-ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid), *trans*-isoferulic acid (*trans*-3-hydroxy-4-methoxycinnamic acid) and mixture of stigmasteryl-3-*O*-β-D-glucopyranoside and β-sitosteryl-3-*O*-β-D-glucopyranoside. Their structures were elucidated on the basis of spectral analysis, including homonuclear and heteronuclear correlation NMR experiments (COSY, HMQC and HMBC).

Thesis

entitled

ISOLATION AND STRUCTURE ELUCIDATION OF SOME COMPOUNDS FROM CRUDE ETHYL ACETATE AND CRUDE METHANOL EXTRACTS OF *SPILANTHES ACMELLA* MURR.

by

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Chapter I

Introduction

Background

For a long history, human have used natural substances, especially plants, to relieve pain, heal wound and maintain health. Thailand is a tropical country enriched with abundance of diverse medicinal plant resources[1].

In Thailand, medicinal plants are still being used widely and legally in traditional medicine due to there were believed to possess less harmful side effect than synthetic drugs. Now, medicinal plant research is being on development. This results in an increase public awareness of medicinal plants to valuable heritage in Thai traditional medicine. There has also been a concerted effort by both government and private sectors to develop safe and effective drugs from Thai medicinal plants [2]. For examples, the development of antiasthmatic drug from plai (*Zingiber cassummunar* Roxb.) and antipeptic ulcer drug from plaunoi (*Croton sublyratus Kurz.*)[3].

However, many Thai medicinal plants have been used for toothache treatment. For examples, *Syzygium aromaticum* linn. Meer & Peery (Kaan Phluu), *Stemona tuberosa* Lour. (Non Taai Yaak), *Ficus religiosa* linn. (pole) and *Spilanthes acmella* Murr. (Phak khraat Huawaen) [4].

Spilanthes acmella Murr. is a toothache plant [5]. In some countries in Asia, it has been used as fresh vegetable and cooking diet[6]. Its flowers and leaves have a pungent taste accompanied with tingling and numbness. It has been used as folk medicine for toothache and headache[7]. The root decoction of *Spilanthes acmella* Murr. has been used as a laxative and diuretic drug[8]. In addition, the whole plant has also been used for treatment of asthma, rheumatism, fever, sore throat and haemorrhoids[9].

Previously, the chemical constituents of *Spilanthes acmella* Murr. were investigated. Compounds found in the whole plant were α - amyrin, β - amyrin, α - amyrin ester, β - amyrin ester, α - amyrin acetate, β - amyrin acetate, daucosterol, lauric acid, linoleic acid, myricyl alcohol, oleic acid, myristic acid, palmetic acid, stigmasterol and spilanthol[9]. In addition, pharmacological activities of the isolates were reported. The main constituent of *Spilanthes acmella* Murr., spilanthol showed strong local anesthetic[5,8] and analgesic actions[10]. In Thailand , pharmacological activities of *Spilanthes acmella* Murr. were reported. It was found that ethanol extracts of *Spilanthes acmella* Murr. possessed local anesthetic activity in guinea pigs and frogs sciatic nerves[11] . In addition, the ethanol extracts of the flowers and stems of *Spilanthes acmella* Murr. inhibited the growth of *Steptococcus pyogenes* with minimum inhibitory concentation (MIC) values of 23 and 100 mg/mL, respectively.[12]

Furthermore, insecticidal, antiinflammatory and cytotoxic activities of *Spilanthes acmella* Murr. were reported. Insecticidal activity of *Spilanthes acmella* Murr. was studied. It was found that the dilution of diethyl ether extract with water (1:100,000) of flower heads of *Spilanthes acmella* Murr. was lethal to mosquito larvae.[13]

Antiinflammatory activity of ethanol extract *of Spilanthes acmella* Murr. on carageenin induced rat pedal edema was studied. The activity was observed at a dose of 2 g/ kg which was much lower than aspirin.[14] In addition , cytotoxic activity of ethanol – water (1:1) extract of *Spilanthes acmella* Murr. was reported as an ED_{50} value of 20 µg/mL against 9KB carcinoma cells.[15]

Although the isolation and pharmacological activities of some chemical constituents of *Spilanthes acmella* Murr. have been reported. However, investigation on the chemical constituents of *Spilanthes acmella* Murr. has not yet been reported in Thailand. Therefore, it is interesting to investigate in detail of *Spilanthes acmella* Murr. in order to isolate and elucidate structures of some pure compounds.

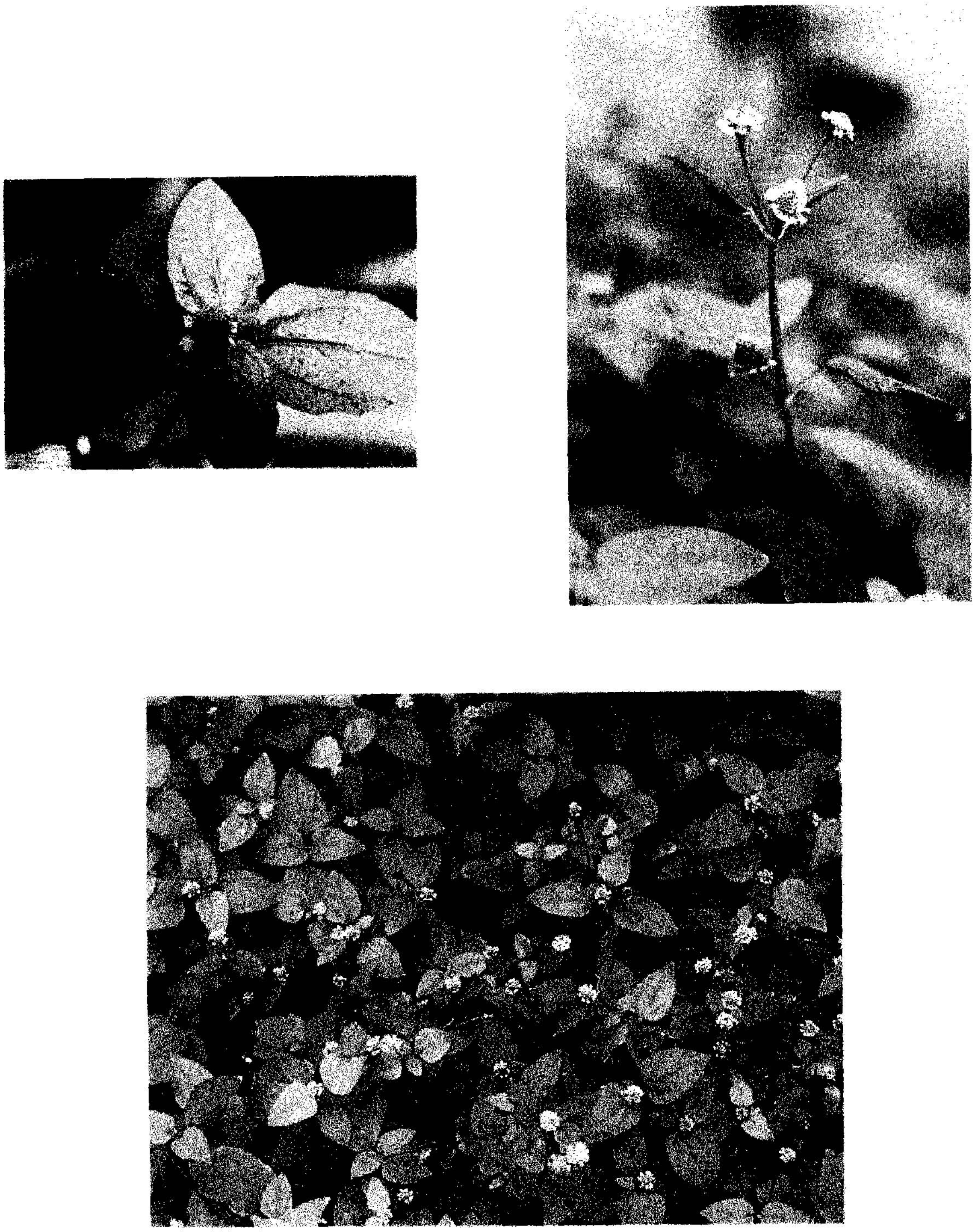


Figure 1 Picture of *Spilanthes acmella* Murr[7].

Objective

1. To isolate and purify some compounds from crude ethyl acetate and crude methanol extracts of *Spilanthes acmella* Murr.

2. To elucidate structures of pure compounds by using spectroscopic methods

Benefit

1. To provide more data of *Spilanthes acmella* Murr. regarding to natural products chemistry

2. To obtain pure compounds for further investigation on biological activity and toxicity

Scope

1. Plant materials, the whole plant of *Spilanthes acmella* Murr. were collected from Thasala District, Nakornsrithammarat Province in June,1999.

2. Sequential extraction of plant materials were carried out in hexane, chloroform, ethyl acetate and methanol respectively.

3. Isolation and purification of some compounds from crude ethyl acetate and crude methanol extracts were carried out by using chromatographic together with recrystallization techniques.

4. Structure elucidation of the obtained pure compounds were performed by using spectroscopic techniques e.g. Ultraviolet Spectroscopy, Infrared Spectroscopy, Nuclear Magnetic Resonance Spectroscopy and Mass Spectroscopy.

Chapter II

Literature Review

Spilanthes acmella Murr. is a plant of family compositae[5], the tribe Helianthease and the subtribe Ectipetinae widely distributed throughout the tropic and subtropic zones.[8] It is commonly found in Thailand, India, Laos, Philippine, Indonesia, Vietnam, Papua New Guinea and Peninsula[6].

Spilanthes acmella Murr. has various local names. In Thailand, it is called " Phak khraat " in central area, " Phak phet " in northern area and " Phak tumhuu " in southern area. In Malaysia, it is known as " getang ", in Philippine as " biri " and in Indonesia as " jotang ", in China as " ueng huai kia, hueng huai kia " [6].

Genus Spilanthes comprise about 75 species and found mainly in Central and South America. Approximately five species have been found in South - East Asia[6], there are *Spilanthes iabadirensis, Spilanthes paniculata, Spilanthes oleracea, Spilanthes toctoria* and *Spilanthes acmella* Murr.. *Spilanthes acmella* Murr. is erect annual and creeping herb, spreads in low open places and requires moist soil[16], having stem branched which reaches a height of 15 - 60 centimetres. Leaves are simple, opposite, ovate or ovate–lanceolate with pointed tip and wedge–shaped base and with toothed margin [17]. Flowers are in the head axillary or terminal, solitary or panicle that they are yellow and head ovate. Fruit is achene about 3 milimetre long, 3 ribbed that having apex slightly emarginate and bristle. This plant is propagated by seeds[9].

Up to date, chemical constituents from some Spilanthes species have been reported such as *Spilanthes oleracea* [18], *Spilanthes ocymifolia* [19], *Spilanthes alba* [20], and *Spilanthes acmella* Murr.[18]. Phytochemical investigation of the genus Spilanthes showed that spilanthol (2*E*,6*Z*,8*E*)-*N*-isobutyl–2,6,8-decatrienamide, (**I**) was firstly obtained in 1903 from *Spilanthes oleracea* Jacq.[18].

In 1984, Borgess-Del-Castillo et al. reported the isolation of *N*–2– phenylethyl-cinnamamide (**II**) from ethanol extract of leaves of *Spilanthes ocymifolia* [19].

Previously in 1945, Gokhale and Bhide reported the isolation of major compound, spilanthol (**I**) from ethanol extract of flower heads of *Spilanthes acmella* Murr.[18]. The spilanthol (**I**) of *Spilanthes acmella* Murr. showed interesting bioactivity such as strong local anesthetic, analgesic and insecticidal activities[9]. It was found that spilanthol (**I**) produced analgesic action at 20 mg/mL by using the tails of abino rats and thermal method[10]. In addition, insecticidal activity of spilanthol showed high acute toxicity against adults of *Periplaneta americana* at LD_{50} value of 2.46 μg/g insect. Moreover, it was more toxic than carbaryl, bioresmethrin and lindane , respectively[21].

In 1975, Krishnaswamy et al. reported that α- and β–amyrin esters (**III a,b**), stigmasterol (**IV**) and myricyl alcohol (**V**) were isolated from light petrol extract of *Spilanthes acmella* Murr.[22]. In addition, ethanol extract of such plant gave sitosterol–*O*-β-D-glucoside (**VI**)[22].

In 1987, Mukharya and Ansari reported the isolation of olean–12-en-3-*O*-β-D-galactopyranosyl (1→4)–*O*-α-L-rhamnopyranoside (**VII**) from ethanol extract of roots of *Spilanthes acmella* Murr.[23].

In 1992, Nakatani and Nagashima reported that spilanthol (**I**) and three alkamides, (2*E*)–*N*–(2–methylbutyl)–2–undecene–8,10–diynamide (**VIII**), (2*E*,7*Z*)–*N*–isobutyl–2,7–tridecadiene–10,12–diynamide (**IX**) and (7*Z*)–*N*–isobutyl–7–tridecene–10,12–diynamide (**X**) were isolated from hexane extract of the flower heads of *Spilanthes acmella* L.var. oleracea Clarke. Among the isolates, compound **IX** was found for the first time in Spilanthes species. However, compounds **VIII** and **X** were new[24].

Recently, in 1999. Ramsiwak et al. reported that hexane extract of dried flower buds of *Spilanthes acmella* L.var. oleracea Clarke. afforded three *N*-isobutylamides. There were spilanthol (**I**), undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide (**XI**) and undeca-2*E*-en-8,10-diynoic acid isobutylamide (**XII**). These compounds were firstly reported to exhibit biological activity against *Aedes aegyptii* larvae and *Heliverpa zea neonates* at the concentrations of 12.5 and 250 μg/mL, respectively[25].

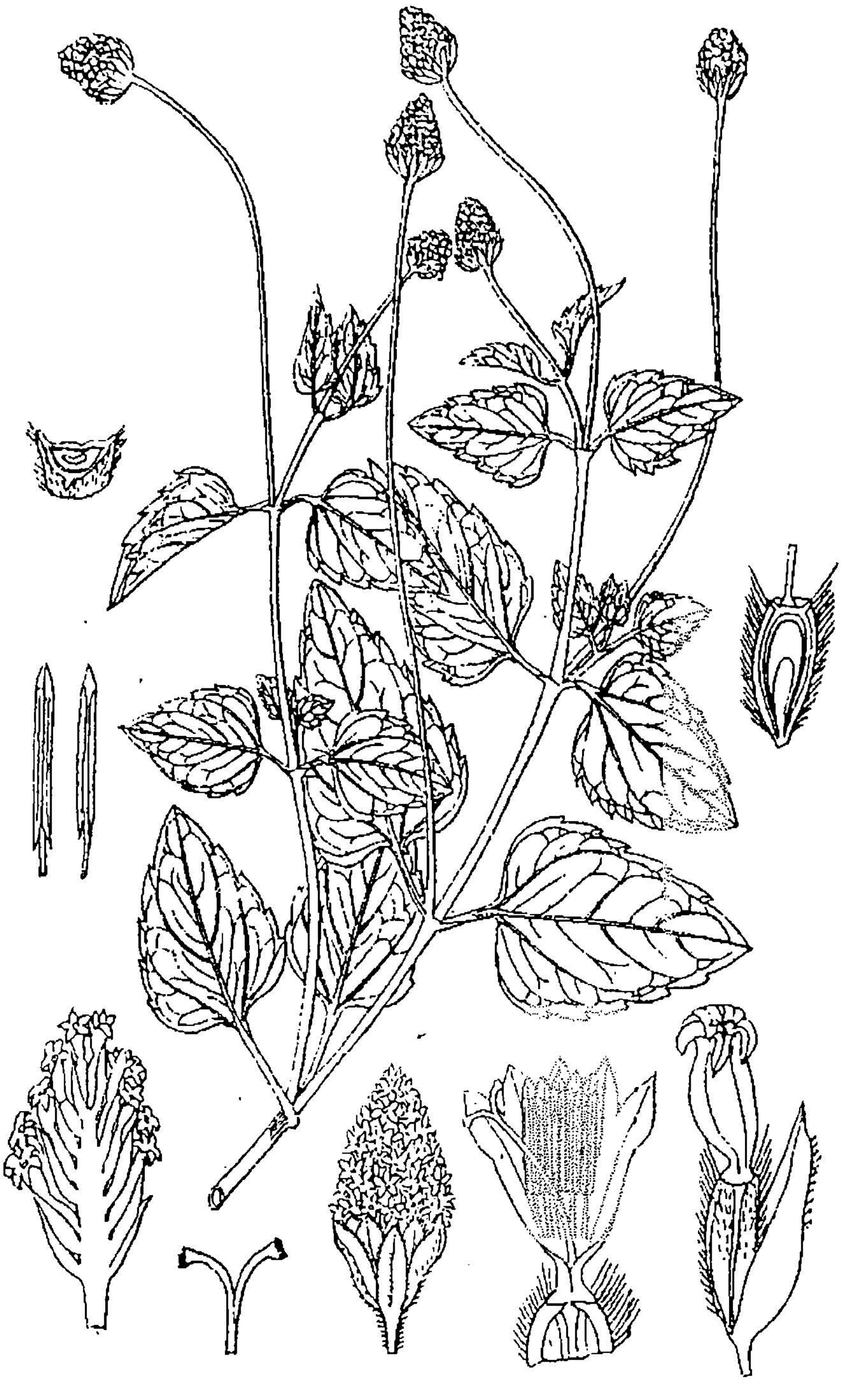
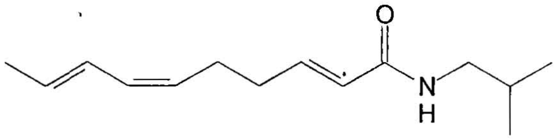
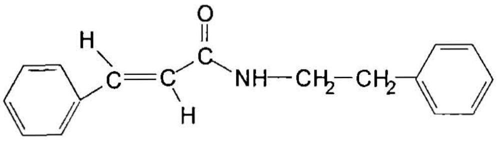


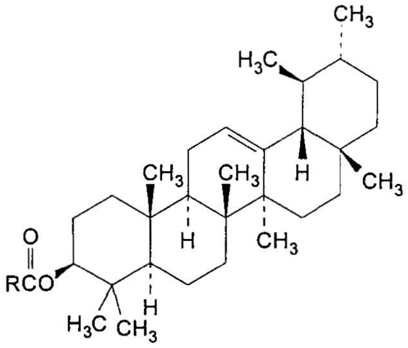
Figure 2 Morphological Feature of *Spilanthes acmella* Murr[4].



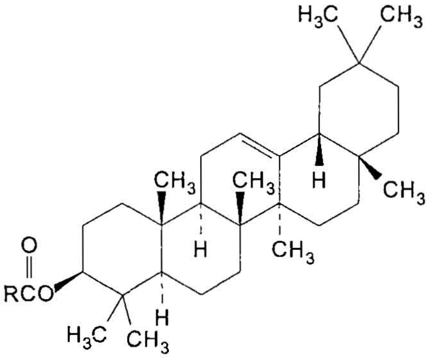
(I) spilanthol



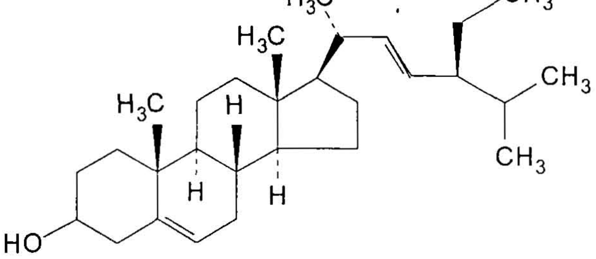
(II) *N* - 2- phenylethylcinnamamide



(IIIa) α- amyrin ester

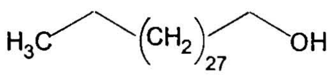


(IIIb) β- amyrin ester

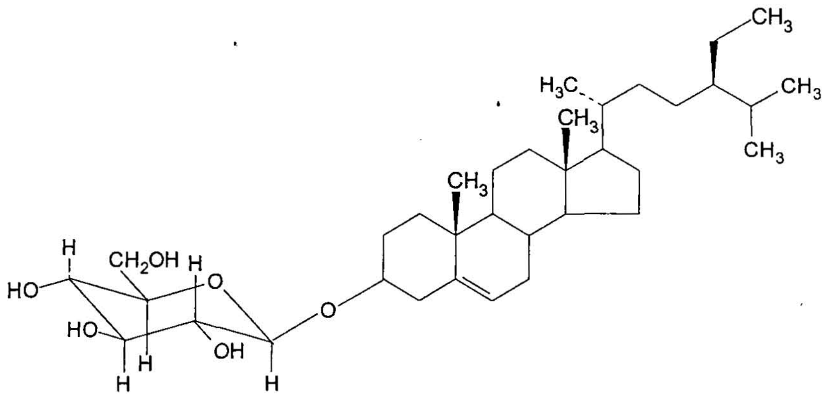


(IV) stigmasterol

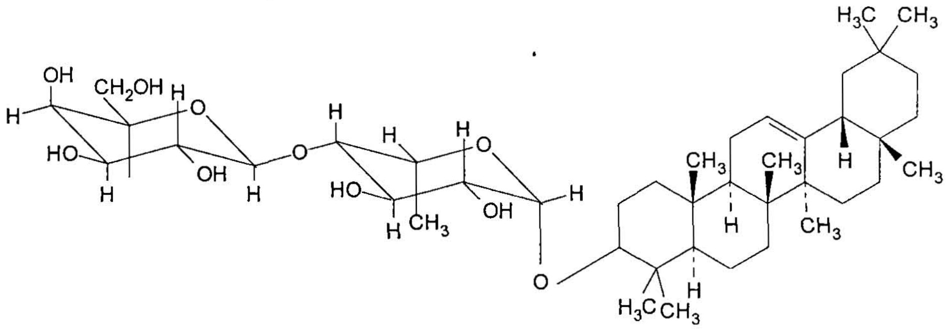
Figure 3 Structures of Compounds **I-IV** from *Spilanthes* species



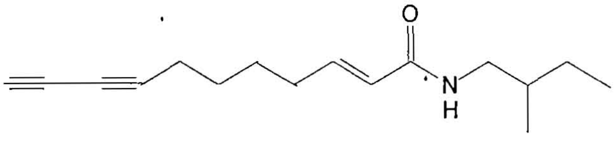
(V) myricyl alcohol



(VI) sitosterol-*O*-β-D-glucoside

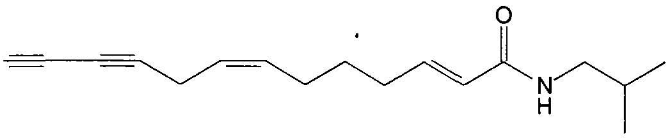


(VII) olean-12-en-3-*O*-β-D-galactopyranosyl(1→4)-*O*-α-L-rhamnopyranoside

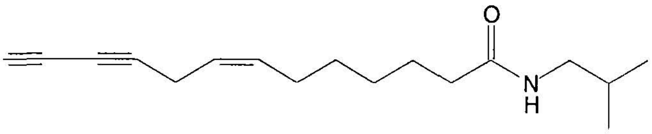


(VIII) (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide

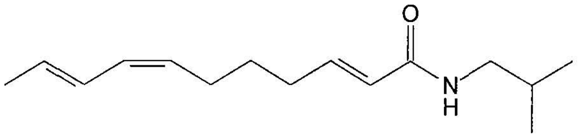
Figure 4 Structures of Compounds **V**- **VIII** from *Spilanthes species*



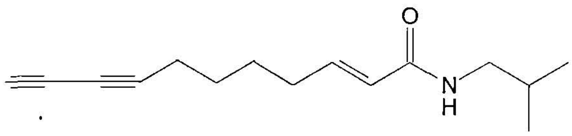
(IX) (2*E*,7*Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide



(X) (7*Z*)-*N*-isobutyl-7-tridecene-10,12-diynamide



(XI) undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide



(XII) undeca-2*E*-en-8,10-diynoic acid isobutylamide

Figure 5 Structures of Compounds **IX-XII** from *Spilanthes* species

Phenolic compounds are natural products of plant origin, possessing various biological activities. They contain carboaromatic rings such as benzene and naphthalene, generally substituted by one or more hydroxy groups. Some may exhibit neutral or acidic character due to the absence or the presence of free phenolic group. However, naturally occuring phenolic compounds are formed by the shikimic acid pathway[26].

The shikimate pathway provides an alternative route to aromatic compounds, particularly aromatic amino acids such as L-phenylalanine, L-tyrosine and L-tryptophan. Such pathway is employed by microorganisms in plants[27].

Biosynthesis of shikimic and chorismic acids began with the condensation of D-erythrose-4-phosphate**1** (**Figure 6**) deriving from the pentosephosphate cycle, and phosphoenolpyruvic acid (PEP) arising from glycolysis. A typical nucleophilic addition of the PEP methylene group to carbonyl group of **1** followed by hydrolytic cleavage of phosphate bond to form enol **1a**. Subsequent intramolecular nucleophilic addition of enol group of **1a** to keto carboxylic resulted in the formation of cyclohexane ring of 5-dehydroquinic acid, **2**. Enzymatic catalysed reaction of 5-dehydroquinic acid **2** produced first route of guinic acid **3** , and second route of 3-dehydroshikimate **4** which was converted to shikimic acid **6** . Rearrangement of the shikimic acid **6** afforded chrorismic acid **7** then prephenic acid**10** which was finally transformed to L-tyrosine**13** and L- phenylalanine **15**[27].

In addition, the shikimic acid pathway also provides many simple benzoic acid derivatives such as gallic acid **5**[27].

Obviously, L- phenylalanine and L-tyrosine **13**, as C_6-C_3 building block and are precursors for wide range of natural products. In plant, a frequent first step is elimination of ammonia from the side chain of **15** to generate appropriate trans *(E)* cinnamic acid **16.** Similarly, the elimination of L-tyrosine **13** provides 4-coumaric acid **14.** All plants appear to have the ability to deaminate phenylalanine *via* the enzyme phenylalanine ammonia lyase (PAL), but the corresponding transformation of tyrosine is more restricted,

Further hydroxylation and methylation reactions of cinnamic acid give some common natural metabolites such as 4-coumaric acid 14, caffeic acid **17**, ferulic acid **18** and sinapic acids **19** as shown in **Figure 7.**

Furthermore, the hydroxylation of cinnamic acid **16** ortho to the side chain as seen in the biosynthesis of salicylic acid, is a crucial step in the formation of a group of cinnamic acid lactone derivative, the coumarin **21**. Whilst the direct 4-hydroxylation of the aromatic ring of the cinnamic acid is generally common. Subsequent *meta*-hydroxylation of **14** produce 2,4-dihydroxylcinnamic acid **22** which undergo lactonization to form umbelliferone **23**. Other coumarins with additional oxygen substitution pattern on the aromatic ring, e.g. aesculetin **24** and scopoletin **25** appear to be derived by modification of umbelliferone **23** as depicted in **Figure 8**[27].

Coumarins are widely distributed in plants and are commonly found in botanical families such as the Umbelliferae and Rutaceae. They occur as free form and as glycoside (scopolin **26**)[20]. Coumarins were first isolated from seed of *Dipteryx odara* and other plants such as *Asperrula odorat*, *Cinnamonum cassia, Lavandum officinalus* and *Myroxylon pereirae*[28]. Coumarins, along with several hydroxy and methoxy derivatives have been shown to exhibit antiturmorgenic and immunological effects in rodent model and human clinical trial. Moreover, coumarins were reported to function by virtue as the protection of plants. For example, they exhibit antimicrobial activity, the ability to deter feeding by insects, autoinhibition of germination and shielding against ultraviolet irradiation[29].

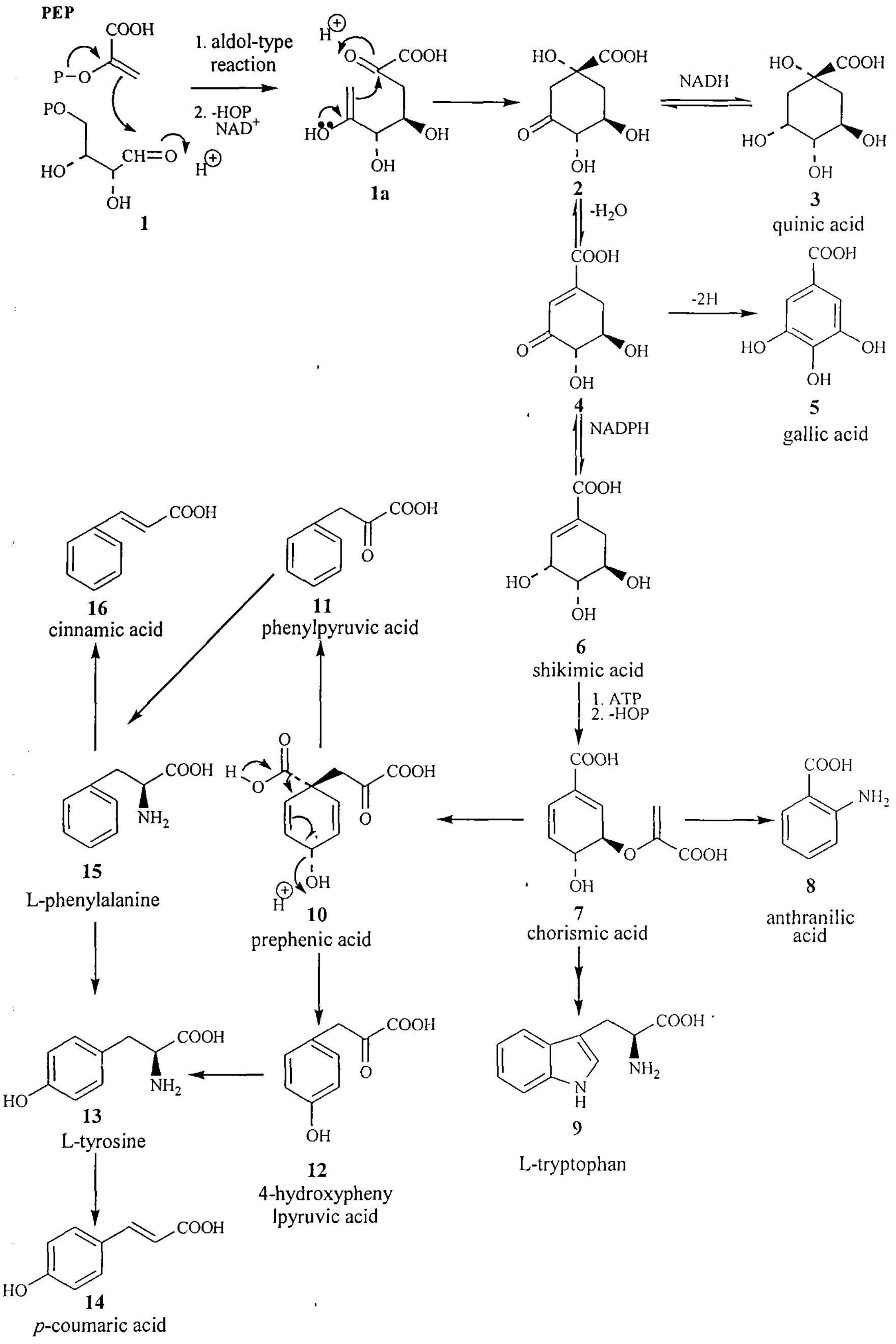


Figure 6 The Shikimic Acid Pathway [27]

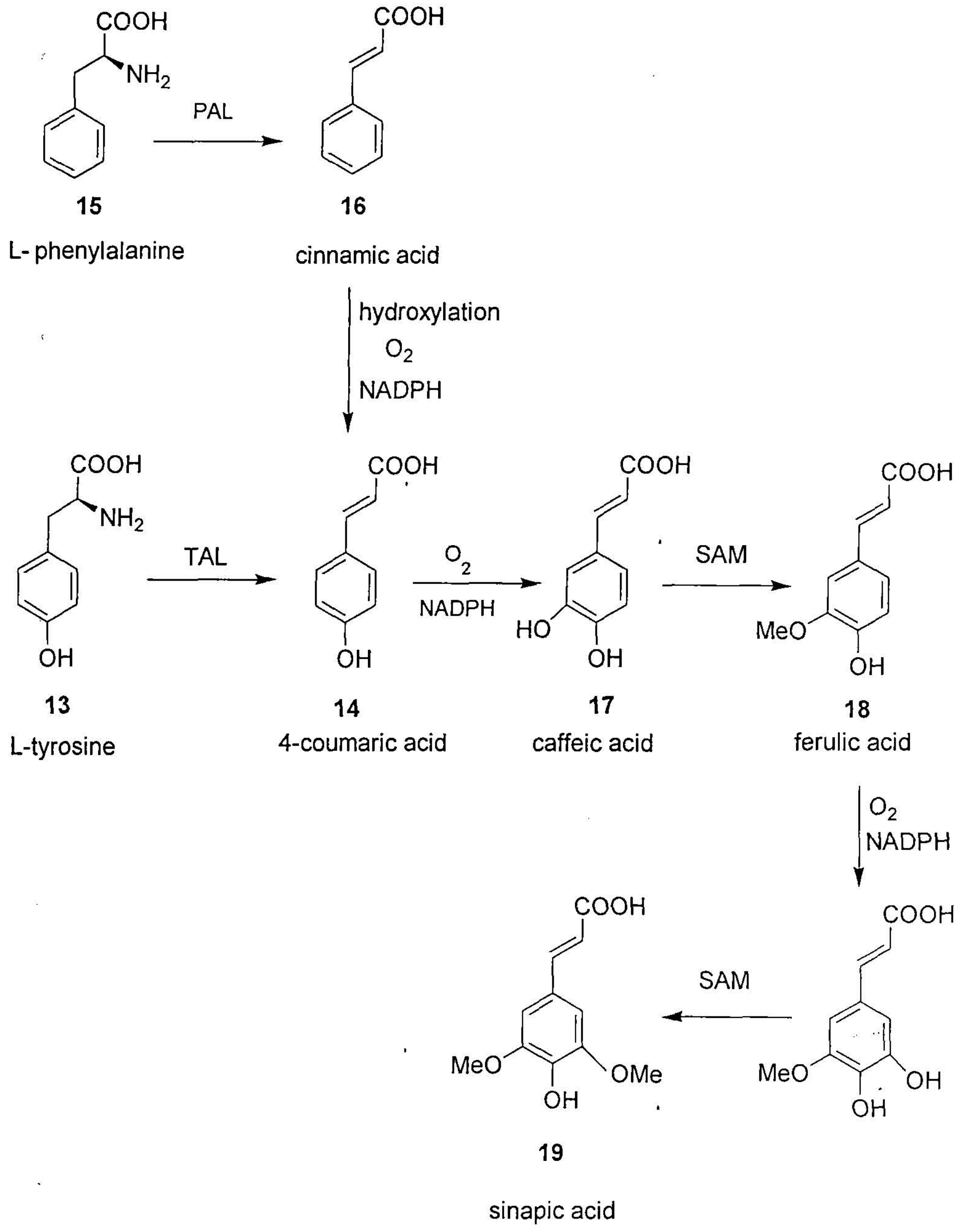


Figure 7 Biosynthesis of cinnamic acids [27]

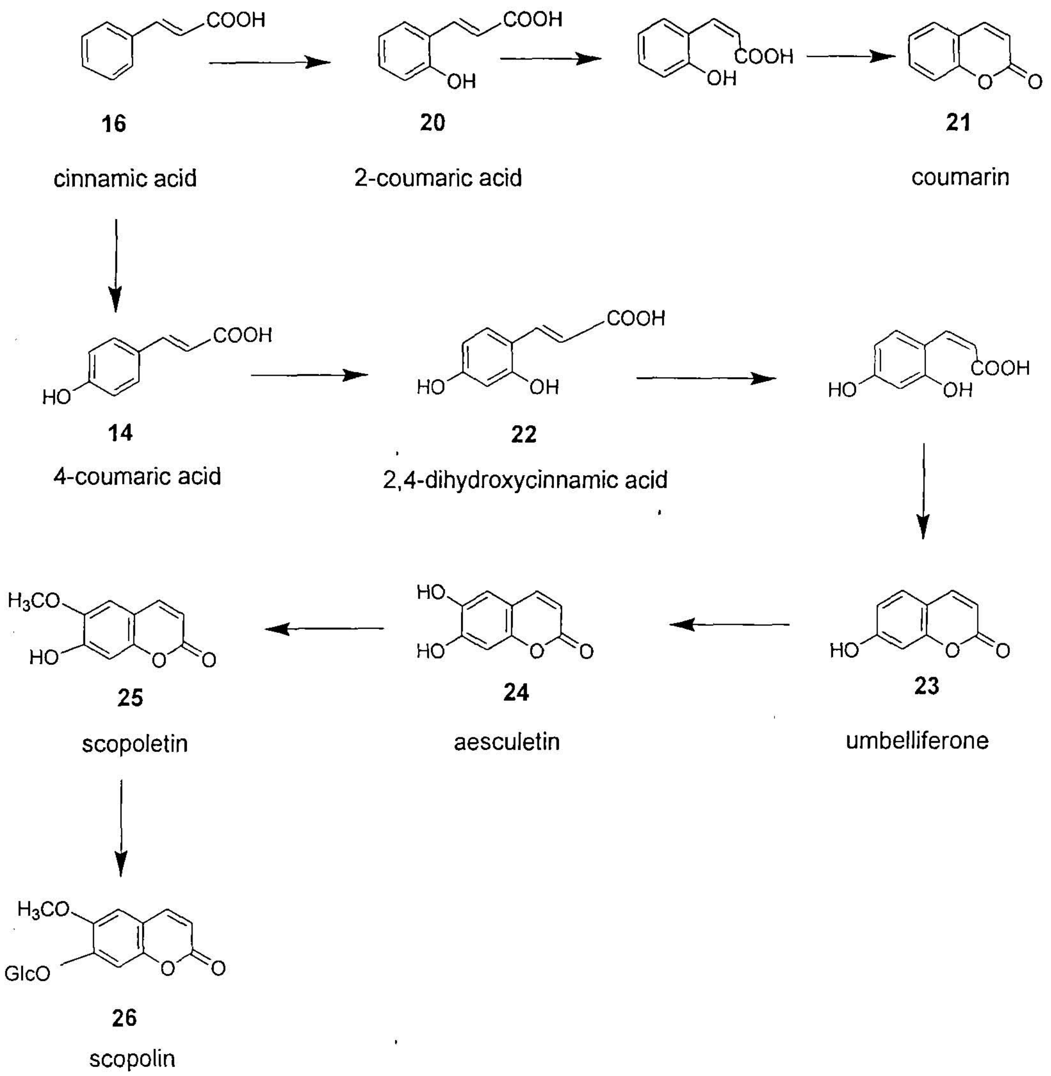


Figure 8 Biosynthesis of coumarins [27]

Chapter III

Experimental

Materials and Methods

Materials

Plant Materials

Spilanthes acmella Murr. were collected from Thasara district, Nakornsrithammarat province,Thailand, in June, 1999.

Chemicals

- Hexane, chloroform, ethyl acetate and methanol (Commercial grade)
- Silica gel for column chromatography
  - Silica gel (< 0.063 mm Merck 1.07729.5000)
  - Silica gel (0.063-0.200 mm Merck 1.07734.2500)
- Silica gel for thin - layer chromatography
  - Silica gel 60GF$_{254}$ (Merck 7730.1000)
- Spray reagent (anisaldehyde : conc.H_2SO_4 : glacial acetic acid : methanol ratio 0.50 : 8.00 : 10.00 : 85.00)

Instruments

- Electrothermal melting point apparatus (Electrothermal 9100)
- Fourier Transform Infrared Spectrophotometer (Perkin Elmer FT-IR spectrum BX)
- Mass Spectrometer (Finnigan MAT 90)
- Nuclear Magnetic Resonance Spectrometer (Bruker AM 400)
- Ultraviolet lamp (Spectroline ENF-260 C/F)
- UV-VIS spectrophotometer (Shimadzu UV-2401PC)

Structure Elucidation

1. Melting point

Melting points were determined on electrothermal melting point apparatus (Electrothermal 9100)

2. Spectroscopy

2.1 Infrared (IR) spectra

Infrared spectra were recorded on Perkin Elmer FT- IR spectrum BX

2.2 Nuclear magnetic resonance (NMR) spectra

^{1}H Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AM 400 with a 400 MHz operating frequency by using tetramethylsilane as internal standard in a solution of deuterochloroform or deuteromethanol .

^{13}C Nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker AM 400 with a 100 MHz operating frequency by using tetramethylsilane as internal standard in a solution of deuterochloroform or deuteromethanol.

2.3 Mass (MS) spectra

Mass spectra were determined by using a Finnigan MAT90 mass spectrometer.

Methods

Extraction

The air – dried and milled *Spilanthes acmella* Murr. (5 kg) was extracted three times by using n-hexane (10L x 7 days x3) at room temperature and followed by filtration. The filtrates were combined and evaporated to dryness under reduced pressure to obtain crude hexane extract (57 g). Similarly, the extraction was carried out by using chloroform, ethyl acetate and methanol to give the corresponding crude chloroform (55 g) , crude ethyl acetate (95 g) and crude methanol extracts (170.70 g) as shown in **Figure 9**.

In our laboratory, previous study on the isolation of the crude hexane extracts of *Spilanthes acmella* Murr. showed the presence of long chain ester compounds.

In this study, crude ethyl acetate and crude methanol extracts were isolated and purified.

Isolation and purification of crude ethyl acetate extract

The crude ethyl acetate extract (50.7 g) of *Spilanthes acmella* Murr. was applied to silica gel (1,500 g) column chromatography (**Figure 10**). Elution was conducted initially with hexane , then gradually increasing polarity with chloroform and ethyl acetate, and finally enriched with methanol. All fractions were collected and combined as approximate by TLC monitoring. The solvents were evaporated to dryness under reduced pressure to afford 14 fractions. Four selected main fractions (5, 6, 8 and 12) were further isolated and purified.

Fraction 5 (3.49 g) was obtained as green residue from 40% ethyl acetate-chloroform elution. The mixture was subjected to silica gel (180 g) column followed by elution with 40% ethyl acetate-hexane . The obtained yellowish green semi-solid (354.2 mg) fractions were purified by silica gel (160 g) column using 20% ethyl acetate-hexane as a mobile phase to give white needle **compound A** (23.7 mg).

Fraction 6 (2.78 g) obtaining from 50% ethyl acetate-chloroform elution was further purified by silica gel (160 g) column which was eluted with increasing 50% ethyl acetate in hexane to afford orange gum (673 mg) . Further purification of the orange gum was carried out by silica gel (28 g) column using gradient elution of methanol in chloroform as follow.

Elution with 5% methanol–chloroform gave white crystalline solid which was recrystallized from chloroform to give **compound B** (3.8 mg).

Elution with 10% methanol–chloroform afforded white semi-solid (143.4 mg) which was subjected to column chromatography on silica gel (10 g). Elution with 5-7 % methanol–chloroform gave unidentified **compound H** (8.1 mg).

Fraction 8 (2.61g) as greenish gum obtained from 70% ethyl acetate-chloroform elution was further purified by silica gel (160 g) column. Elution with hexane then increasing amounts of ethyl acetate was carried out as the following.

Elution with 20% ethyl acetate-hexane gave white semi-solid (27.1 mg) which was further purified by repeated preparative TLC on silica gel developing by 8% ethyl acetate- hexane for 5 times to give **compound C** (4 mg).

Fraction 12 (3.16 g) as greenish gum obtained from ethyl acetate elution was further purified by silica gel (278 g) column. Elution with 12% methanol–chloroform afforded white semi-solid powder (98.4 mg). Further purification by silica gel (9 g) column using 12% methanol–chloroform as an eluting solvent gave unidentified **compound I**(3.4mg)

Isolation and purification of crude methanol extract (flash column)

The crude methanol extract (40.0 g) of *Spilanthes acmella* Murr. was first subjected to coarse separation by flash column chromatography on silica gel (140 g) as shown in **Figure 11.** Elution was conducted initially with chloroform then increasing polarity with methanol. Fractions were collected and combined as approximate according to TLC chromatograms. The solvents were evaporated to dryness under reduced pressure to afford 5 fractions. The main fractions 2 and 3 were further purified as the following.

Fraction 2 (3.12 g), dark brown gum eluted by 15% methanol–chloroform, was further subjected to column chromatography on silica gel (110 g). Gradient elution was performed by using chloroform containing methanol up to solely methanol.

Elution with 10% methanol-chloroform afforded brown gum (405.9 mg) which was further separated by column chromatography on silica gel (27 g) using 7% methanol-chloroform as an eluent. The combined fractions (103.1mg) were rechromatographed on silica gel (10 g) column. Gradient elution with 3-5% methanol-chloroform gave **compound D** (3.2 mg).

Fraction 3 (4.25 g) as brown gum from 25 % methanol-chloroform fractions was purified by silica gel (110 g) column. Elution with 15 % methanol-chloroform gave brown semi-solid (1.02 g) which was further purified by silica gel (50 g) column chromatography . Elution with 10% methanol- chloroform provided impured compound (160 mg). Recrystallization from chloroform gave **compound E** (5.1 mg).

Isolation and purification of crude methanol extract (conventional column)

The crude methanol extract (123.4 g) of *Spilanthes acmella* Murr. was isolated by silica gel (1,700 g) column chromatography as shown in **Figure 12**. Elution was conducted initially with chloroform then gradually increasing with enriched methanol. All fractions were collected and combined as approximate based on TLC chromatograms. The solvents were evaporated to dryness under reduced pressure to afford 6 fractions which were further purified. The main fractions 2 and 3 were purified further.

Fraction 2 (642.40 mg) as greenish gum was obtained from 10-12% methanol-chloroform elution . The greenish gum was further subjected to column chromatography on silica gel (29 g). Gradient elution with increasing amount of methanol in chloroform up to methanol was carried out as the following.

Elution with 7% methanol–chloroform gave brownish solid (30.7 mg) which was recrystallized from chloroform to give **compound F** (12 mg).

Further elution with 10 % methanol–chloroform provided white powder (102.5mg). Recrystallization from acetone gave unidentified **compound J** (10 mg).

Fraction 3 (313.5 mg) as greenish semi-solid was obtained from 20% methanol -chloroform. Some amount of greenish semi-solid (80.2 mg) was recrystallized from methanol to give **compound G** (29 mg).

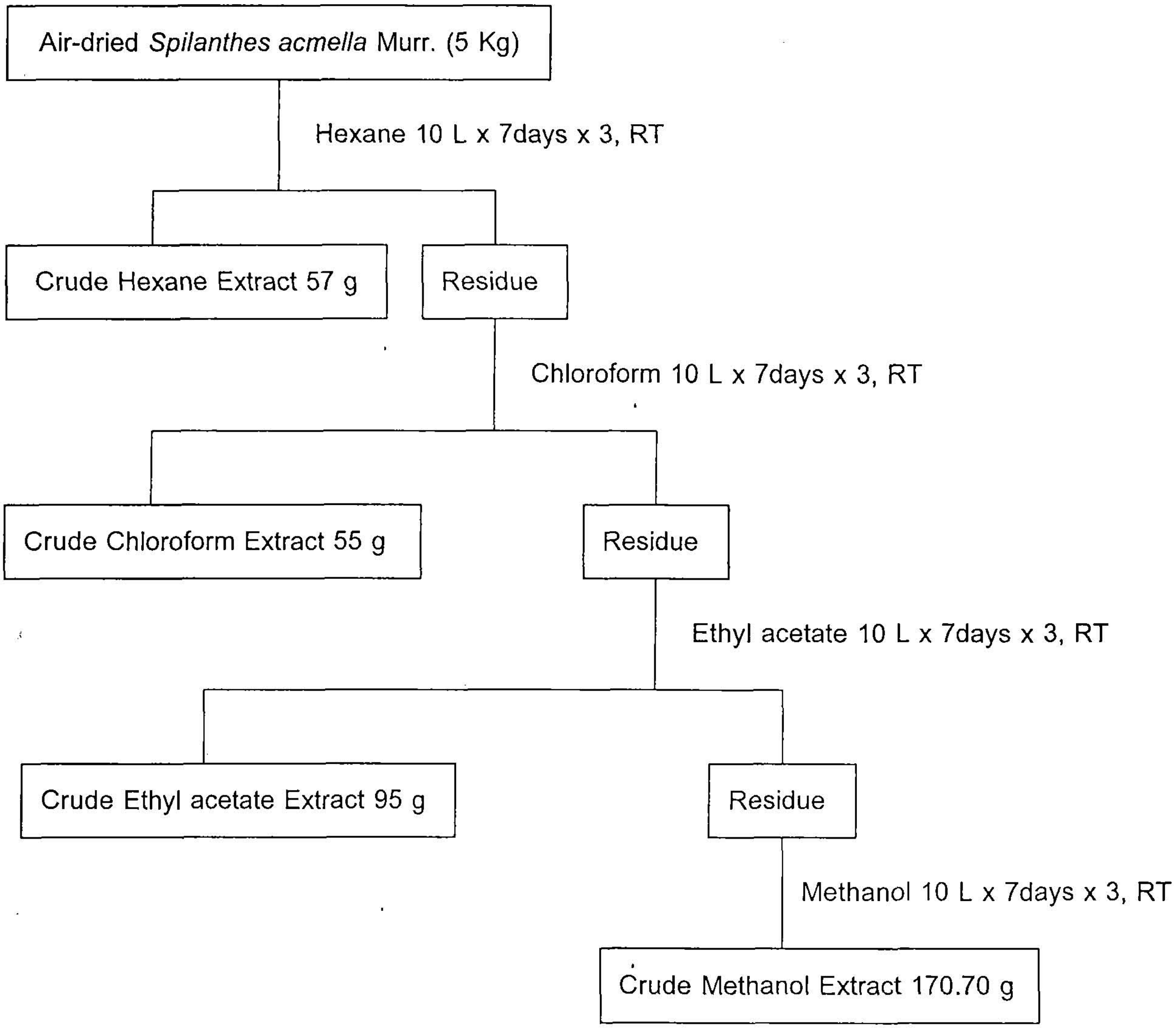
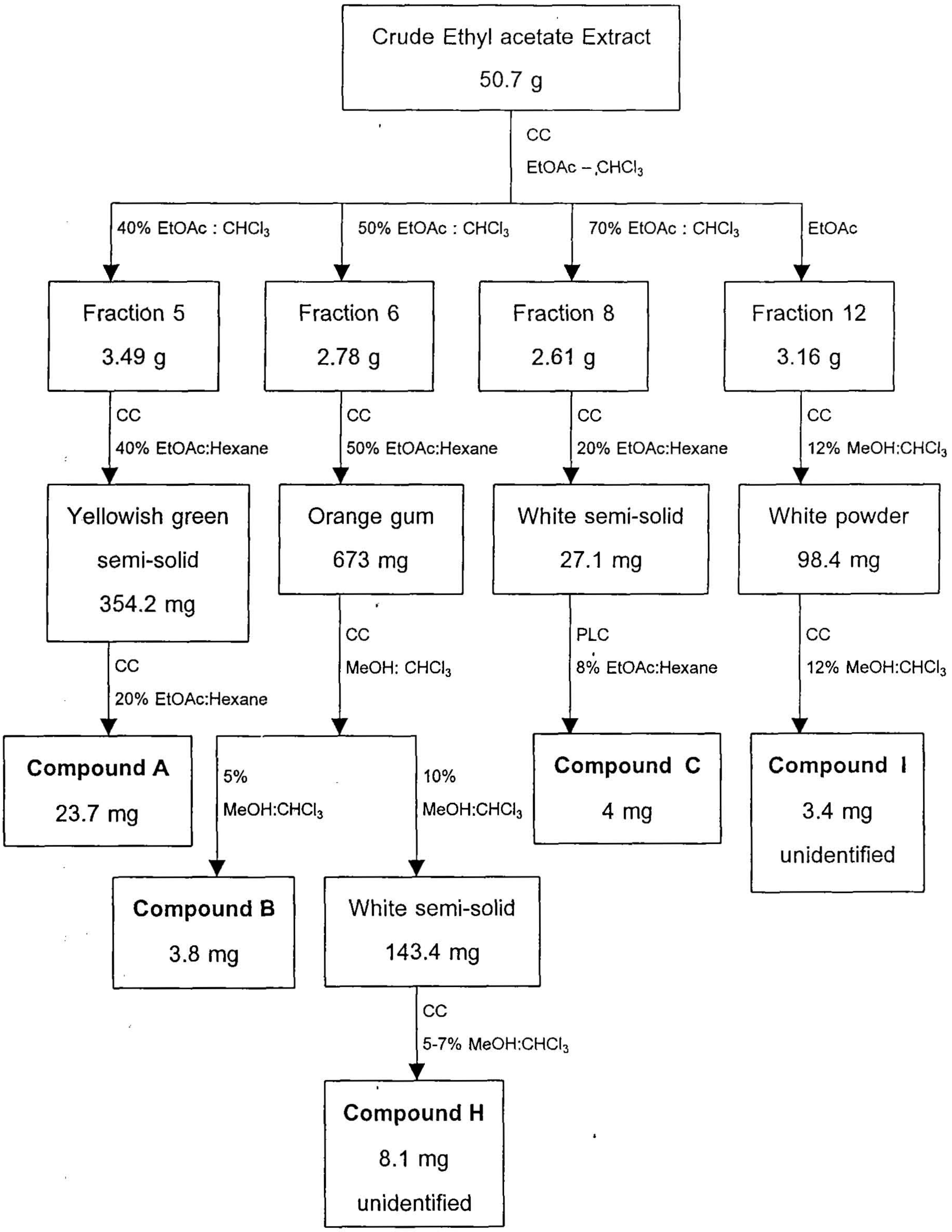
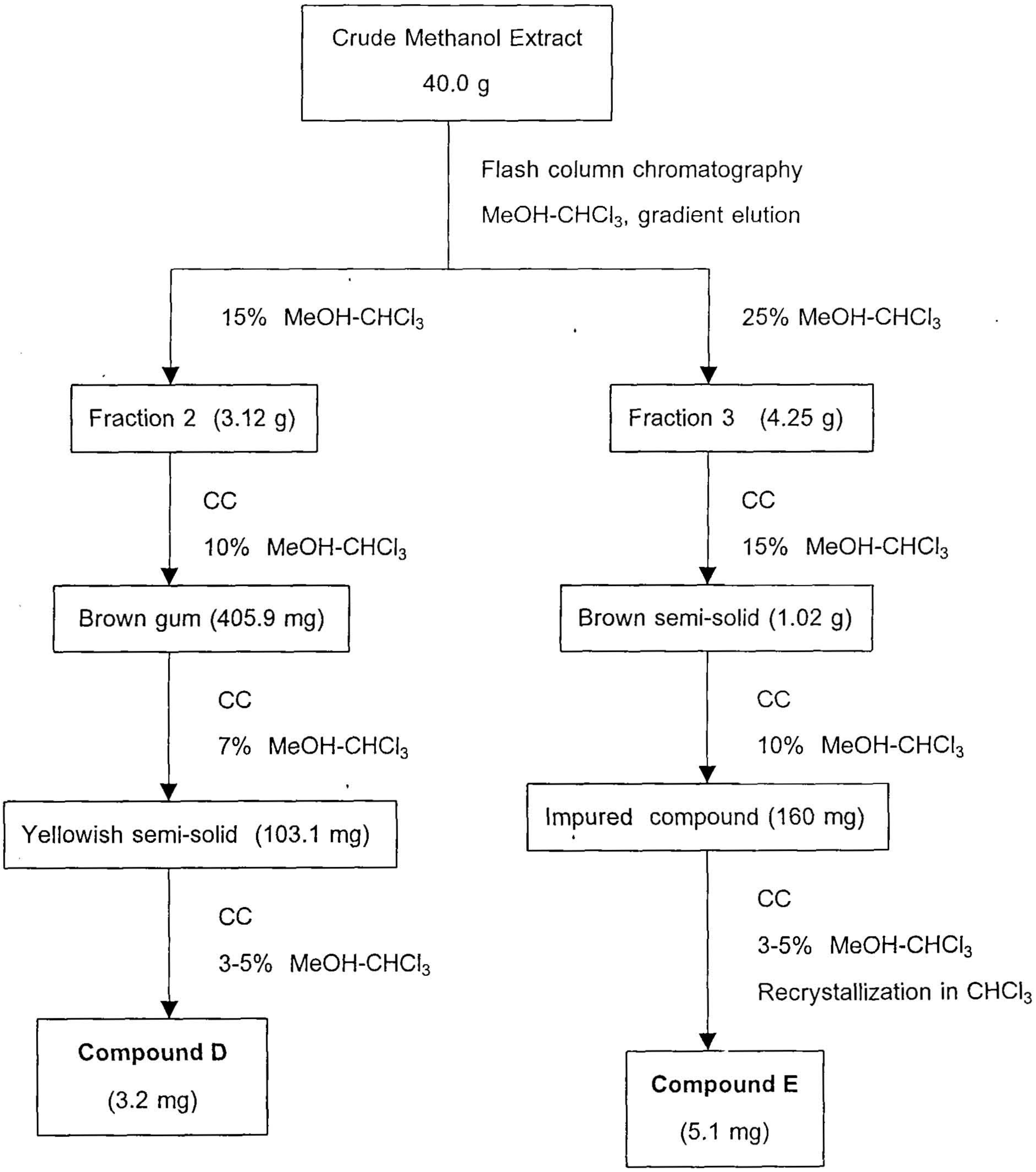


Figure 9 Crude extracts from *Spilanthes acmella* Murr.



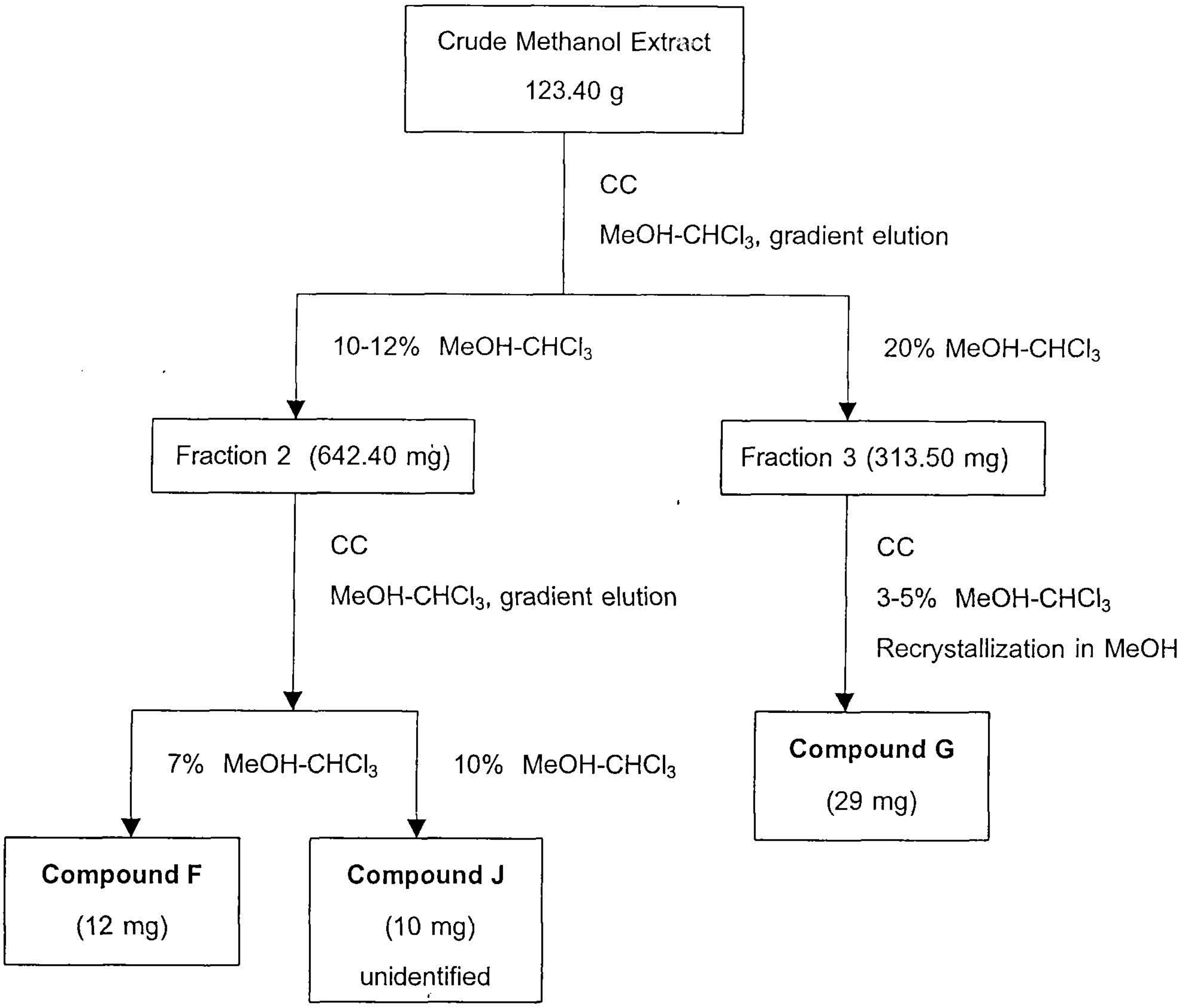
CC = Column chromatography

Figure 10 Isolation and purification of crude ethyl acetate extract of *Spilanthes acmella* Murr. by column chromatography



CC= Column chromatography

Figure 11 Isolation and purification of crude methanol extract of *spilanthes acmella* Murr. by flash column chromatography



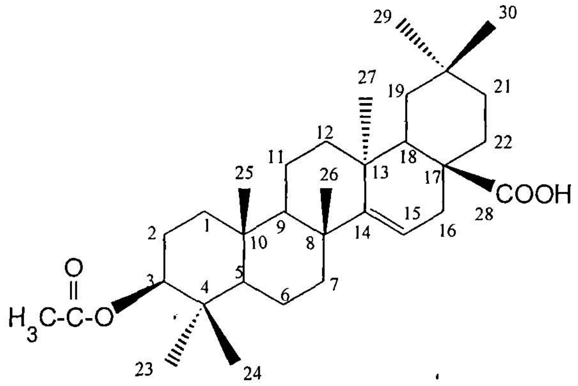
CC= Column chromatography

Figure 12 Isolation and purification of crude methanol extract of *spilanthes acmella* Murr. by conventional column chromatography.

Experimental Data of Isolated Compounds

Compound A

[**3-acetylaleuritolic acid**, 3-*β*-*O*-acetyltaraxer-14-en-28-oic]



Physical characteristic: white crystal from methanol

m.p. 299-300°C, (lit[30] 302-304 °C, lit[31] 304-305 °C)

FT IR ν_{max}^{KBr} cm^{-1} :

3435, 2935,1734,1686,1364,1242,1026 (lit[31] 3416, 2938,1732,1685, 1470,1365, 1295, 1245,1028)

^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.47(1H, *dd*,*J* = 3.40, 7.90 Hz,H-15), 4.39(1H, *dd*, *J* =5.50, 10.00 Hz, H-3), 1.97(3H, *s*,$COOCH_3$), 0.86(3H,*s*, H-24), 0.81(3H,*s*, H-27), 0.78 (3H,*s* H-25), 1.18(3H,*s* H-26)

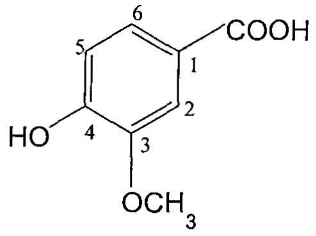
^{13}C NMR ($CDCl_3$,100 MHz) δ 37.88(C-4), 39.00(C-8), 37.87(C-10), 37.37(C-13), 160.53 (C-14), 116.59(C-15), 51.44(C-17), 29.22(C-20), 171.06 ($COOCH_3$), 184.03 (COOH), 80.88(CH-3), 55.59(CH-5),49.03(CH-9),41.58 (CH-18), 40.83(CH_2-7), 37.37 (CH_2-1), 35.33(CH_2-19), 33.64(CH_2-12), 33.30(CH_2-21), 31.43(CH_2-16), 30.76(CH_2-22), 23.42(CH_2-2),18.64(CH_2-6), 17.26(CH_2-11) 31.92(CH_3- 29), 28.65(CH_3-30), 27.91(CH_3-23),26.02(CH_3-26), 22.46(CH_3-27), 21.21($COOCH_3$), 16.52 (CH_3-24), 15.52 (CH_3-25)

MS *m/z* (% relative intensity):

329(3), 269(7), 234(7), 189(100) 133(21),119(50) [lit[30] 498 (M^+,0.2),483(1), 438 (4), 248(33),234(100),189(69)]

Compound B

[**vanillic acid** , 4-hydroxy-3-methoxybenzoic acid]



Physical characteristic: white crystal from chloroform

m.p. 210-212 °C (lit[32] 213-214 °C)

UV λ_{max}^{MeOH} nm (log ε):

253(3.41), 286(3.47) (lit[33] in ethanol 260, 290)

FT IR ν_{max}^{KBr} cm^{-1}:

3485,2955,1686,1598,1547,1523,1473,1299,1239 ,1205,1113, 918, 882, 819 (lit[32] 3745, 2940,1682,1600,1247,1212)

^{1}H NMR (CD$_3$OD+CDCl$_3$, 400 MHz) δ 7.55 (1H, *d*, *J* =1.90 Hz, H-2), 7.59 (1H, *dd*, *J* = 1.90, 8.20 Hz ,H-6), 6.88(1H, d, *J* =8.20 Hz, H-5), 3.92(3H, *s*, OCH$_3$)

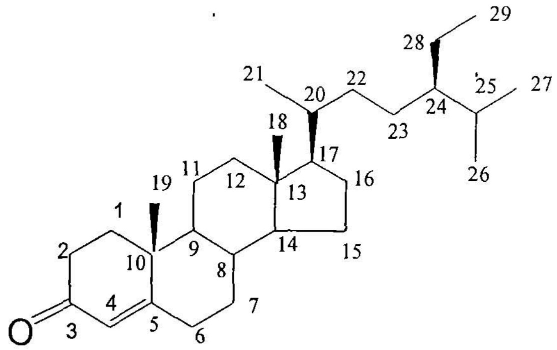
^{13}C NMR (CD$_3$OD+CDCl$_3$,100 MHz) δ 169.11(CO),150.81 (C-1),147.00 (C-3), 124.22 (C-6), 121.90(C-4), 114.56(C-5), 112.63(C-2), 55.68(OCH$_3$)

MS *m/z* (% relative intensity):

168(M$^+$,100),153(72),125(35),97(55) 77(5) [lit[34] 168 (M$^+$,100),153(77),125(23), 77(55)]

Compound C

β-sitostenone (stigmast-4-en-3-one, 24α-ethyl-cholest-4-en-3-one)



Physical characteristic: white crystal from methanol

m.p. 97-99°C, (lit[35] 95-96°C)

FT IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}:

2936,1681,1464,1378,1228 (lit[36] 2959, 2983,2874,1663, 1614,1467,1378,1232)

^{1}H NMR ($CDCl_3$,400 MHz) δ 5.74(1H,*s*,H-4), 0.71(3H,*s*,H-18), 0.80-1.10 (*m*,H-21,26,27, 29),1.18 (3H,*s*,H-19).

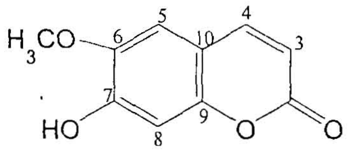
^{13}C NMR ($CDCl_3$,100 MHz) δ 29.64(CH-25), 35.60(CH-8), 36.07(CH-20),45.81(CH-24), 53.79(CH-9), 55.85(CH-14), 55.99(CH-17),123.69 (CH-4),11.14(CH_3-29), 11.90 (CH_3-18), 18.65(CH_3-19),18.98(CH_3-21),19.75(CH_3-27), 20.99(CH_3-26),21.10 (CH_2-11), 23.04(CH_2-28), 24.14(CH_2-15), 26.08(CH_2-23), 28.13(CH_2-16), 32.91 (CH_2-7), 33.86 (CH_2-6), 33.93 (CH_2-2), 35.65 (CH_2-22), 36.06 (CH_2-1), 38.57 (CH_2-12), 39.59(C-10), 42.35(C-13), 171.64(C-5),199.58 (C=O)

MS *m/z* (% relative intensity):

412(M^+,13), 397(27),370(13),288(26),271(39),229(92),187(26),173(46),147(57), 124(100) [lit[36] 412(M^+13), 397(2), 370(5), 288 (8), 271(7), 245(3), 229(45),187 (13),173 (12),147(32),124(100)]

Compound D

[**scopoletin** , 7-hydroxy-6-methoxycoumarin]



Physical characteristic: yellowish needle crystal from chloroform

m.p.205-206 °C (lit[37] 203-204°C)

UV λ_{max}^{MeOH} nm (log ε):

294(3.68), 344(4.07) [lit[37] in methanol 297 (3.68), 345(4.04)]

FTIR ν_{max}^{KBr} cm^{-1}:

3333, 1702, 1566, 1437 (lit[38] 3330, 1670, 1615, 1595, 1550, 1490)

^{1}H NMR (CD_3OD,400 MHz) δ 6.23(1H, *d*,*J* = 9.42 Hz, H-3), 7.89(1H,*d*,*J* = 9.42 Hz,H-4), 6.82 (1H, *s*, H-8), 7.14(1H, *s*, H-5), 3.91(3H, *s*, OCH_3), 8.10(1H, *s*, OH)

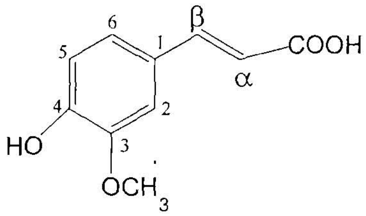
^{13}C NMR (CD_3OD,100 MHz) δ 163.00(CO),150.00(C-9),149.00(C-7),146.00(C-6),144.91 (C-4), 111.15(C-3),110.00(C-10),108.63(C-5), 102.57 (C-8), 55.51(OCH_3)

MS *m/z* (% relative intensity):

192(M^+,100),177(28),164(41),121(37) [lit[39] 192(M^+,100),180(40),154(48),135(52) (121(18)]

Compound E

[***trans*-ferulic acid** , *trans*-4-hydroxy-3-methoxycinnamic acid]



Physical characteristic: browish crystal from chloroform

m.p.168-169 °C (lit[40] 168-169 °C)

UV λ_{max}^{MeOH} nm (log ε):

289 (3.83), 318 (3.86) [lit [33] in ethanol 298.3 (sh), 322.5)]

FT IR ν_{max}^{KBr} cm^{-1}:

3437,1691,1665,1517 (lit[41] 1671,1641,1512, 1323,1264,1209,1137,1025, 818)

^{1}H NMR (CD$_3$OD, 400 MHz) δ 7.18(1H, *d*, *J*=1.93 Hz, H-2), 7.07 (1H, *dd*, *J*=1.93, 8.23 Hz,H-6), 6.82(1H, *d*, *J*=8.23 Hz, H-5),6.31(1H, *d*, *J*=15.88 Hz, H-α), 7.59 (1H, *d*, J=15.88 Hz, H-β), 3.89 (3H, *s*, OCH$_3$)

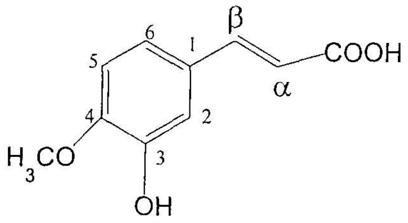
^{13}C NMR (CD$_3$OD, 100 MHz) δ,171.19 (CO), 151.50 (C-3), 149.90 (C-4) , 127.76 (C-1), 123.97(C-6), 116.46(C-5),115.89 (C-α),111.64(C-2),146.95(C-β), 56.45(OCH$_3$)

MS *m/z* (% relative intensity):

194(M$^+$,100), 179(16), 161(5), 148(6), 133(17), 105(5), 77(6)

Compound F

[trans-isoferulic acid ,*trans*-3-hydroxy-4-methoxycinnamic acid]



Physical characteristic: brownish crystal from chloroform

m.p. 230-232 °C (lit[40] 230 °C)

UV λ_{max}^{MeOH} nm (log ε):

289 (3.93), 313 (3.97) (lit[33] in ethanol 295,323)

FT IR ν_{max}^{KBr} cm^{-1}:

3437, 2968, 1692, 1665, 1620, 1600,1517,1277,1206,1178 (lit [41]3400, 1672, 1614, 1512, 1324 1268, 1209, 1137, 1025, 819)

^{1}H NMR (CD_3OD+$CDCl_3$, 400 MHz) δ 6.26 (1H, *d*, *J*=15.90 Hz, H-α), 7.61 (1H, *d*, *J*=15.90 Hz, H-β), 7.07(1H, *d*, *J* =1.67 Hz,H-2), 7.05(1H, *dd*, *J* = 8.00,1.67 Hz, H-6), 6.87 (1H, *d*, *J* = 8.00 Hz, H-5), 3.91(3H, *s*, OCH_3).

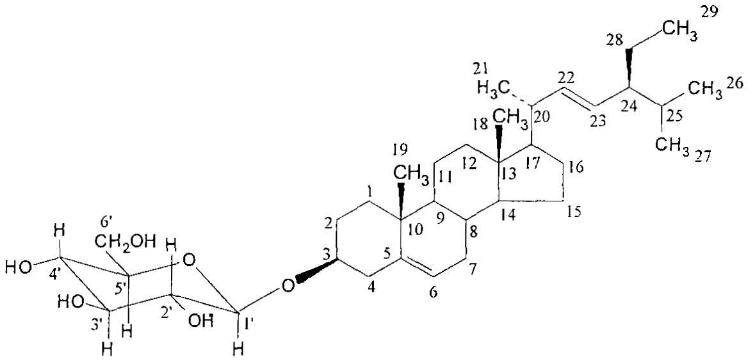
^{13}C NMR (CD_3OD+$CDCl_3$,100 MHz) δ 110.50(CH-2), 115.28(CH-α), 115.57(CH-5), 123.28(CH-6),126.83(C-1),146.11(CH-β), 170.25(CO), 148.98(C-3), 147.97 (C-4), 56.04(OCH_3).

MS *m/z* (% relative intensity):

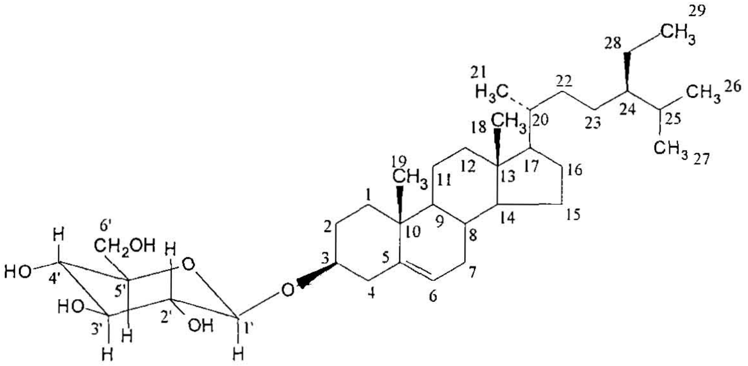
194(M^+,100), 193(28), 179(23), 177(12), 148(6), 133(28), 105(14), 77(12)

Compound G

Mixture of Stigmasteryl-3-*O*-*β*-D-glucopyranoside and *β*-sitosteryl-3-*O*-*β*-D-glucopyranoside



Stigmasteryl-3-*O*-*β*-D-glucopyranoside



β-sitosteryl-3-*O*-*β*-D-glucopyranoside

Physical characteristic: white powder from methanol,

m.p. 261-262 °C (lit[42] 264-266 °C, lit [43] 278-290 °C)

FT IR ν_{max}^{KBr} cm^{-1} :

3406,2935, 1654, 1459,1368, 1024

^{1}H NMR ($CDCl_3$+CD_3OD,400 MHz) δ 0.64-2.50 (*m*,CH, CH_2,CH_3 of steroid) 3.20- 3.40 , 3.72-3.88 (m, glucosidic protons), 3.56-3.64 (*m*, 1H, H-3), 4.42 (1H, *d*, *J* = 7.83 Hz, H-*β*-anomeric), 5.03(1H, *dd*, *J* = 15.66, 8.75 Hz, H-22$^+$), 5.17 (1H, *dd*, *J* = 15.63, 8.62 Hz, 23$^+$), 5.38 (1H, *t*, *J* = 3.59, H-6)

^{13}C NMR ($CDCl_3$+CD_3OD,100 MHz) δ [69.52(CH-3),121.93(CH-6), 49.94(CH-9), 31.76

(CH-8),56.61(CH-14), 55.81(CH-17), 40.28(CH-20),138.11(CH-22^{+}),129.05 (CH-23^{+}),51.02 (CH-24),33.69 (CH-25),11.76 (CH_3-18),19.51(CH_3-19), 20.93 (CH_3-21),19.02(CH_3-26),20.80(CH_3-27),11.95(CH_3-29),36.99(CH_2-1),29.30 (CH_2-2),41.97(CH_2-4),31.63(CH_2-7), 22.81 (CH_2-11), 39.51(CH_2-12), 24.04 (CH_2-15),28.89 (CH_2-16), 33.69(CH_2-22^{++}), 27.99(CH_2-23^{++}), 25.17(CH_2-28), 140.05(C-5), 36.47(C-10),42.08 (C-13),100.84(C-1′), 73.26(C-2′), 76.14(C-3′), 69.52(C-4′),76.67(C-5′), 61.22(C-6′)

MS *m/z* (% relative intensity):

414(6), 412(7), 393(57),394(84), 381(33),300 (75), 287(66), 255(100), 227(37), 213(61), 147(87), 145 (90),131(49), 105(66), 91(87), 79(44)

$^{+}$ ^{1}H, ^{13}C of Stigmasteryl-3-*O*-*β*-D-glucopyranoside

$^{++}$ ^{1}H, ^{13}C of *β*-sitosteryl-3-*O*-*β*-D-glucopyranoside

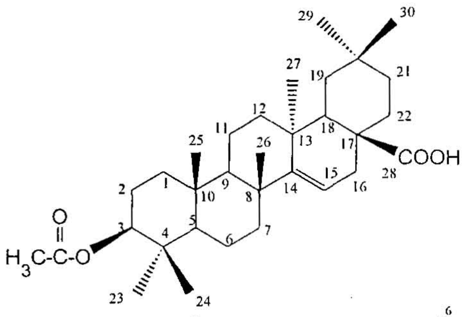
Chapter IV

Results and Discussion

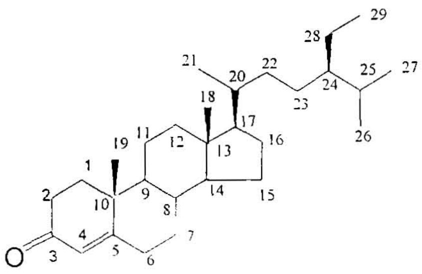
Previous study in our laboratory on the isolation of the crude hexane extracts of *Spilanthes acmella* Murr. showed the presence of esters of long chain hydrocarbons.

Chemical constituents of all crude extracts of *Spilanthes acmella* Murr. were preliminary screened by TLC chromatograms. Based on the TLC chromatograms, thus, the interesting crude extracts of ethyl acetate and methanol were isolated and purified.

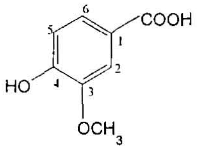
The ethyl acetate extract (50.7 g) of *Spilanthes acmella Murr.* was separated by silica gel column using gradient elution of chloroform-ethyl acetate with increasing polarity. Several fractions were collected from the column and examined by ^{1}H and ^{13}C NMR spectroscopy. Thus, the interesting selected fractions were further purified by repeated silica gel column and preparative TLC together with recrystallization to obtain three compounds, **A-C**.



A

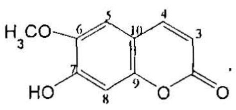


C

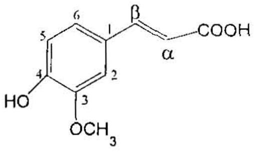


B

The crude methanol extract (170.70 g) of *Spilanthes acmellla* Murr. was available in hand. Initially, 40 g of the crude methanol extract of *Spilanthes acmellla* Murr. was subjected to coarse separation by flash column chromatography on silica gel using gradient elution of chloroform-methanol with increasing polarity. Several fractions were collected and examined by ^{1}H and ^{13}C NMR spectroscopy. The selected fractions were further purified by repeated silica gel column and recrystallization to afford two compounds, **D** and **E**.

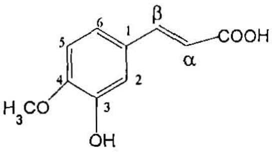


D

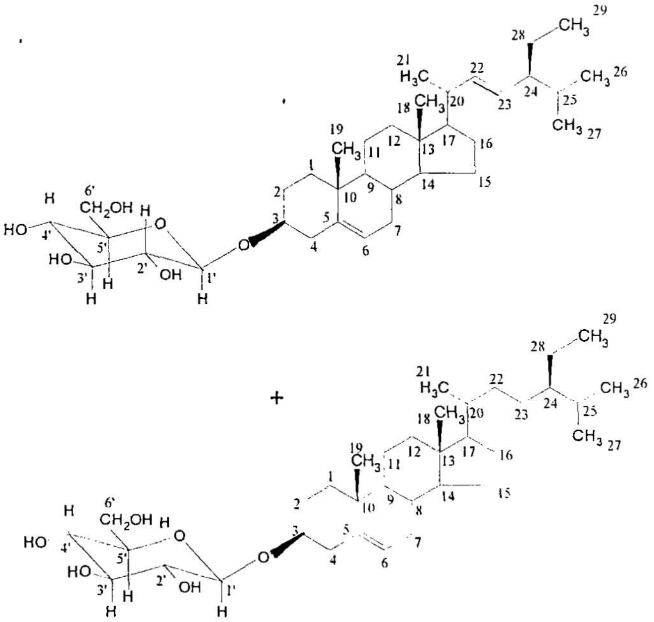


E

Further separation of the rest of crude methanol extract (123.4 g) of *Spilanthes acmellla* Murr. was carried out by silica gel column using gradient elution of chloroform-methanol with increasing polarity by enriched with methanol. Many crude fractions were collected and examined by ^{1}H and ^{13}C NMR spectroscopy, The selected fractions were further purified by repeated silica gel column and recrystallization to give two compounds, **F** and **G**.

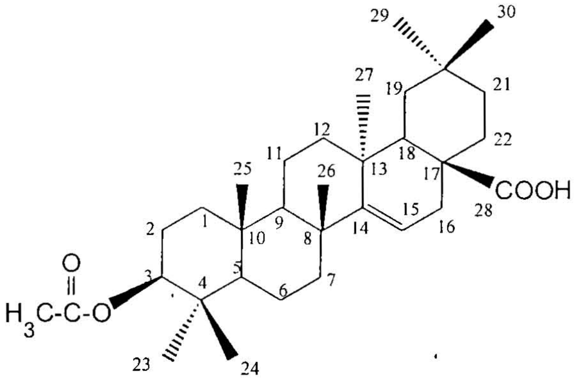


F



G

Identification of compound A



Compound **A** was isolated as white crystalline solid, m.p. 299-300 °C. The structure of compound **A** was elucidated by spectroscopic method. The mass spectrum did not show the molecular ion, only the base peak was observed at *m/z* 189, corresponding to the bond cleavage between C-11, C-12 and C-8, C-14 followed by the loss of carboxyl group as shown in **Figure 13**.

The IR spectrum of compound **A** exhibited characteristic absorption bands at 3435 cm^{-1} (OH), 2935 cm^{-1} (CH),1734 cm^{-1} (ester, CO), 1686 cm^{-1} (carboxylic, CO),1242 cm^{-1} (C-O).

The 100 MHz ^{13}C NMR spectrum of compound A exhibited 32 carbon signals consisting of 8 methyl, 10 methylene, 5 methine and 9 quaternary carbons. The DEPT spectrum (DEPT-90 and DEPT-135) in **Table 3** established the existence of 5 methines [at δ 116.59(CH-15), 80.88(CH-3), 55.59(CH-5),49.03(CH-9), and 41.58 (CH-18)] , 8 methyl carbons [at δ 31.92(CH$_3$-29), 28.65(CH$_3$-30), 27.91 (CH$_3$-23), 26.02 (CH$_3$-26), 22.46 (CH$_3$-27), 21.21(COOCH$_3$),16.52 (CH$_3$-24),15.52 (CH$_3$-25)], 10 methylene carbons [at δ 40.83(CH$_2$-7), 37.37(CH$_2$-1), 35.33(CH$_2$-19), 33.64(CH$_2$-12) 33.30(CH$_2$-21), 31.43 (CH$_2$-16), 30.76(CH$_2$-22), 23.42(CH$_2$-2), 18.64(CH$_2$-6), 17.26(CH$_2$-11) and 9 quaternary carbons at [δ 37.88(C-4), 39.00(C-8), 37.87(C-10), 37.37(C-13), 51.44(C-17), 29.22(C-20), 160.53(C-14), 171.06(COOCH$_3$) and 184.03(COOH)] .

The 400 MHz ^{1}H NMR of compound A showed signals at δ 5.47(1H, *dd, J*=3.40,7.90 Hz,H-15), 4.39(1H,*dd,J*=5.50,10.00 Hz,H-3), 1.97(3H,s, COOCH$_3$) and three singlets at δ 0.78(H-25),0.81(H-27)and 1.18(H-26) indicated the presence of 3 methyl groups. Doublet of doublets at δ 4.39(*J*=5.50,10.00 Hz) was observed suggesting the presence of oxymethine proton bonded[48] to an *O*-acetyl group most likely at C-3.

Additionally, doublet of doublets at δ 5.47(1H,*J*=3.40,7.90 Hz) together with olefinic carbon signal at δ 116.59 and 160.53 indicated a taraxer-14-en skeleton[44].

This assignment was confirmed by HMBC experiment as shown in **Table 6**.

Based on the spectral data of compound **A** and comparison of its ^{1}H and ^{13}C NMR with 3-acetylaleuritolic acid shown in **Table 1 and Table 2**, thus, the structure of compound **A** was identified to be 3-acetylaleuritolic acid. (lit[30] m.p.302-304 °C). The compound **A** has been first isolated from *Spilanthes acmella* Murr.

Previously, 3-acetylaleuritolic acid was isolated from various plants such as the seeds of *Phytolacca americana*[44], the barks of *Alerurites montana*[31] and the barks of *Croton urucurana* Baillon.[30].

In 1994, Pengsuparp et al. reported that 3-acetylaleuritolic acid was isolated from *Maprounea africana* and showed potent inhibitory effect against HIV-1 reverse transcriptase at IC_{50} value of 136.0 μM[45].

In 1997, Peres et al. reported the isolation of 3-acetylaleuritolic acid from methanol extract of the barks of *Croton urucurana* Baillon. Such compound inhibited the growth of *Staphylococcus aureus* and *Samonella typhimurium* with the minimum inhibitory concentration (MIC) values of 0.1 mg/mL[46].

Recently in 2000, an antiinflammatory activity of 3-acetylaleuritolic acid was also investigated using the following test: paw edema induced in the rats by carageenin, dextrin and histamine. It was found that 3-acetylaleuritolic acid inhibited antiinflammatory action at a dose of 50 μg/kg[47].

Table 1 ^{1}H NMR spectral data of compound **A** and 3-acetylaleuritolic acid

Position	Compound **A** $^*\delta_H$, mult, *J* (Hz)	3-acetylaleuritolic acid $^*\delta_H$, mult, J(Hz)[44]
H-2	0.80 (*m*)	–
H-3	4.39 (1H, *dd* , 5.50, 10.00)	4.38 (1H, *dd,* 5.80, 10.10)
H-15	5.47 (1H, *dd* , 3.40, 7.90)	5.45 (1H, *dd,* 3.30, 7.90)
H-16	2.21 (1H, *dd* , 3.00, 13.80)	2.21 (1H, *dd* , 3.00, 13.80)
	2.30 (1H, *m*)	2.26 (1H, *m*)
H-24	0.86 (3H, *s*)	-
H-25	0.78 (3H, *s*)	0.79 (3H, *s*)
H-27	0.81 (3H, *s*)	0.84 (3H, *s*)
H-26	1.18 (3H, *s*)	1.18 (3H, *s*)
CH_3COO-	1.97 (3H ,*s*)	1.97 (3H ,*s*)

*^{1}H NMR (400 MHz) in $CDCl_3$

Table 2 ^{13}C NMR spectral data of compound **A** and 3-acetylaleuritolic acid

Position	Compound **A** $*\delta_C$	3-acetylaleuritolic acid[48] $*\delta_C$
1	37.37 (CH_2)	37.34 (CH_2)
2	23.42. (CH_2)	23.43 (CH_2)
3	80.88 (CH)	80.84 (CH)
4	37.88 (C)	37.65 (C)
5	55.59 (CH)	55.64 (CH)
6	18.64. (CH_2)	18.78 (CH_2)
7	40.83 (CH_2)	40.68 (CH_2)
8	39.00 (C)	39.00 (C)
9	49.03 (CH)	49.03 (CH)
10	37.87 (C)	37.90 (C)
11	17.26 (CH_2)	17.28 (CH_2)
12	33.64 (CH_2)	33.62 (CH_2)
13	37.37 (C)	37.27 (C)
14	160.53 (C)	160.50 (C)
15	116.59 (CH)	116.86 (CH)
16	31.43 (CH_2)	31.24 (CH_2)
17	51.44 (C)	51.52 (C)
18	41.58 (CH)	41.30 (CH)
19	35.33 (CH_2)	35.28 (CH_2)
20	29.22 (C)	29.30 (C)
21	33.30 (CH_2)	33.28 (CH_2)
22	30.76 (CH_2)	30.63 (CH_2)
23	27.91 (CH_3)	27.13 (CH_3)
24	16.52 (CH_3)	16.58 (CH_3)
25	15.52 (CH_3)	15.64 (CH_3)
26	26.02 (CH_3)	26.20 (CH_3)

Table 2 (continued)

Position	Compound **A** *δ_C	3-acetylaleuritolic acid[48] *δ_C
27	22.46 (CH_3)	22.46 (CH_3)
28	184.03 (COOH)	184.54 (COOH)
29	31.92 (CH_3)	31.80 (CH_3)
30	28.65 (CH_3)	28.63 (CH_3)
CO ester	171.06 ($COOCH_3$)	171.00 ($COOCH_3$)
CH_3 ester	21.21 (CH_3)	21.29 (CH_3)

* ^{13}C NMR (100 MHz) in $CDCl_3$

Table 3 ^{13}C and DEPT NMR spectral data of compound **A**

δ_C	DEPT- 90	DEPT- 135
37.37 (CH_2)	-	37.37 (CH_2)
23.42. (CH_2)	-	23.42. (CH_2)
80.88 (CH)	80.88 (CH)	80.88 (CH)
37.88 (C)	-	-
55.59 (CH)	55.59 (CH)	55.59 (CH)
18.64 (CH_2)	-	18.64. (CH_2)
40.83 (CH_2)	-	40.83 (CH_2)
39.00 (C)	-	-
49.03 (CH)	49.03 (CH)	49.03 (CH)
37.87 (C)	-	-
17.26 (CH_2)	-	17.26 (CH_2)
33.64 (CH_2)	-	33.64 (CH_2)
37.37 (C)	-	-
160.53 (C)	-	-
116.59 (CH)	116.59 (CH)	116.59 (CH)
31.43 (CH_2)	-	31.43 (CH_2)
51.44 (C)	-	-
41.58 (CH)	41.58 (CH)	41.58 (CH)
35.33 (CH_2)	-	35.33 (CH_2)
29.22 (C)	-	-
33.30 (CH_2)	-	33.30 (CH_2)
30.76 (CH_2)	-	30.76 (CH_2)
27.91 (CH_3)	-	27.91 (CH_3)
16.52 (CH_3)	-	16.52 (CH_3)
15.52 (CH_3)	-	15.52 (CH_3)
26.02 (CH_3)	-	26.02 (CH_3)

Table 3 (continued)

δ_C	DEPT- 90	DEPT- 135
22.46 (CH_3)	-	22.46 (CH_3)
184.03 (COOH)	-	-
31.92 (CH_3)	-	31.92 (CH_3)
28.65 (CH_3)	-	28.65 (CH_3)
171.06 ($COOCH_3$)	-	-
21.21 (CH_3)	-	21.21 (CH_3)

Table 4 COSY correlation among some protons of compound **A**

δ_H (ppm)		Proton correlated with δ_H (ppm)
1.52 (H-1)	↔	4.39 (H-3)
4.39 (H-3)	↔	0.80 (H-2)
5.47 (H-15)	↔	2.21 (H-16)
2.21 (H-16)	↔	0.84 (H-22)

Table 5 ^{1}H - Detected Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectral data of compound **A**

δ_C (ppm)	δ_H (ppm)	assignment
15.52	0.78	CH_3-25
16.52	0.86	CH_3-24
21.21	1.97	CH_3-ester
22.46	0.81	CH_3-27
26.02	1.18	CH_3-26
80.88	4.39	CH-3
116.59	5.47	CH-15

Table 6 Heteronuclear Multiple Bond Connectivity (HMBC) NMR spectral data of compound **A**

δ_C (ppm)		Proton correlated with δ_H (ppm)
80.88	↔	1.52 (H-1), 0.86 (H-24)
160.53	↔	1.18 (H-26)
116.59	↔	2.21(H-16)
51.44	↔	2.21 (H-16)
16.52	↔	4.39 (H-3)
171.06	↔	1.97 ($COOCH_3$)

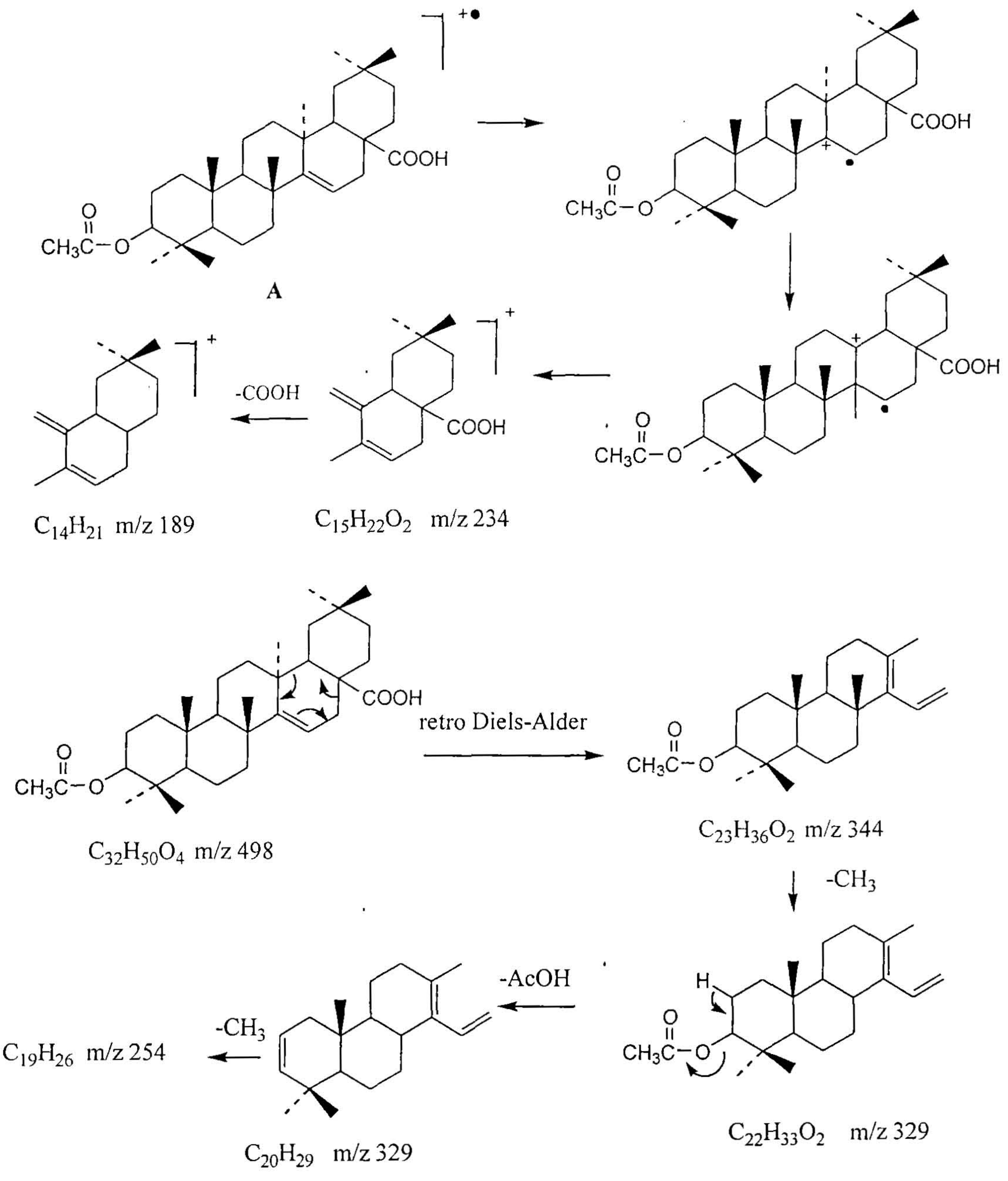
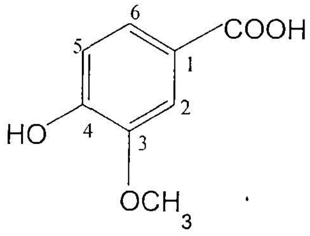


Figure 13 Mass fragmentation pattern of compound A[49]

Identification of compound B



Compound **B** was isolated as white crystal, m.p. 210-212 °C. The structure of compound **B** was elucidated by spectroscopic method. Its molecular formula $C_8H_8O_4$ was established by EIMS. The mass spectrum showed the molecular ion as the base peak at *m/z* 168. The IR spectrum of compound **B** exhibited characteristic broad absorption band for carboxylic group at 2300-3060, hydroxy group at 3486 cm^{-1}. The absorption bands belonging to C=C stretching vibration of aromatic moiety were detected at 1598, 1523, 1434 cm^{-1}. Additionally, C-O stretching were found at 1299, 1239, 1205 cm^{-1}. The UV spectrum showed maximum absorption at 253 and 286 nm.

The 100 MHz ^{13}C NMR of compound **B** exhibited 8 signals. The DEPT spectral data in **Table 8** (DEPT- 90 and DEPT- 135) established the existence of three methine carbons [δ 124.22(CH-6), 114.56(CH-5), 112.63 (CH-2)], four quaternary carbons [δ 169.11(CO), 150.81(C-1), 147.00(C-3),121.90(C-4)] and one methoxy carbon at δ 55.68.

The 400 MHz ^{1}H NMR of compound **B** showed one proton singlet at δ 3.92 and was assigned to be methoxy group (OCH$_3$). In addition, three protons belonging to 1,3,4–trisubstitued benzene ring system were observed at δ 7.55(1H, *d,J*=1.90 Hz, H-2), 7.59(1H, *dd,J*=1.90 ,8.20 Hz, H-6), 6.88(1H, *d,J*=8.20 Hz, H-5). This assignment was confirmed by ^{1}H-^{1}H correlation in cosy spectral data (**Table 9**) including the correlation between H-2 (δ 7.55) with C-2 (δ112.63), H-5 (δ 6.88) with C-5 (δ114.56) and H-6 (δ 7.59) with C-6 (δ 124.22) in HMQC spectral data as shown in **Table 10** . In addition, the connectivity of C-H bond through two and three bonds was observed in HMBC experiment as shown in **Table 11**.

By careful analysis of the obtained spectral data of compound **B** and comparison of ^{1}H and ^{13}C NMR (**Table 7**) including the melting point, thus , compound **B** was determined to be vanillic acid (lit[32] m.p 213-214 °C). This compound **B** has been first isolated from *Spilanthes acmella* Murr.

Vanillic acid exhibited various bioactivities such as antiinflammatory and antioxidant activities[27]. In 1993, Huang et al. reported that resting cells of *Rhodotorula rubra* converted *trans*-ferulic acid to vanillic acid then guaiacol and protocatechuic acid under aerobic condition[34].

Table 7 ^{1}H and ^{13}C NMR spectral data of compound **B** (CD_3OD+$CDCl_3$) and vanillic acid

Position	Compound **B**	Compound **B**	Vanillic acid[34]
	δ_H, mult, *J* (Hz)	δ_C	$*\delta_C$
1	-	150.81 (C)	150.30 (C)
2	7.55 (1H, *d,* 1.90)	112.63 (CH)	113.80 (CH)
3	-	147.00 (C)	148.60 (C)
4	-	121.90 (C)	123.10 (C)
5	6.88 (1H, *d,* 8.20)	114.56 (CH)	113.80 (CH)
6	7.59 (1H, *dd,* 1.90, 8.20)	124.22 (CH)	125..20 (CH)
OCH_3	3.92 (3H, *s*)	55.68 (CH_3)	56.40 (CH_3)
COOH	-	169.11 (CO)	170.00 (CO)

$*^{13}$C NMR in CD_3OD

Table 8 ^{13}C and DEPT NMR spectral data of compound **B**

δ_C	DEPT- 90	DEPT- 135
169.11 (COOH)	-	-
150.81 (C)	-	-
147.00 (C)	-	-
124.22 (CH)	124.22 (CH)	124.22 (CH)
121.90 (C)	-	-
114.56 (CH)	114.56 (CH)	114.56 (CH)
112.63 (CH)	112.63 (CH)	112.63 (CH)
55.68 (OCH_3)	-	55.68 (OCH_3)

Table 9 COSY correlation among some protons of compound **B**

δ_H (ppm)		Proton correlated with δ_H (pp
6.88 (H-5)	↔	7.59 (H-6)
7.55 (H-2)	↔	7.59 (H-6)

Table 10 ^{1}H - Detected Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectral data of compound **B**

δ_C (ppm)	δ_H (ppm)	assignment
124.22	7.59	CH-6
114.56	6.88	CH-5
112.63	7.55	CH-2
55.68	3.92	OCH_3

Table 11 Heteronuclear Multiple Bond Connectivity (HMBC) NMR spectral data of compound **B**

δ_C (ppm)		Proton correlated with δ_H (ppm)
169.11	↔	7.55 (H-2), 7.59 (H-6)
150.81	↔	6.88 (H-5), 7.55 (H-2), 7.59 (H-6)
147.00	↔	3.92 (OCH_3), 6.88 (H-5), 7.55 (H-2)
124.22	↔	7.55 (H-2)
121.90	↔	6.88 (H-5), 7.55 (H-2)
112.63	↔	7.59 (H-6)

Shown only two or three bonds correlation

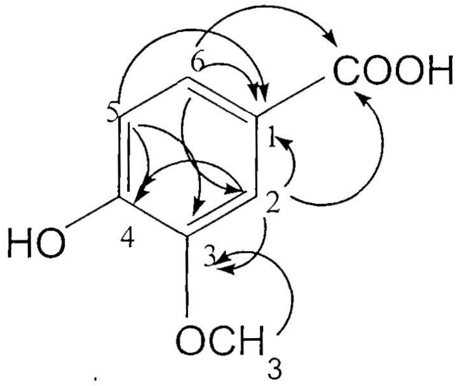
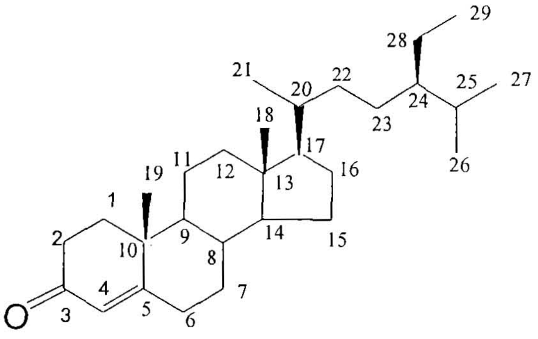


Figure 14 HMBC of compound **B**

Identification of compound C



Compound **C** was isolated as white crystal solid, m.p. 97-99 °C. The structure of compound **C** was elucidated by spectroscopic method. Its molecular formula $C_{29}H_{50}O$ was established by EIMS showing the molecular ion at *m/z* 412, the base peak at *m/z* 124, and typical ions of 4-en-3-ketosteroids[36] at m/z 397, 370, 288, 271, 229 as shown in **Figure 15** . The IR spectrum of compound **C** exhibited characteristic absorption band for α, β- unsaturated carbonyl group at 1654 cm^{-1} .

The 100 MHz ^{13}C NMR of compound **C** exhibited 29 carbon signals consisting of 6 methyl,11 methylene, 8 methine and 4 quaternary carbons. The DEPT spectral data in **Table 13** (DEPT-90 and DEPT-135) established the existence of 8 methines δ 29.64(CH-25), 35.60(CH-8), 36.07(CH-20), 45.81(CH-24), 53.79(CH-9), 55.85(CH-14), 55.99(CH-17) and 123.69 (CH-4), 6 methyls at δ 11.14(CH_3-29), 11.90(CH_3-18), 18.65 (CH_3-19),18.98(CH_3-21),19.75(CH_3-27), 20.99(CH_3-26), 11 methylene carbons at δ 21.10(CH_2-11), 23.04(CH_2-28), 24.14(CH_2-15), 26.08(CH_2-23), 28.13(CH_2-16), 32.91 (CH_2-7), 33.86(CH_2-6), 33.93(CH_2-2), 35.65(CH_2-22),36.06(CH_2-1), and 38.57(CH_2-12) and 4 quaternary carbons at δ 39.59(C-10), 42.35(C-13), 171.64(C-5),199.58 (C=O).

The 400 MHz ^{1}H NMR of compound **C** showed a broad singlet signal of olefinic proton (H-4) at δ 5.74 connecting to C-4 at δ 123.69 (HMQC spectrum). HMBC spectrum showed C-H connectivity through two and three bonds (**Table 16**) . It was observed that H-4 (δ 5.74) related with C-2 (δ 33.93) and C-6 (δ 33.86), H-2(δ 2.39) and H-6 (δ 2.37) related with C-4 (δ123.69), H-1 (δ 1.68) related with CO (δ 199.58) and C-5 (δ 171.64). Additionally, C-5 related with CH_3 (δ1.18 at C-19) and H-7 (δ 2.27) These data implied that the olefinic moiety was conjugated with CO group.

Two singlets of CH_3 at C-18 and C-19 appeared at δ 0.71 and 1.18 respectively. Multiplet signals at δ 0.80-1.10 was corresponded to four methyls at C-21,26,27 and 29. Another multiplet signals at δ 1.20-2.50 suggested the presence of methylene protons, some of these are adjacent to α, β- unsaturated carbonyl group.

By careful analysis of the obtained spectral data of compound **C** and comparison of its ^{13}C NMR with β-sitostenone shown in **Table 12**, including the mass fragmentation (**Figure 15**), it was indicated that compound **C** was β-sitostenone of m.p.97-99 °C (lit[35] 95-96 °C). Our study show that compound **C** has been first isolated from *Spilanthes acmella* Murr.

Previously in 1974, Sheikh and Djearassi reported that β-sitostenone was isolated from *Stelleta clarella*[50] of the sponge and mature wheat straw[36].

In 1994, Hirano et al. investigated the effect of steroidal components stringing nettle roots on activity of Na^+,K^+-ATPase on Benign Prostatic Hyperplasia (BPH). It was found that β-sitostenone showed an inhibitory effect at concentrations ranging from 10^{-6}-10^{-4} M on BPH Na^+,K^+- ATPase activity [51].

Recently in 2000, Balde et al. reported that β-sitostenone was isolated from stem bark of *Harrisonia obyssinica* [52].

Table 12 ^{13}C NMR spectral data of compound **C** and β-sitostenone

Position	Compound **C**	β − sitostenone[53]
	$*\delta_C$	$*\delta_C$
1	36.06 (CH_2)	35.68 (CH_2)
2	33.93. (CH_2)	33.89 (CH_2)
3	199.58(CO)	198.92 (CO)
4	123.69 (CH)	123.64 (CH)
5	171.64 (C)	171.01 (C)
6	33.86 (CH_2)	32.86 (CH_2)
7	32.91 (CH_2)	32.07 (CH_2)
8	35.60 (CH)	35.73 (CH)
9	53.79 (CH)	53.84 (CH)
10	39.59 (C)	38.58 (C)
11	21.10 (CH_2)	21.13 (CH_2)
12	38.57 (CH_2)	39.48 (CH_2)
13	42.35 (C)	42.35 (C)
14	55.85 (CH)	55.94 (CH)
15	24.14 (CH_2)	24.12 (CH_2)
16	28.13 (CH_2)	28.10 (CH_2)
17	55.99 (CH)	56.63 (CH)
18	11.90 (CH_3)	12.85 (CH_3)
19	18.65 (CH_3)	17.38 (CH_3)
20	36.07 (CH)	36.10 (CH)
21	18.98 (CH_3)	18.72 (CH_3)
22	35.65 (CH_2)	34.01 (CH_2)
23	26.08 (CH_2)	25.99 (CH_2)
24	45.81 (CH)	45.80 (CH)
25	29.64 (CH)	29.11 (CH)
26	20.99 (CH_3)	19.81 (CH_3)

Table 12 (continued)

Position	Compound **C**	β – sitostenone[53]
	$*\delta_C$	$*\delta_C$
27	19.75 (CH_3)	19.18 (CH_3)
28	23.04 (CH_2)	23.10 (CH_2)
29	11.14 (CH_3)	11.14 (CH_3)

$*^{13}C$ NMR (100 MHz) in $CDCl_3$

Table 13 ^{13}C and DEPT NMR spectral data of compound **C**

δ_C	DEPT- 90	DEPT- 135
36.06 (CH_2)	-	36.06 (CH_2)
33.93. (CH_2)	-	33.93. (CH_2)
199.58(CO)	-	-
123.69 (CH)	123.69 (CH)	123.69 (CH)
171.64 (C)	-	-
33.86 (CH_2)	-	33.86 (CH_2)
32.91 (CH_2)	-	32.91 (CH_2)
35.60 (CH)	35.60 (CH)	35.60 (CH)
53.79 (CH)	53.79 (CH)	53.79 (CH)
39.59 (C)	-	-
21.10 (CH_2)	-	21.10 (CH_2)
38.57 (CH_2)	-	38.57 (CH_2)
42.35 (C)	-	-
55.85 (CH)	55.85 (CH)	55.85 (CH)
24.14 (CH_2)	-	24.14 (CH_2)
28.13 (CH_2)	-	28.13 (CH_2)
55.99 (CH)	55.99 (CH)	55.99 (CH)
11.90 (CH_3)	-	11.90 (CH_3)
18.65 (CH_3)	-	18.65 (CH_3)
36.07 (CH)	36.07 (CH)	36.07 (CH)
18.98 (CH_3)	-	18.98 (CH_3)
35.65 (CH_2)	-	35.65 (CH_2)
26.08 (CH_2)	-	26.08 (CH_2)
45.81 (CH)	45.81 (CH)	45.81 (CH)
29.64 (CH)	29.64 (CH)	29.64 (CH)
20.99 (CH_3)	-	20.99 (CH_3)

Table 13 (continued)

δ_C	DEPT- 90	DEPT- 135
19.75 (CH_3)	-	19.75 (CH_3)
23.04 (CH_2)	-	23.04 (CH_2)
11.14 (CH_3)	-	11.14 (CH_3)

Table 14 COSY correlation among some protons of compound **C**

δ_H (ppm)		Proton correlated with δ_H (ppm)
2.39 (H-2)	↔	1.68 (H-1)
2.27 (H-7)	↔	2.03 (H-8)
2.03 (H-8)	↔	1.08 (H-9)

Table 15 ^{1}H - detected Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectral data of compound **C**

δ_C (ppm)	δ_H (ppm)	assignment
36.06	1.68	CH_2-1
33.93	2.39	CH_2-2
123.69	5.74	CH-4
33.86	2.37	CH_2-6
32.91	2.27	CH_2-7
35.60	2.03	CH-8
53.79	1.08	CH-9
55.85	1.13	CH-14
11.90	0.71	CH_3-18
18.65	1.18	CH_3-19
45.81	0.94	CH-24
29.64	1.27	CH-25

Table 16 Heteronuclear Multiple Bond Connectivity (HMBC) NMR spectral data of compound **C**

δ_C (ppm)		Proton correlated with δ_H (ppm)
36.06	↔	5.74 (H-4)
33.93	↔	5.74 (H-4)
123.69	↔	2.39 (H-2), 2.37 (H-6)
171.64	↔	1.68 (H-1), 2.27(H-7),1.18(H-19)
33.86	↔	5.74 (H-4), 2.03(H-8)
199.58	↔	1.68 (H-1)

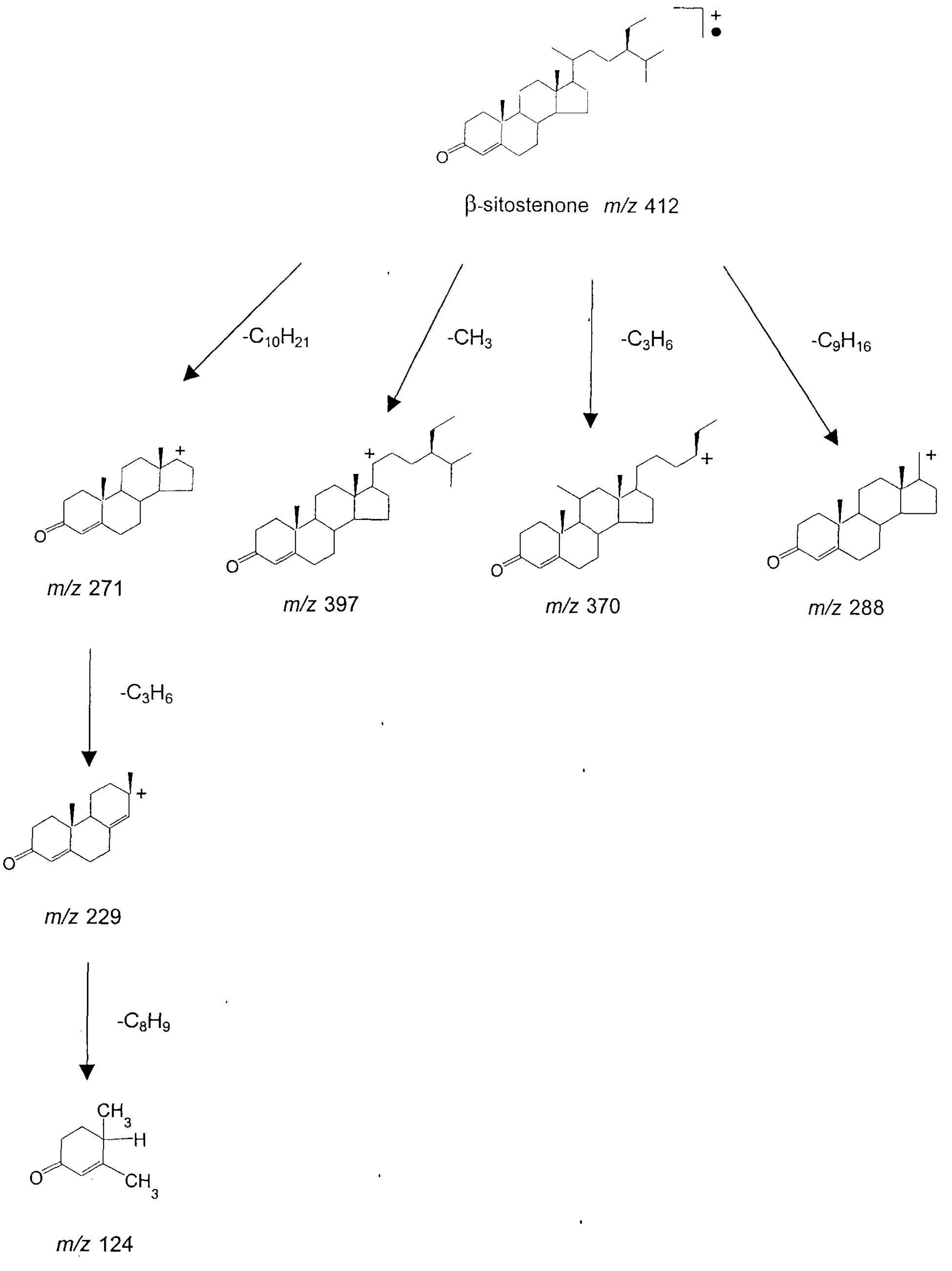
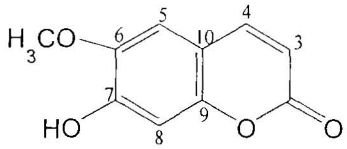


Figure 15 Mass fragmentation pattern of compound **C**

Identification of compound D



Compound **D** was isolated as yellowish needle crystal of m.p. 205-206 °C. The structure of compound **D** was elucidated by spectroscopic methods. Its molecular formula $C_{10}H_8O_4$ was established by EIMS showing the molecular ion as the base peak at m/z 192. The ion peak at m/z 177 was resulted by the loss of methoxy group as shown in **Figure 17.** This suggested the presence of methoxy group in the molecule of compound **D**. The UV spectrum showed maximum absorption at 294 and 344 nm.

The IR spectrum of compound **D** exhibited characteristic absorption band of hydroxy group at 3333 cm^{-1}, and conjugated carbonyl group of lactone at 1702 cm^{-1}. The absorption bands belonging to C=C stretching vibration of aromatic moiety were detected at 1566 and 1437 cm^{-1}.

The 100 MHz ^{13}C NMR of compound **D** exhibited 10 signals. The DEPT spectral data (DEPT-90 and DEPT-135) in **Table 19** established the existence of four methine carbons at δ 144.91(CH-4), 111.15(CH-3), 108.63(CH-5), 102.57(CH-8) and five quaternary carbons at δ 163.00(CO), 150.00(C-9), 149.00(C-7), 146.00(C-6), 110.00 (C-10), one methoxy carbon at δ 55.51(OCH_3).

The 400 MHz ^1H NMR of compound **D** showed two doublets signals at δ 6.23 (1H, *d,J*=9.42 Hz, H-3) and 7.89(1H, *d,J*=9.42 Hz,H-4) implying that the substituents were located on an aryl ring instead of an unsaturated lactone ring. Two singlet signals of aromatic protons appeared at δ 6.82 (1H,*s*,H-8) and 7.14(1H,*s*, H-5). The sharp singlet with integration of three protons was detected at δ 3.91 assigning for methoxy protons.

This assignment was confirmed by ^1H-^1H correlation in cosy spectral data (**Table20**) including the connectivity of H-8 (δ 6.82) with C-8 (δ 102.57) and H-5 (δ 7.14) with C-5 (δ 108.63) in HMQC experiment as shown in **Table 21.** In addition, the connectivity of C-H through two or three bonds was observed in HMBC spectral data as shown in **Table 22** .

Based on the spectral data of compound **D** and comparison of its ^1H and ^{13}C NMR with scopoletin shown in **Table 17** and **Table 18**. The structure of compound **D** was concluded to be scopoletin or 7-hydroxy-6-methoxycoumarin which its m.p. was reported to be 203-205 °C[37].

Scopoletin was found in fungi and in many botanical families including important crops, for example, cereals of compositae and legumes of solnaceae[54].

In 1999, Hyun Kang et al. reported that scopoletin was isolated from water extract of *Artemisia feddei* which beloging to family of compositae. It was found that scopoletin inhibited nitric oxide (NO) synthesis in murine macrophage-like RAW 264.7 cells at dose of 50 µg/mL[39]. In addition, Fojioka reported that scopoletin exhibited antiproliferative activity against B6F10 (murine melanoma) cell at value of 14.2 µg/mL[55].

Recently in 2000, Obidoa and Ezeanyika et al. reported the study of comparative effects of scopoletin and cyanide on glucose-6-phosphatase and glutathione-s-transferase activities of rat liver microsomes. The results showed that scopoletin decreased the glucose-6-phosphatase and glutathione-s-transferase activities while the cyanide increased these activities[56].

Table 17 ^1H NMR spectral data of compound **D** (in CD_3OD) and scopoletin

Position	Compound **D**	Scopoletin
	δ_H, mult, *J* (Hz)	*δ_H, mult, *J* (Hz)[36]
3	6.23 (1H, *d*, 9.42)	6.28 (1H, *d*, 10.00)
4	7.89 (1H, *d*, 9.42)	7.70 (1H, *d*, 10.00)
5	7.14 (1H, *s*)	7.10 (1H, *s*)
8	6.82 (1H, *s*)	7.05 (1H, *s*)
OH	8.10 (1H, *s*)	10.10 (1H, *s*)
OCH_3	3.91(1H, *s*)	3.79 (1H, *s*)

*^1H NMR (60 MHz) in Pyridine-d_5

Table 18 ^{13}C NMR spectral data of compound **D** (in CD_3OD) and scopoletin.

Position	Compound **D**	Scopoletin[57]
	δ_C	$*\delta_C$
2	163.00 (CO)	160.20 (CO)
3	111.15 (CH)	112.50 (CH)
4	144.91 (CH)	142.30 (CH)
5	108.63 (CH)	107.00 (CH)
6	146.00 (C)	143.20 (C)
7	149.00 (C)	149.20 (C)
8	102.57 (CH)	102.50 (CH)
9	150.00 (C)	149.80 (C)
10	110.00 (C)	110.50 (C)
OCH_3	55.51 (CH_3)	55.20 (CH_3)

$*^{13}C$ NMR (100 Hz) in DMSO-d_6

Table 19 ^{13}C and DEPT NMR spectral data of compound **D**

δ_C	DEPT- 90	DEPT- 135
144.91 (CH)	144.91 (CH)	144.91 (CH)
111.15 (CH)	111.15 (CH)	111.15 (CH)
108.63 (CH)	108.63 (CH)	108.63 (CH)
102.57 (CH)	102.57 (CH)	102.57 (CH)
55.51 (OCH_3)	-	55.51 (OCH_3)

Table 20 COSY correlation among some protons of compound **D**

δ_H (ppm)		Proton correlated with δ_H (ppm)
6.23 (H-3)	↔	7.89 (H-4)

Table 21 ^{1}H - Detected Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectral data of compound **D**

δ_C (ppm)	δ_H (ppm)	assignment
144.91	7.89	CH-4
111.15	6.23	CH-3
108.63	7.13	CH-5
102.57	6.82	CH-8
55.51	3.92	OCH_3

Table 22 Heteronuclear Multiple Bond Connectivity (HMBC) NMR spectral data of compound **D**

δ_C (ppm)		Proton correlated with δ_H (ppm)
163.00	↔	6.23 (H-3), 7.89 (H-4)
146.00	↔	3.91(OCH_3), 6.82 (H-8)
150.00	↔	7.89 (H-4), 7.14 (H-5),6.82 (H-
144.91	↔	6.82 (H-8), 7.14 (H-5)
108.63	↔	7.89 (H-4)

Shown only two or three bonds correlation

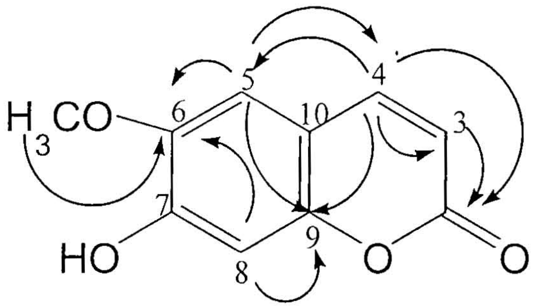


Figure 16 HMBC of compound **D**

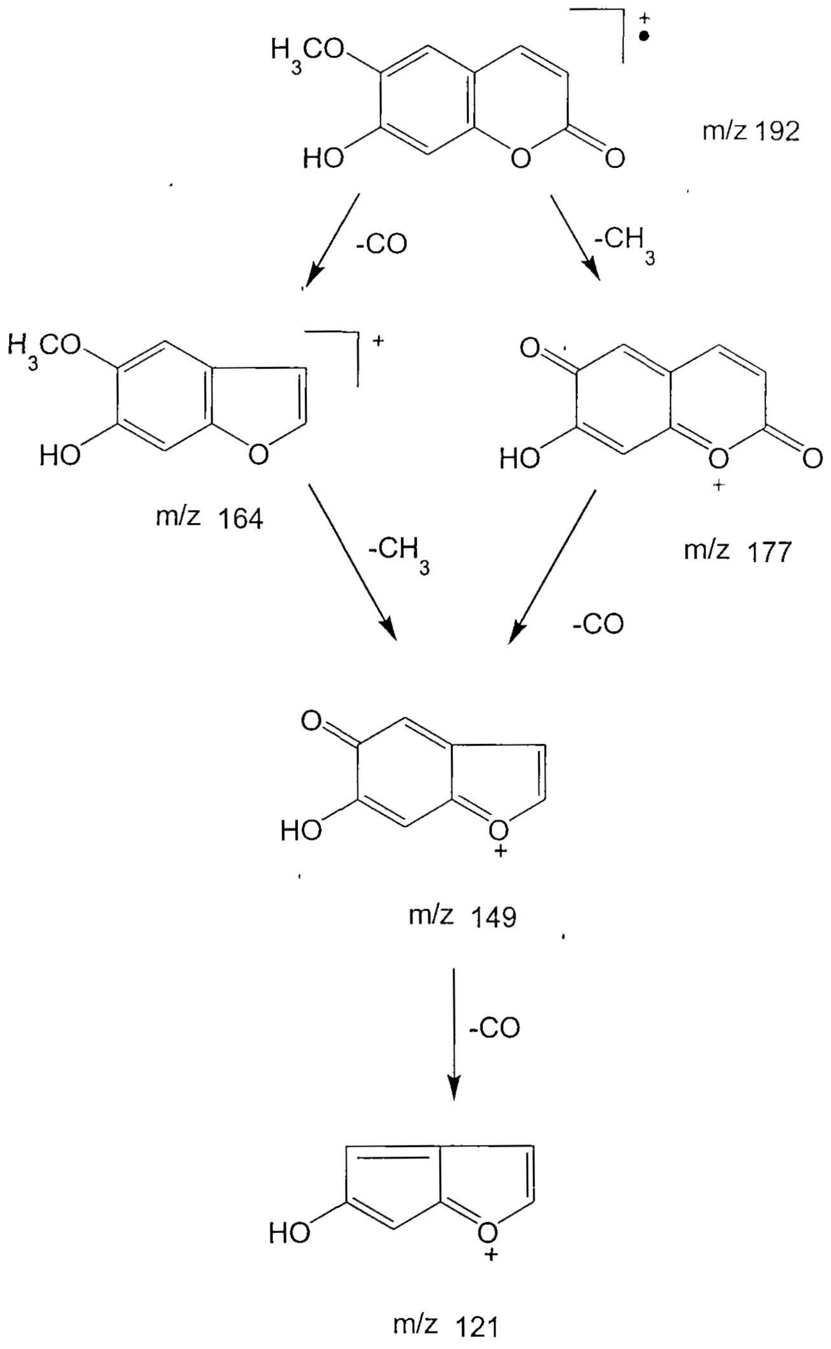
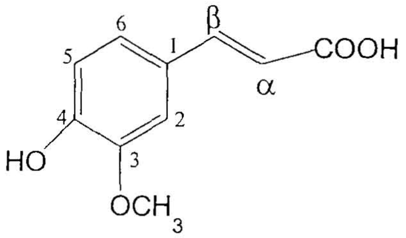


Figure 17 Mass fragmentation pattern of compound **D**

Identification of compound E



Compound **E** was isolated as brownish crystal of m.p.168-169°C. The structure of **E** was elucidated by spectroscopic method. Its molecular formula $C_{10}H_{10}O_4$ was established by EIMS showing the molecular ion as the base peak at *m/z* 194. The prominent ion peaks at *m/z* 179 and 177 which were formed by loss of methyl and hydroxy group, respectively as depicted in **Figure 19**. The IR spectrum of compound **E** exhibited characteristic absorption band for carboxylic group at 3437 cm^{-1} and conjugated carbonyl group at 1691 cm^{-1}. The absorption bands belonging to C=C stretching vibration of aromatic moiety were detected at 1665 and 1517 cm^{-1}. The UV spectrum showed maximum absorption at 289 and 318 nm.

The 100 MHz ^{13}C NMR of compound **E** exhibited 10 signals. The DEPT spectral data in **Table 25** (DEPT-90 and DEPT-135) established the existence of five methine carbons at δ 123.97 (CH-6), 116.46 (CH-5), 115.89 (CH-α), 111.64(CH-2) and 146.95 (CH-β). Four quaternary carbons were observed at δ 171.19(CO), 151.50(C-3), 149.90 (C-4) and 127.76(C-1). One methoxy carbon was shown at δ 56.45.

The 400 MHz ^{1}H NMR of compound **E** showed three protons belonging to 1,3,4-trisubstituted benzene ring system at δ 7.18(1H, *d,J*=1.93 Hz, H-2), 7.07(1H, *dd,J*=1.93, 8.23 Hz, H-6), 6.82 (1H, *d*, *J*=8.23 Hz, H-5). Ortho and meta coupling constants (8.23 and 1.93 Hz) for aromatic protons were observed implying 1,3,4–trisubstitution pattern for the benzene ring. Two doublets at δ 6.31 (*J*=15.88 Hz, H-α) and 7.59 (*J*=15.88 Hz,H-β) were observed suggesting the presence of *trans* olefinic protons. In addition, a singlet of one methoxy was shown at δ 3.89.

In cosy experiment ^{1}H-^{1}H correlation were observed (**Table 26**), H-5 at δ 6.82 correlated with H-6 at δ 7.07, H-α at δ 6.31 correlated with H-β at δ 7.59. Additionally, the meta coupling of H-6 (δ 7.07) and H-2 (δ7.18) was also noted. Assignment of direct C-H connectivity was well correlated as observed in HMQC spectral data shown in **Table 27**. HMBC spectral data of compound **E** showed C-H connectivity through two and three bonds. In case of C-1 at δ 127.76 which connected with H-α and H-β through two bonds and three bonds, respectively as depicted in **Table 28**. According to the available spectral data of compound **E** and comparison of its ^{1}H and ^{13}C NMR with *trans*-ferulic acid as shown in **Table 23** and **Table 24**, thus, we concluded that compound **E** was *trans*-ferulic acid (lit[40] m.p.168-169 °C). However, our isolated *trans*-ferulic acid is first found in *Spilanthes acmella* Murr.

Trans-ferulic acid, a secondary plant metabolite, occurs primarily in seeds and leaves both in free form and covalently linked to mono and disaccharide[58].

At present, *trans*-ferulic acid has gained popularity as a synthetic antioxdants for food preservation. Interestingly, it is currently used as an active ingredient in many lotion for photoprotection[58].

In 1995, Hirabayashi et al. reported an inhibitory effect of both *trans*-ferulic acid and *trans*- isoferulic acid on murine interleukin-8 (IL-8) production in response to influenza virus infections. The results showed that IL-8 levels were reduced to 43% and 56% of the control in the presence of 100 μg/mL of *trans*- ferulic and *trans*-isoferulic acid, respectively[59].

In 1996, antioxidant activity of cinnamic acid and *trans*-ferulic acid was studied by measuring their protectives actions toward linoleic acid peroxidation in micelles of sodium dodecyl sulfate in buffer solution at pH 7.4. It was found that *trans*-ferulic acid showed low antioxidant activity with RAE (relative antioxidant efficient) value of 0.04 by comparison with other cinnamic acids[60].

Recently,in 2001. Pretorius and Watt reported that *trans*-ferulic acid was isolated from methanolic crude extract of *Carpobrotus edulis* L. The *trans*-ferulic acid exhibited antimicrobial activity against *Bacillus subtilis* and *Streptococcus pneumoniae* at a concentration of 10 μg/mL[61].

Table 23 ^{1}H NMR spectral data of compound **E** and *trans*-ferulic acid

Position	Compound **E**	*trans*-ferulic acid
	$*\delta_H$, mult, *J* (Hz)	$*\delta_H$, mult, *J* (Hz)[62]
2	7.18 (1H, *d*, 1.93)	7.24 (1H, *d*, 2.40)
5	6.82 (1H, *d*, 8.23)	6.84 (1H, *d*, 8.50)
6	7.07 (1H, *dd,* 1.93, 8.23)	7.13 (1H, *dd,2.40*,8.50)
α	6.31 (1H, *d,* 15.88)	6.21 (1H, *d,* 16.00)
β	7.59 (1H, *d,* 15.88)	7.79 (1H, *d,* 16.00)
OCH_3	3.89 (3H,*s*)	3.91 (3H,*s*)

$*{}^{1}$H NMR (400 MHz) in CD_3OD

Table 24 ^{13}C NMR spectral data of compound **E** and *trans*-ferulic acid

Position	Compound **E**	*trans*-ferulic acid[62]
	$*\delta_C$	$*\delta_C$
1	127.76 (C)	127.69 (C)
2	111.64 (CH)	111.94 (CH)
3	151.50 (C)	150.81 (C)
4	149.90 (C)	149.38 (C)
5	116.46 (CH)	116.53 (CH)
6	123.97 (CH)	124.36 (CH)
α	115.89 (CH)	114.53 (CH)
β	146.95 (CH)	148.16 (CH)
OCH_3	56.45 (OCH_3)	56.52 (OCH_3)
COOH	171.19 (COOH)	170.65 (COOH)

$*^{13}C$ NMR (100 MHz) in CD_3OD

Table 25 ^{13}C and DEPT NMR spectral data of compound **E**

δ_C	DEPT- 90	DEPT- 135
171.19 (COOH)	-	-
151.50 (C)	-	-
149.90 (C)	-	-
146.95 (CH)	146.95 (CH)	146.95 (CH)
127.76 (C)	-	-
123.97 (CH)	123.97 (CH)	123.97 (CH)
116.46 (CH)	116.46 (CH)	116.46 (CH)
115.89 (CH)	115.89 (CH)	115.89 (CH)
111.64 (CH)	111.64 (CH)	111.64 (CH)
56.45 (OCH_3)	-	56.45 (OCH_3)

Table 26 COSY correlation among some protons of compound **E**

δ_H (ppm)		Proton correlated with δ_H (ppm)
6.31 (H-α)	↔	7.59 (H-β)
6.82 (H-5)	↔	7.07 (H-6)
7.07 (H-6)	↔	6.82 (H-2)

Table 27 ^{1}H - Detected Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectral data of compound **E**

δ_C (ppm)	δ_H (ppm)	assignment
171.19	-	COOH
151.50	-	C-3
149.90	-	C-4
146.95	7.59	CH-β
127.76	-	C-1
123.97	7.07	CH-6
116.46	6.82	CH-5
115.89	6.31	CH-α
111.64	7.18	CH-2
56.45	3.89	OCH_3

Table 28 Heteronuclear Multiple Bond Connectivity (HMBC) NMR spectral data of compound **E**

δ_C (ppm)		Proton correlated with δ_H (ppm)
171.19	↔	6.31 (H-α), 7.59 (H-β)
151.50	↔	6.82(H-5), 3.89(OCH_3)
149.90	↔	7.18(H-2), 7.07(H-6)
146.95	↔	6.31 (H-α), 7.07 (H-6), 7.18 (H-2)
127.76	↔	6.31 (H-α), 6.82 (H-5), 7.18 (H-2), 7.59 (H-β)
123.37	↔	6.82 (H-5), 7.18 (H-2), 7.59 (H-β)
116.46	↔	7.07 (H-6), 7.59 (H-β)
115.89	↔	7.07 (H-6), 7.59 (H-β)
111.64	↔	7.07 (H-6), 7.59 (H-β)

Shown only two or three bonds correlation

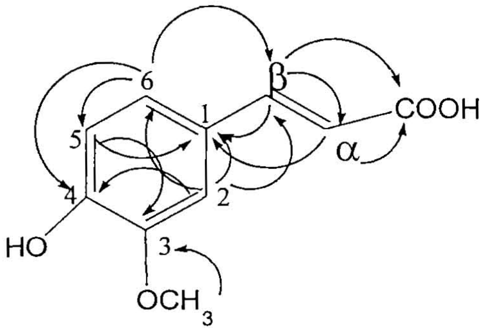


Figure 18 HMBC of compound **E**

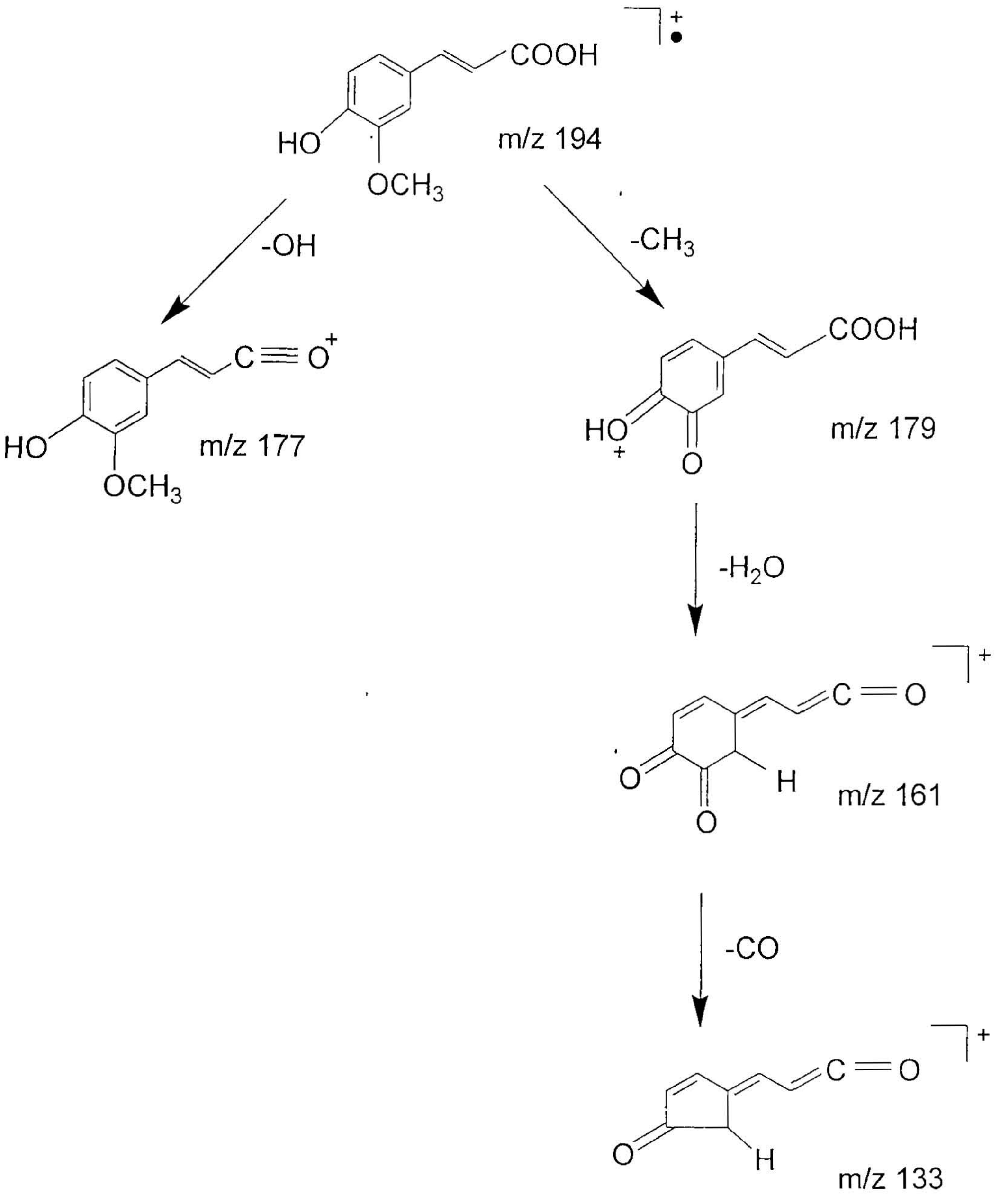
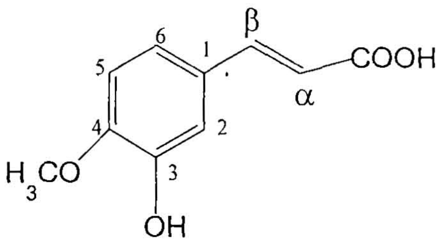


Figure 19 Mass fragmentation pattern of compound **E**

Identification of compound F



Compound **F** was isolated as brownish crystal, m.p. 230-232 °C. The structure of compound **F** was elucidated by spectroscopic method. Its molecular formula $C_{10}H_{10}O_4$ was established by EIMS showing the molecular ion at m/z 194 as the base peak. The prominent ion peak at m/z 179 and 177 which were formed by loss of methyl and hydroxy group shown in **Figure 21**. The UV spectrum showed maximum absorption at 289 and 313 nm.

The IR spectrum of compound **F** exhibited characteristic absorption bands for hydroxy group (3438 cm^{-1}) and conjugated carbonyl group at 1692 cm^{-1}. The absorption bands belonging to C=C stretching vibration of aromatic moiety were detected at 1665, 1621 and 1517 cm^{-1}. The C-O stretching were observed at 1277,1206 and 1178 cm^{-1}.

The 100 MHz ^{13}C NMR of compound **F** exhibited 10 signals. The DEPT spectral data in **Table 31** (DEPT-90 and DEPT-135) established the existence of five methine carbons at δ 110.50(CH-2), 115.28(CH-α), 115.57(CH-5), 123.28(CH-6),146.11(CH-β). The signals of four quaternary carbons were shown at δ 170.25 (CO), 148.98 (C-3), 147.97 (C-4) and 126.83(C-1), One methoxy carbon appeared at δ 56.04 (OCH_3).

The 400 MHz ^1H NMR of compound **F** showed three protons belonging to 1,3,4-trisubstitued benzene ring system at δ 7.07 (1H, *d, J= 1.67* Hz, H-2), 7.05 (1H, *dd, J=* 8.00, 1.67 Hz, H-6), 6.87 (1H, *d,J*=8.00 Hz, H-5). Two doublets at δ 6.26 (*J*=15.90 Hz, H-α) and 7.61 (*J*=15.90 Hz, H-β) were observed suggesting the presence of double bond which compatible with trans olefinic protons. In addition, a singlet of one methoxy was shown at δ 3.91.

In cosy experiment, H-5 at δ 6.87 was well correlated with H-6 at δ 7.05. In addition, the correlation of H-α (δ 6.26) and H-β (δ 7.61) was observed as shown in **Table 32**. Assignment of direct C-H connectivity was well correlated as observed in

HMQC spectral data shown in **Table 33** . The HMBC spectral data of compound **F** showed C-H connectivity through two or three bonds. In case of C-3 at (δ 148.98) which connected with H-2 (δ 7.07) and H-5 (δ 6.87) through two bonds and three bonds, respectively as depicted in **Table 34**.

According to the available spectral data of compound **F** and comparison of its ^{1}H and ^{13}C NMR with *trans*-isoferulic acid as shown in **Table 29** and **Table 30**, thus, we concluded that compound **F** was *trans*-isoferulic acid (lit[40] m.p.230°C). This compound **F** has been first isolated from *Spilanthes acmella* Murr.

Previously, *trans*-isoferulic acid was isolated from *veronicastrum sibiricum* (L.) , Pennel., and possessed antiinflammatory and analgesic activities. In addition, *trans*-isoferulic acid is a main active ingradient of the rhizoma of *Cimicifuga beracleifolia*, generally used in Japanese traditional medicines as an antiinflammatory drug. It was revealed that *trans*-isoferulic acid inhibited the production of macrophage inflammatory protein-2(MIP-2). This may contribute to the pathogenesis of inflammatory diseases and immune effector cells. Furthermore, Sakai et al. reported that administration of *trans*-isoferulic acid markedly improved the survival rate of influenza virus-infected mice at a dose of 0.5 mg/day. The result showed that *trans*-isoferulic acid was a better inhibitor than dexamethasone which is a potent inhibitor for inflammatory cytokines[63].

Recently in 2000, Liu et al. reported that *trans*-isoferulic acid showed antihyperglycemic action at a dose of 5.0 mg/kg, which decreased the plasma glucose in steptozotocin-induced diabetic rats[64].

Table 29 ^{1}H NMR spectral data of compound **F** (in CD_3OD+$CDCl_3$) and *trans* -isoferulic acid

Position	Compound **F**	*trans*- isoferulic acid
	δ_H , mult, *J* (Hz)	*δ_H , mult, *J* (Hz)[62]
2	7.07 (1H, *d* , 1.67)	7.14 (1H, *d* , 2.50)
5	6.87 (1H, *d* , 8.00)	6.97 (1H, *d* , 8.20)
6	705 (1H, *dd,1.67* ,8.20)	7.11 (1H, *dd,2.50* ,8.20)
α	6.26 (1H, *d,* 15.90)	6.47 (1H, *d,* 16.00)
β	7.61 (1H, *d,* 15.90)	7.75 (1H, *d,* 16.00)
OCH_3	3.91 (3H, *s*)	3.90 (3H,*s*)

*^{1}H NMR (400 MHz) in CD_3OD

Table 30 ^{13}C NMR spectral data of compound **F** (in $CD_3OD+CDCl_3$) and *trans* - isoferulic acid

Position	Compound **F** δ_C	*trans* -isoferulic acid[62] $*\delta_C$
1	126.83 (C)	128.91 (C)
2	110.50 (CH)	115.32 (CH)
3	148.98 (C)	148.08 (C)
4	147.97 (C)	151.74 (C)
5	115.57 (CH)	112.64 (CH)
6	123.28 (CH)	123.06 (CH)
α	115.28 (CH)	114.96 (CH)
β	146.11(CH)	147.82 (CH)
COOH	170.25 (COOH)	170.09 (COOH)
OCH_3	56.04 (OCH_3)	56.43(OCH_3)

$*^{13}$C NMR (100 MHz) in CD_3OD

Table 31 ^{13}C and DEPT NMR spectral data of compound **F**

δ_C	DEPT- 90	DEPT- 135
170.25 (COOH)	-	-
148.98 (C)	-	-
147.97 (C)	-	-
146.11 (CH)	146.11 (CH)	146.11 (CH)
126.83 (C)	-	-
123.28 (CH)	123.28 (CH)	123.28 (CH)
115.57 (CH)	115.57 (CH)	115.57 (CH)
115.28 (CH)	115.28 (CH)	115.28 (CH)
110.50 (CH)	110.50 (CH)	110.50 (CH)
56.04 (OCH_3)	-	56.04 (OCH_3)

Table 32 COSY correlation among some protons of compound **F**

δ_H (ppm)		Proton correlated with δ_H (ppm
6.26 (H-α)	↔	7.61 (H-β)
6.87 (H-5)	↔	7.05 (H-6)
7.05 (H-6)	↔	6.87 (H-5)
7.61 (H-β)	↔	6.26 (H-α)

Table 33 ^{1}H - Detected Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectral data of compound **F**

δ_C (ppm)	δ_H (ppm)	assignment
170.25	-	CO
148.98	-	C-3
147.97	-	C-4
146.11	7.61	CH-β
126.83	-	C-1
123.28	7.05	CH-6
115.57	6.87	CH-5
115.28	6.26	CH-α
110.50	7.07	CH-2
56.04	3.91	OCH_3

Table 34 Heteronuclear Multiple Bond Connectivity (HMBC) NMR spectral data of compound **F**

δ_C (ppm)		Proton correlated with δ_H (ppm)
170.25	↔	6.26 (H-α), 7.61 (H-β)
148.98	↔	6.87 (H-5), 7.07(H-2)
147.97	↔	3.91 (OCH_3),7.07(H-2), 7.05(H-6)
146.11	↔	6.26 (H-α), 7.07(H-2), 7.05(H-6)
126.83	↔	6.26 (H-α), 6.87 (H-5), 7.07(H-2), 7.61 (H-β)
123.27	↔	6.26 (H-α), 6.87 (H-5), 7.07(H-2), 7.61 (H-β)
115.57	↔	7.07(H-2), 7.05(H-6), 7.61 (H-β)
115.28	↔	7.07(H-2), 7.05(H-6), 7.61 (H-β)
110.50	↔	6.87 (H-5), 7.05(H-6), 7.61 (H-β)

Shown only two or three bonds correlation

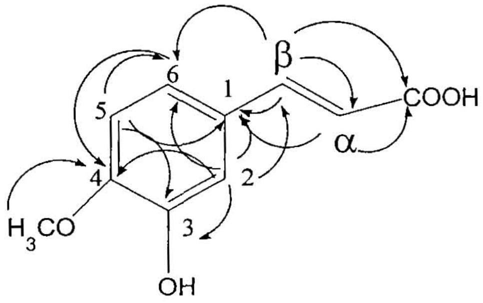


Figure 20 HMBC of compound **F**

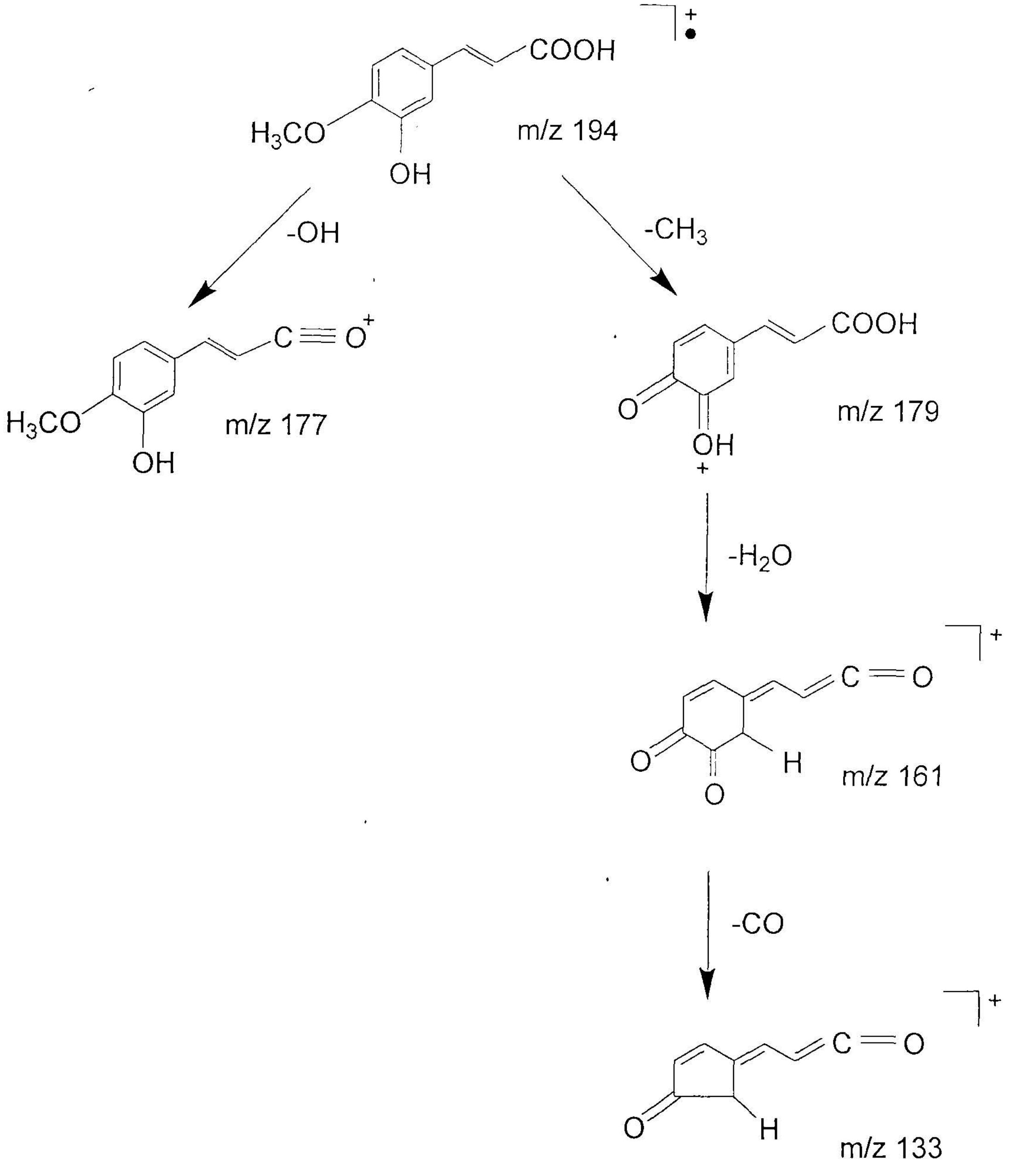
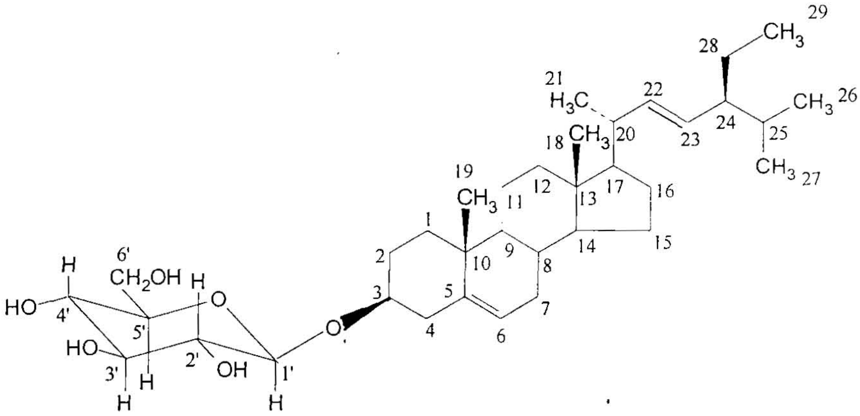
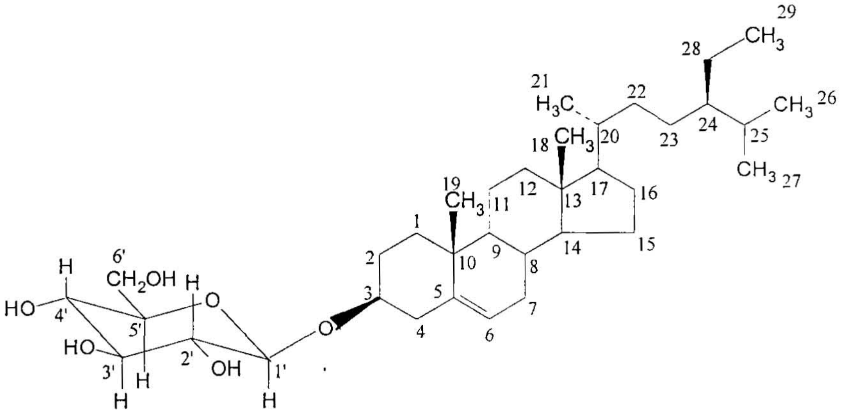


Figure 21 Mass fragmentation pattern of compound **F**

Identification of compound G



Stigmasteryl-3-*O*-*β*-D-glucopyranoside



β-sitosteryl-3-*O*-*β*-D-glucopyranoside

Compound **G** was isolated as white powder, m.p. 261-262 °C. The structure of **G** was elucidated by spectroscopic method.

The IR spectrum of **G** exhibited characteristic absorption band of hydroxy group at 3406 cm^{-1}. The absorption band at 1024 cm^{-1} was assigned for C-O stretching of the hydroxy group. The weak signal of anomeric axial proton of *β*-sugar was observed at 890 cm^{-1}. The 400 MHz ^1H NMR spectrum of compound **G** showed the signals of methyl, methylene and methine groups of steroids at δ 0.64- 2.50 ppm. The multiplet signal at δ 3.20-3.90 ppm were assigned to oxymethine protons (2′,3′,4′,5′) and oxymethylene proton (6′) of the sugar. The methine proton (H-3) on carbon attached to sugar moiety appeared as multiplet signal at δ 3.56-3.64. The doublet signal at δ 4.42 with coupling constant of 7.83 Hz belonged to the *β*-anomeric proton of D-glucose. A typical doublet of doublet at 5.03 (1H, *dd*, *J* = 15.66, 8.75 Hz), 5.17 (1H, *dd*, *J* = 15.63, 8.62 Hz) were assigned to disubstituted vinyl proton (CH=CH) at 22,23 –position, a triplet at 5.38 (1H, *t*, *J* = 3.59) belonged to olefinic proton at C-6.

The 100 MHz ^{13}C NMR spectrum of compound **G** exhibited 37 carbon signals consisting of 6 methyl , 16 methine, 12 methylene and 3 quaternary carbons. The DEPT spectral data in **Table 36** (DEPT-90 and DEPT-135) established the existence of 6 methyls at [δ11.76(CH_3-18),19.51(CH_3-19),20.93(CH_3-21),19.02(CH_3-26),20.80(CH_3-27), 11.95(CH-29), 16 methine carbons at [δ 69.52(CH-3), 121.93(CH-6), 49.94(CH-9), 31.67(CH-8), 56.61(CH-14), 55.81(CH-17), 40.28(CH-20),138.11(CH-22^{+}),129.05(CH-23^{+}), 51.02(CH-24),33.69 (CH-25), 100.84(CH-1′), 73.26(CH-2′), 76.14(CH-3′), 69.52 (CH-4′) , 76.67(CH-5′), 12 methylene carbons at [δ 36.99(CH_2-1), 29.30(CH_2-2), 41.97 (CH_2-4), 31.63(CH_2-7),22.81 (CH_2-11), 39.51(CH_2-12), 24.04(CH_2-15),28.89(CH_2-16), 33.69(CH_2-22^{++}), 27.99(CH_2-23^{++}),25.17(CH_2-28), 61.22(CH_2-6′). 3 quaternary carbons at [δ140.05(C-5), 36.47(C-10), 42.08(C-13). It is noted that the signals at δ 11.58-56.60 ppm were appeared to be the signals of methyl, methylene and methine groups of steroids. The carbon signals at δ 140.05 ,121.93 and 138.11, 129.05 were characteristic of the olefinic proton at C-5,6 and C-22,23, respectively, while some carbon signals at δ 33.69, 27.99 ppm were assigned to the methylene groups of C-22,23 as shown in **Table 35**. In addition, six carbon signals appeared at δ100.84 (C-1′), 76.14(C-3′) 76.67(C-5′), 73.26(C-2′),69.52(C-4′), and 61.22(C-6′) ppm were very similar to those of D-glucose.

The mass spectrum did not show the molecular ion of this compound, perhaps due to the breaking of molecule. However the dominant fragmentation ion peaks were observed at *m/z* 414 and 412 including other fragments at *m/z* 396, 394, 255, 233. A suggested fragmentation ion pattern of compound **G** is given as shown in **Figure 22**.

Based on the available spectral data of compound **G** and comparison of its ^{13}C NMR with stigmasteryl-3-O-*β*-D-glucopyranoside and *β*-sitosteryl-3-O-*β*-D-glucopyranoside, thus, compound **G** was concluded to be a mixture stigmasteryl-3-O-*β*-D-glucopyra--noside and *β*-sitosteryl-3-O-*β*-D-glucopyranoside.

Table 35 ^{13}C NMR spectral data of compound **G** (in $CD_3OD+CDCl_3$), stigmasteryl-3-O-β-D-glucopyranoside and β–sitosteryl-3-O-β-D-glucopyranoside

Position	Compound **G** δ_C	stigmasteryl-3-O-β-D-glucopyranoside[42] $*\delta_C$	β–sitosteryl-3-O-β-D-glucopyranoside[43] $*\delta_C$
1	36.99 (CH_2)	37.40 (CH_2)	36.57 (CH_2)
2	29.30 (CH_2)	31.70 (CH_2)	29.26 (CH_2)
3	69.52 (CH)	71.80 (CH)	70.01 (CH)
4	41.97 (CH_2)	42.40 (CH_2)	42.93 (CH_2)
5	140.05 (C)	140.00 (C)	139.05 (C)
6	121.93 (CH)	121.10 (CH)	119.47 (CH)
7	31.63 (CH_2)	31.90 (CH_2)	33.24 (CH_2)
8	31.67 (CH)	31.90 (CH)	33.91 (CH)
9	49.94 (CH)	50.30 (CH)	48.71CH)
10	36.47 (C)	36.60 (C)	35.93 (C)
11	22.81 (CH_2)	21.10 (CH_2)	22.53 (CH_2)
12	39.51 (CH_2)	39.80 (CH_2)	39.91 (CH_2)
13	42.08 (C)	42.40 (C)	42.93 (C)
14	56.61 (CH)	57.00 (CH)	55.41 (CH)
15	24.04 (CH_2)	24.40 (CH_2)	25.60 (CH_2)
16	28.89 (CH_2)	28.90 (CH_2)	28.73 (CH_2)
17	55.81 (CH)	56.00 (CH)	54.41 (CH)
18	11.76 (CH_3)	12.20 (CH_3)	11.77 (CH_3)
19	19.51 (CH_3)	19.40 (CH_3)	19.72 (CH_3)
20	40.28 (CH)	40.50 (CH)	39.90 (CH)
21	20.93 (CH_3)	21.10 (CH_3)	21.01 (CH_3)
22^{+}	138.10 (CH)	138.40 (CH)	-
22^{++}	33.69 (CH_2)	-	33.89 (CH_2)
23^{+}	129.05 (CH)	129.40 (CH)	129.40 (CH)

Table 35 (continued)

Position	Compound **G** δ_C	stigmasteryl-3-O-β-D-glucopyranoside[42] $*\delta_C$	β-sitosteryl-3-O-β-D-glucopyranoside[43] $*\delta_C$
23^{++}	27.99 (CH_2)	-	27.49 (CH_2)
24	51.02 (CH)	51.20 (CH)	54.41 (CH)
25	33.69 (CH)	31.90 (CH)	29.08 (CH)
26	19.02 (CH_3)	19.00 (CH_3)	19.00 (CH_3)
27	20.80 (CH_3)	21.00 (CH_3)	21.01 (CH_3)
28	25.17 (CH_2)	25.40 (CH_2)	25.60 (CH_2)
29	11.95 (CH_3)	12.00 (CH_3)	11.64 (CH_3)
1'	100.84(CH)	100.69(CH)	100.83(CH)
2'	73.26(CH)	73.43(CH)	73.48(CH)
3'	76.14(CH)	76.93(CH)	76.75(CH)
4'	69.52(CH)	70.06(CH)	70.06(CH)
5'	76.67(CH)	76.69(CH)	76.69(CH)
6'	61.22(CH_2)	61.05(CH_2)	61.05(CH_2)

$^{+}$(-CH=CH- ; C-22,23)

$^{++}$ (-CH_2-CH_2- ; C-22,23)

*300 MHz in DMSO-d_6

Table 36 ^{13}C and DEPT NMR spectral data of compound **G**

δ_C	DEPT- 90	DEPT- 135
36.99 (CH_2)	-	36.99 (CH_2)
29.30 (CH_2)	-	29.30 (CH_2)
69.52 (CH)	69.52 (CH)	69.52 (CH)
41.97 (CH_2)	-	41.97 (CH_2)
140.05 (C)	-	-
121.93 (CH)	121.93 (CH)	121.93 (CH)
31.63 (CH_2)	-	31.63 (CH_2)
31.67 (CH)	31.67 (CH)	31.67 (CH)
49.94 (CH)	49.94 (CH)	49.94 (CH)
36.47 (C)	-	-
22.81 (CH_2)	-	22.81 (CH_2)
39.51 (CH_2)	-	39.51 (CH_2)
42.08 (C)	-	-
56.61 (CH)	56.61 (CH)	56.61 (CH)
24.04 (CH_2)	-	24.04 (CH_2)
28.89 (CH_2)	-	28.89 (CH_2)
55.81 (CH)	55.81 (CH)	55.81 (CH)
11.76 (CH_3)	-	11.76 (CH_3)
19.51 (CH_3)	-	19.51 (CH_3)
40.28 (CH)	40.28 (CH)	40.28 (CH)
20.93 (CH_3)	-	20.93 (CH_3)
138.11 (CH)	138.11 (CH)	138.10 (CH)
33.69 (CH_2)	-	33.69 (CH_2)
129.05 (CH)	129.05 (CH)	129.05 (CH)
27.99 (CH_2)	-	27.99 (CH_2)
51.02 (CH)	51.02 (CH)	51.02 (CH)

Table 36 (continued)

δ_C	DEPT- 90	DEPT- 135
33.69 (CH)	33.69 (CH)	33.69 (CH)
19.02 (CH_3)	-	19.02 (CH_3)
20.80 (CH_3)	-	20.80 (CH_3)
25.17 (CH_2)	-	25.17 (CH_2)
11.95 (CH_3)	-	11.95 (CH_3)
100.84(CH)	100.84(CH)	100.84(CH)
73.26(CH)	73.26(CH)	73.26(CH)
76.14(CH)	76.14(CH)	76.14(CH)
69.52(CH)	69.52(CH)	69.52(CH)
76.67(CH)	76.67(CH)	76.67(CH)
61.22 (CH_2)	-	61.22 (CH_2)

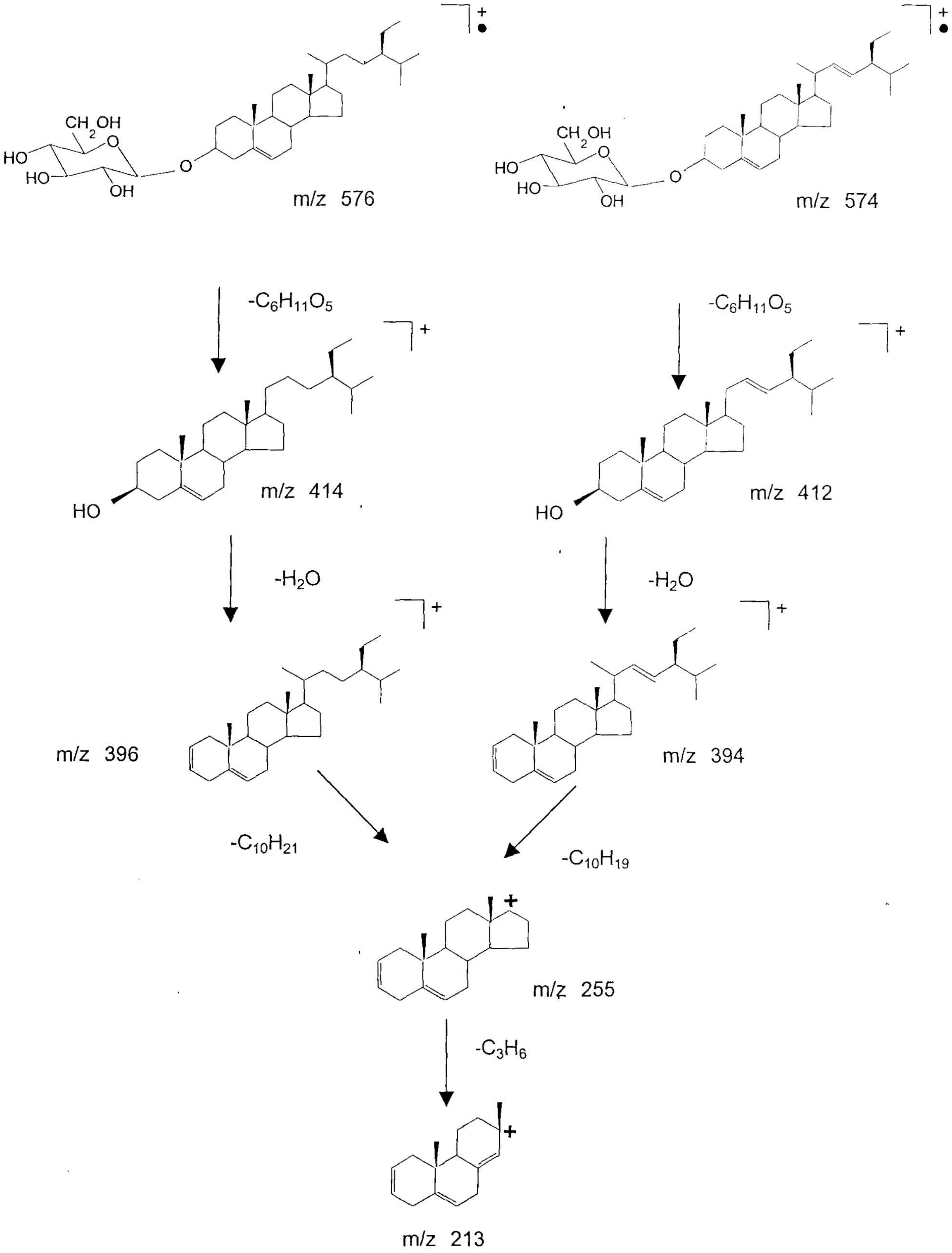


Figure 22 Mass fragmentation pattern of compound **G**

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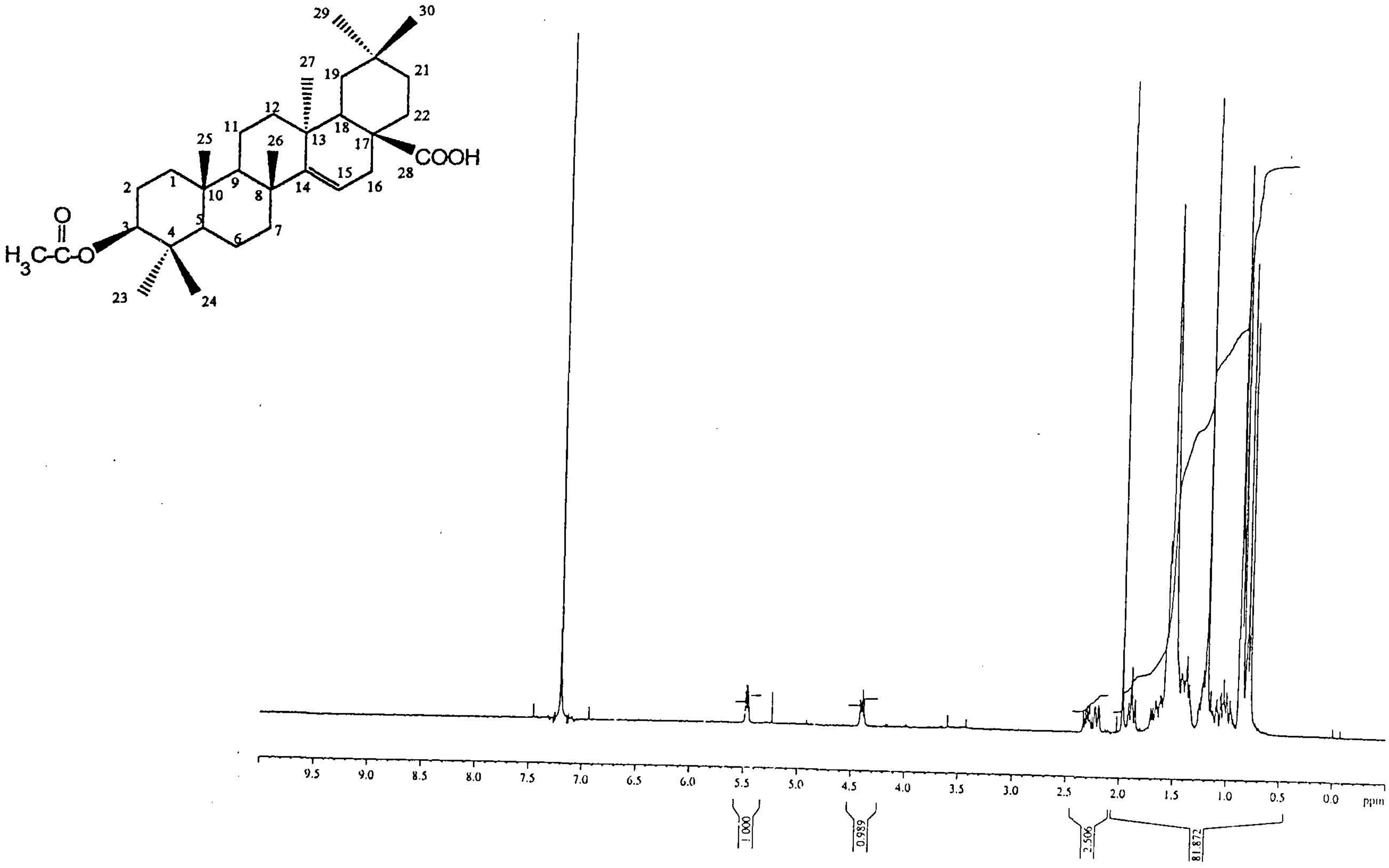
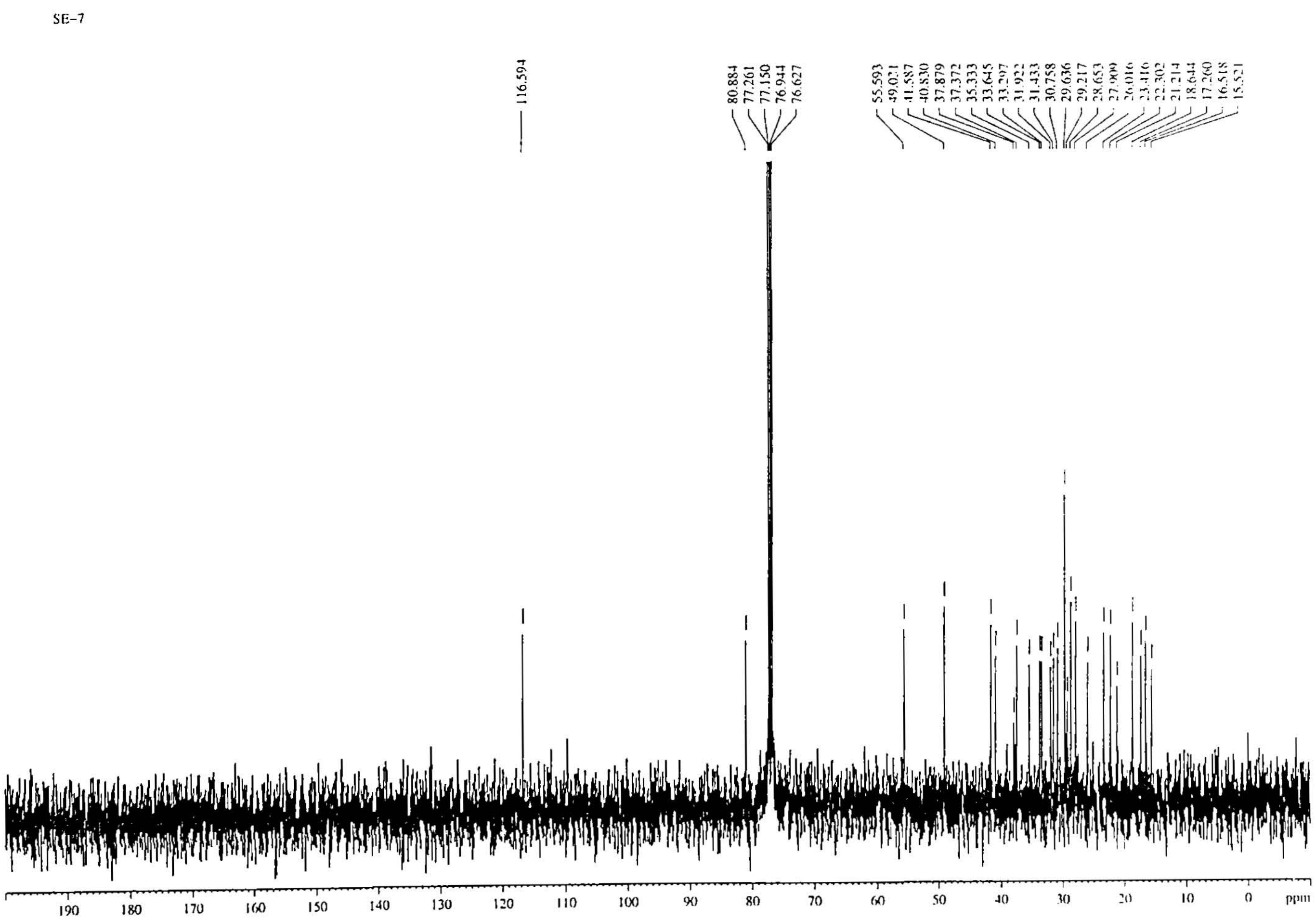
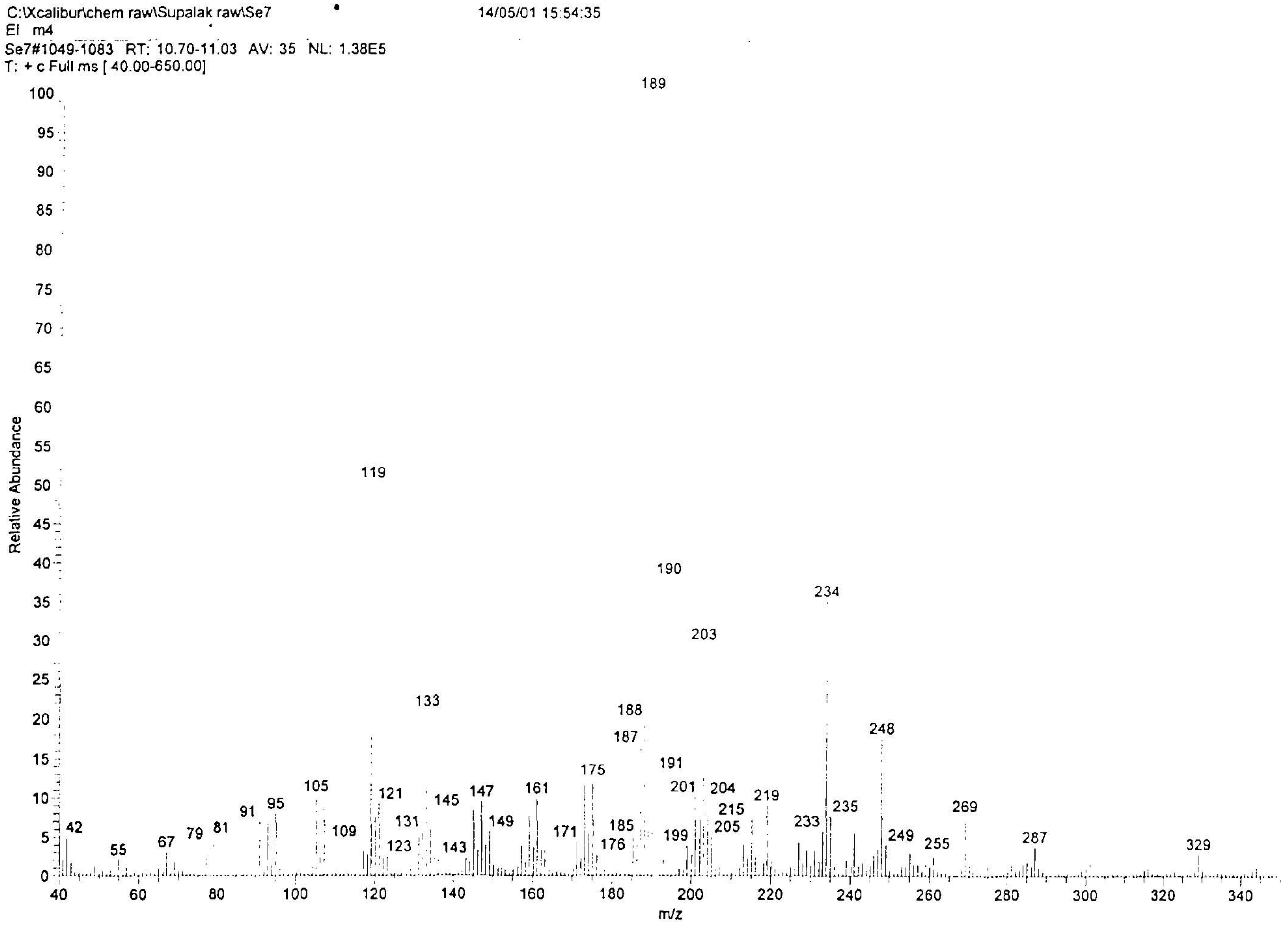
APPENDIX



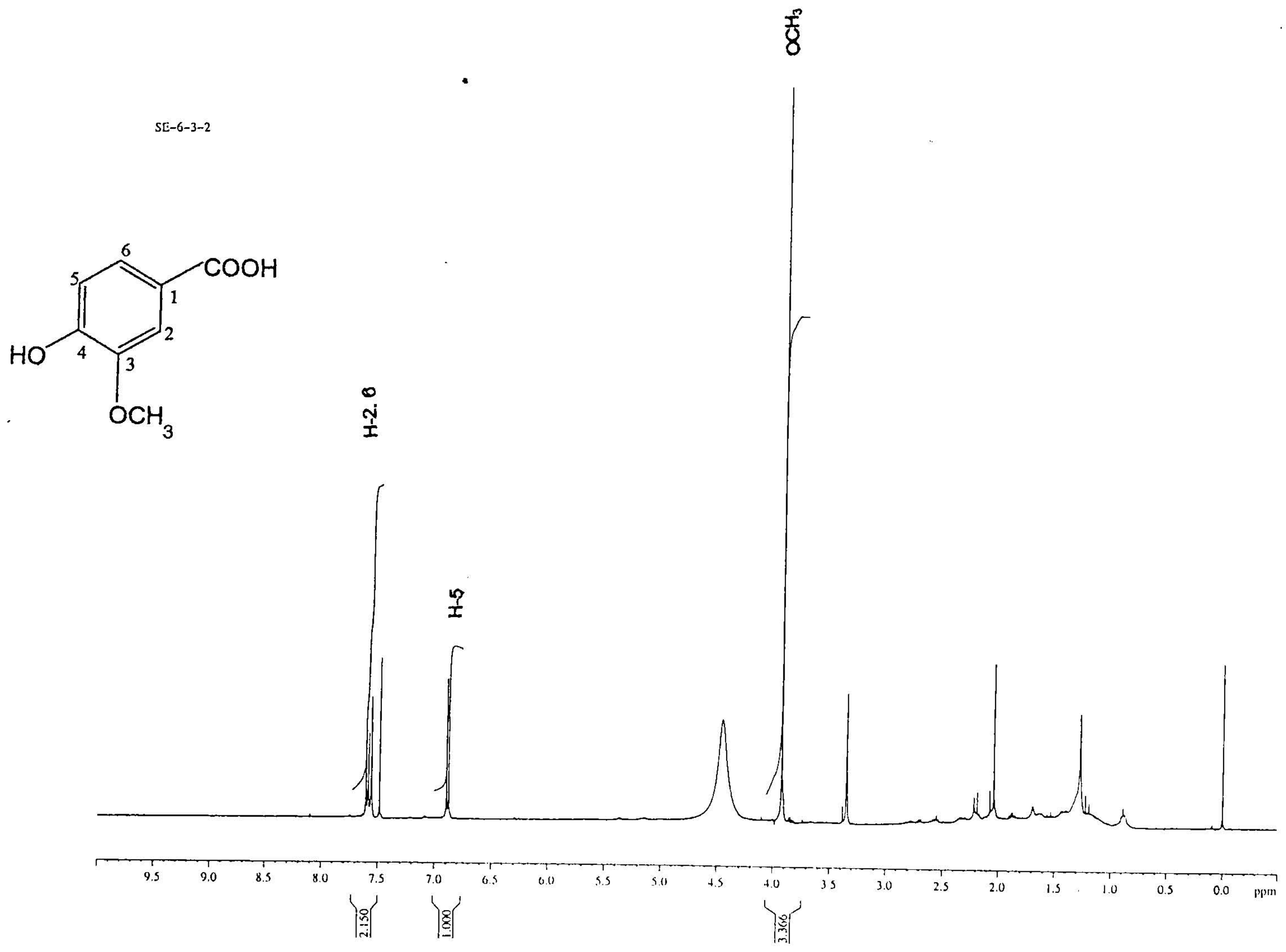
^{1}**H NMR** spectrum (400 MHz, CD_3OD) of **compound A**



^{13}C **NMR** spectrum (100 MHz, CD_3OD) of **compound A**



MS spectrum of **compound A**



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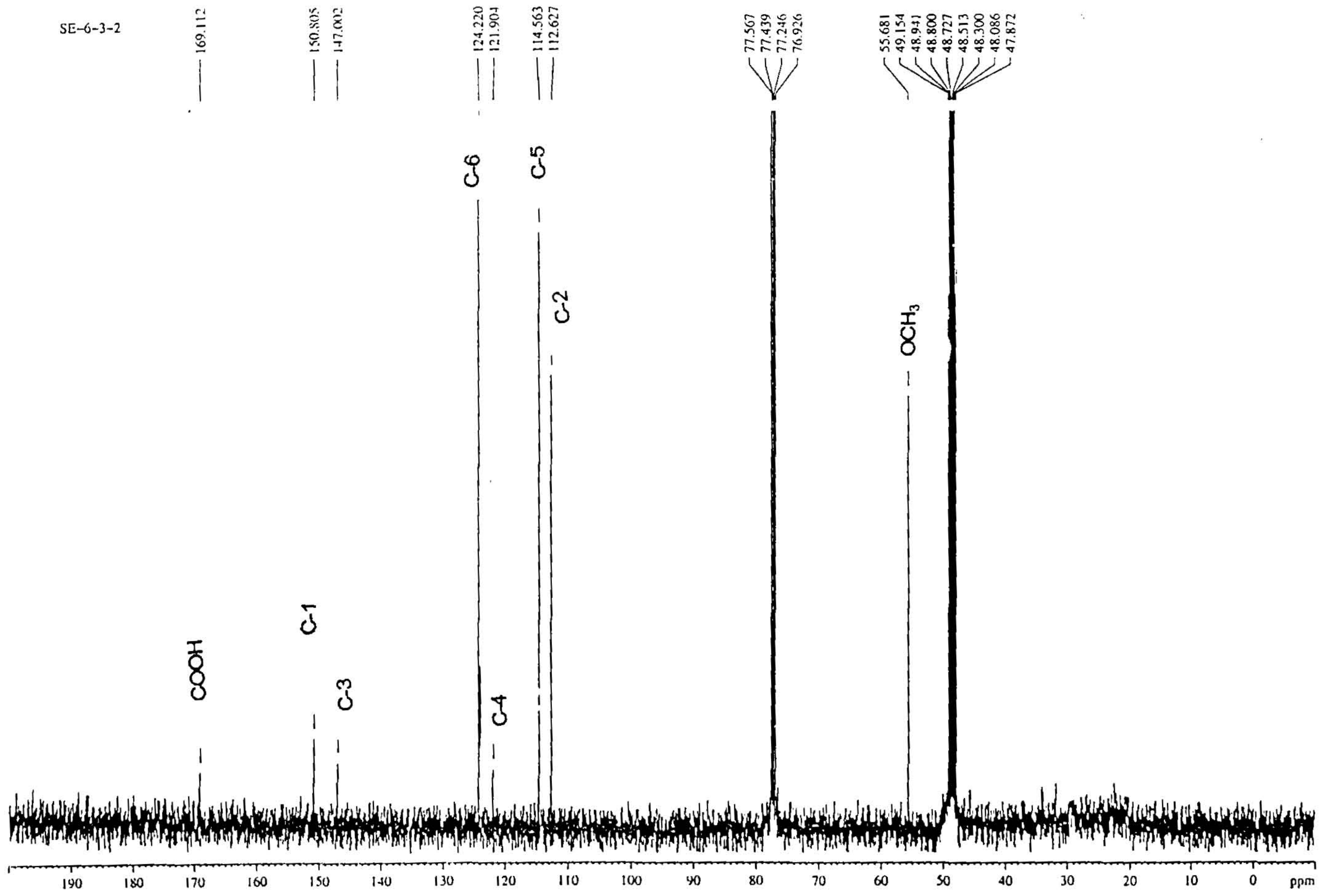

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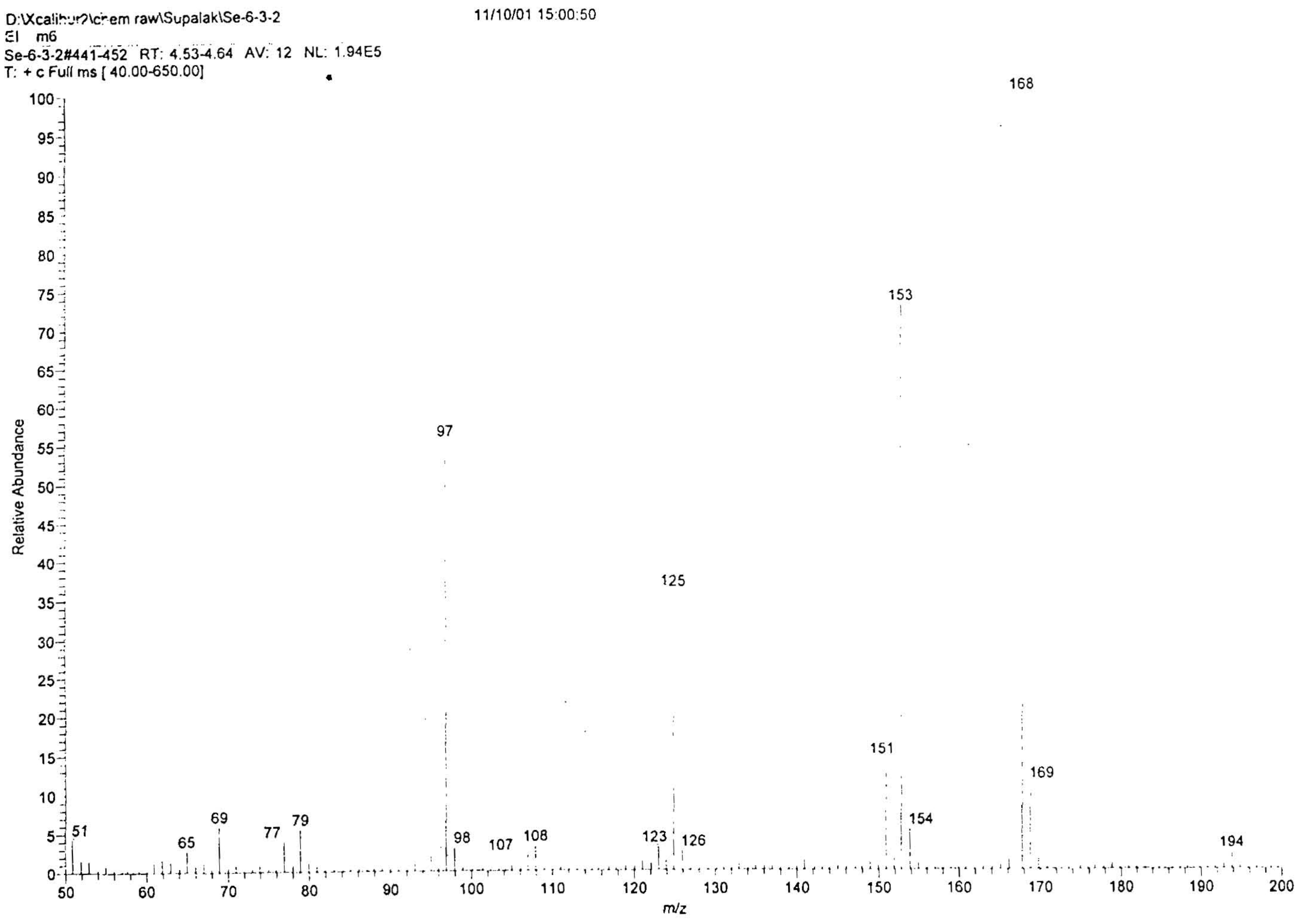

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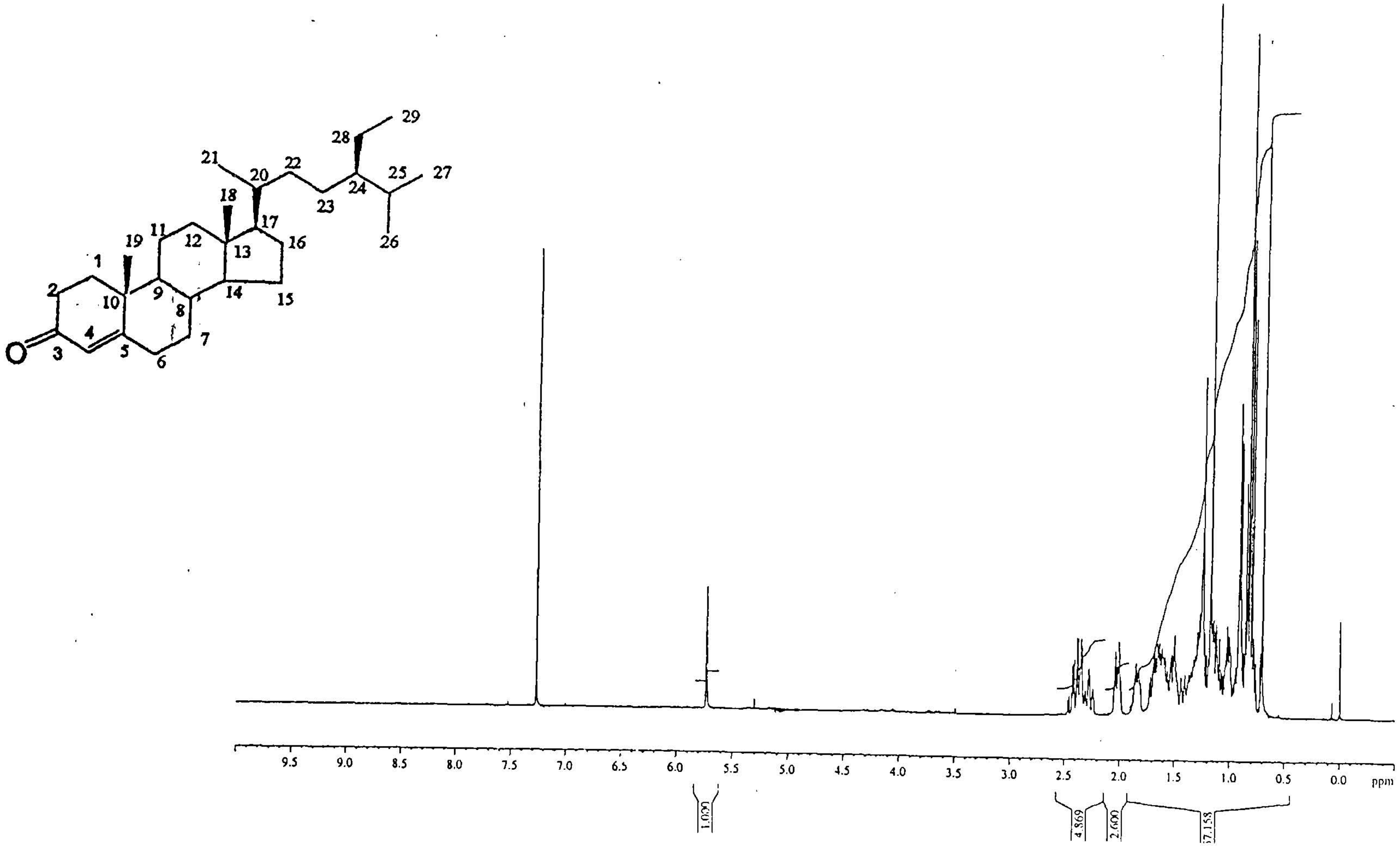
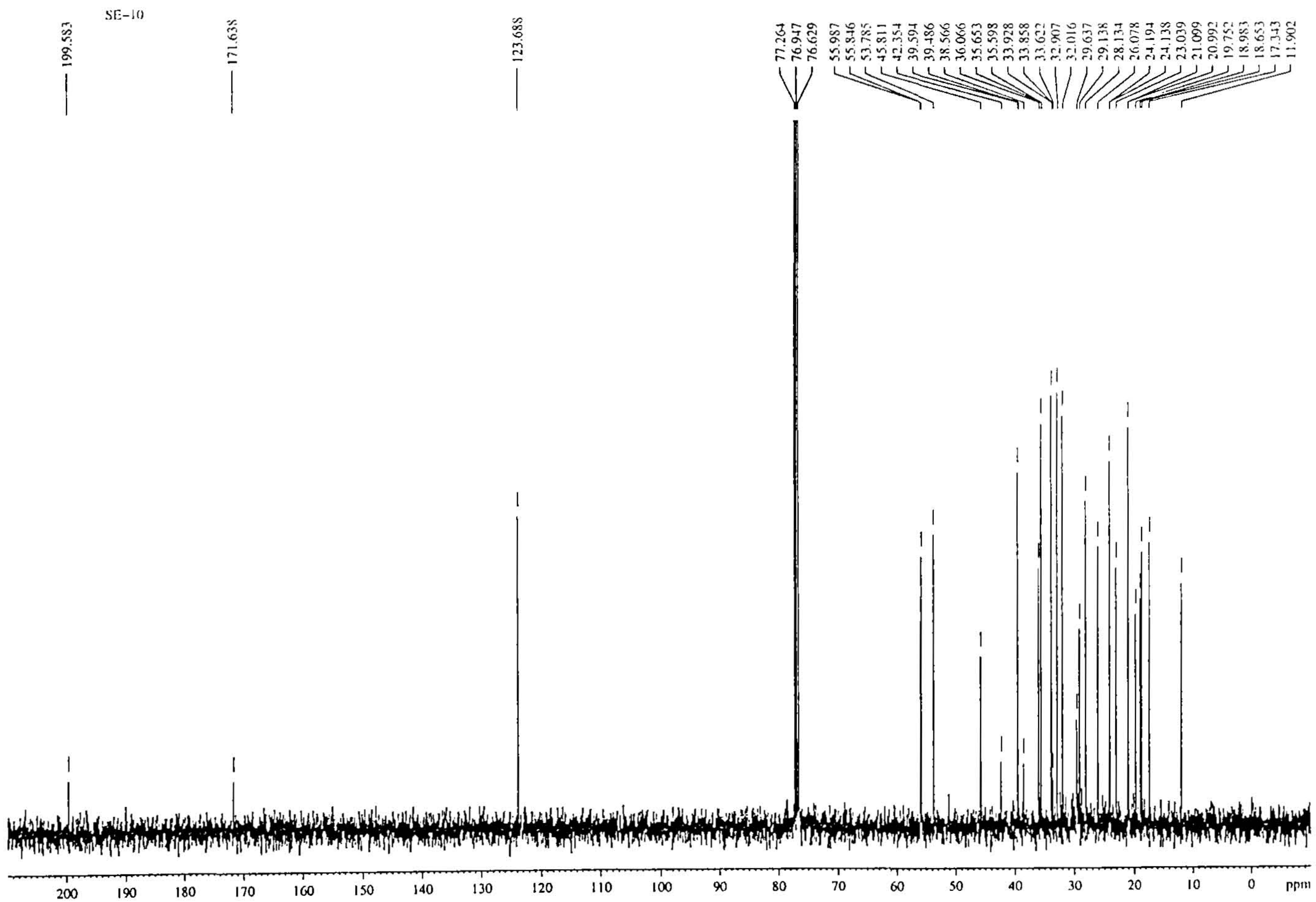


^{1}H NMR spectrum (400 MHz, $CDCl_3$) of **compound C**



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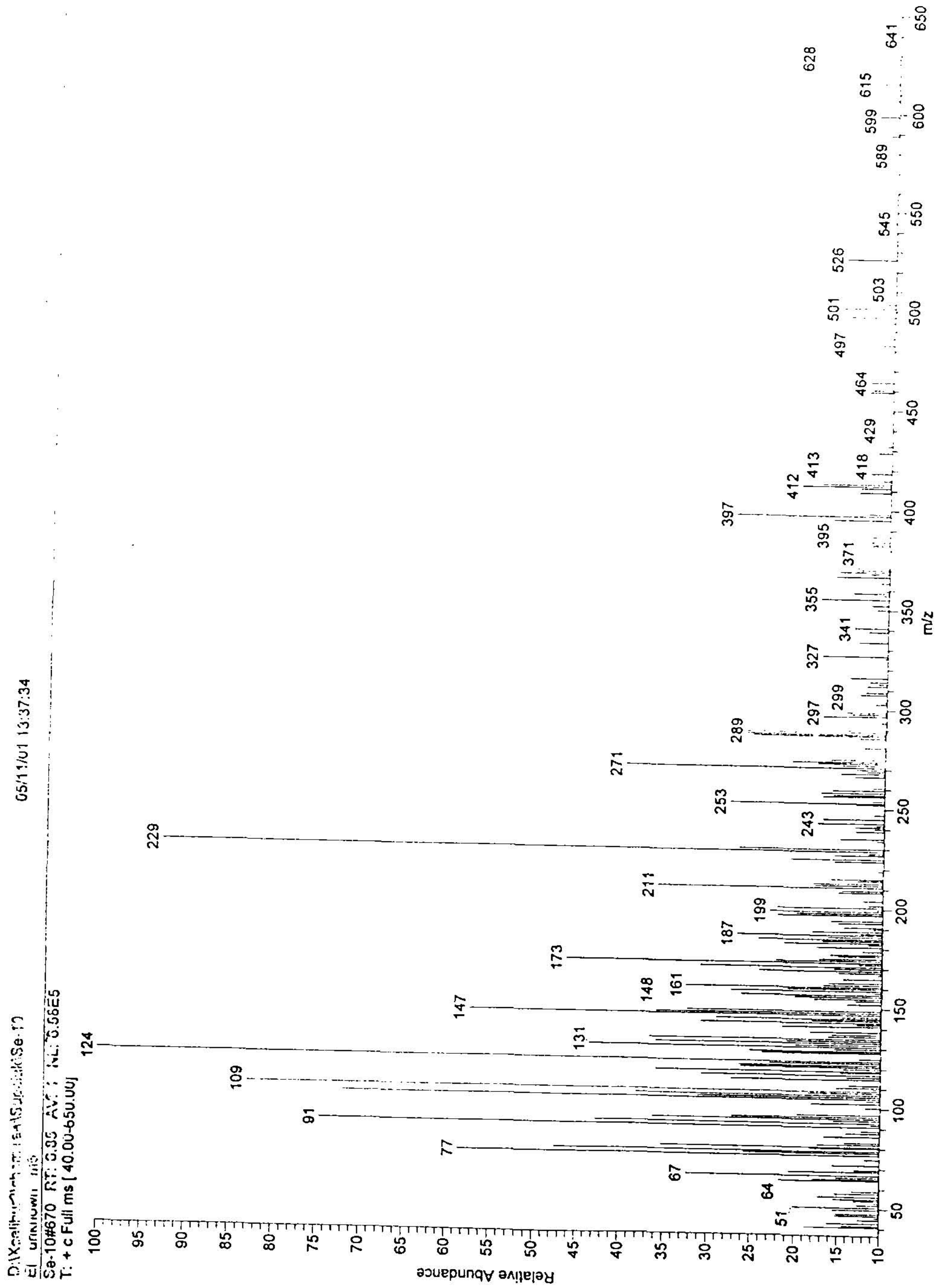
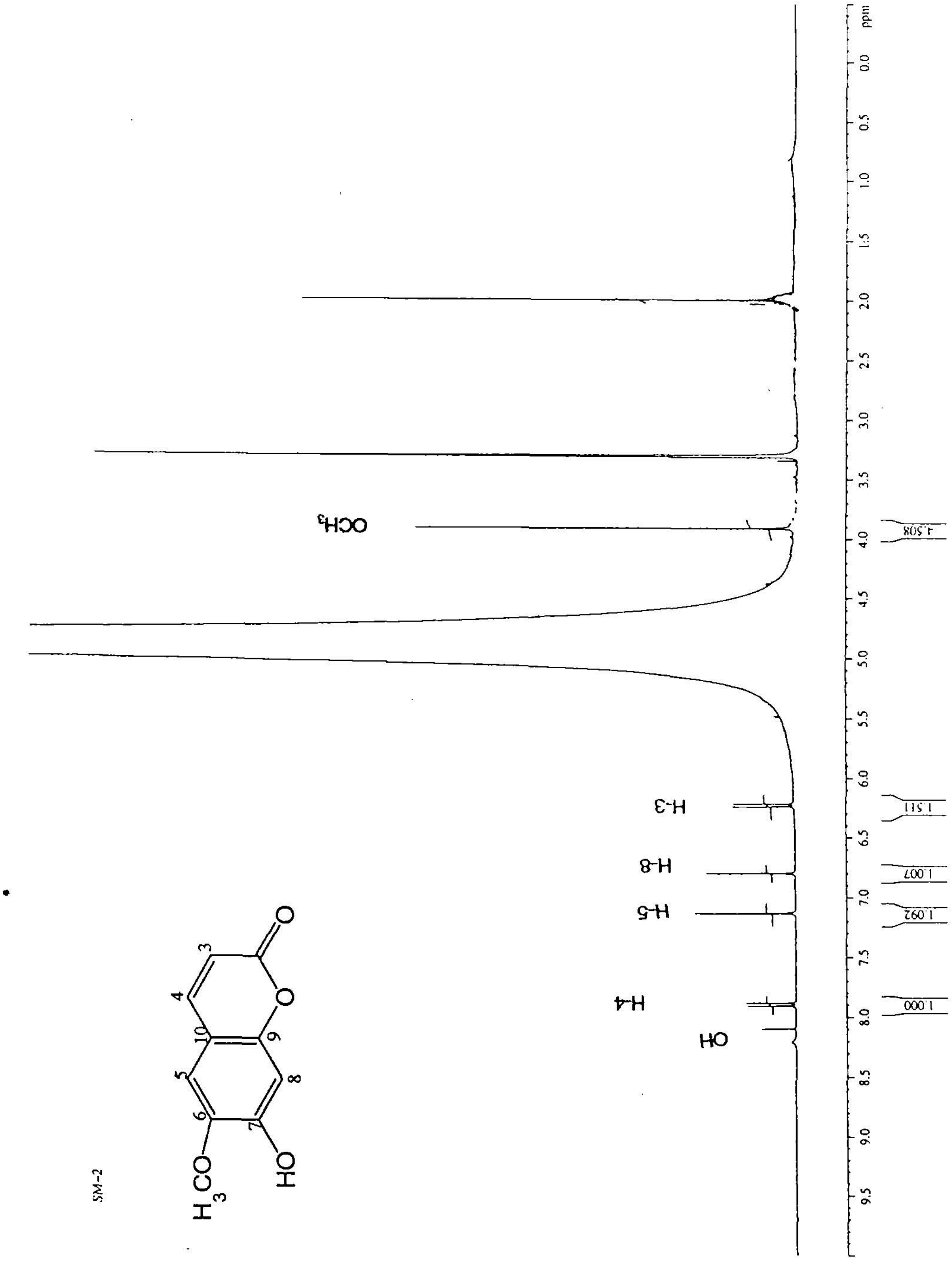
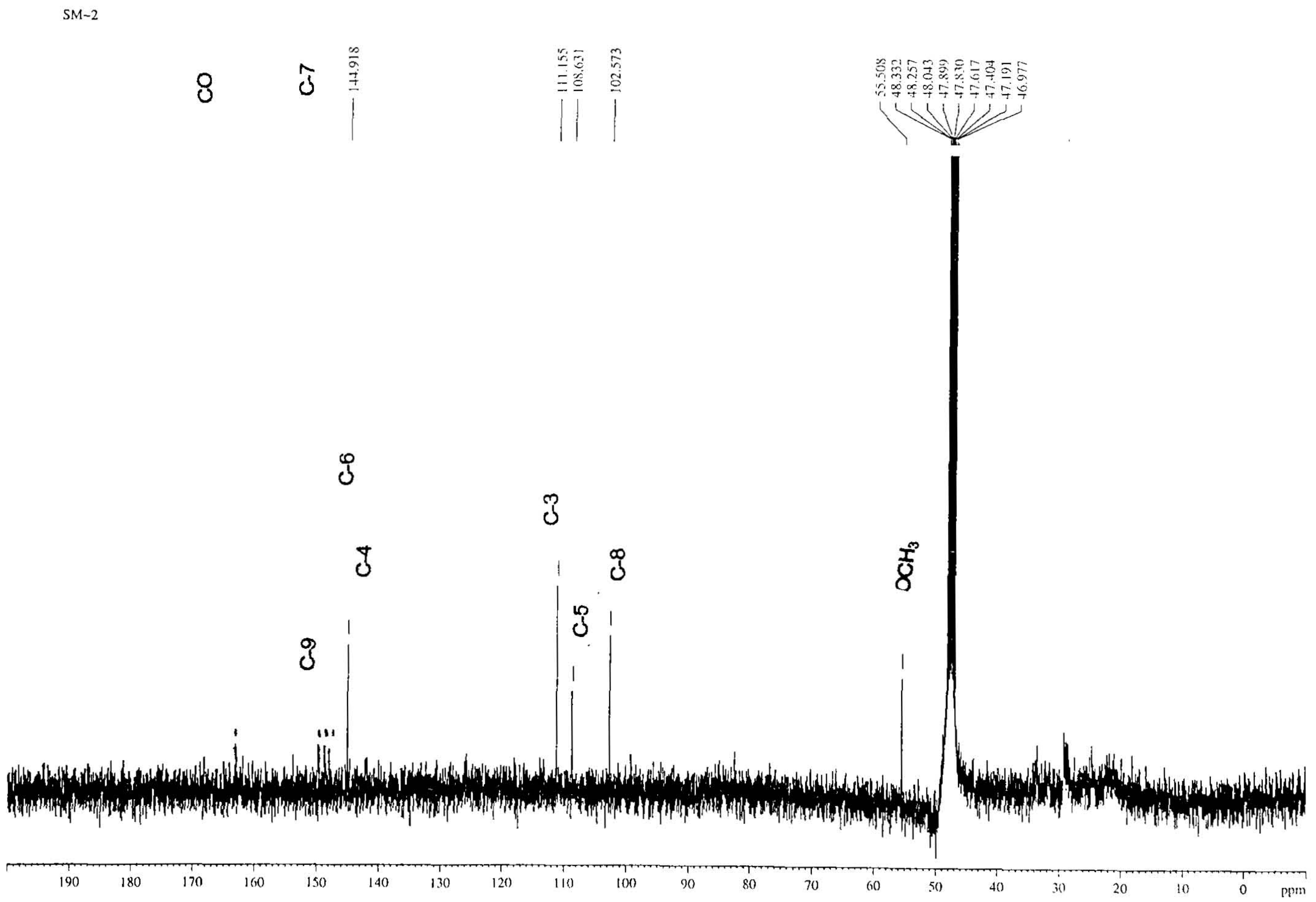


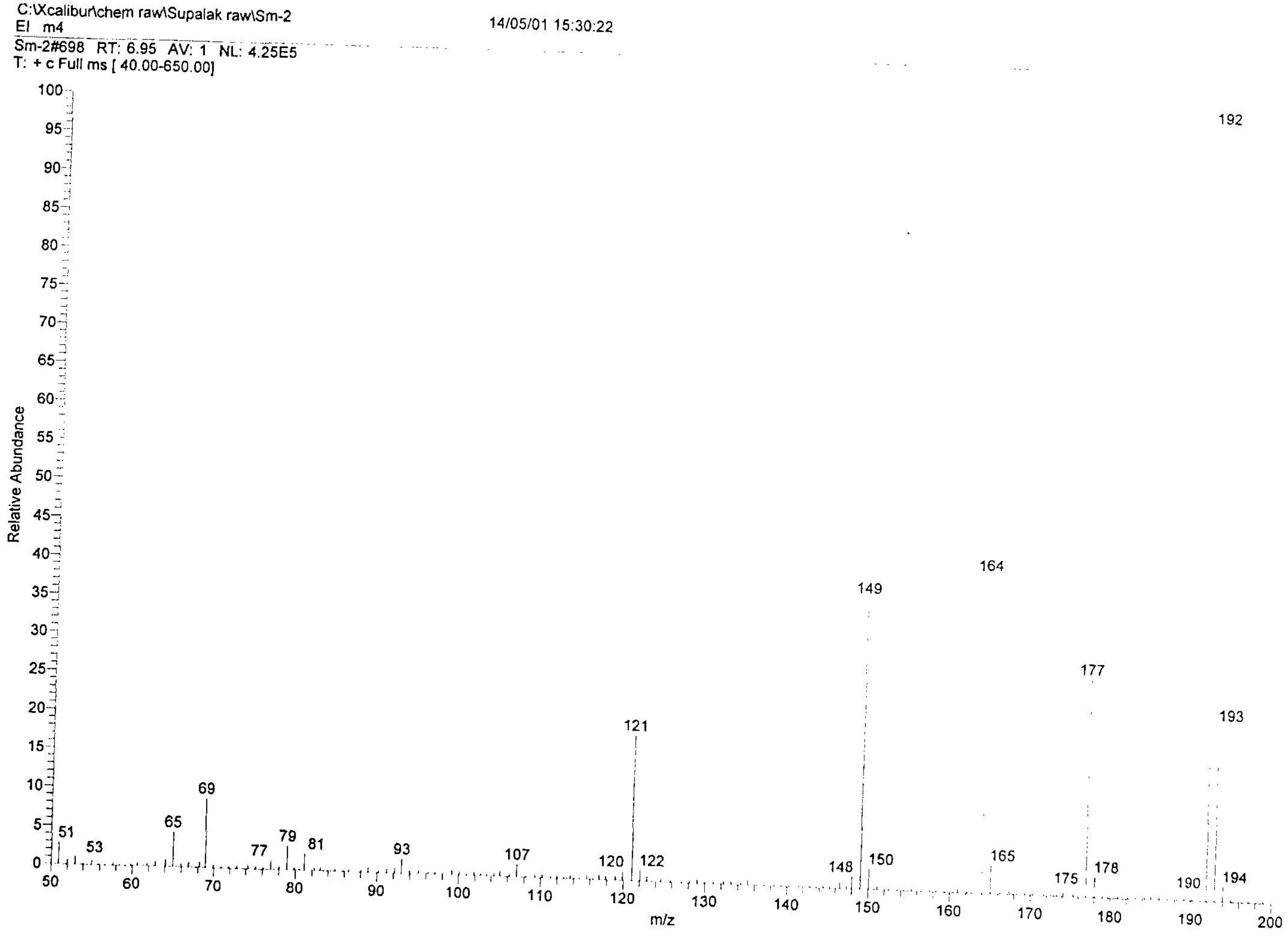
MS spectrum of **compound C**



^{1}**H NMR** spectrum (400 MHz, CD_3OD) of **compound D**



^{13}C **NMR** spectrum (100 MHz, CD_3OD) of **compound D**



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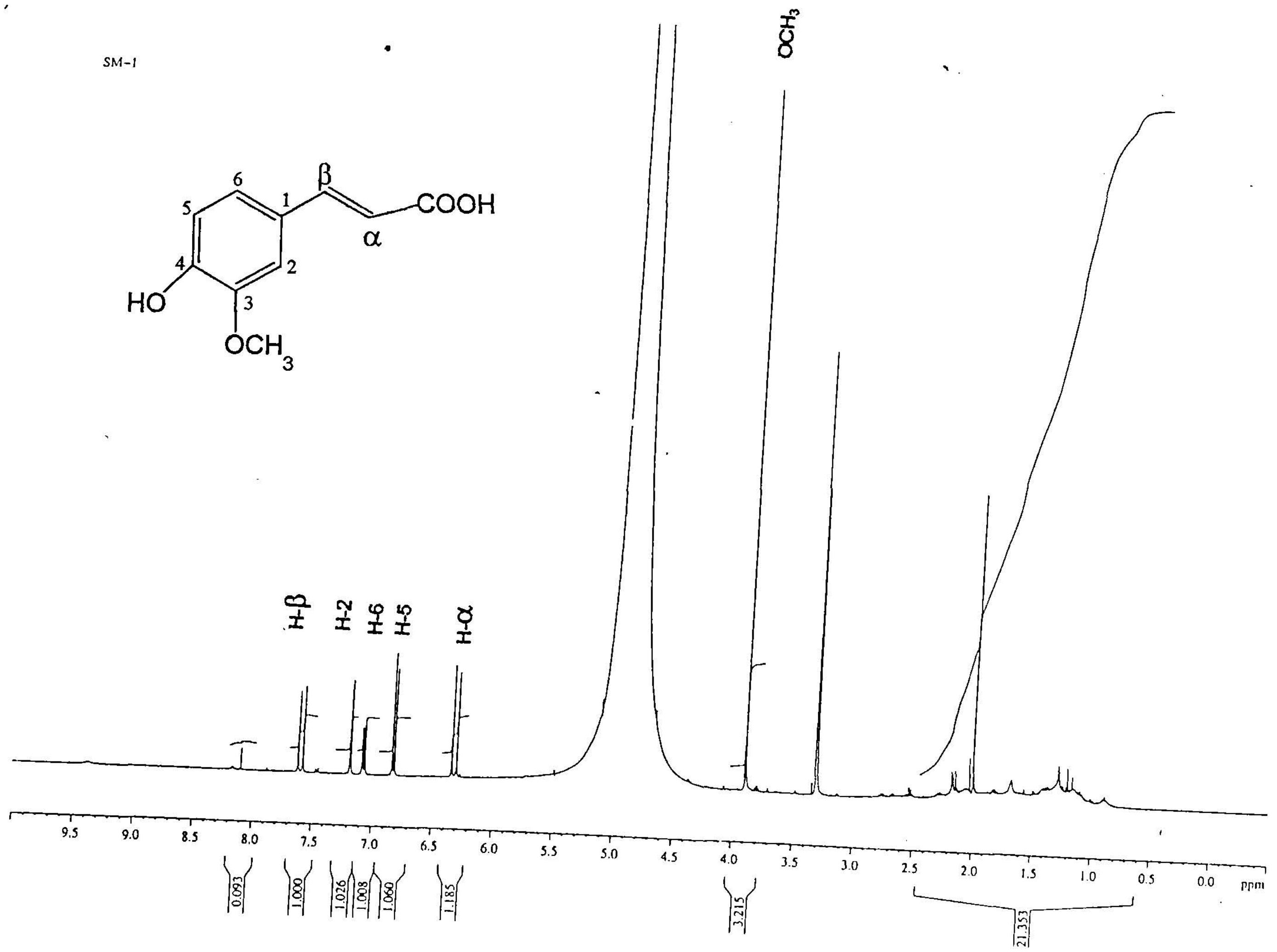

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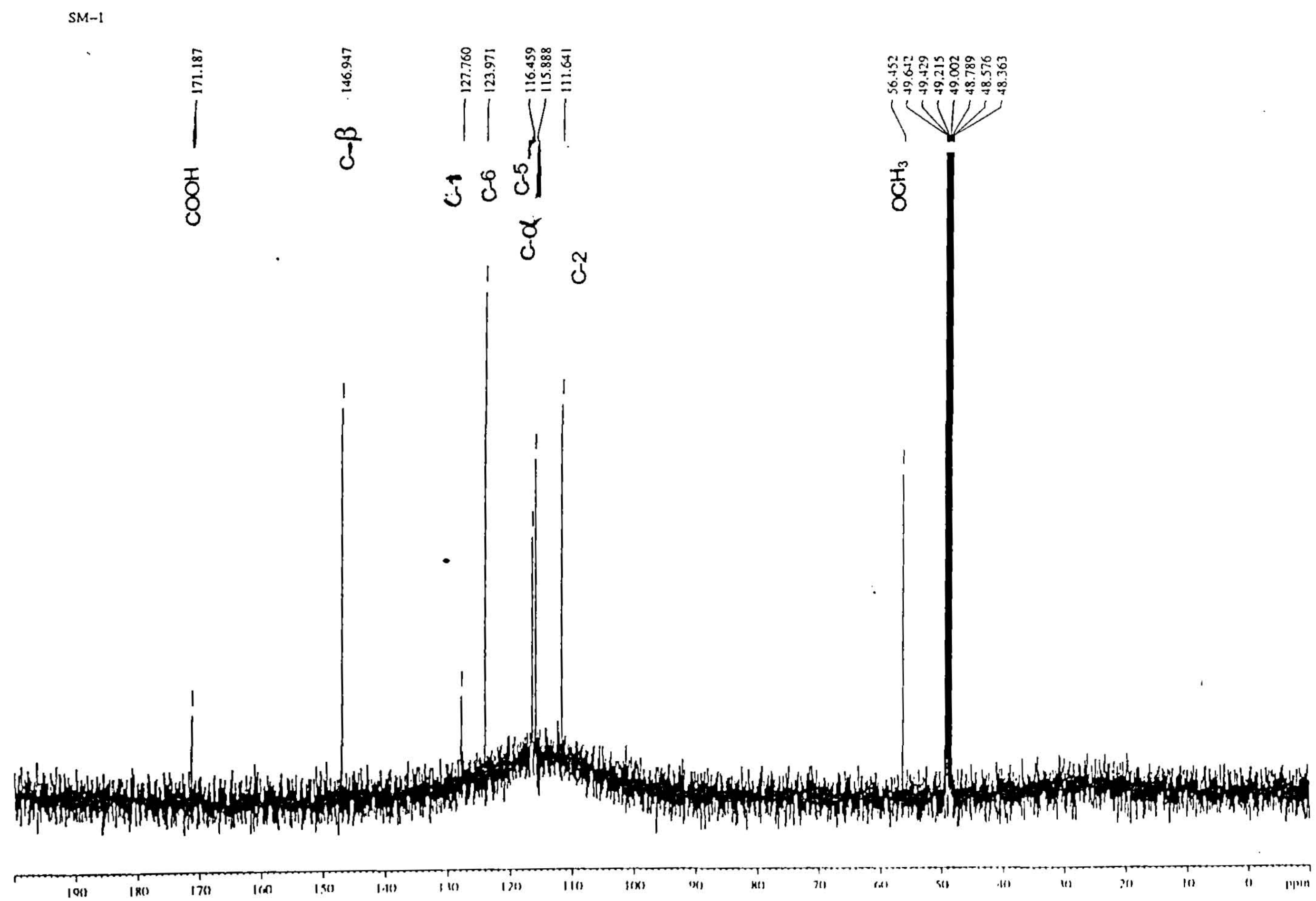

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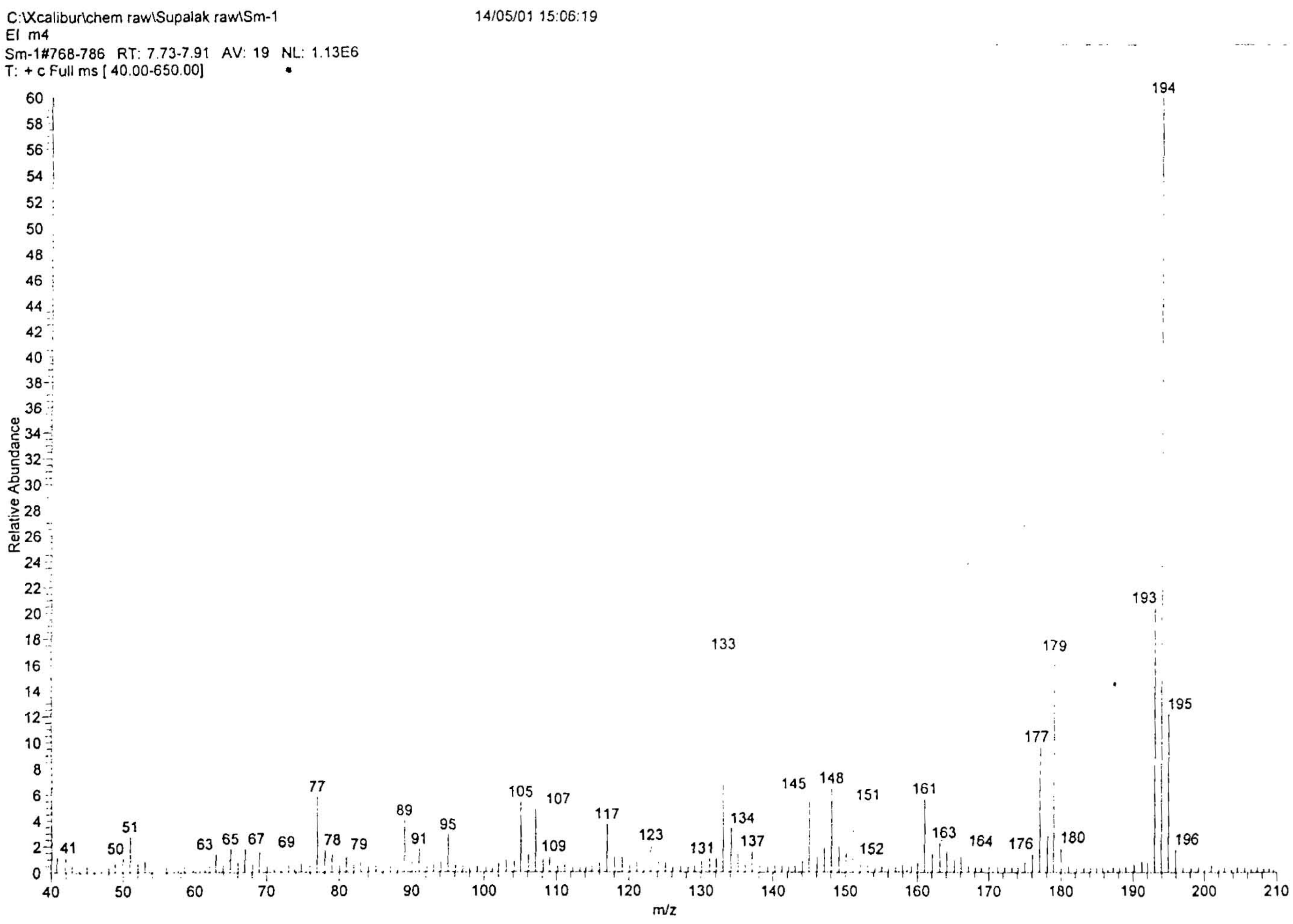
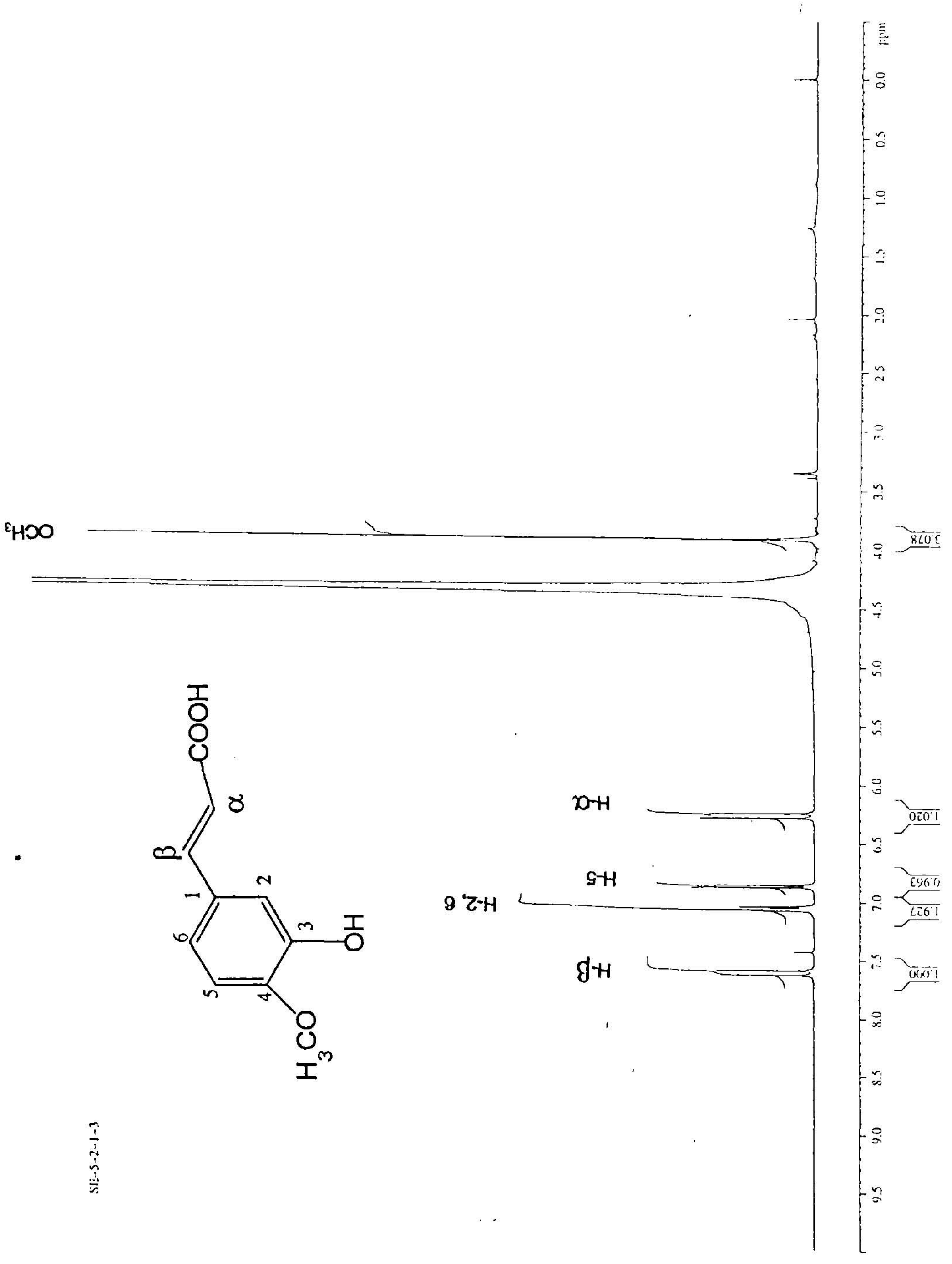
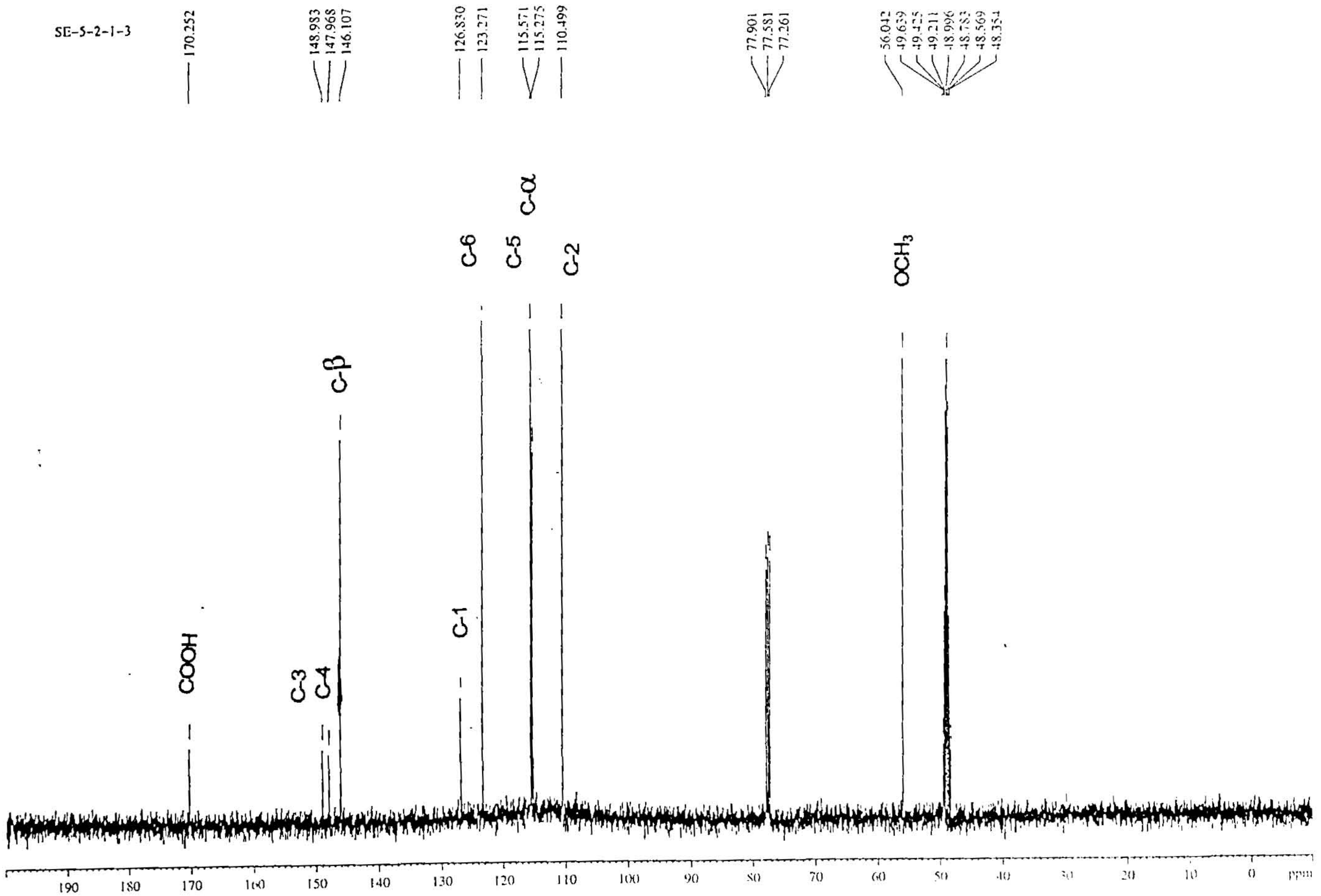


MS spectrum of **compound E**



^{1}H NMR spectrum (400 MHz, $CD_3OD+CDCl_3$) of **compound F**



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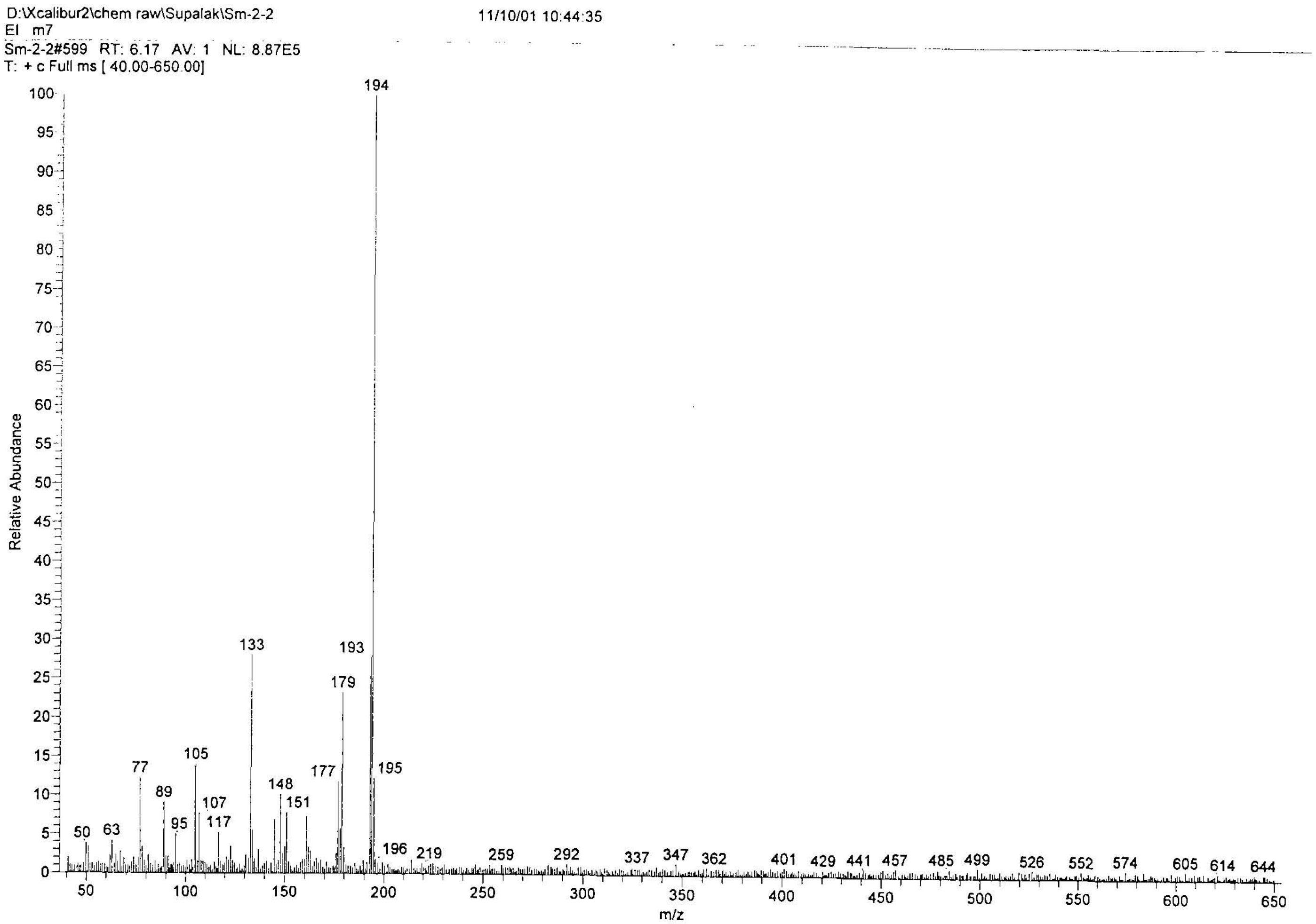
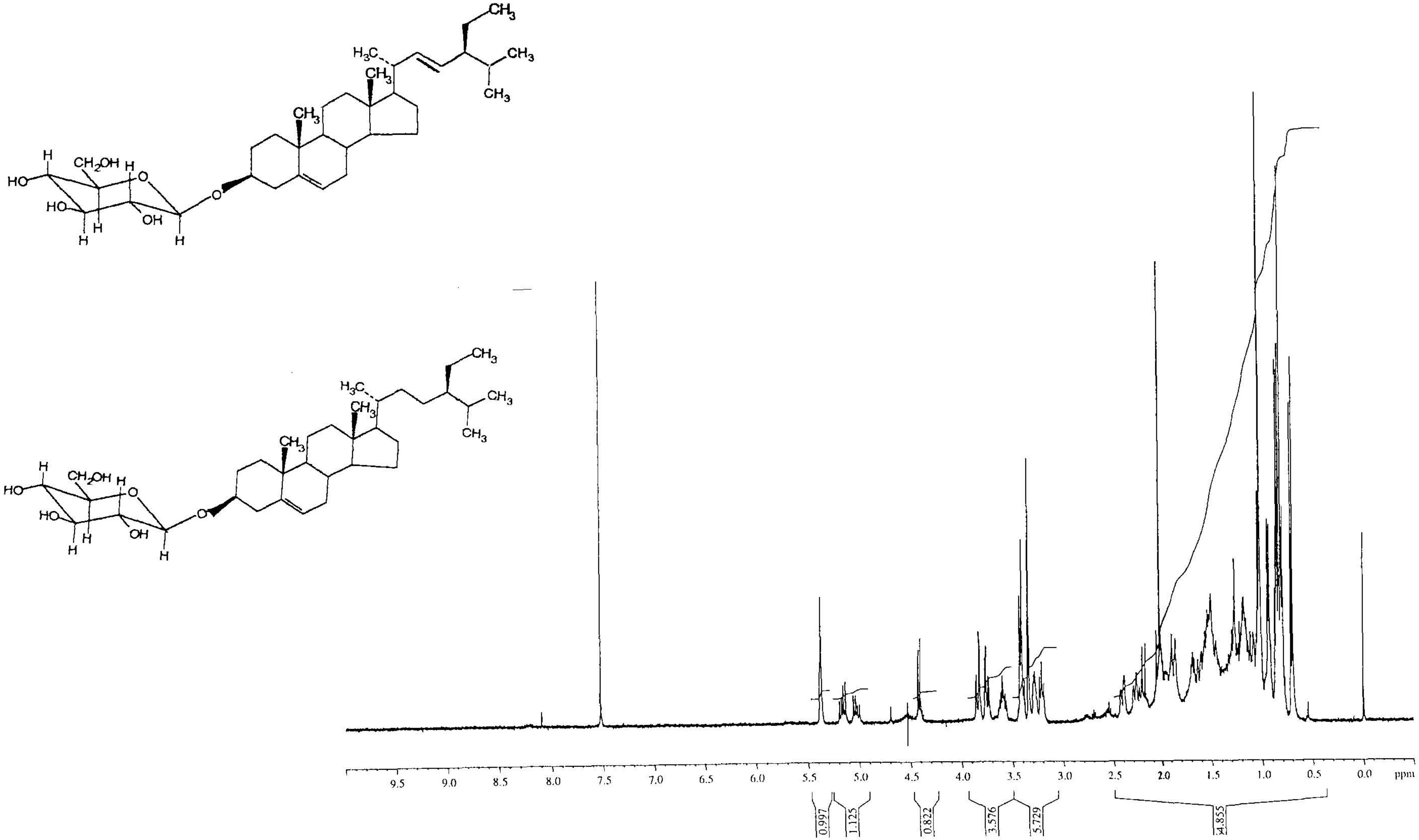
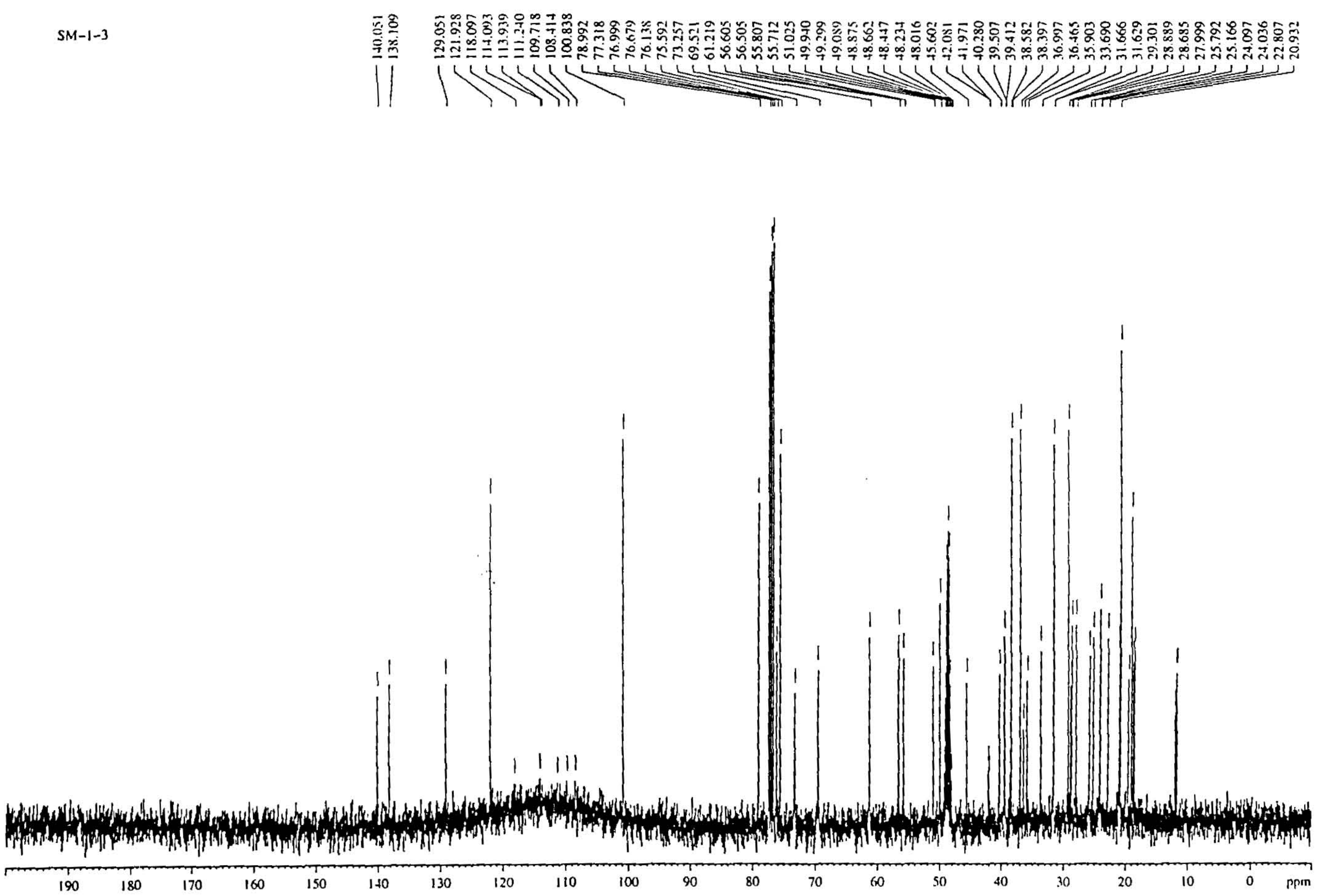


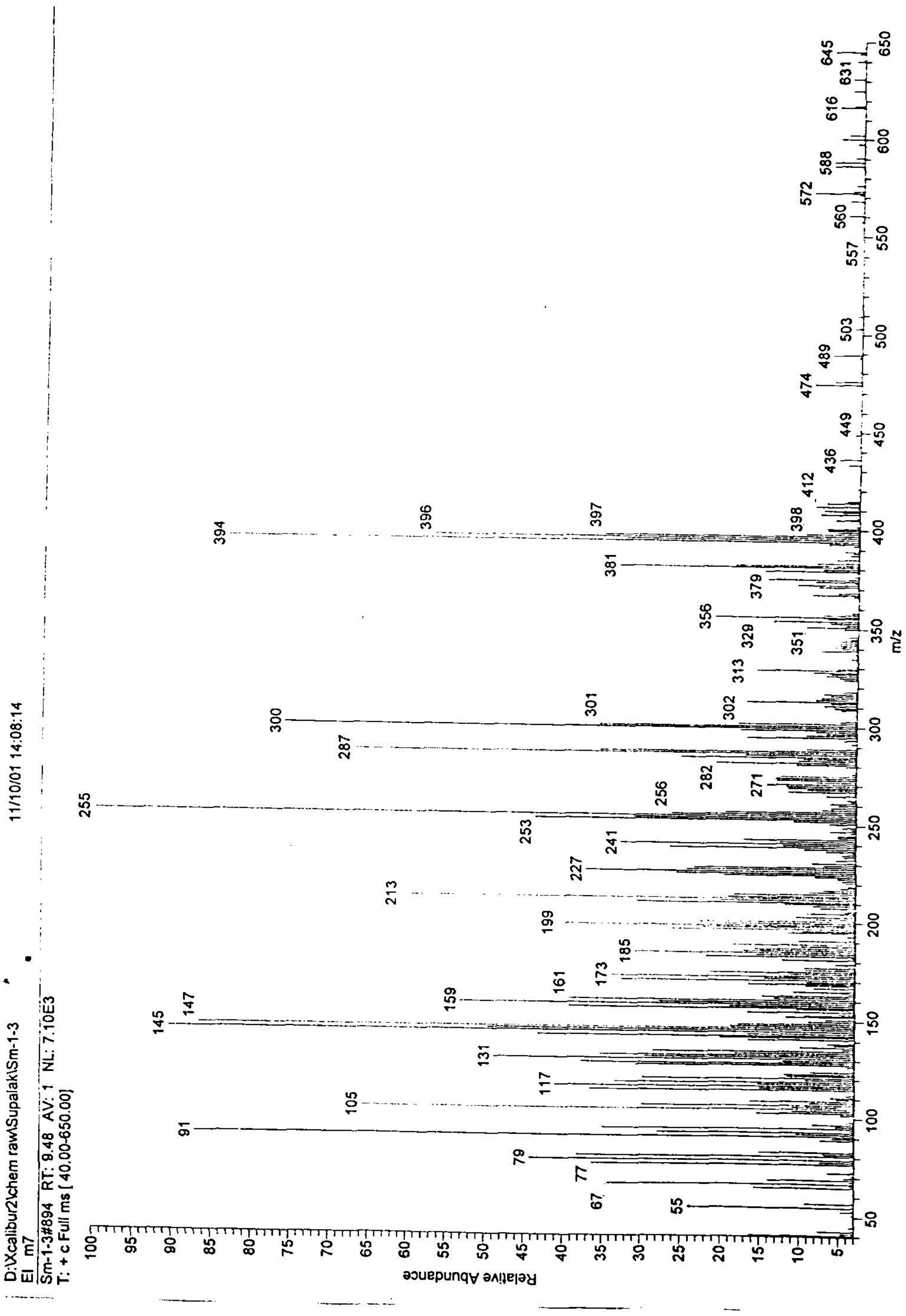
MS spectrum of compound F



^{1}**H NMR** spectrum (400 MHz, CD_3OD+$CDCl_3$) of **compound G**



^{13}C **NMR** spectrum (100 MHz, CD_3OD+$CDCl_3$) of **compound G**



MS spectrum of compound G

GLOSSARY

GLOSSARY

s	singlet
d	doublet
dd	doublet of doublet
m	multiplet
br s	broad singlet
br d	broad doublet
J	coupling constant
EIMS	electron impact mass spectra
IR	infrared spectrum
m/z	a value of mass divided by charge
°C	degree celcius
g	gram
mg	milligram
mL	millilitre
h	hour
%	percent
m.p.	melting point
cm^{-1}	wave number
TMS	tetramethylsilane
Hz	hertz
TLC	thin layer chromatography
Lit	literature
ν_{max}	maximum absorption frequencies
$CDCl_3$	deuterochloroform
CD_3OD	deuteromethanol
DMSO-d_6	deuterodimethylsulfoxide
^{1}H NMR	proton nuclear magnetic resonance
^{13}C NMR	carbon nuclear magnetic resonance
^{1}H-^{1}H COSY	^{1}H-relayed correlated spectroscopy

DEPT	distortionless enhancement by polarization transfer
HMBC	heteronuclear multiple bond connectivity
HMQC	^{1}H- detected heteronuclear multiple quantum coherence

BIOGRAPHY

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POSITION & OFFICE	1996-present, Teacher Khururatrungsarit High School, Ratchaburi, Thailand.