CHEMICAL STUDIES OF LEUCAS MARTINICENSIS

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MASTER OF SCIENCE IN CHEMISTRY

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ESTIFANOS ELE
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ADDIS ABABA UNIVERSITY
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Dedicated to: My parents, my wife and my son.

DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for any degree in any other university and that all sources of material used for the thesis have been duly acknowledged.

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This thesis has been submitted for examination with my approval as a university advisor.

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Date and place of submission: Office of Research and Graduate Programs

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ABSTRACT

CHEMICAL STUDIES OF *LEUCAS MARTINICENSIS*

Advisors: Dr. Tarekegn G/Yesus
Dr. Wendimagegn Mammo

Chemical studies were carried out on *Leucas martinicensis* of the family *Labiatae.*

Chloroform extract of the leaves afforded triacontane (**E2'**), tetracosane (**E2'-C**) and lutein (**E45**) while methanol extract of the leaves afforded cholesterol (**E24**).

Elucidations of the structures of the compounds were based on their ^{1}H NMR, ^{13}C NMR, and IR spectra. In the case of cholesterol comparison of the material with an authentic sample was carried.

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INTRODUCTION

1.1 General

The high diversity of plants is an available source of useful compounds. People extracted and used them for their advantages. Primitive man found these extracts efficient as medicines for the relief of pain or alleviation of the symptoms of disease, as poisons for use in warfare and hunting, as effective agents for euthanasia and capital punishment. They were also used as narcotics, hallucinogens, or stimulants to relieve tedium, or alleviate fatigue and hunger. Many of these natural products are still used today and usually for the same general purpose [1].

Natural products are organic compounds of natural origin that are unique to one organism or common to a small number of closely related organisms. In most instances they appear to be nonessential to the plant, insect or microorganism producing them, in contrast to steroids, fatty acids and the polymers derived from them, which are essential and have structural role in most organisms. Substances that have no apparent utility to the organism that produces them are commonly known as secondary metabolites.

The function of natural products in living organisms has been a controversial issue for a long time. It has been suggested that secondary metabolites are formed in a living organism as metabolic byproducts. Some investigators believe that the secondary metabolites are a measure of the fitness of the organism to survive. The ability to synthesize an array of secondary

metabolites, which may repel or attract other organisms, has evolved as one facet of the organism's strategy for survival.

The use of natural products as medicines, poisons, hallucinogens, stimulants, perfumes, flavoring agents, insecticides, insect antifeedants, fungicides, plant growth regulating hormones, molluscicides, etc., is well known. It is not therefore difficult to understand what motivates chemists to isolate and characterize natural products. The characterization of new compounds is usually followed by their synthesis and the study of their biological activity and biosynthesis. Interest in natural products is also turning to chemotaxonomy. One of the most exciting things that emerged in the last few years is the realization that natural products that have been considered useless do have functions in the organisms from which they originate. It is recognized that many of them have vital roles as mediators of ecological interactions, thereby ensuring the continued survival of a particular organism.

Despite the vast number and structural diversity of metabolites, almost all arise by one of three biosynthetic pathways or by a combination of two or more of these pathways. These are known as the acetate, mevalonate and shikmate pathways [2-4].

2.0 LITERATURE REVIEW

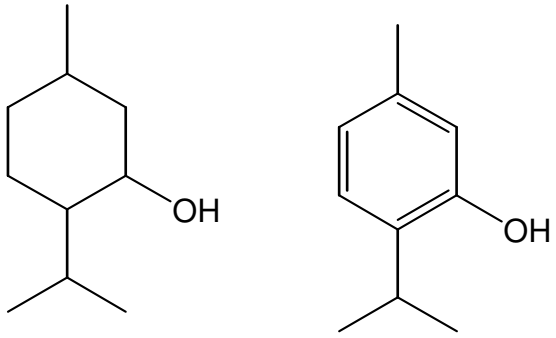
2.1 The family *Labiatae/Lamiaceae*

Labiatae is a large family of about 252 genera and 6700 species world wide, with 37 genera and 235 species indigenous or naturalized in South Africa. The *Labiatae* are important and many are of great economic importance. They are widely used in many traditional systems of medicines and horticultures.

Plants belonging to the family *Labiatae* occur almost throughout the world, with the exception of the coldest polar regions. *Labiatae* are particularly well represented in tropical and temperate areas especially those with a seasonal climate, such as the Mediterranean region and in tropical upland savannas. While some species are characteristic of semi-arid conditions, many others are adapted to wet habitats, in seasonally flooded areas or along riverbanks in forests.

Essential oils are commercially extracted from many species belonging to the *Labiatae*. Menthol **(1),** which is used in medicine and as a flavoring material, is one of the most important constituents of the oils. Thymol **(2)**, extracted from thyme, is used medicinally as very powerful antiseptic [5-8].

In general, plants belonging to the family *Labiatae* have been exploited for their antiviral, antibacterial, immuno-modulating, antifungal, insecticidal and ornamental properties [9, 10].



1 **2**

2.2 The genus *Leucas*

The genus *Leucas* belongs to the family *Labiatae/ Lamiaceae*. With about 150 species, the genus *Leucas* is native mainly to Africa and Asia, with 7 species found in South Africa and 18 species in Ethiopia.

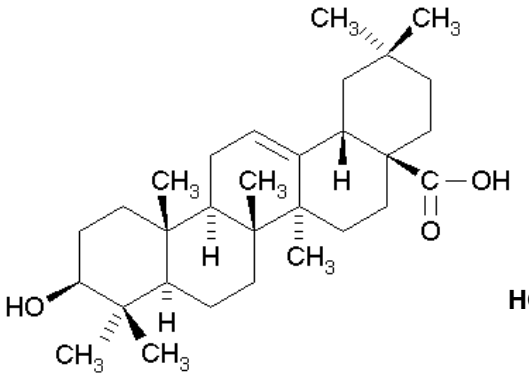
Leucas species have been reported to have woundhealing, antimycobacterial, anti-inflammatory, insecticidal, antifungal, sedative, antidiarrhoeal, etc., properties.

2.2.1 Review of The Chemistry of *Leucas* Species

Of the 150 species of *Leucas*, about seven species have so far been chemically investigated. A number of secondary metabolites have been isolated and characterized. From a number of *Leucas* species compounds like diterpenes, triterpenes, flavones, alkaloids, glycosides, sitosterols, chromon, sterol, oleanolic acid, ursolic acid, leucolacton, stigmasterol, campesterol, isopimarane, rhamnoglucoside, etc. have been isolated and characterized.

2.2.1.1 Leucas aspera

Leucas aspera is a plant which has been used in Indian traditional medicinal system in the treatment of various skin diseases and also as insecticides. The leaves are said to be useful in the treatment of chronic rheumatism and skin eruptions. As a result, chemical compounds of biological importance were investigated from the roots, shoots, and the leaves of the plant. Some of the compounds that have been isolated from the roots, shoots and leaves of the plant are oleanolic acid **(3)**, ursolic acid **(4)**, leucolactone **(5)**, acyl leucolactone **(6)**, diketo leucolactone **(7)**, 3β-acetyloleanan-28-13β-olide **(8),** sitosterol **(9)**, stigmasterol **(10)**, campesterol **(11)**, 1-dotriacontanol **(12)**, 1-hydroxytetratriacontan-4-one **(13)**, 32-methyltetratriacontan-8-ol **(14)**, 28-hydroxypentatriacontan-7-one **(15)**, 7-hydroxydotriacontan-2-one **(16)**, [11-13].



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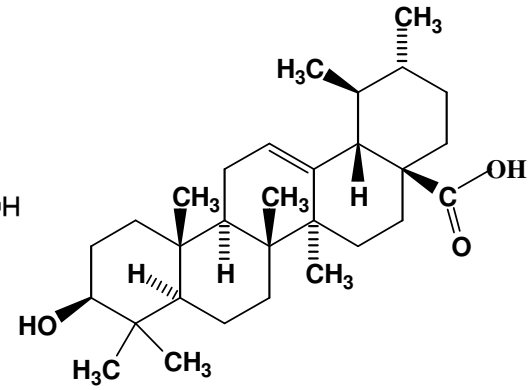

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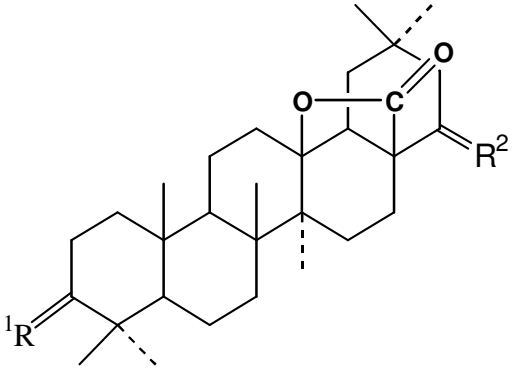
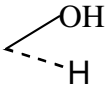
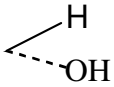
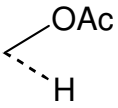
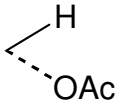
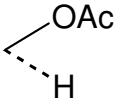
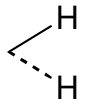
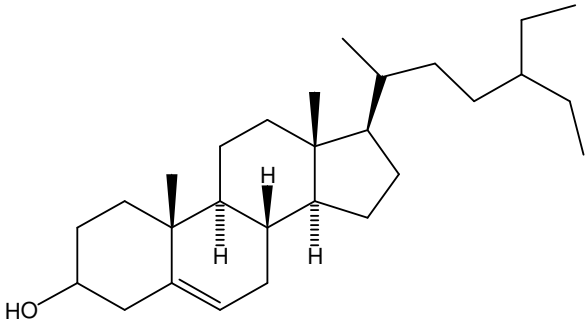


5 ^{1}R=  R^2= 

6 ^{1}R=  R^2= 

7 ^{1}R= R^2= O

8 ^{1}R=  R^2= 



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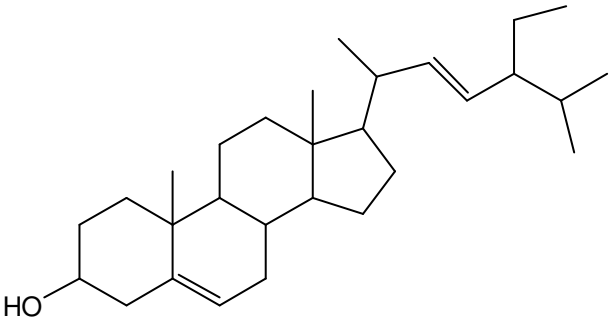

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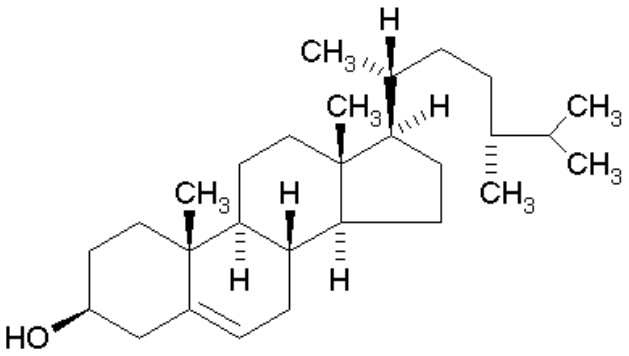


11

$CH_3\text{-}CH_2\text{-}(CH_2)_{28}\text{-}CH_2\text{-}CH_2\text{-}OH$

12

$Me\text{-}(CH_2)_{27}\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}OH$

13

$$Me\text{-}CH_2\text{-}\underset{\displaystyle Me}{\underset{|}{C}}H\text{-}CH_2\text{-}(CH_2)_{21}\text{-}CH_2\text{-}\underset{\displaystyle OH}{\underset{|}{C}}H\text{-}CH_2\text{-}(CH_2)_5\text{-}Me$$

14

$$Me\text{-}(CH_2)_6\text{-}\underset{\displaystyle OH}{\underset{|}{C}}H\text{-}(CH_2)_{20}\text{-}CO\text{-}(CH_2)_5\text{-}Me$$

15

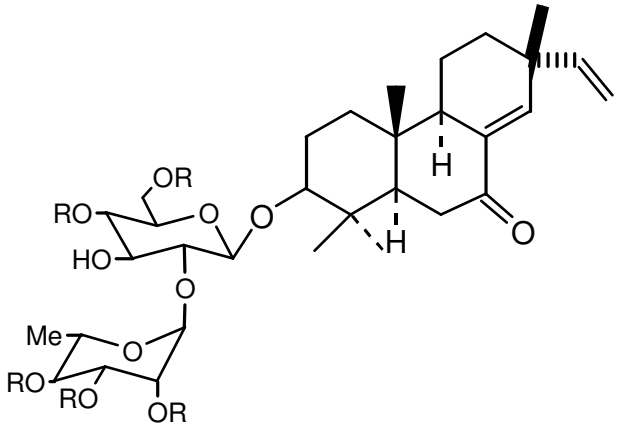
$$Me\text{-}CO\text{-}(CH_2)_4\text{-}\underset{\displaystyle OH}{\underset{|}{C}}H\text{-}(CH_2)_{24}\text{-}Me$$

16

2.2.1.2 *Leucas linifolia*

Leucas linifolia is an herbaceous annual weed which grows abundantly in field, pastures and wastelands of India. It has a strong flavor and is reputed for its use as sedative, vermifuge and dermatosis. Compounds that have been isolated from this plant are; linifolioside **(17)**, acetate

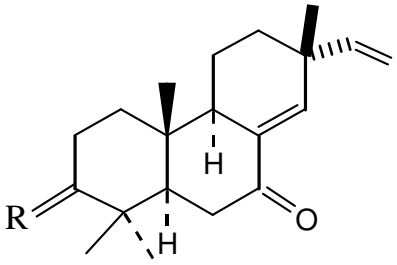
of linifolioside **(18)**, permethylate **(19)**, linifoliol **(20)**, acetate of linifoliol **(21)** and diketolinifoliol **(22)** [14].



17 R= H

18 R= Ac

19 R= Me



20 R= OH, H

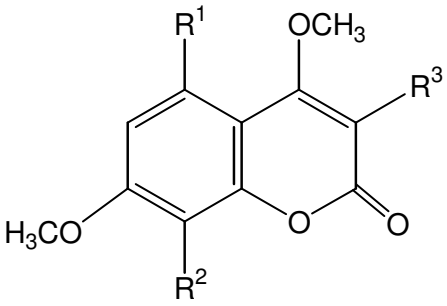
21 R= OAc, H

22 R= O

2.2.1.3 *Leucas inflata*

Leucas inflata is indigenous to the United Arab Emirates. The analgestic activity of the methanol and acetone extracts of the plant was evaluated. It was concluded that the crude methanol and acetone extracts of *L.inflata* have CNS depressant properties which manifested as anti-inflammatory and anti-pyretic actions. The two crude extracts were phytochemically analyzed and a few constituents were isolated and characterized. These include; siderin **(23)**,

coumarsabin **(24)**, 8-methoxycoumarsabin **(25),** coumarleucasin **(26)**, leucason **(27)**, chromon **(28)** and stigmasterol **(10)**. [15, 16]

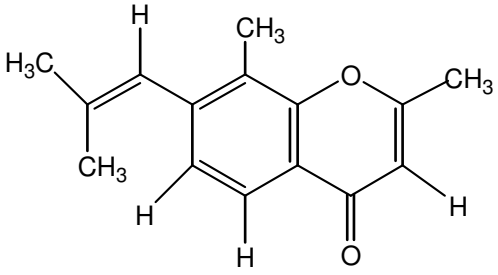


23 R^1= Me; R^2= H; R^3= H

24 R^1= Me; R^2= H; R^3= Me

25 R^1= Me; R^2= OMe; R^3= Me

26 R^1= CHO; R^2= OMe; R^3= Me



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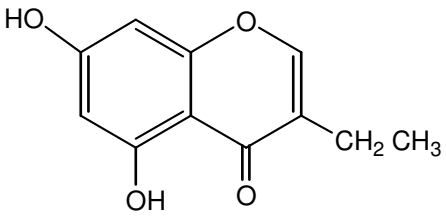

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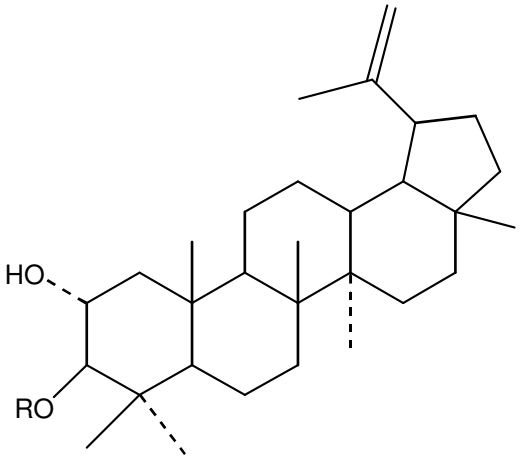
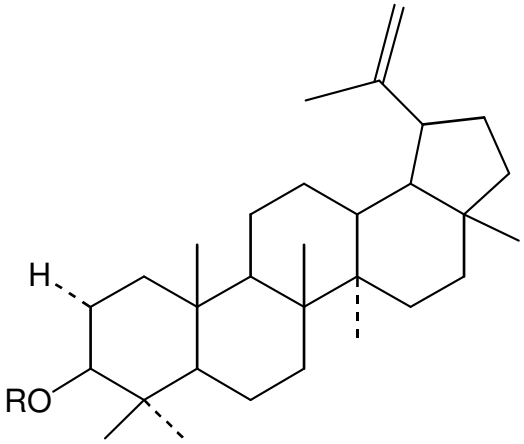
2.2.1.4 *Leucas nutans*

Leucas nutans is distributed in Sindh, Punjab and the North West frontier provinces of Pakistan. Compounds that have been isolated from this plant are the saponin leucasin **(29)**, prosapgenin **(30)**, glycone **(31)**, lupeol palmitate **(32)** [17].



29 R = Gly(1-2)Gly-

30 R = Gly-

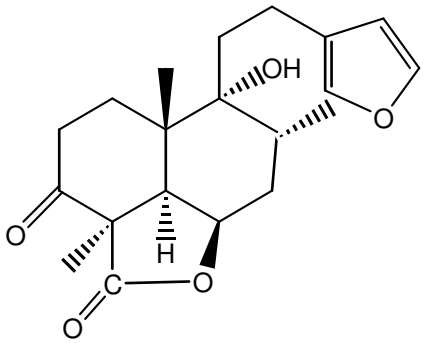


33 R= H

34 R= CO $(CH_2)_{14}CH_3$

2.2.1.5 *Leucas neufliseana*

Leucas neufliseana is a rare annual herb that grows wild in southeast Egypt. Compounds that have been isolated from the plant are peregrinone **(33)** and 9α,13α,15,16-bisepoxy-3-oxo-labdan-6β,19-olide **(34)** [18].



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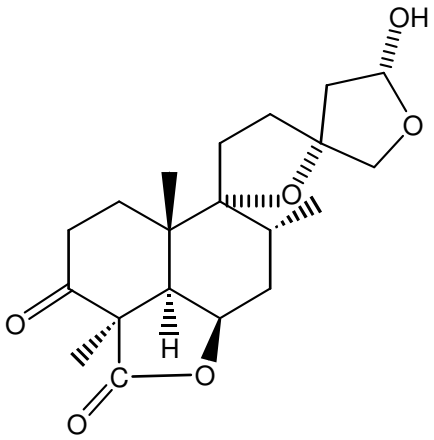

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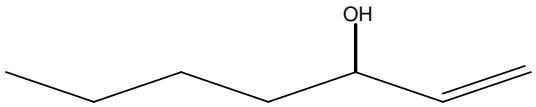
2.2.1.6 *Leucas lavandulaefolia*

Leucas lavandulaefolia Rees is a well known plant in India. Analysis of the ethanolic and methanolic extracts of *L. lavandulaefolia* showed significant properties like anti-diarrhoea wound healing, antipyretic and muscle relaxant activities [19-21].

2.2.1.7 *Leucas martinicensis*

Leucas martinicensis is a plant which is distributed in the tropical parts of the world. The essential oils obtained from the leaves and flowers of *L. martinicensis* were found to contain 1-hepten –3 ol **(35)** and germacrene **(36)** [22].



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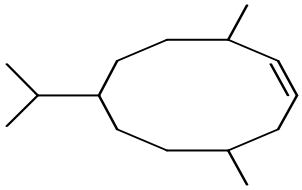

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The volatile oil obtained from *L. martinicensis* was reported to be suitable for use in pharmaceuticals, cosmetics and food products, e.g., lotions, creams, soaps, shampoos, rinses, gargles, candies [23]. Its antibacterial and antifungal properties have also been reported [24].

3.0 OBJECTIVES

Leucas martinicensis is one of the cultural medicinal plants found in the southern part of Ethiopia around Yabello town. The main objectives of this research work are, to isolate compounds from the leaves of *L. martinicensis* and elucidate their structures by the use of chemical and spectroscopic methods and to study their biological activity.

4 RESULTS AND DISCUSSIONS

4.1 Specimen collection and identification

Leucas martinicensis is an erect herb growing to 0.8 m in height. It has green leaves ovate to elliptic with toothed margins. The flowers are white with spiny calces, in tight whorls borne in the axils of the upper leaves [25].

It grows in disturbed bushland and grassland; 0-2500 m. It is widely distributed in the tropical parts of Africa, Arabia, Asia and America [26].

The leaves of *L. martinicensis* were collected in August 2002, from the southern part of Ethiopia around Yabello. The collection was carried out from two localities called Gerbi-Minch and Dida-Yaballo 2 Km North-East and 5 Km East of Yabello respectively. The plant specimen collected was identified by Prof. Sebsibe Demissew of the Biology Department (AAU). Voucher specimen was deposited at the National Herbarium under the voucher number Estifanos 3.

4.2 Ethnobotanical information on *Leucas martinicensis*

Leucas martinicensis is very common in Southern Ethiopia around Yabello town. It is frequently found in the valleys and flat lands. It is not common on hills and around hillsides. It grows on farmlands as weed. Semi arid weather condition of the area allows the plant to grow only during the rainy seasons.

L. martinicensis is known by the name “Mat-burisa” in Yabello area. The plant is well known for its medicinal value by the communities around Yabello. It is taken mainly to prevent diarrhea. The water extract of the leaves (about 30 mL) is taken only once. This amount of crude extract effectively cures the disease.

4.3 Extraction and isolation

4.3.1 Extraction

The air dried and powdered leaves of *L. martinicensis* (500 g) was first soaked in 1.2 L petrol for 24 h, followed by 1.0 L chloroform for 48 h and 1.0 L methanol for 48 h. The extracts were concentrated under vacuum to afford 7.69 g of petrol extract, 18 g of chloroform extract and 30 g of methanol extract.

4.3.2 Isolation

Isolation of the pure compounds was performed by column chromatography of the extracts on silica gel. The progress of the separation was monitored by TLC. The components were detected by exposing TLC plates to UV light and by spraying with vanillin-sulfuric acid reagent. For the compounds which have been isolated, ^{1}H NMR, ^{13}C NMR, IR and MP data were used in their identification. One compound was identified based on comparison of the spectra and TLC behaviors with an authentic material.

4.3.2.1 Fractionation of Chloroform Extract

The black, gummy residue from the chloroform extract (10 g) of the leaves of *L. martinicensis* was dissolved in chloroform and applied over a column packed with 300 g silica gel (Scheme I). The elution process was carried out by the use of chloroform and chloroform/ethyl acetate mixtures by increasing the amount of ethyl acetate. A total of 20 fractions, each 100 mL, were collected. Based on TLC analysis, those fractions that showed similar R_f values and identical

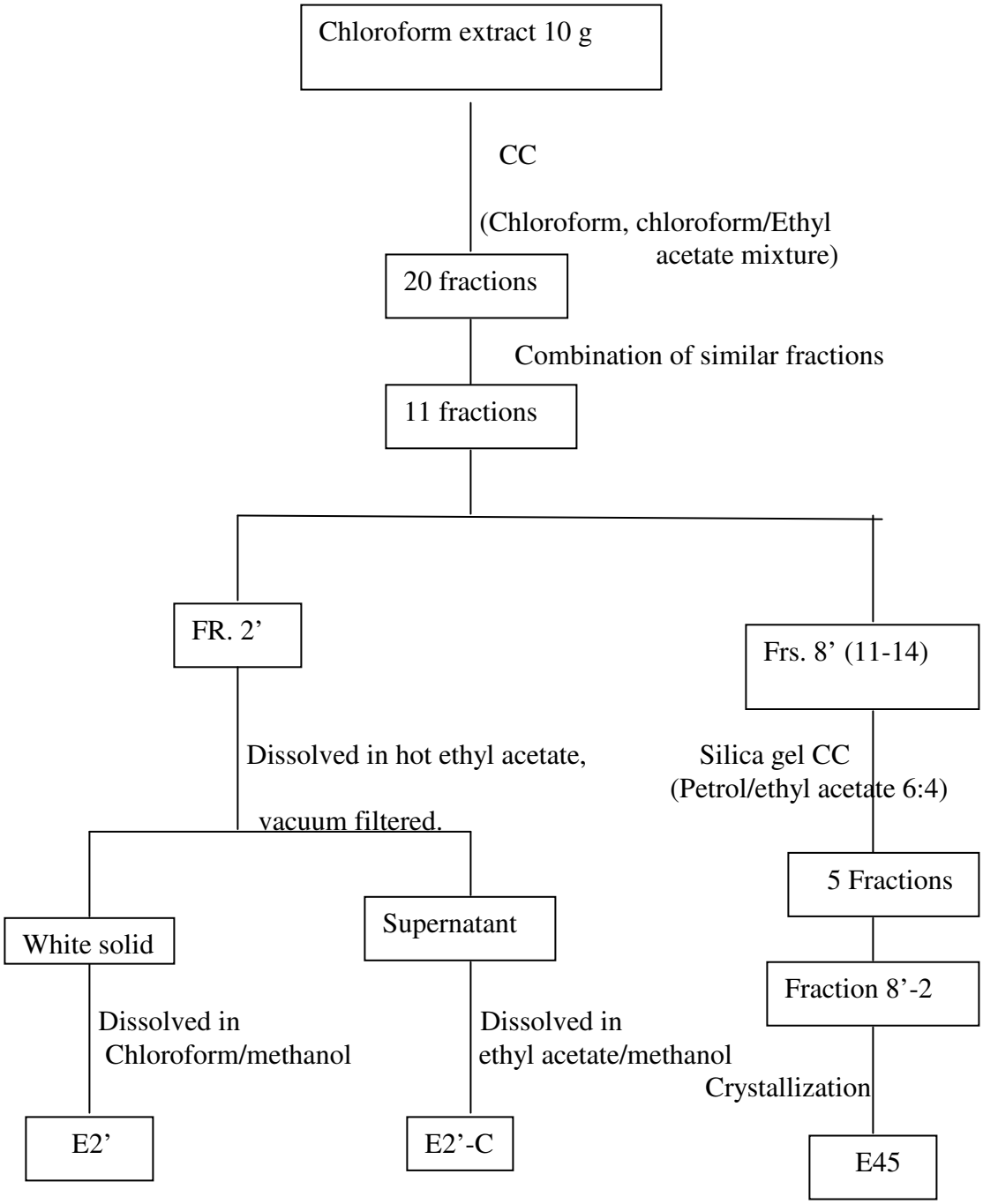
characteristic color were combined to give a total of 11 fractions as described in the Experimental part.

Fraction 2' was concentrated under vacuum and afforded 0.91 g yellowish material. The TLC (silica gel) analysis showed two spots with R_f 0.9 and 0.26 using petrol as developing solvent. The mixture was dissolved in hot ethyl acetate and allowed to cool: a white suspended material was formed. The white solid was separated by filtration. The yellowish supernatant solution was concentrated under vacuum and kept aside. The white solid (42 mg) was dissolved in hot chloroform/methanol and allowed to cool. The white suspended solid material (**E2'**) was separated by filtration and analyzed by both chemical and spectroscopic methods as discussed below.

The yellow solid that was obtained from the supernatant solution was dissolved in hot ethyl acetate/methanol. When the solution was cooled, a white solid material separated (**E2'-C**). The white solid was collected by filtration and was analyzed by chemical as well as spectroscopic methods, as discussed below.

A dark precipitate (0.5 g) from fraction 8' was analyzed by TLC and was found to show a major spot with R_f 0.3 (petrol/ethyl acetate 6:4). It was applied on a column packed with 100 g silica gel and was eluted with petrol/ethyl acetate (6:4). Five fractions were collected. Fraction number 2 (30 mg) was found to show a single spot on TLC with R_f 0.3 (petrol/ethyl acetate 6:4). It was crystallized from chloroform methanol to afford reddish crystals. The identification of this material is discussed below.

Scheme-I Fractionation of the chloroform extract

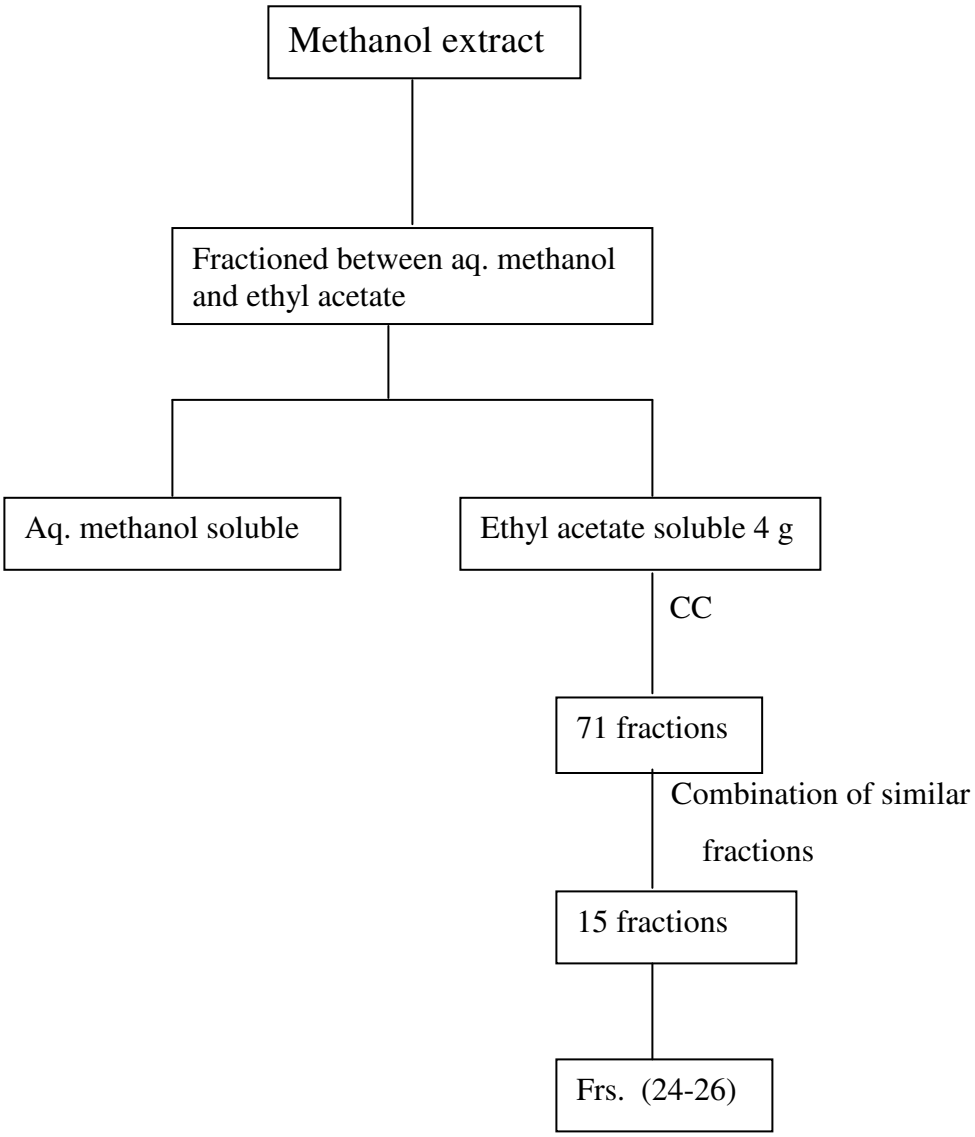

Chloroform extract 10 g
CC
(Chloroform, chloroform/Ethyl acetate mixture)
20 fractions
Combination of similar fractions
11 fractions
FR. 2'
Frs. 8' (11-14)
Dissolved in hot ethyl acetate,
vacuum filtered.
Silica gel CC
(Petrol/ethyl acetate 6:4)
5 Fractions
White solid
Supernatant
Fraction 8'-2
Dissolved in
Chloroform/methanol
Dissolved in
ethyl acetate/methanol
Crystallization
E2'
E2'-C
E45

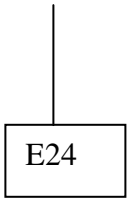
4.3.2.2 Fractionation of the methanol extract

The methanol extract (20 g) was dissolved in water and extracted with ethyl acetate (Scheme II). The ethyl acetate soluble material was concentrated and gave 4 g of a dark gummy material. This crude material was adsorbed on 30 g silica gel and was applied on a column packed with 120 g silica gel. N-hexane, n-hexane/ethyl acetate and ethyl acetate/methanol were used for gradient elution. 71 fractions, each of 100 mL, were collected. Fractions were combined in to a total of 15 fractions based on their TLC behaviors.

Fractions 24-26 were combined and concentrated under vacuum. When the mixture was taken-up in methanol, a white precipitate formed, which was recovered by filtration. TLC analysis of the white precipitate showed a single spot R_f 0.24 (petrol/ethyl acetate 8:2) with impurity at the baseline. The material was repeatedly washed with hot methanol until the impurity at the baseline was removed. The pure compound was analyzed by NMR. Comparisons of the NMR data and TLC behavior of this compound is discussed below.

Scheme-II Fractionation of the methanol extract.





4.4 Identification of compounds isolated from the leaves of *L. martinicensis*

The investigation of the chloroform and methanol extracts of the leaves of *L. martinicensis* resulted in the identification of four compounds. These are triacontane **(E2')**, tetracosane **E2'-C)**, lutein **(E45)** and cholesterol **(E24)**.

Triacontane **(E2')**, tetracosane **(E2'-C)** and lutein **(E45)** were identified based on their IR, ^{1}H NMR, ^{13}C NMR and MP data. Cholesterol **(E24)** was identified by direct comparison with an authentic sample.

The elucidation of the structures of compounds **E2'**, **E2'-C, E45** and **E24** are discussed below.

4.4.1 E2'

Compound **E2'** was obtained in pure form after column chromatography on silica gel and precipitated out from chloroform/methanol as described in the Experimental section.

E2' had a melting point of 66-68 ^{0}C. The compound was not soluble in cold conc. H_2SO_4. But, it gave a violet color on exposure to iodine. According to the "iodine charge transfer" and "insolubility in cold conc. H_2SO_4" tests **E2'** did not contain unsaturated sites nor did it contain functional groups having oxygen or nitrogen.

The IR spectrum displayed the presence of a band at 720 cm^{-1} attributable to long chain CH_2 rocking vibration. The bands at 1470 and 1370 cm^{-1} are due to CH_2 bending and bands at 2890 cm^{-1} and 2960 cm^{-1} are due to CH_2 and CH_3 stretching (Appendix 1). Based on this spectrum it was proposed that **E2'** could be a straight chain aliphatic compound.

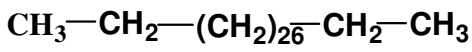
The ^{1}H NMR spectrum showed a triplet at δ 0.7 integrating for three protons and a broad peak at δ 1.2 integrating for 28 protons. Thus, the presence of one methyl group and 14 methylene groups could be inferred from the ^{1}H NMR spectrum. ^{13}C NMR spectrum showed a total of six carbon resonances in the range δ 14.5-32.3. The DEPT-135 spectrum indicated the presence of one methyl carbon (δ 14.5 ppm) and five methylene carbon atoms (Appendix 2 and 3).

Both the proton ^{1}H and ^{13}C NMR spectra are in good agreement with the assumption that **E2'** could be a straight-chain alkane.

The ^{1}H NMR and ^{13} C NMR data revealed that the compound may be symmetrical with a total of 2 methyl and 28 methylene carbons. Comparison of the melting point of the isolated compound with that of a straight-chain compound containing a total of 30 carbons revealed a good agreement (66-68 ^{0}C and lit [27] 65-67). This was also supported by the violet iodine test

and insolubility of the compound in cold concentrated sulfuric acid, which is a characteristic behavior of saturated compounds [28].

Thus, it was concluded that **E2'** is most likely triacontane ($C_{30}H_{62}$). Table 1 shows the ^{13}C NMR assignment of **E2'**.



E2'

Table 1 ^{13}C NMR assignments of compound **E 2'**

C	δ
1 & 30	14.47
2 & 29	23.07
3 & 28	32.31
4 & 27	29.74
5 & 26	30.04
6 – 25	30.08

4.4.2 E2'-C

This white solid material was isolated from the less polar fractions of the column chromatography of the chloroform extract. It had a melting point of 51-54^{0}C. The IR spectrum displayed absorption band at 740 cm^{-1} attributable to long chain CH_2 rocking vibration. The bands at 1480 and 1390 cm^{-1} are due to CH_2 bending, while bands between 2860 and 3000 cm^{-1} are due to stretching vibration of CH_2 and CH_3 groups (Appendix 4).

The ^{1}H NMR spectrum showed a triplet at δ 0.7 integrating for 3 protons and a peak at δ 1.2 integrating for 22 protons. These upfield proton shifts could be due to methyl and methylene protons. The ^{13}C NMR spectrum showed a total of five carbon resonances of which one is found at δ 14.4 and others are found in the range of δ 23-32.3.

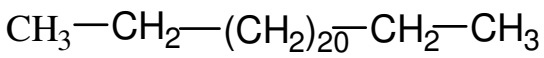
The DEPT-135 spectrum showed the presence of one methyl carbon and four methylene carbons (Appendix 5 and 6). The absence of methine and quaternary carbons suggests that the compound could be a straight chain alkane.

E2'-C gave a violet color with iodine and turned out to be insoluble in cold concentrated sulfuric acid. This showed that the compound has no unsaturation or functional group containing oxygen or nitrogen

The ^{1}H NMR and ^{13} C NMR data revealed that the compound can be symmetrical with a total of 2 methyl and 22 methylene carbons. Comparison of the melting point of **E2'-C** with that of a straight chain alkane containing 24 carbons revealed a good agreement (51-54^{o}C, lit. [29] 49-

52). The assumption was also supported by the violet iodine test and insolubility of the compound in cold concentrated sulfuric acid.

Thus, it was concluded that **E2'-C** is most likely the alkane tetracosane. Table 2 shows the ^{13}C NMR assignments of **E2'-C.**



E2'-C

Table 2 ^{13}C NMR assignments of compound E **2'-C**

C	δ
1 & 24	14.48
2 & 21	23.07
3 & 20	32.32
4 & 19	29.75
5-18	30.091

4.4.3 E45

Compound **E45** was obtained from the polar fraction of the chromatography of the chloroform extract as described in the experimental section. Crystallization from chloroform-methanol afforded **E45** as red crystals (mp 193-196 ^{0}C).

The ^{1}H NMR spectrum revealed a one proton multiplet at δ 3.9 and a one proton broad singlet at δ 4.2 assignable to protons on oxygenated carbons. The proton resonances in the range δ 5.5-6.7 are due to 15 protons attached on olefinic carbons. The signals in the range δ 0.5-2.5 are due to a total of one methine, 6 methylene and 30 methyl protons (Appendix 7)

The ^{13}C NMR spectrum showed a total of 40 carbon resonances indicating that **E45** could be a tetraterpene. 22 carbon signals are found in the olefinic region indicating the presence of at least eleven double bonds. The remaining carbon resonances are in the range δ 12-67 ppm. Resonances at δ 65 and 66 are due to oxygenated carbons (Appendix 8).

From the DEPT-135 spectrum, a total of nine quaternary carbons were identified, among which seven are found in the olefinic region and the remaining two are found at δ 37 and 34. The spectrum also revealed the presence of four methylene carbons and a total of 27 methine and methyl carbons.

Based on the spectral data, especially the distribution of proton resonances around δ 4.0 and the olefinic region, the total number of carbon atoms, the number of double bonds, the number of quaternary carbons in the olefinic and aliphatic regions and the number of methylene

carbons, **E45** could possibly be a cyclic carotenoid. Comparison of the ^{13}C NMR spectral data of **E45** with that reported for lutein in the literature showed a very close agreement as shown in the table below Table 3.

Table 3 Comparison of ^{13}C NMR spectral data of **E45** with that of lutein.

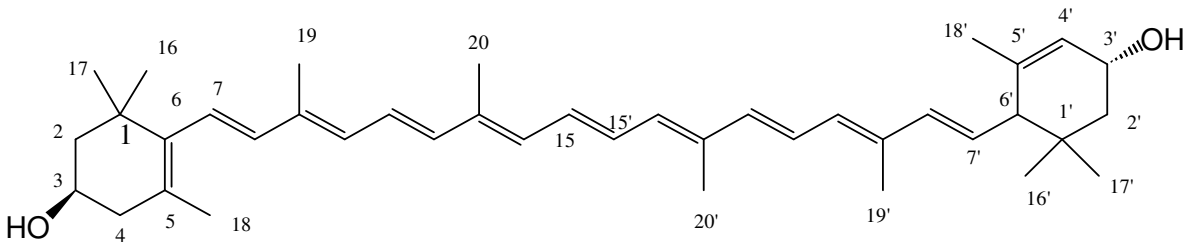
	LUTEIN		E 45	
C.No	Beta end	Alpha end	Beta end	Alpha end
1.	37.1	34.0	37.5	34.4
2.	48.4	44.7	48.8	45
3.	65.1	65.9	65.5	66.3
4.	42.5	125.6	42.9	125.9
5.	126.2	137.8	126.5	138.3
6.	137.6	55.0	138.1	55.4
7.	125.6	128.6	125.3	129.1
8.	138.5	138.5	138.8	138.8
9.	135.6	135.0	136.0	135.4
10.	131.3	130.8	131.6	131.2
11.	124.9	124.5	125.2	124.9
12.	137.6	137.6	137.9	138.1

Table 3 Comparison of ^{13}C NMR spectral data of **E45** with that of lutein (contd.)

13.	136.5	136.5	136.8	136.8
14.	132.5	132.6	132.9	132.9
15.	130.0	130.0	130.4	130.4
1-Me's	28.7, 30.2	24.3, 29.5	29.1, 30.6	24.7, 29.8
5-Me	21.6	22.8	21.9	23.2

9-Me	12.7	13.2	13.1	13.4
13-Me	12.7	12.7	13.1	13.1

The spectrum reveals the presence of ten methyl singlets between δ 0.8 and 1.9. Proton resonances due to H-2', H-6', H-2 and H-4 appear in the region between δ 1.4 and 2.4. The broad singlets at δ 4.2 and 5.5 are assignable to H-3' and H-4' respectively. The one-proton multiplets at δ 3.9 and 5.35 are due to H-3 and H-7'. The olefinic proton resonances appear between δ 5.5 and 6.4.



β-end **E45** **α-end**

Table 4 ^{1}H NMR data of **E45**

δ ppm	H
0.5-2.0 (singlets)	3H, Me(16, 16',17, 17', 18, 18', 19, 19', 20, 20')

Table 4 ^{1}H NMR data of **E45** (contd.)

2.3 (doublet of doublets)	2H, H-C(2'), H-C(2)
3.9 (multiplet)	1H, H-C(3)

4.2 (broad singlets)	1H, H-(3')
5.35 (doublets of doublet)	1H, H-C(7')
5.5 (singlet)	1H, H-C(4')
6.0 (doublet)	5H, H-C(8, 8', 10, 10', 7)
6.15 & 6.25 (doublets)	4H, H-C(12, 12', 14, 14')
6.5 (doublets of doublet)	4H, H-C(11, 11', 15, 15')

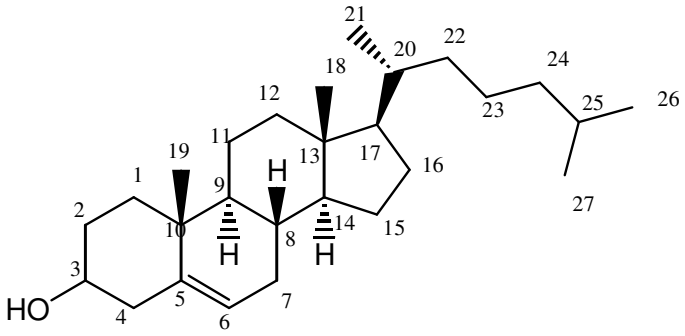
The above spectroscopic data and comparison of these with those reported in the literature for lutein allowed the identification of **E45** as the known carotenoid lutein.

4.4.4 E24

This white solid material obtained from the methanol extract showed a violet color upon spraying with vanillin-sulfuric acid reagent. The ^{1}H NMR data shows, a one proton doublet at δ 5.3 which can be due to olefinic proton, a one proton multiplet at δ 3.5 which is probably due to a proton attached to an oxygenated carbon and complex proton resonances in the range δ 2.5-0.5 (Appendix 10).

Although the ^{1}H NMR spectrum of **E24** appeared to be due to a pure compound, a close look at the integration of the proton resonances revealed that **E24** is a 1:2 mixture of two compounds. The signals due to the major compound had the characteristic feature of a triterpene with one double bond and one oxygenated carbon atom. Thus, the ^{1}H and ^{13}C NMR

spectra of **E24** were compared with those of authentic cholesterol to reveal a very close resemblance (Appendices 12 &13). In addition TLC comparison (in five different solvent systems) of **E24** with authentic cholesterol showed that the major component of **E24** is identical to cholesterol (Appendix 15). Table 5 compares the ^{13}C chemical shifts of authentic cholesterol with those of **E24**.



E24

Table 5 Comparison of the ^{13}C NMR of cholesterol with those of **E24**

	Literature value	Ref. cpd	**E24**
C No	δ	δ	δ
1	37.5	37.6	37.6
2	31.6	32.0	32.0
3	71.3	72.2	72.2
4	42.2	42.6	42.6
5	141.2	141.1	141.1
6	121.3	122.1	122.1
7	32.0	32.2	32.2
8	32.3	32.3	32.3
9	50.5	50.5	50.5

Table 5 Comparison of the ^{13}C NMR of cholesterol with those of **E24** (contd.)

10	36.5	36.9	36.9
11	21.2	21.2	21.4
12	28.3	28.3	28.6
13	42.4	42.6	42.6
14	56.9	57.1	57.1
15	24.3	24.7	24.7
16	40.0	40.1	40.1
17	56.5	56.4	56.4
18	12.0	12.2	12.2
19	19.4	19.7	19.7
20	35.8	36.5	36.5
21	18.8	19.1	19.1
22	36.4	37.6	37.6
23	24.1	24.1	24.7
24	39.6	39.6	40.1
25	28.0	28.6	28.6
26	22.5	21.6	21.6
27	22.8	23.4	23.4

5.0 EXPERIMENTAL

5.1 General

Instruments

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer at 400.13 and 100.06 MHz, respectively.

IR Spectra were recorded using Pye-Unicam SP 200 Infrared Spectrometer in the range 4000-200 cm^{-1}.

Melting points were recorded on Leica A-1170 melting point microscope and Thomas Hoover Capillary melting point apparatus and are uncorrected.

Chromatography

Analytical TLC: Silica gel with fluorescent indicator 254 nm on aluminum cards with layer thickness 0.2 mm.

Alumina with fluorescent indicator 254 nm on aluminum cards with layer thickness 0.2 mm.

Column Chromatography:

Silica gel 60 (Merck), particle size 0.063-0.200 (70-230 mesh ASTM).

Sephadex LH-20 ($CHCl_3$: MeOH 2:1)

Plant material

Leucas martinicensis was collected from "Dida-Yaballo and Gerbi-Minch" in Borena, Ethiopia, during August 2002. The plant specimen was identified by Prof. Sebsibe Demissew of the Biology Department AAU. Voucher specimen of the plant was deposited in the AAU National Herbarium under voucher number Estifanos 3.

5.2 Extraction

The extraction of air dried and powdered leaves (500 g) of *L.martinicensis* was done by first soaking in 1.2 L petrol for 24 h, followed by 1.0 L chloroform for 48 h and 1.0 L methanol for 48 h. The extracts were concentrated under vacuum to afford 7.69 g of petrol extract, 18 g of chloroform extract and 30 g of methanol extract.

5.3 Isolation

The crude (10 g) chloroform extract was applied on a column (46 cm x 4 cm) packed with 300 g silica gel. It was eluted with 200 mL chloroform followed by chloroform/ethyl acetate mixtures with increasing amounts of ethyl acetate. A total of 20 fractions (each 100mL) were collected as follows.

Solvent system	**Ratio**	**Fractions**	**Volume**
Chloroform-Ethyl acetate	10:0	1 & 2	200 mL
"	9:1	3 & 4	200 mL
"	8:2	5 – 11	600 mL
"	6:4	12 – 14	300 mL
"	4:6	15 & 16	200 mL
"	2:8	17	100 mL
"	1:9	18	100 mL
"	0:10	19 & 20	200 mL

All fractions were analyzed by TLC and those that showed similar R_f values and characteristic color were combined.

Fractions combined	**Code**	**Weight**

1	1’	35 mg
2	2’	0.91 g
3 & 4	3’	52 mg
5 & 6	4’	2 g
7	5’	-
8	6’	0.2 g
9 & 10	7’	0.7 g
11-14	8’	0.5 g
15&16	9’	-
17&18	10’	1.2 g
19&20	11’	-

To isolate pure compounds, analysis was done on promising fractions. Some of the fractions were very small in quantity and others showed a number of spots on TLC. Detail analyses done were described below.

5.3.1 Fraction 2’

Fraction 2’ was concentrated under reduced pressure and gave 0.91 g of a mixture of compounds. It was analyzed by TLC in petrol, where two spots with R_f 0.9 and 0.26 were observed. While concentrating the dissolved material in open-air, a solid was formed on the top layer of the liquid. It was separated by decanting the liquid and washed with 10-15 mL acetone and then the same amount of methanol. Part of the yellow color was removed during

washing. The solid material was dissolved in hot ethyl acetate and then allowed to cool. A precipitate formed; it was separated by filtration and was washed with ethyl acetate until the yellow color was completely removed. The yellow solution was concentrated under vacuum and kept aside. The white amorphous solid (42 mg) was dissolved in hot chloroform-methanol. On cooling of the solution a white material was formed. It was separated from the solution by filtration. It was then analyzed by chemical and spectroscopic methods and was identified to be triacontane (**E2'**).

Triacontane (**E2'**): White solid; R_f 0.9 (petrol); **mp**.(66-68 ^{0}C lit. [27] 65-67); IR ν_{max} (KBr) cm^{-1}: 720, 1370, 1470, 2890 and 2960; ^{1}H **NMR** (400 MHz, $CDCl_3$): δ 0.7 (6H, **t**, methyl) and δ 1.2 (58H, **br. S**, methylene), ^{13}C **NMR** (100 MHz, $CDCl_3$), δ 14.5 (methyl carbon) and δ 23.1-32.3 (methylene carbons) see Appendix 1, 2 and 3.

5.3.2 Supernatant solution from fraction 2'

The supernatant solution was concentrated under vacuum and gave a yellowish solid material. It was dissolved in hot ethyl acetate/methanol. A white solid material was formed on cooling of the solution. It was allowed to settle down and then vacuum filtered. Finally it was analyzed by chemical and spectroscopic methods and found to be tetracosane (**E2'-C**).

Tetracosane (**E2'-C**): white solid; R_f 0.92 (petrol); **mp** (51-54^oC lit. [29] 49-52); **IR** ν_{max} (KBr) cm^{-1}: 740, 1390, 1480, 2860-3000; ^1H **NMR** (400 MHz, $CDCl_3$): δ 0.7 (6H, **t**, methyl), δ 1.2 (44H, **br. S**, methylene); ^{13}C **NMR** (100 MHz, $CDCl_3$), δ 14.4 (methyl carbon), δ 23.0-32.3 (methylene carbons) see Appendix 4, 5 and 6.

5.3.3 Combined fraction 8' (Fraction 11-14)

The mixture of compounds (0.5 g) obtained from fraction 8' was chromatographed over a column (27 cm x 2.6 cm) packed with 100 g silica gel using petrol-ethyl acetate (6:4). Five fractions were collected and coded as E44, **E45**, E46, E47 and E48. **E45** was analyzed by TLC and showed a single spot with R_f 0.3 in petrol-ethyl acetate (6:4). **E45** (30 mg) was crystallized from chloroform-methanol repeatedly. Reddish crystals were formed which were analyzed by NMR. **E45** was identified as the known carotenoid lutein.

Lutein (**E45**): reddish crystals R_f 0.3 (petrol-ethyl acetate 6:4), mp 193-198, ^1H **NMR** (400 MHz, $CDCl_3$) δ 4.2 (1H, s, H-3'), δ 5.5 (1H, s, H-4'), δ 3.9 (1H, m, H-3), δ 5.35 (1H, dd, H-7'), δ 6.15 (2H, d, H-12, 12'), δ 6.25 (2H, d, H-14, 14'), δ 2.3 (2H, m, H4), δ 6.5 (4H, dd, H-11, 11', 15, 15'), δ 6.05 (5H, m, H-8, 8', 10, 10', 7), ^{13}C **NMR** (100 MHz, $CDCl_3$), δ 125-140 (22C, olefin), δ 126.5, 138.1, 138.3, 135.4, 136, 136.8, 136.8 (7C, quaternary) δ 65, 66 (2C, hydroxylated), δ 55.4 (α-ring C-6), δ 42.9, 45, 48.8, (3C, methylene, C-2, 2', 4), δ 37.5, 34.4 (2C, quaternary, C-1, 1') see appendix 7 and 8.

5.4 Methanol extract

The methanol extract was fractionated between ethyl acetate and water. The dark residue (4 g) obtained from the ethyl acetate extract was chromatographed over a column (30 cm x 3 cm) packed with silica gel (120 g) and was eluted with n-hexane, n-hexane/ethyl acetate and ethyl acetate/methanol mixtures and 100 mL fractions were collected as shown below.

Solvent system		Fraction collected
Hexane		1-4
Hexane/ethyl acetate	(9.5:0.5)	5-13
"	(9:1)	14-24
"	(8:2)	25-33
"	(7:3)	34-39
"	(6:4)	40-43
"	(5:5)	44-47
"	(4:6)	48-50
"	(2:8)	51-56
"	(1:9)	57-60
Ethyl acetate		61-63
Ethyl acetate/methanol	(9:1)	64-67
"	(8-2)	68-71

Fractions were analyzed by TLC and similar fractions were combined. Based on the quantity of the fractions and TLC spot separation, attempts were made to isolate pure compounds. As a result one compound was isolated and characterized.

5.4.1 Fractions (24-26)

Fraction 24-26 were combined and concentrated under vacuum. The mixture (70 mg) was dissolved in methanol, and a white precipitate settled down. The material was separated by decantation. It was analyzed by TLC and showed a single spot with impurity at the base. The mixture was taken up in hot methanol and the undissolved white material was separated by filtration. The white solid (**E24**) was analyzed by NMR.

Cholesterol (**E24**): white solid; R_f 0.25 (petrol/ethyl acetate 8:2); mp. 138-145 ^{0}C, (lit. [30], 148.5 ^{0}C), ^{1}H NMR (400 MHz, $CDCl_3$), δ 5.3 (1H, d, H-6), δ 3.5 (1H, m, H-3), ^{13}C NMR (100 MHz), see Table 5 and Appendix 10 and 11.

6.0 REFERENCES

1 Mann, J., *Secondary metabolism*, 2nd ed., 1995, Oxford University Press, New York., Introduction.

2 Mann, J., *Chemical Aspect of Biosynthesis,* 1st ed., 1994, Oxford University Press, New York, Preface.

3 Solomon, H.*, MSc. Thesis*, AAU, 1997, pp 1.

4 Alemayehu, G., *Ph. D. Thesis,* AAU, 1999, p 1.

5 Hooker W.J., *Niger Flora*, 1996, Wheldon & Wesley Ltd., New York, N.Y., P. 489.

6 Hutchinson and Daiziel, *Flora of West Tropical Africa,* 2nd ed., 1954, Crown Agents for Oversea Governments and Administrations, Millbank, London, S. W. 1., p. 470.

7 Riley, H.P., *Family of Flowering Plants of Southern Africa,* 1963, University of Kentucky Press, p.188-190.

8 http://www. Museums. Org. Za/ bio/ plants/ lamiaceae.

9 Chem. Abst., 1999, **131**, **No.7**, p92498t.

10 Chem. abst., 1989, **110**, 92122g

11 Pradhan B. P., Chakraborty, D.K., Subba, G. C., *Phytochemistry,* 1990, **29,**1693-1695

12 Misra, T.N., Singh, R.S., Prasad, C., Singh, S., *Phytochemistry,* 1993, **32,** 199-201

13 Misra, T. N., Singh, R. S., Pandey, H. S., Singh, S., *Phytochemistry,* 1992, **31,** 1809-1810.

14 Mahato, S. B., Pal, B. C., *Phytochemistry*, 1986, **25,** 909-912**.**

15 Al Yousuf, M. H., Bashir, A. K., Blunden, G., Yang, M., Patel, A. V., *Phytochemistry,* 1999, 51, 95-98.

16 http://www.ncbi.nlm.nih.gov/enterz/query.fcgi?cmb=Retrieve&db=pubmed&list_uids=12410559&dopt=abstract

17 Hasan, M., Burdi, D. K., Ahmad, V. U., *Phytochemistry,* 1991, **30,** 4181-4183

18 Khalil, A. T., Gedara, S. R., Lahloub, M. F. and Halim, A. F., *Phytochemistry*, 1996, **41**, 1569-1571.

19 Mukherjee, P. K, Saha, K., Murugesan, T., Mandal, S.C., Pal, M., Saha, B. P., *Journal of Ethnopharmacology,* 1998, **60**, 85-89

20 http://www.ncbi.nlm.nih.gov/enterz/query.fcgi?cmd=Retrieve&db=pubmed&list_uids=9174975&dopt=abstract

21 http://www.ncbi.nlm.nih.gov/enterz/query.fcgi?cmd=Retrieve&db=pubmed&list_uids=12403158&dopt=abstract

22 *Chem. Abst.*, 1999, **130,** 227525b

23 http:/uzweb.uz.ac.zw/medicine/pharmacy/pubs/19912.html

24 Personal communication with Ato Melaku Wendafrash, AAU, Herbarium

25 Reinhard Fichtl and Admasu Adi, Hony Bee Flora of Ethiopia, Ded. Margraf Verlag, 1994, pp109.

26 Muhayimana, A., Chalchat, J. C., Jerry, R. P., .*Journal of Essentials Oil Research*, 1998, **10**, 251-259.

27 http://www.sigmaaldrich.com/cgi-bin/hsrun/Distributed/HahtShop/HAHTpage/frmCatalogSearchPost?Brand=

ALDRICH&ProdNo=263842

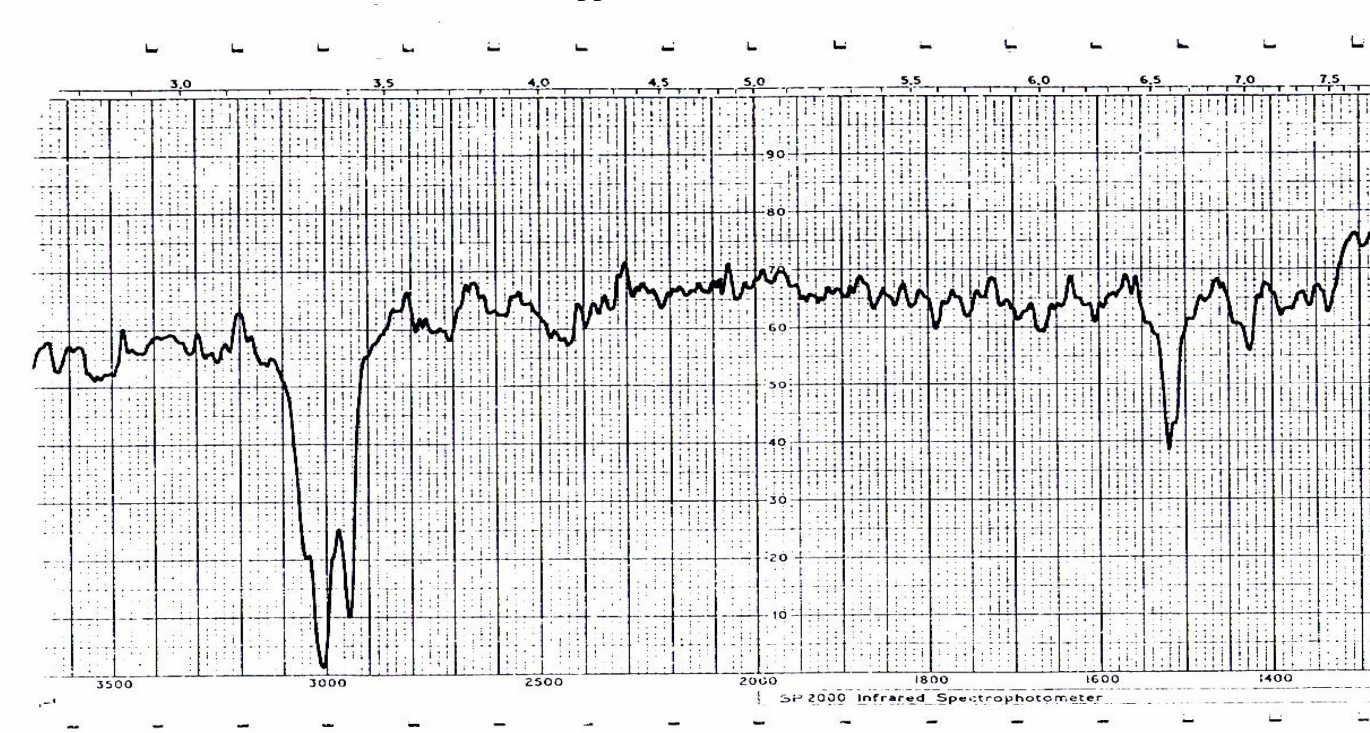
28 Pasto, D. J., Johnson, C. R., *Organic structure determination*, Prentice-Hall, Inc., Englewood Cliffs, N.J. 1969, pp. 327-328.

29 http://www.sigmaaldrich.com/cgi-bin/hsrun/Distributed/HahtShop/HAHTpage/frmCatalogSearchPost?Brand=ALDRICH&ProdNo=T8752

30 Chapman and Hall, *Dictionary of Natural products*, Vol. 1 A-C, Great Britain University Press, Cambridge, 1994, pp. 1011.

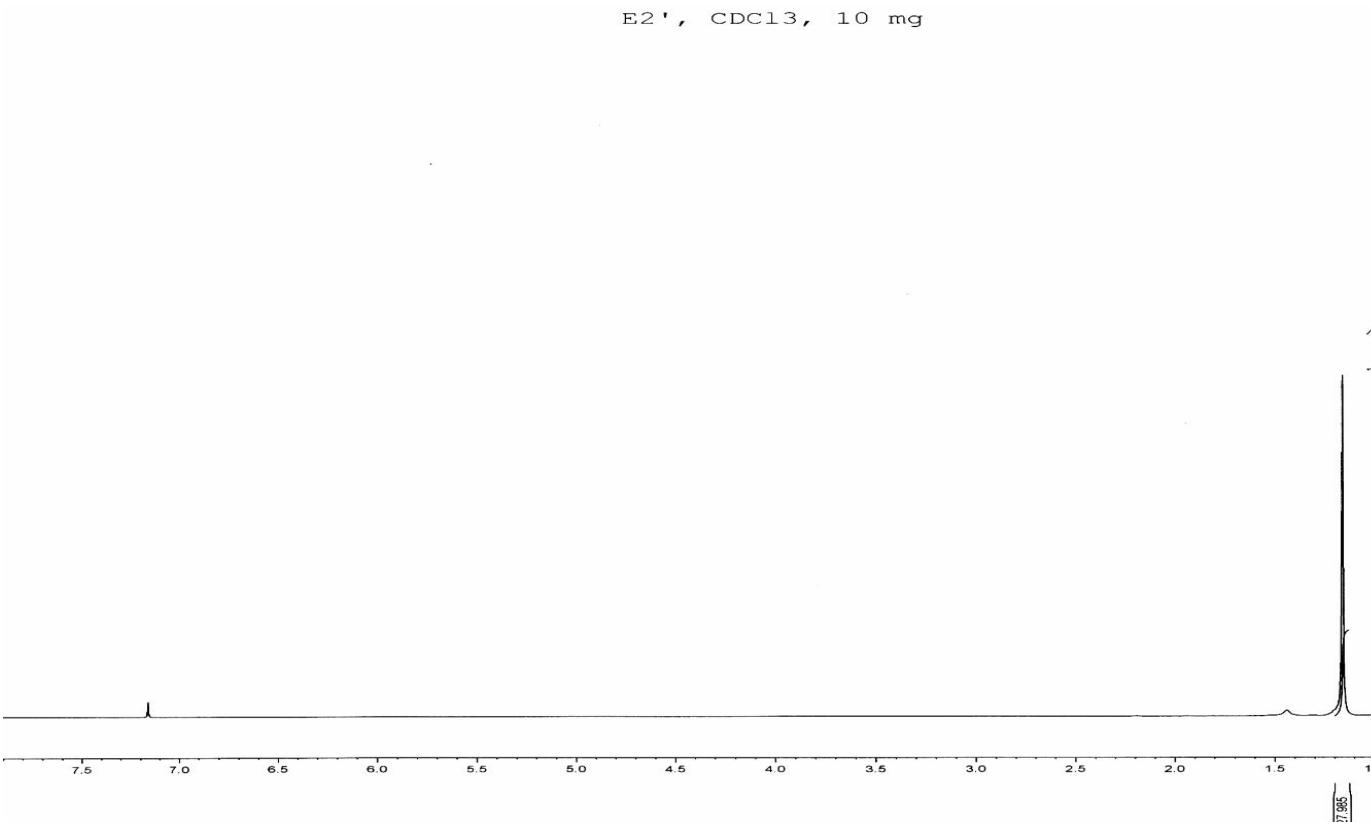
APPENDICES

Appendix 1



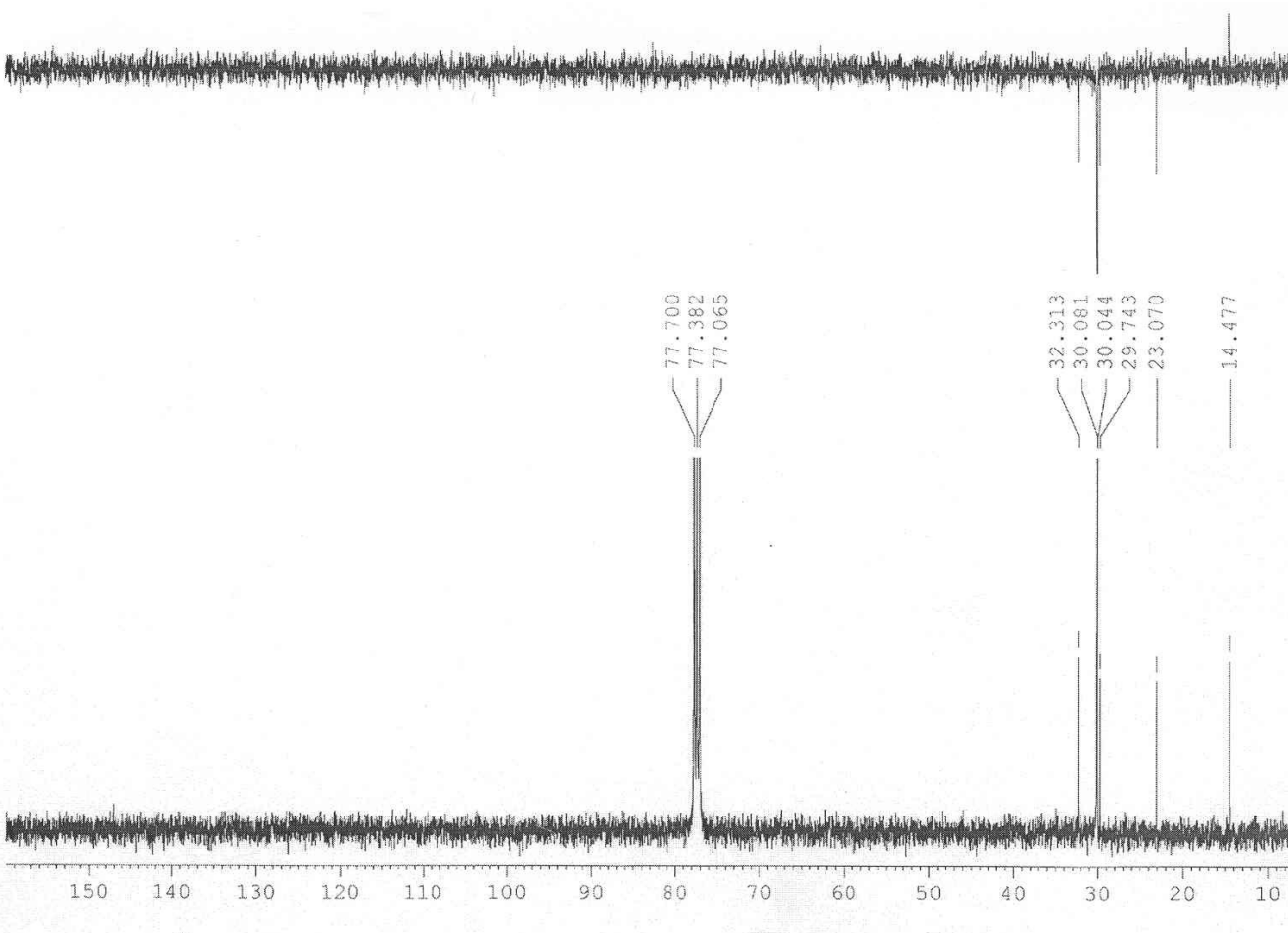
E2’ (IR spectrum)

Appendix 2



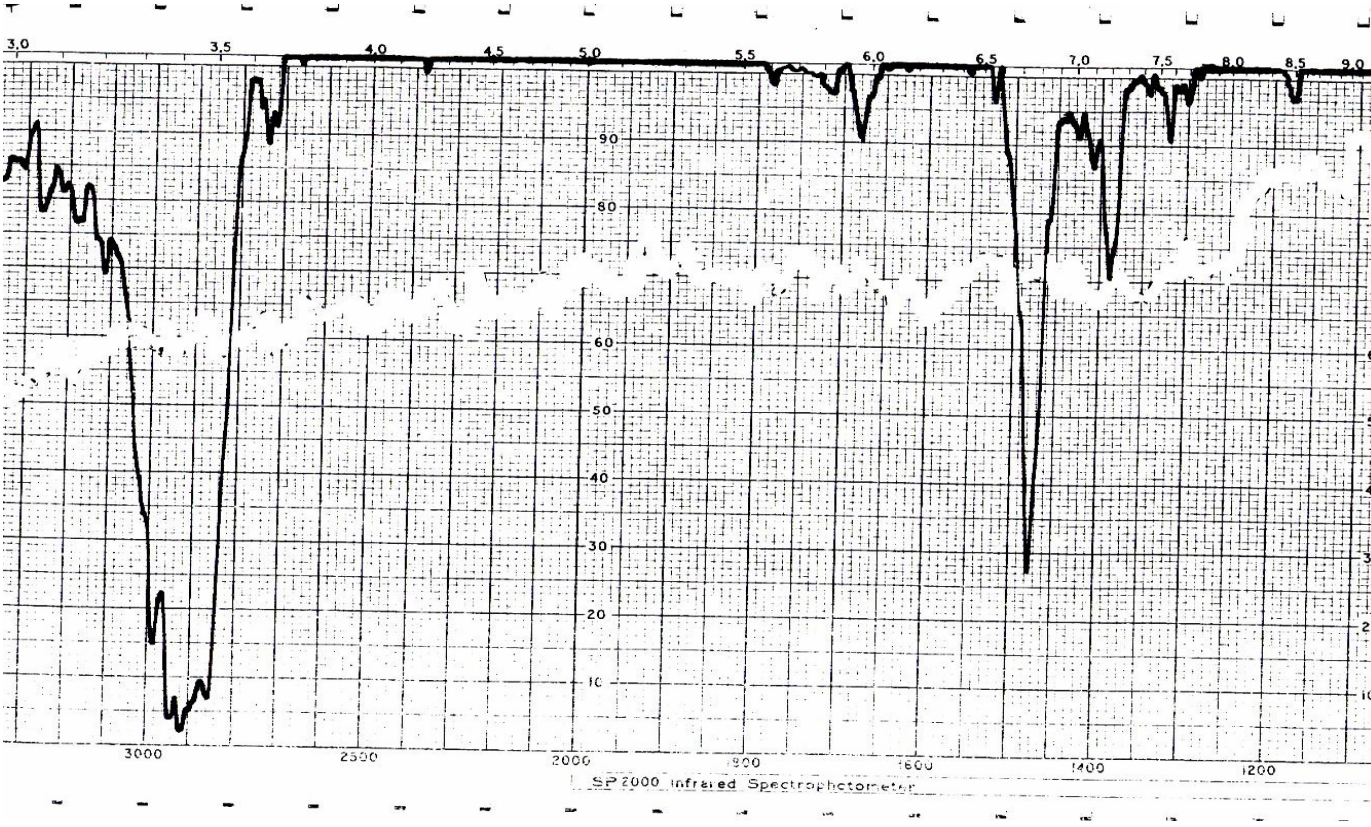
E2' (^{1}H NMR spectrum)

Appendix 3



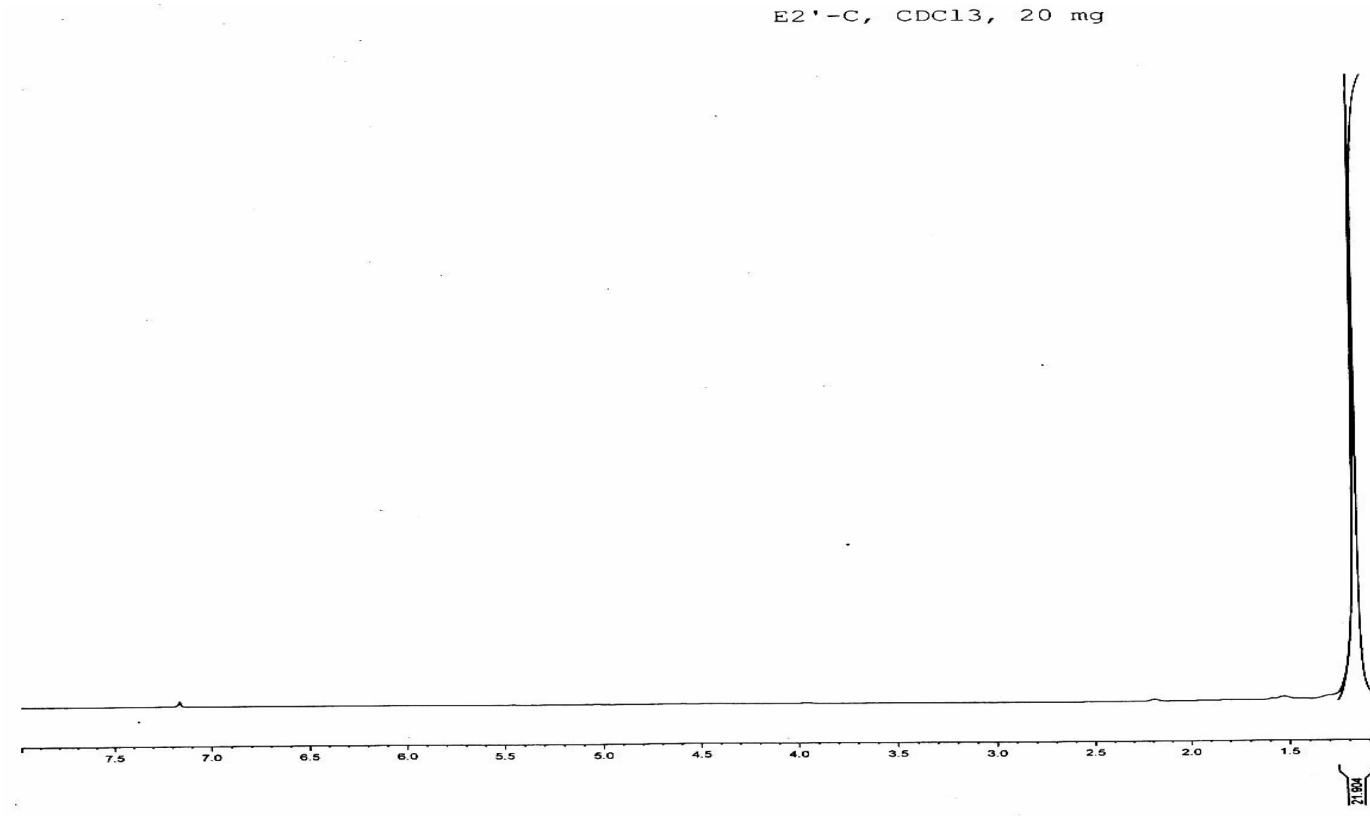
E2', $CDCl_3$ (^{13}C NMR and DEPT-135 Spectra

Appendix 4



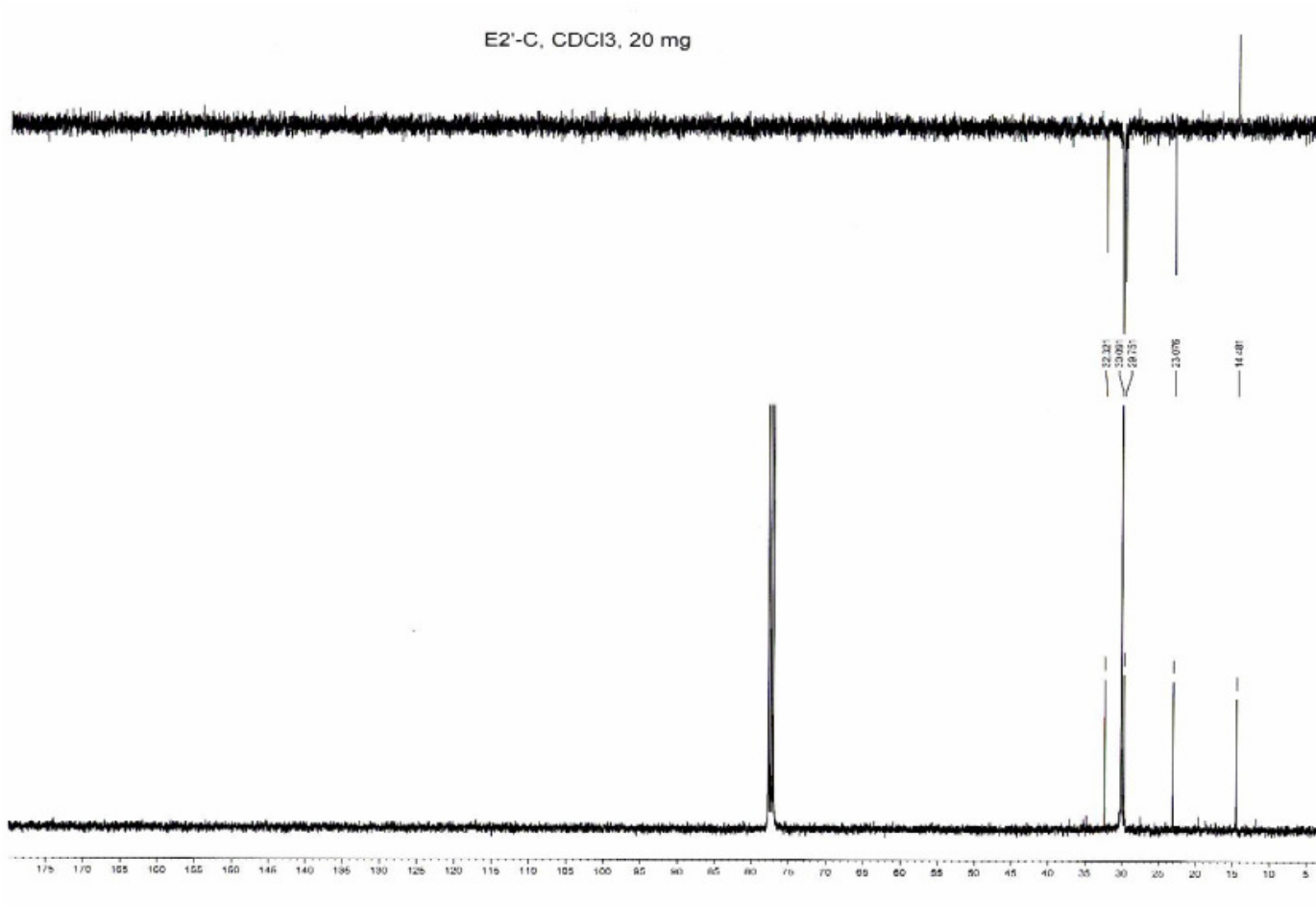
E2’-C (IR spectrum)

Appendix 5



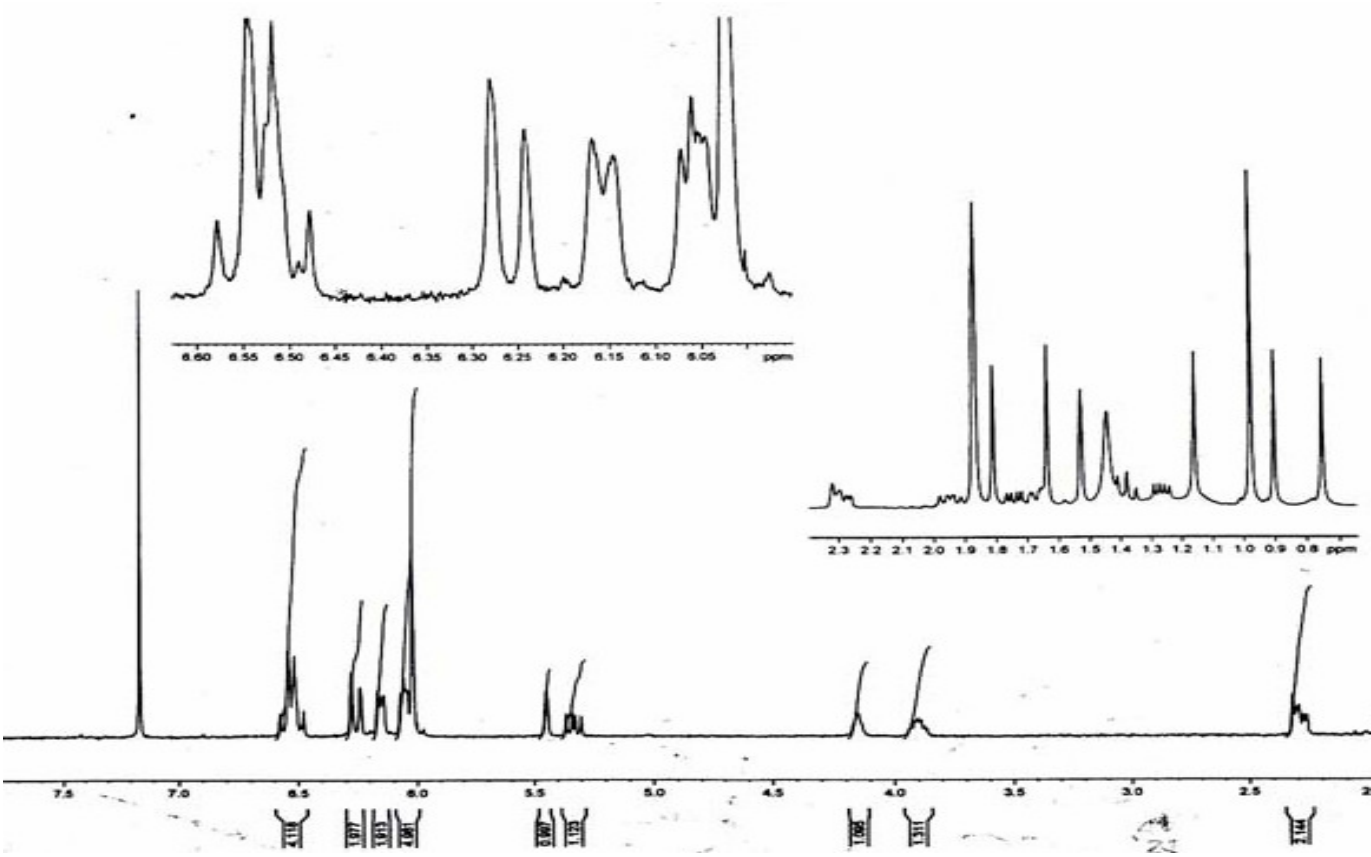
E2'-C (^{1}H NMR spectrum)

Appendix 6



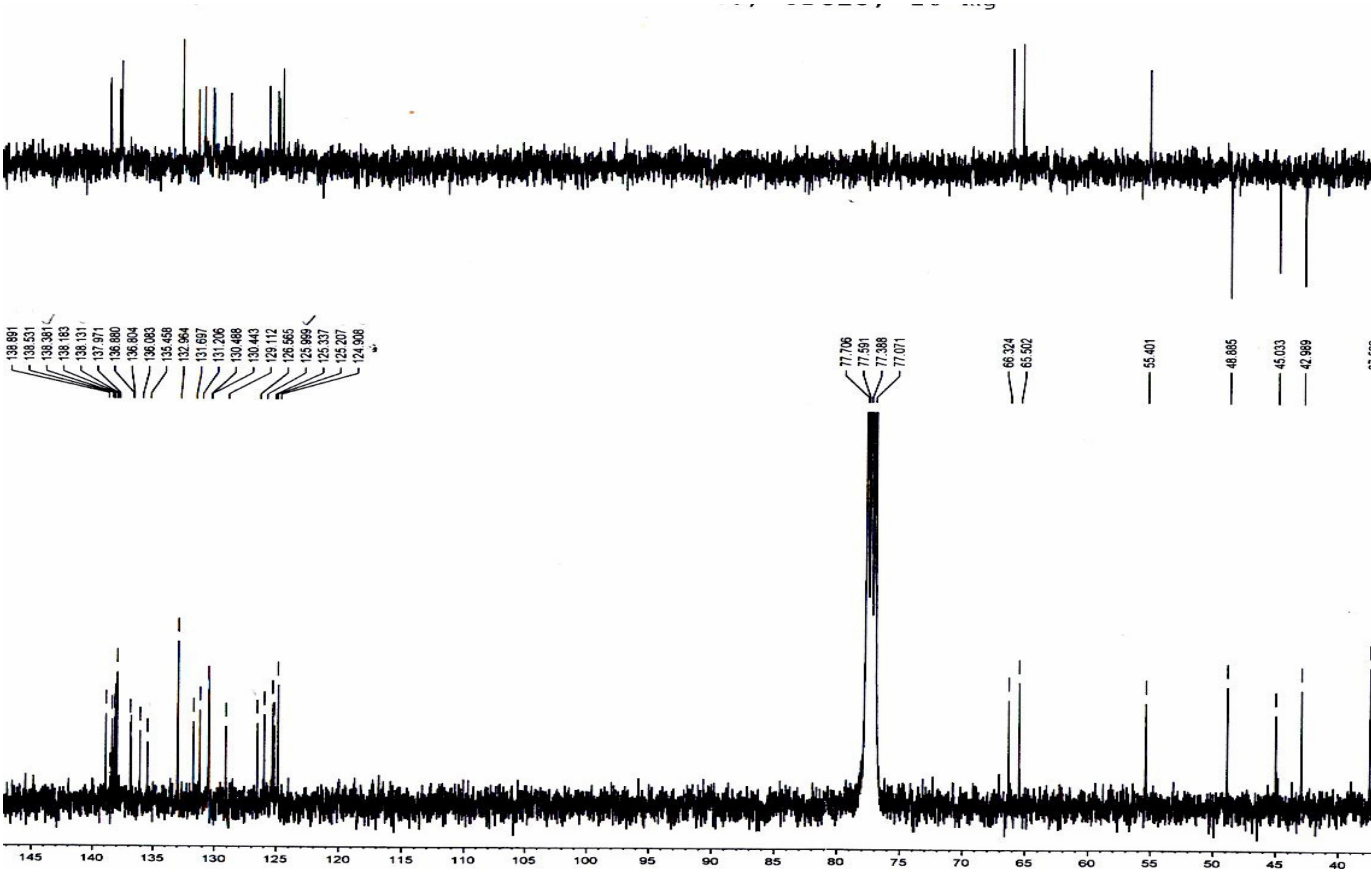
E2'-C (^{13}C NMR & DEPT spectra)

Appendix 7



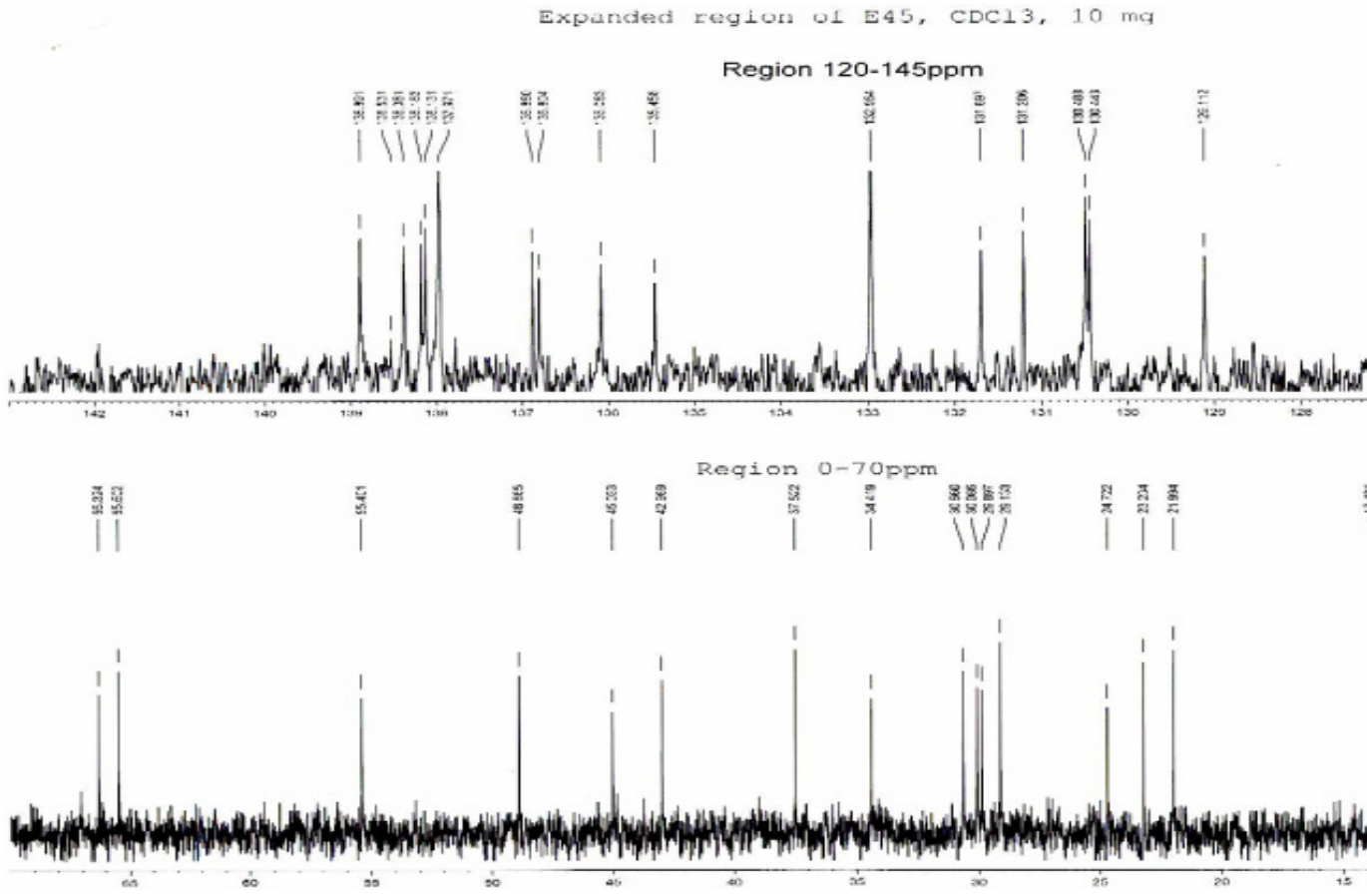
E45 (^{1}H NMR spectrum)

Appendix 8

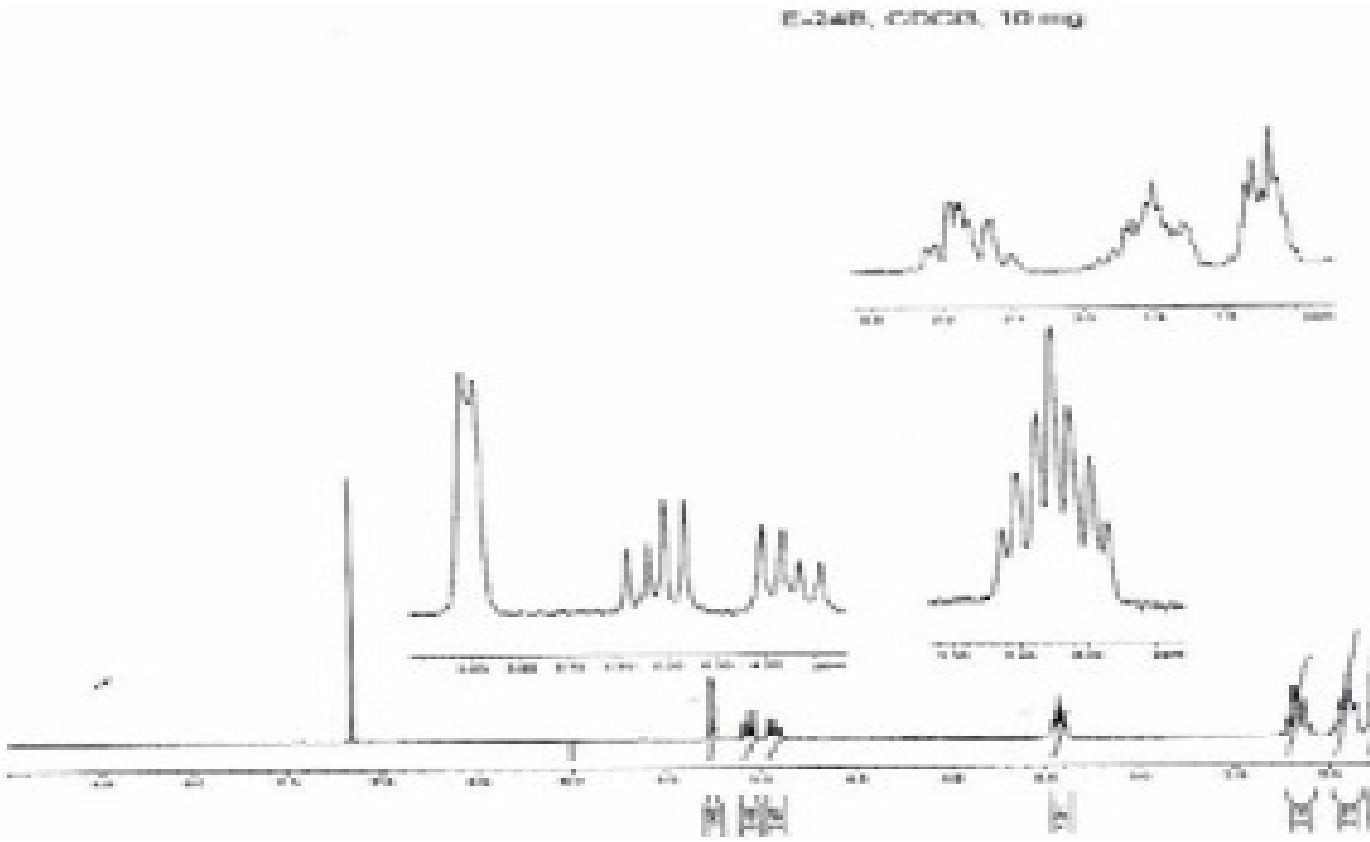


E45 (^{13}C NMR & DEPT spectra)

Appendix 9

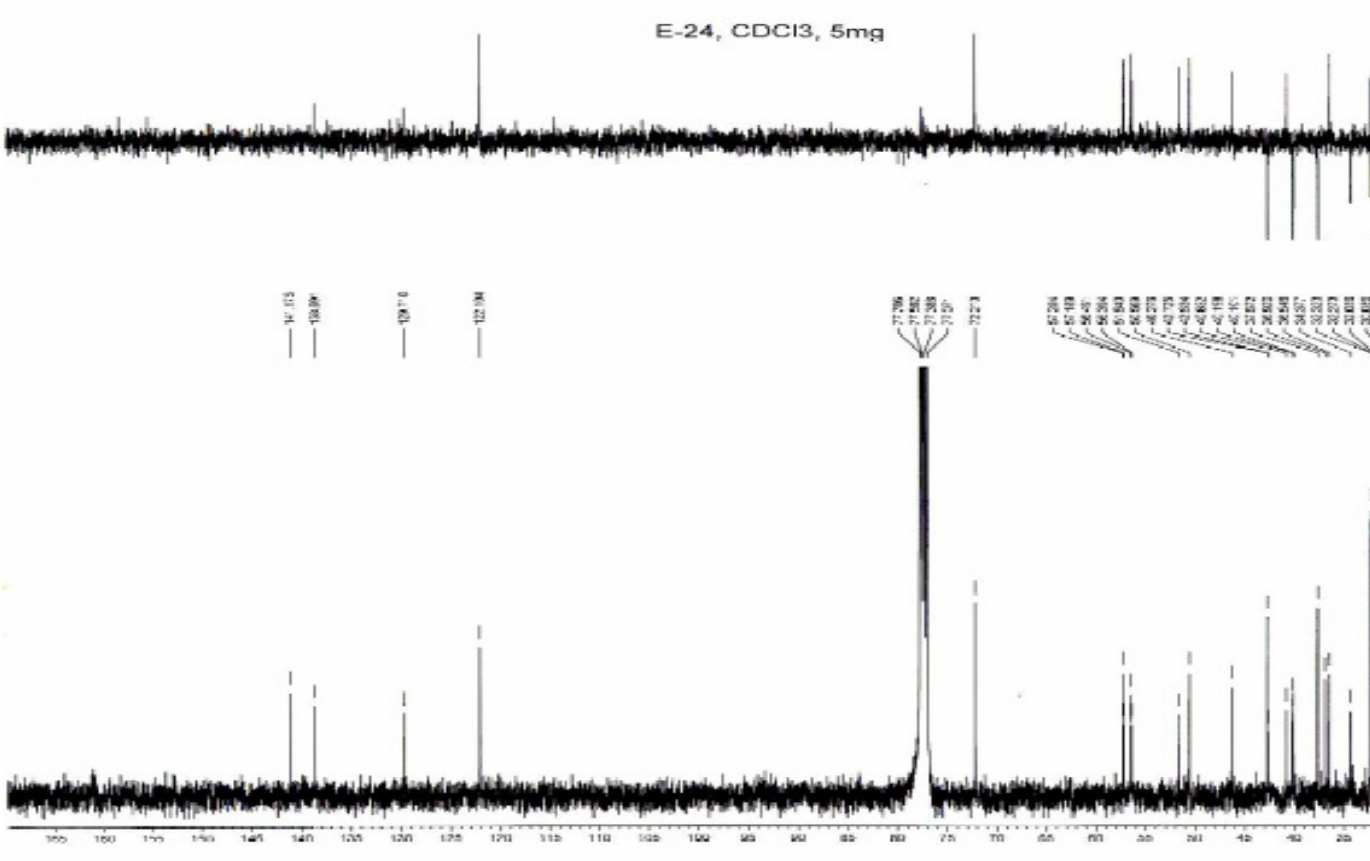

Expanded region of E45, CDCl3, 10 mg
Region 120-145ppm
Region 0-70ppm

Appendix 10



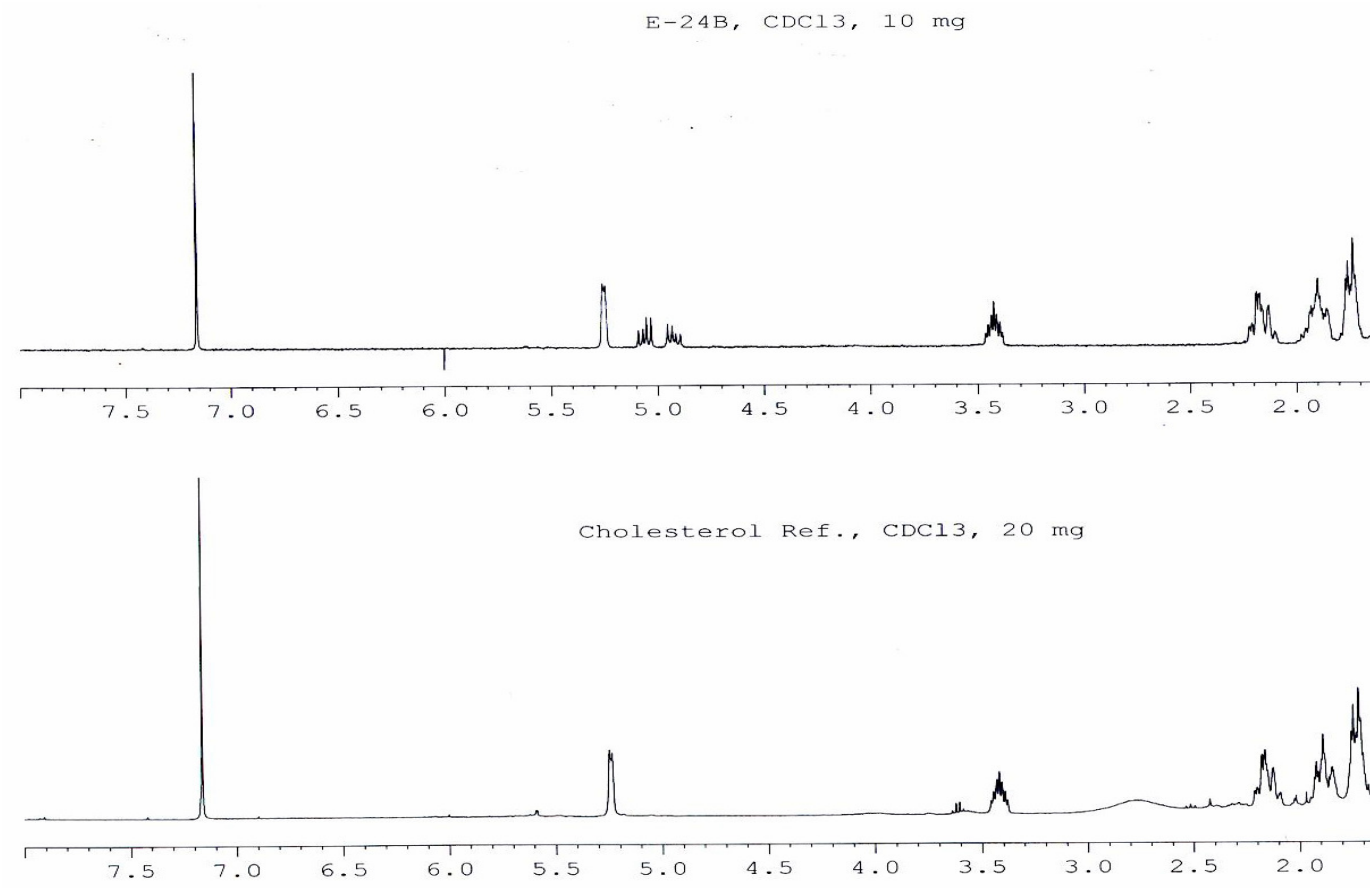
E24 (^{1}H NMR spectrum)

Appendix 11



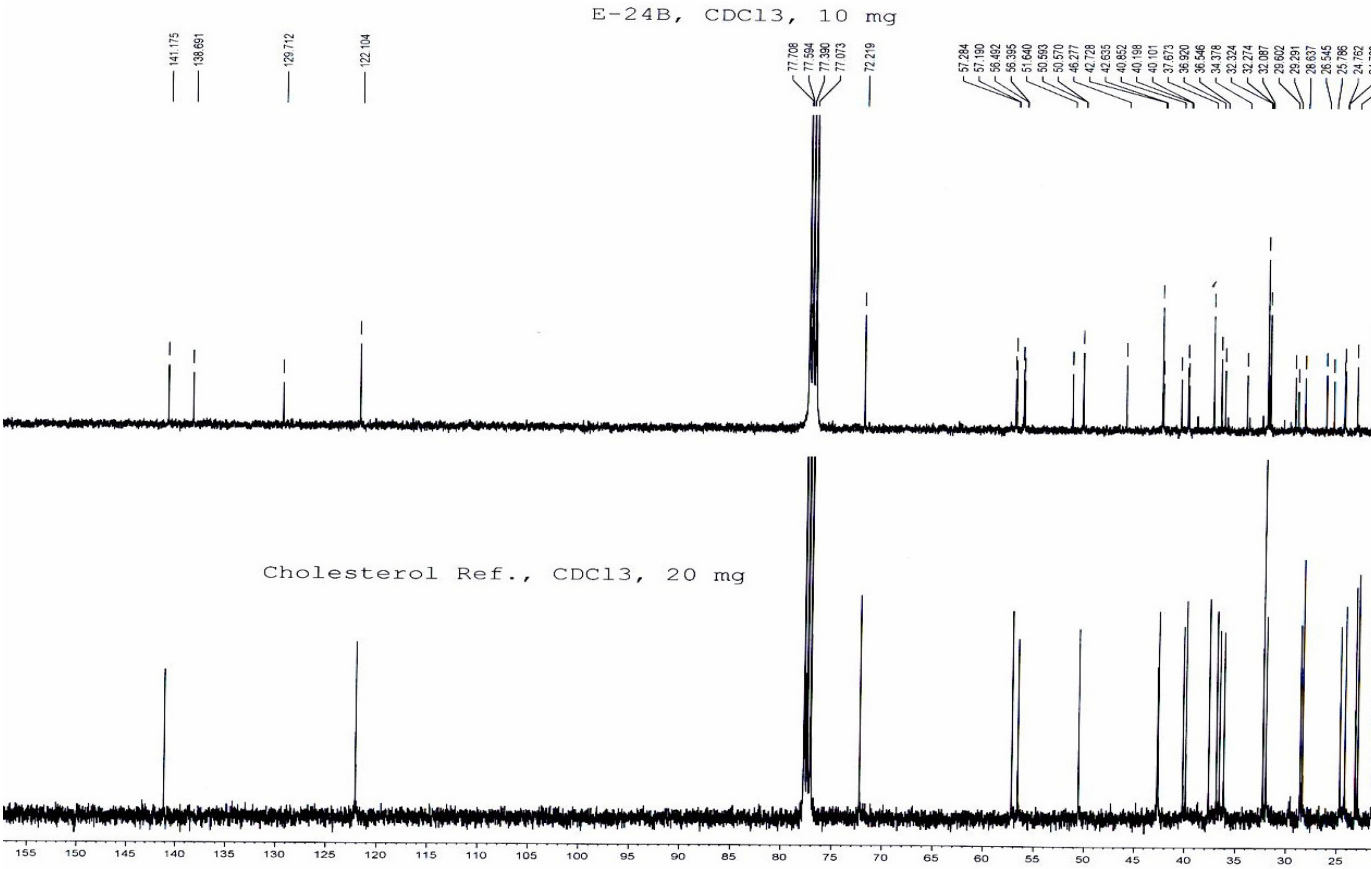
E24 (^{13}C NMR & DEPT spectra)

Appendix 12



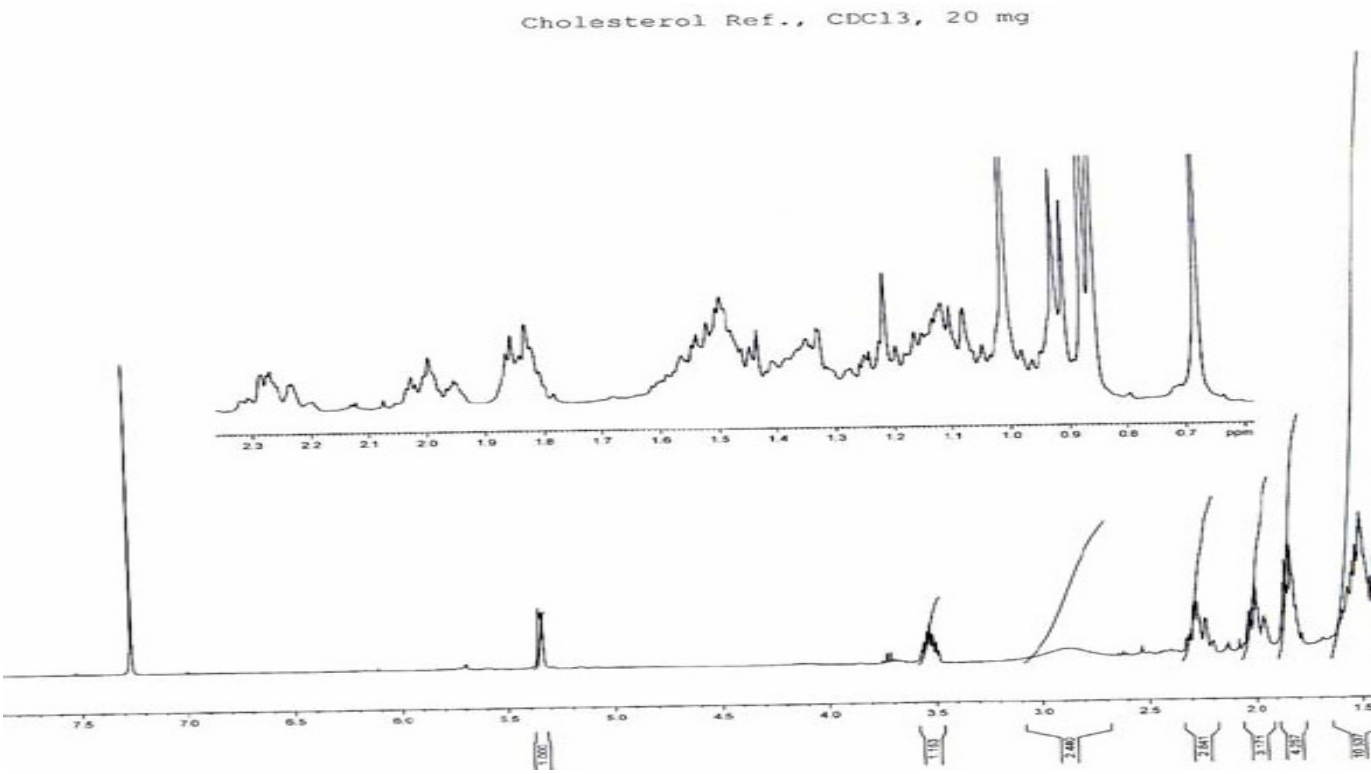
Comparison of proton spectra of cholesterol

Appendix 13



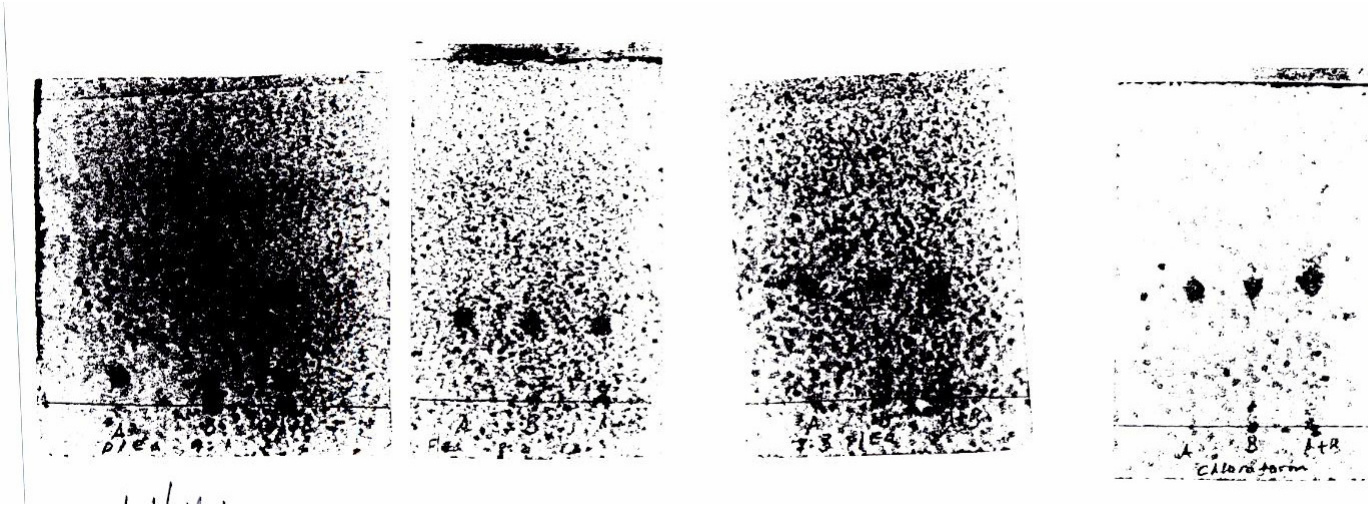
Comparison of ^{13}C spectra of cholesterol

Appendix 14



^{1}H NMR spectrum of cholesterol (Ref)

Appendix 15



TLC spot tests of E24 and cholesterol (Ref)