ADDIS ABABA UNIVERSITY
THE SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF CHEMISTRY

PHYTOCHEMICAL INVESTIGATION ON THE SEEDS OF *Azadirachta indica*

By
Getahun Asmare

A graduate project Submitted to the School of Graduate Studies
In partial Fulfillment of the Requirements
for the Degree of Masters of Science
in Chemistry

July 2006

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Abstract

From hexane extract of seeds of azadirachta indica (neem), azadiradione was isolated. Its structure was elucidated through spectroscopic techniques.

1. Introduction

Azadirachta Indica, commonly referred to in many countries as the neem tree, is a member of the meliaceae family. This broad-leaved evergreen can reach heights of 30 meters with a trunk girth of 2.5 meters. Its deep root system is well adapted to retrieving water and nutrients from the soil profile, but this deep root system is very sensitive to water logging. The neem tree thrives in hot, dry climates where shade temperatures often reach 50 degrees Celsius and annual rainfall ranges from 400 to 1,200 millimeters. The tree can withstand much environmental diversity including drought and infertile, stony, shallow or acidic soils. The neem produces ellipsoidal drupes, which are about two centimeters in length, borne on auxiliary clusters. These fruits contain kernels that have high concentrations of secondary metabolites (1).

1.1. Azadirachta Indica

The neem tree, Azadirachta indica is a large evergreen tree. It's well spread out branches form a wide crown and is a favored avenue tree throughout India. The leaf, 20 - 38 cm long is made of 8-19 leaflets arranged alternately on opposite sides. The yield of leaves per tree is about 350 Kg. It is a fast growing tree producing fruits in four to five years and becoming fully productive in about ten years. The fruits are small (1.3 to 1.8 cm long and 1 cm wide), green when tender and yellow when ripe with a juicy bittery sweet pulp with a single seed inside. The fruits, which mature in May to August contain 24 % skin and 48 % pulp, the remaining being the seed 28 %. A single tree can yield up to 30 Kg of seed per year. The kernel yields about 45 % oil by weight, which is nonedible.

Almost every part of the tree finds use in folk medicine and in day-to-day life. Products and preparations of almost every part of the tree are used as soil fertilizer, insect repellent, insecticide, animal fodder, dye wax, fuel, lubricant, soap, mouth hygiene products and in traditional medicine. The major part of the neem research activities concerns its use in agriculture as a biopesticide. Though neem has been used in traditional medicine since a long time, it is only recently that a lot of work is being done to systematically screen, them for various activities of

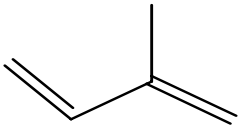
pharmacological importance. Crude fractions from various parts of the neem tree and certain isolated limonoids have been found to have anti-inflammatory, anti-rheumatic, antiarthritic, antipyretic, antimalarial, antimicrobial, CNS-depressive, antiulcer, antidiabetic, antitumor, antifertility, immuno- stimulatory and cardiovascular effects(3).

Over 40 commercial products of neem origin are available in the market. These include soaps, toiletries, medicinals, pesticides, fertilizers, and soil conditioners. However, world attention to neem is essentially due to insect antifeedant and ecdysis inhibiting activities against over 200 agricultural pests, attributed mainly to azadirachtin.

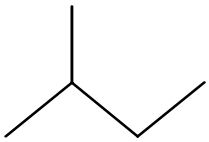
Azadirachtin ($C_{35}H_{44}0_{16}$) is a limonoid present in the neem seed kernel and its concentration varies from 0.1% to 0.5% by weight. From early biological studies to the more recent work on azadirachtin, neem has proved to be an important and exciting pesticidal substance which might eventually lead to new methods in insect pest management. Neem contains at least 200 compounds of which azadirachtin is biologically the most active compound.

1.2 Terpenes

The terpenes are among the most widespread and chemically diverse groups of natural products. Fortunately, despite their structural diversity, they have a simple unifying feature by which they are defined and by which they may be easily classified. Terpenses are a unique group of hydrocarbon-based natural products whose structure may be derived from isoprene, giving rise to structures which may be divided into isopentane (2-methylbutane) units (Figure 1.1)



isoprene,
(2-methyl-1,3-butadiene)



isopentane
(2-methylbutane)

Figure 1.1The terpenes are comprised of isoprene units.

Terpense are thus classified by the number of 5-carbon units they contain:

Hemiterpenes **C_5 ,** Monoterpene **C_{10},** Sesquiterpene **C_{15},** Diterpene **C_{20} ,** Sesterterpene **C_{25}** , Triterpene **C_{30},** Tertraterpenese **C_{40}**

Like all natural products, within this simple classification lies an enormous amount of structural diversity which leads to a wide verity of trerpene- like (or terpenoid) compounds. Note that the simplest examples of the terpense are technically hydrocarbons, though they are considered separately here because of their common structural features. Not surprisingly, the terpenses are of a similar biogenetic origin, in which isopoenteny pyrophosphate and dimethyally pyrophosphate combine to yield geranyl pyrophosphate, leading to monoterpenes. Similarly, compounds derived from farnesyl pyrophosphate lead to sesquiterpene and triterpenes are formed from two equivalents of farnesyl pyrophosphate. These various combinations and oxidations give rise to a large variety of terpenese, which will be surveyed briefly here.

The function of terpenses in plants is generally considered to be both ecological and physiological, Many of them inhibit the growth of competing plants (allelopathy). Some are known to be insecticidal; others are found to attract insect pollinators. The plant hormone, abscissic acid, is one of the sesquiterpenes. One, gibberellic acid, is another one of the major plant hormones (over 90 gibber-ellins have been identified). The variety of structures that the terpenses possess is vast.

1.2.1 Hemiterpenese: C_5

Isoprene itself does not occur free in nature but several five- crobin compounds are known which contain the isopentane skeleton, including isoamyl alcohol, isovaleraldhyde, tiglic acid, angelic acid, and β-furoci acid. Sev eral common plant hemiterpense are shown in figure 1.2

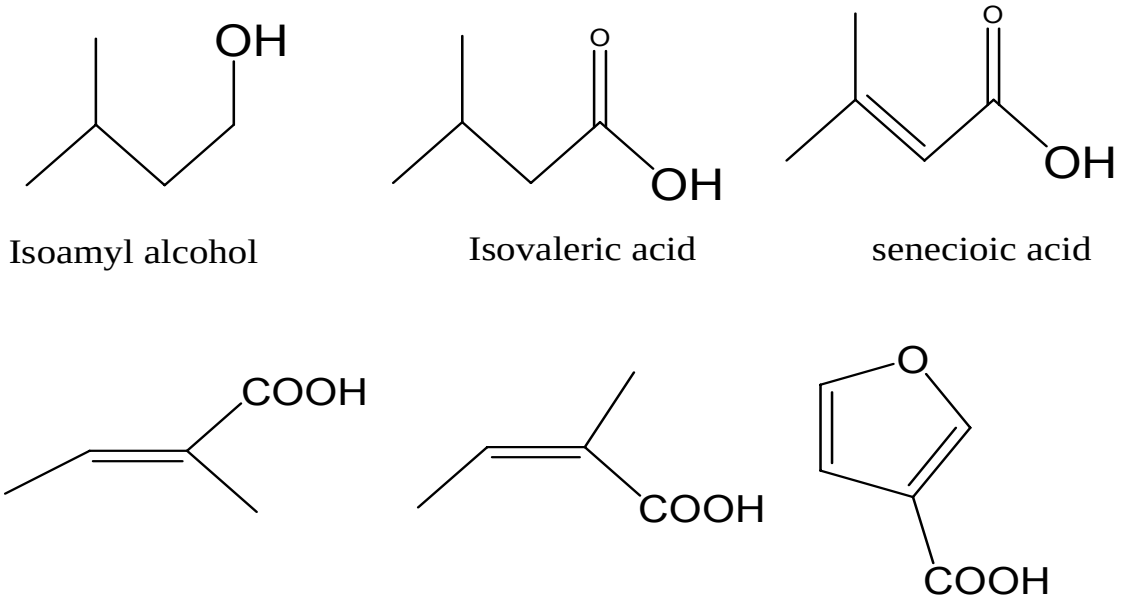
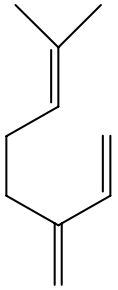


Figure 1.2 Some common plant hemiterpenenes

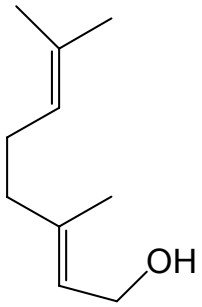
1.2.2 Monoterpenes: C_{10}

Nearly all possible decane arrangements appear to exist in nature. This gives the term *terpenoid* a particularly elastic meaning and is remiscent of some of the current combinatorial efforts employed in the pharmaceutical industry (12). The **monoterpenoids** are the major component of many essential oils and, as such, have economic importance as flavors and perfumes. Common aliphatic examples include myrcene, geraniol, and linalool, Open chain structures include many well-known compounds, including menthol, camphor, pinene, and limonene. A variety of common monoterpenes are shown in figure 1.3.

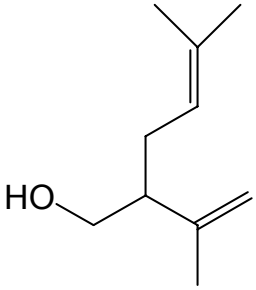
Most of the monoterpense listed in figure 1.3 come from common sources with which most of us are familiar. Myrcene is found in the essential oil of bay leaves as well as hops. It is used as an intermediate in the manufacture of perfumes. Geraniol, which is isomeric with linalool, constitutes the major part of the oil of roses and is also found in essential oils of citronella, lemon grass, and others. Menthol is a well- known monoterpene which is found in the essential oil of peppermint and other members of the mint family. Carvone is a common monoterpene which is one of the main odoriferous components of caraway seed (Carum carvi). Linalool is one of the principle constituents of coriander (Coriandrum sativum), a common spice.



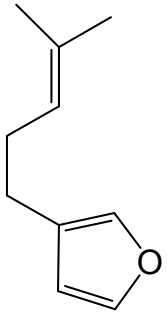
Myrcene



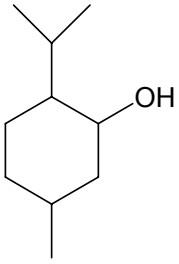
Geraniol



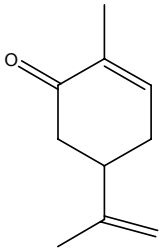
Lavandulol



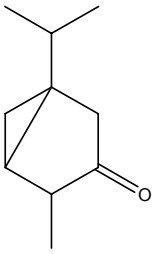
Perillene



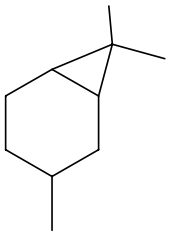
Menthol



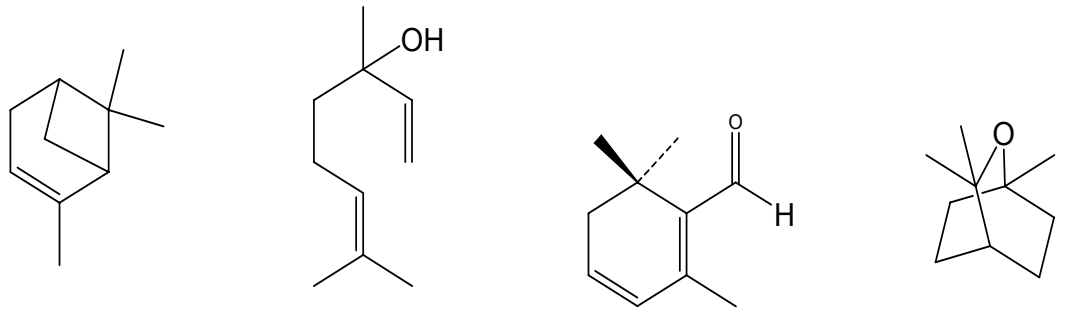
Carvone



Thujone



Carane



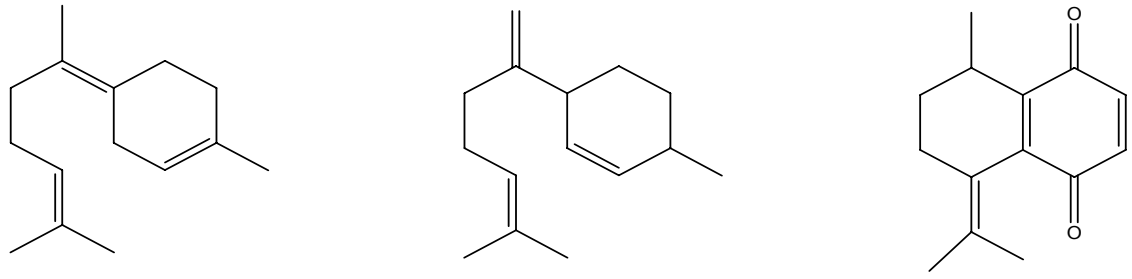
.α-Pinene Linalool Safranal Eucalyptol

Figure 1.3 Some common plant monoterpenes

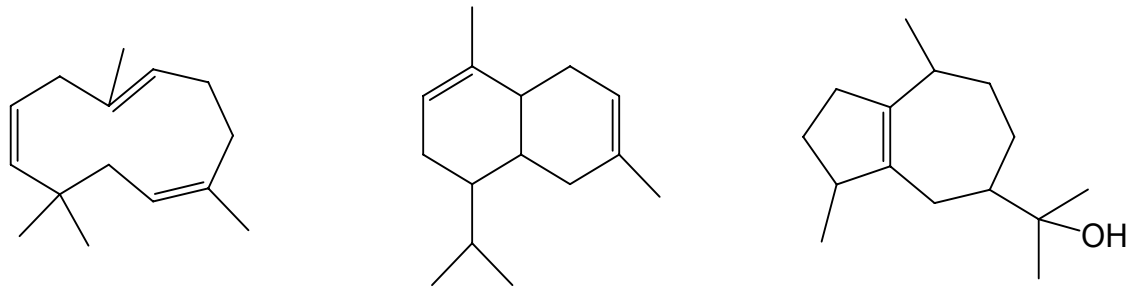
Safranal is chiefly responsible for the characteristic odor of saffron C(Crocus sativus). Eucalyptol, also known as cineole, is the main component of the essential oil of eucalyptus leaf (Eucalyptus spp).

1.2.3 Sesquiterpenese: C_{15}

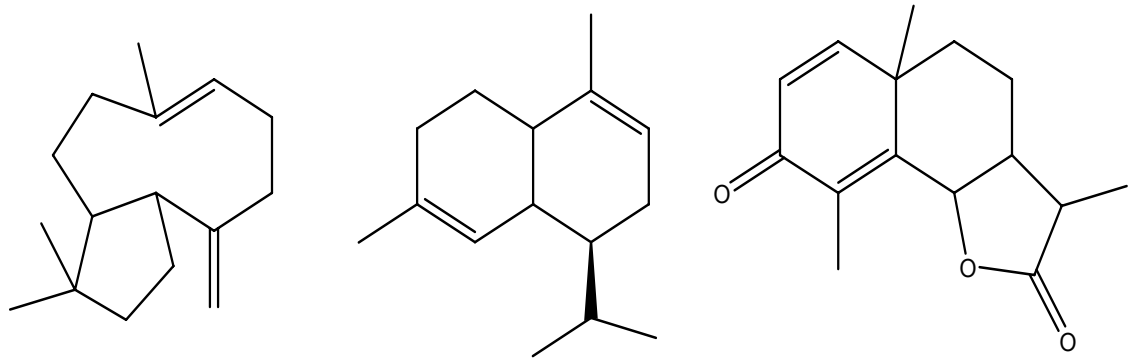
Derived from three isoprene units, the C_{15} sesquiterpenes exist in a wide variety of forms, including liner, bicyclic, and tricycles frameworks. Like the monoterpenes, most of the sequiterpenes are considered to be essential oils because they belong to the steam distillable fraction often containing the characteristic odoriferous components of the plant. An important member of this series is farnesol whose pyrophosphate services as a key intermediate in terpenoid biosynthesis. Some common sequiterpense are shown in figure 1.4



γ-Bisabolene Lanceol Perezone



Humulene α-Cadinene Guaiol



Caryophyllene α-Cadinene Santonin

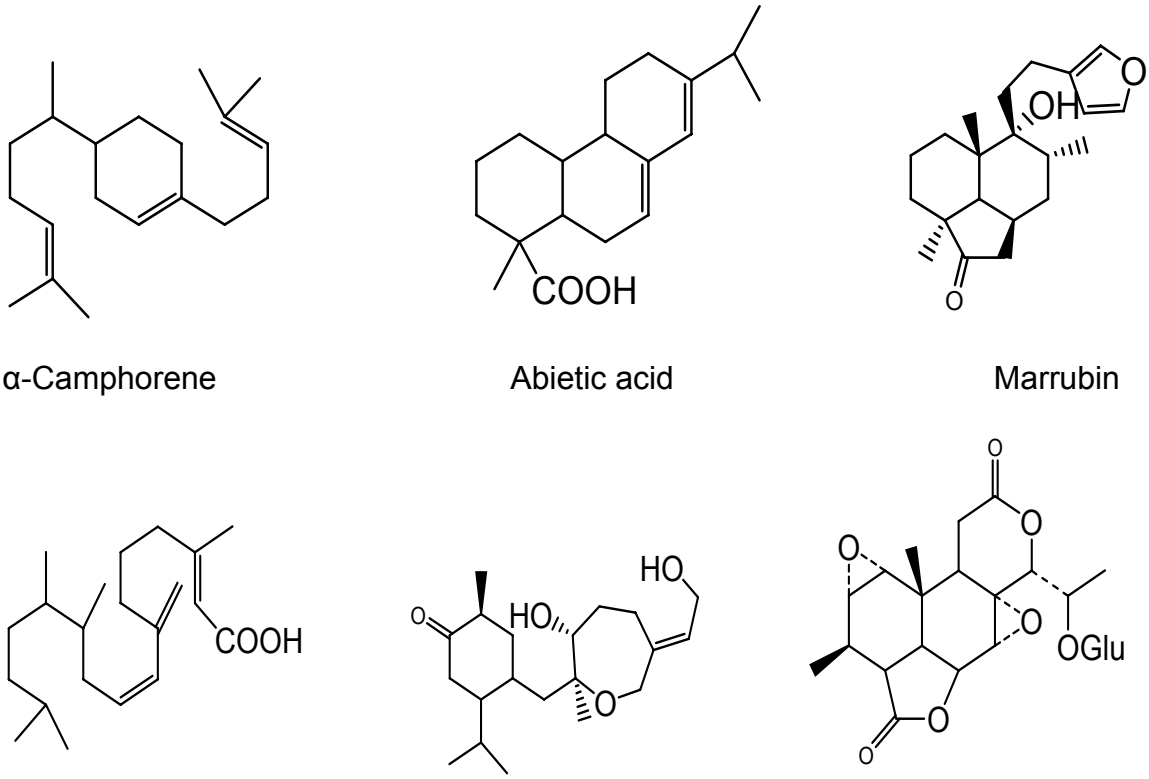
Figure 1.4 Some common plant sesquiterpenes

The cadinenes occur as essential oils from juniper and cedar trees and santonin is an antithelmintic that is isolated from wormwood (Artemisia maritime). Caryophyllene, first synthesized in 1964, is one of the principal components of oil of cloves(14). The sesquiterpene α-cdinene is one of the more than 70 isolated components from the esseitnal oil of juniper berries. It has been used as a diuretic and antiseptic.

1.2.4 Diterpenes: C_{20}

The diterpenes are a widely varied group of compounds based on four isoprene groups, most of which are of limited distribution in the plant kingdom. Because of their higher boiling points, they are not considered to be essential oils, instead, they are classically considered to be resins, the material that remains after steam

distillation of a plant extract. The diterpenes exist in a variety of structural types (shown in Figure 1.5).



α-Camphorene Abietic acid Marrubin

Phytol Zoapatanol Inumakilactone

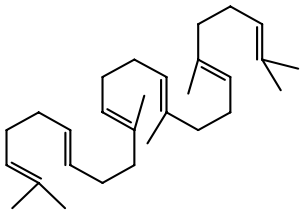
Figure 1.5 Some common plant diterpenes

Many interesting examples may be mentioned. The cyclic ether zoapatonol is derived from the Mexican plant montanoa tomentosa and has been used as an abortificient. A verity of cythotoxic lactones have been isolated from podocarpus species(15). These podolactones have plant regulatory properties as well as antileukemic activity. Marrubin is a diterpene lactone from white horehound (Marrubium vulgare), which has been used as a bitter and choleretic in digestive and bilitary complaints.

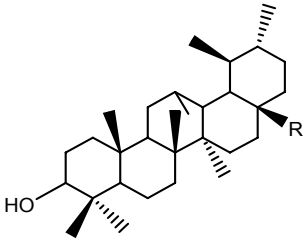
1.2.5 Triterpenes: C_{30}

The C_{30} terpenes are based on six isoprene units and are biosynthetically derived from squalene. They are often high- melting colorless solids and are widely

distributed among plant resin, cork, and cutin. There are several important groups of triterpenes, including common trieterpenes, steroids, saponins, sterolins, and cardiac glycosides. Among these is azadirachtin, a powerful insect antifeedant, isolated from Neem oil. Several triterpenes are shown in figure 1.6

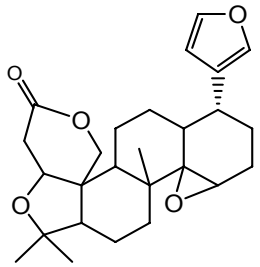


Squalene

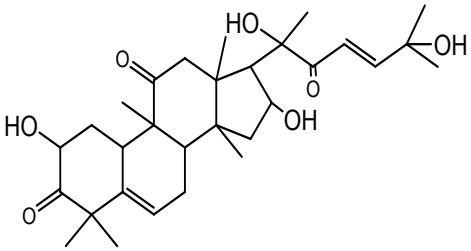


α-Amyrin(R=Me)

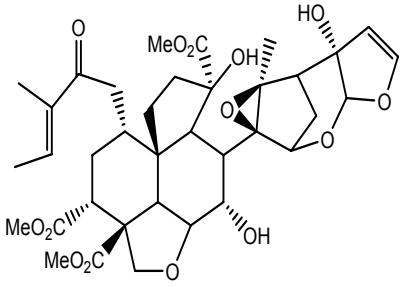
Ursolic acid (R=COOH)



Limonin



Cucurbitacin D



Azadirachtin

Figure 1.6 Some common plant triterpene natural products

1.2.6 Secondary Modification of Triterpenes

Introduction of additional hydroxyl and olefinic groups oxidation of alcohol functions to carbonyl groups and side chain alkylation by S-adenosyl nethionine are common modifications. The cucurbitanes are characterized by extensive oxidation but with retention of the basic triterpenoid skeleton. In other cases extensive degradation has taken place as for the quassinoids, the bitter principles of Simaroubaceae, for the limonoids, bitter principles of citrus species, belonging to the Rutaceae family, and the meliacins of Meliaceae. The compounds are typical for these botaniceal families. The quassinoid skeleton is formed by oxidative

cleavage of the side chain and opening of ring D of a tetracyclic triterpene e.g. apotirucallol. Feeding experiments with 2-^{14}C,(4R)-^{3}H-and 5-^{14}C-mevalonic acids in the seeds of simarouba glauca gave labelled glaucarubinone as shown below. The β-4-methyl is oxidatively decarboxylated via formation of an intermediary 3-β-keto ester as shown by loss of 3-^{3}H and the formation of inactive acetic acid from $C^{4.10.13}$ methyls on Kuhn-Roth oxidation of glaucarubinone derived from (4R)-^{3}H-and 2-^{14}C mevalonic acids. Hydrolysis of active glaucarubinone gave inactive 2-hydroxy-2-methylbutyric acid which is derived from isoleucine. Selective degradation confirmed the labeling pattern expected from the tetracyclic triterpene precursor. The finding that 9-^{3}H is retained excludes a precursor with a C^{8} double bond.

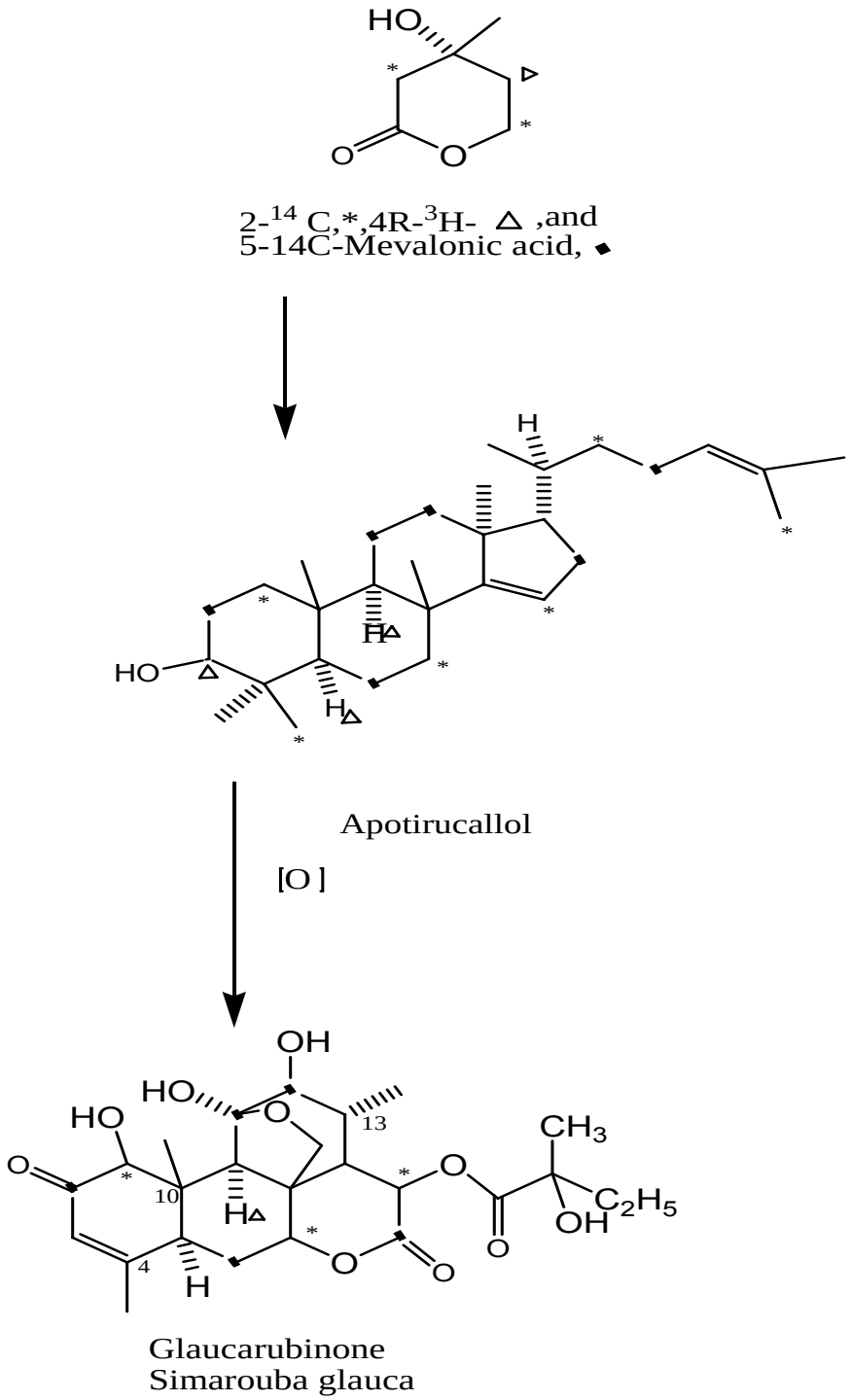
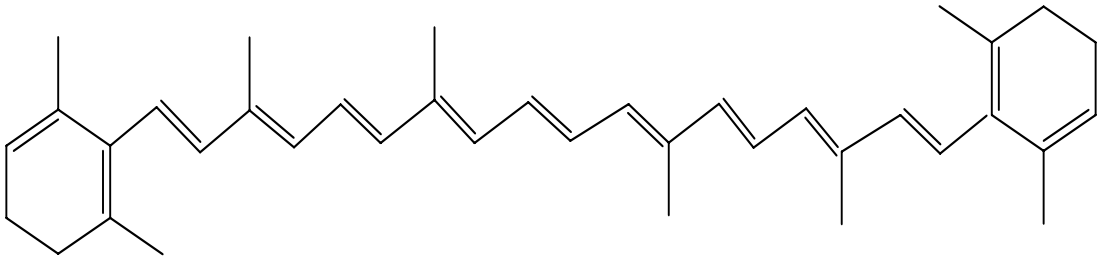


Figure 1.7 Partially degraded triterpenes of Simaroubaceae, Rutaceae and Meliaceae.

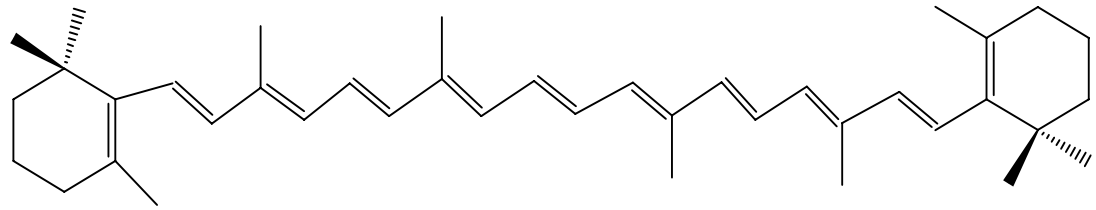
In the limonoids the side chain is transformed to a furan. The constituents of Meliaceae are closely related to the limonoieds.The fundamental secondary modificaton that leads to the steroids is selective C_4 and C_{14} demethylation.

1.2.7 Tetraterpenes: C_{40}

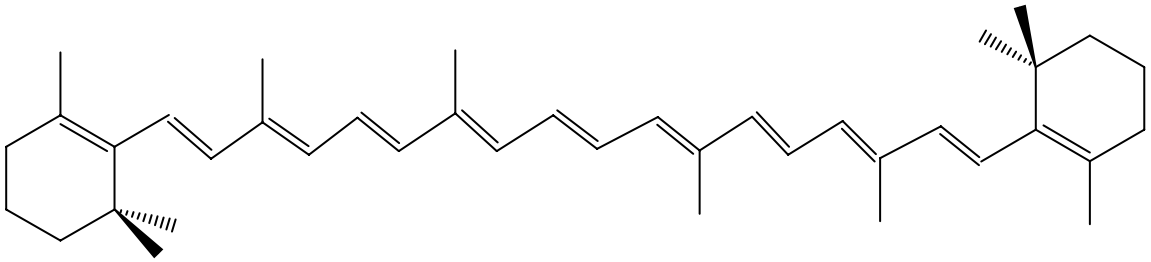
The most common tetraterpenoids are the carotenoids, a widely distributed group of C_{40} compounds. Whereas the structures of the di-and triterpenes can have a wide variety of fascinating structures, the carotenoids are generally derived from lycopene. Cyclization at one end gives γ- carotene and at both ends provides β-carotene. This pigment, first isolated in 1831, is by far the most common of all of these pigments and virtually universal in the leaves of higher plants. As is evident from this polyene structure, numerous double-bond isomers are possible for these basic structures, all of which can provide brightly colored pigments. Thus, in plants, carotenoids serve both as necessary pigments in photosynthesis and as coloring agents in flowers and fruits. This normally results in colors varying from yellow to red. They are also believed to protect plants from over oxidation catalyzed by other light absorbing pigments such as the chlorophylls. Some selected tetraterpenes are shown in figure 1.7



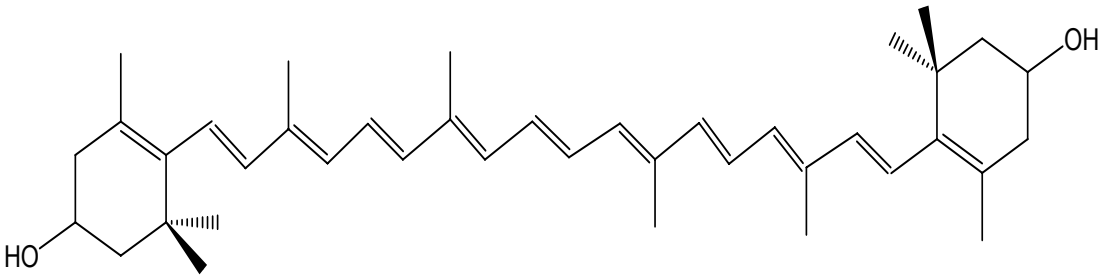
Lycopene



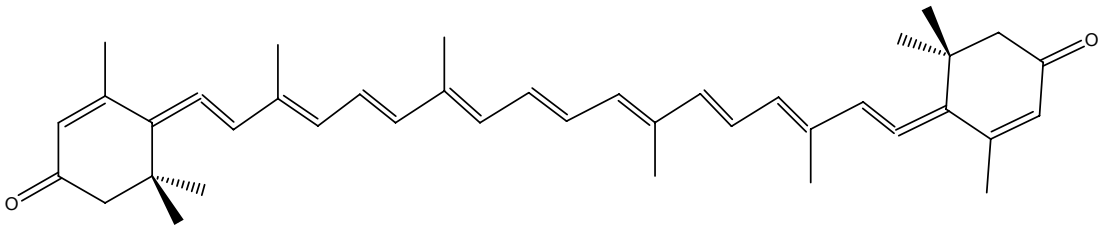
β-Carotene



.α-Carotene



Lutein



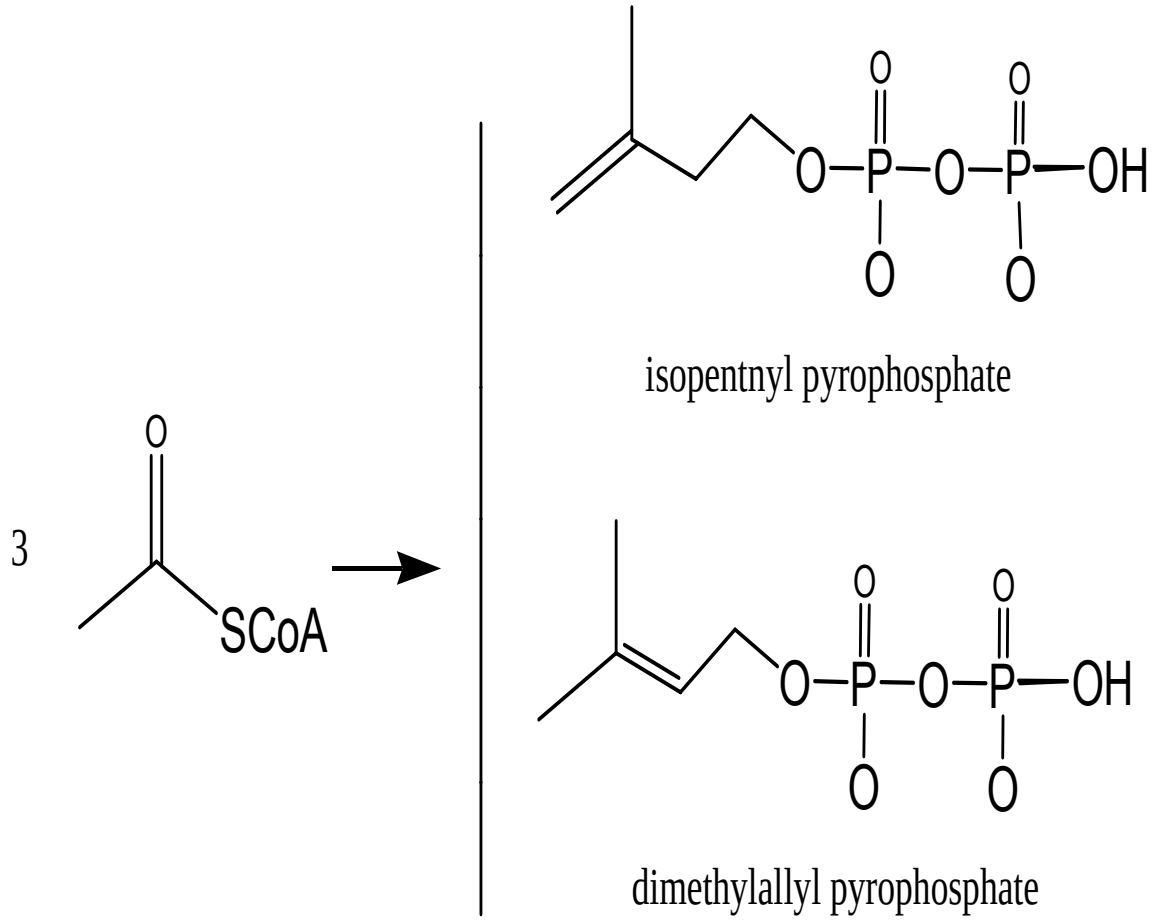
Rhodoxanthin

Figure 1.8 Representative plant tetraterpenes

1.3. Biosynthesis of terpenes

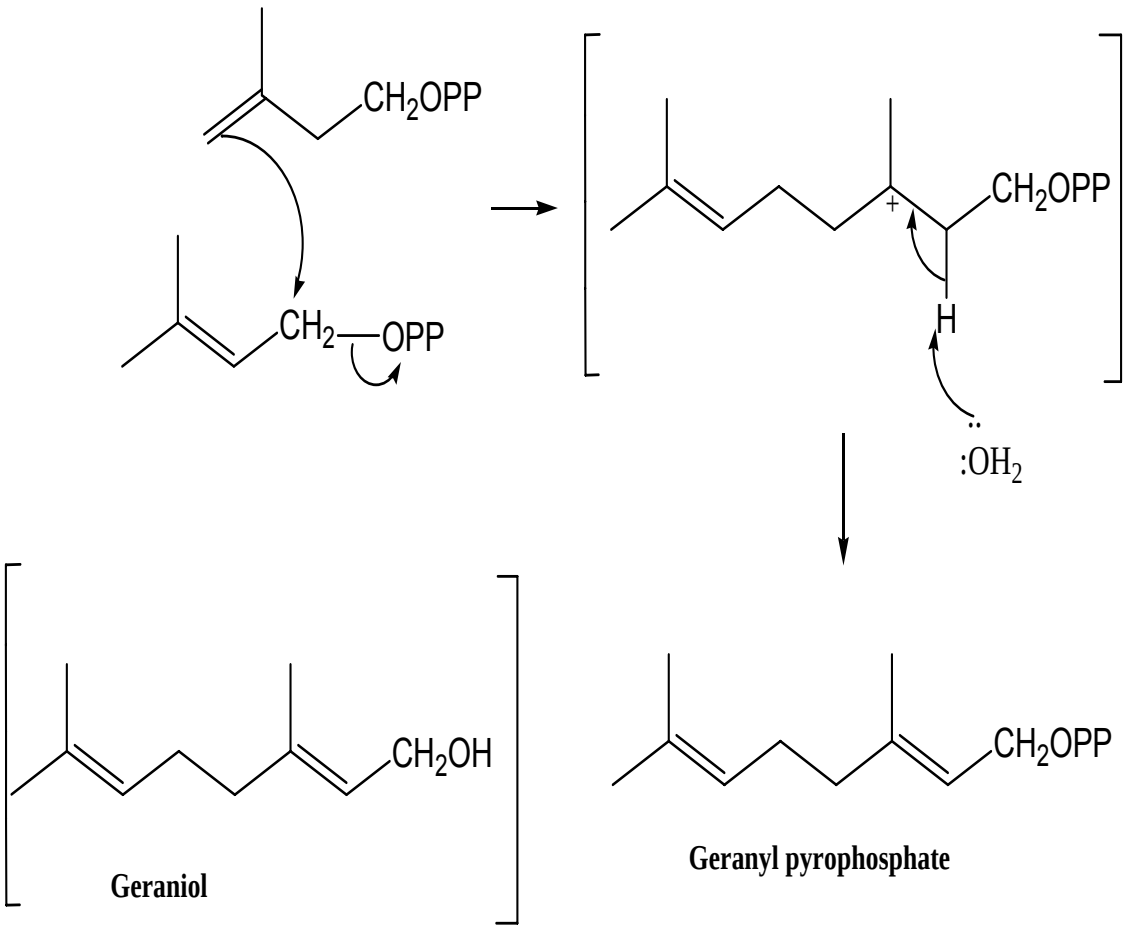
The pyrophosphate ester of an unsaturated five-carbon alcohol, 3-methyl-3-buten-1-ol, is the structural building block for naturally occurring terpenes pyrophosphoric acid is an anhydride of phosphoric acid and seems to be natures tool for creating good leaving groups. 3-Methyl-3-buten-1-yl pyrophosphate, known in the biochemical literature as isopentenyl pyrophosphate, is isomerized enzymatically to 3-methyl-2-buten-1-yl (dimethylallyl) pyrophosphate in a reaction that may be regarded as a protonation at one sp^2 -hybridized carbon atom and a deprotonation of the incipient carbocation at another site to give the more highly substituted

alkene. The participation of an enzyme, a highly specific biological catalyst ensures that no high-energy intermediate is formed at any point of reaction. These five-carbon molecules are themselves made from condensation of three acetyl CoA units



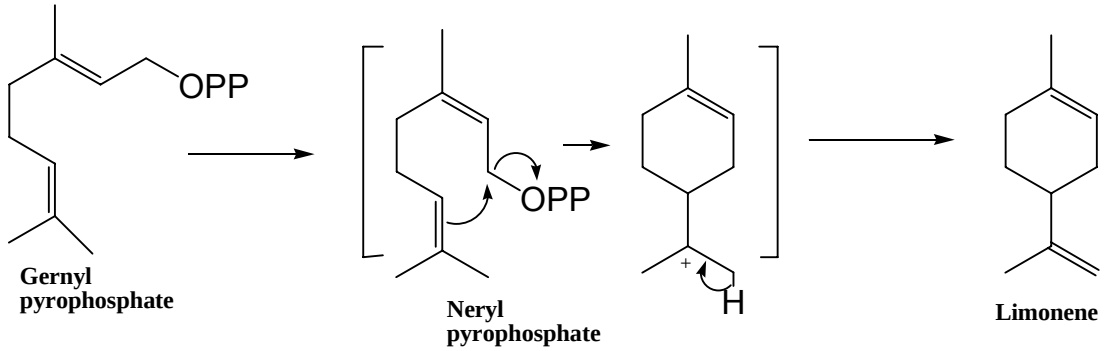
Scheme 1

Dimethylallyl pyrophosphate is effective alkylating agent en S_n2 like reaction because the primary allylic pyrophosphate (abbreviated OPP) can be displaced as a leaving group. Thus displacement of the pyrophosphate group by the nucleophilic C=C bond of isopentenpl pyrophosphate followed by loss of a proton from the carbocation reaction intermediat leads of the head -to-tail coupled 10-carbon unit geranyl pyrophosphate .The corresponding alcohol geraniol is itself a fragrant terpene that occurs in rose oil.



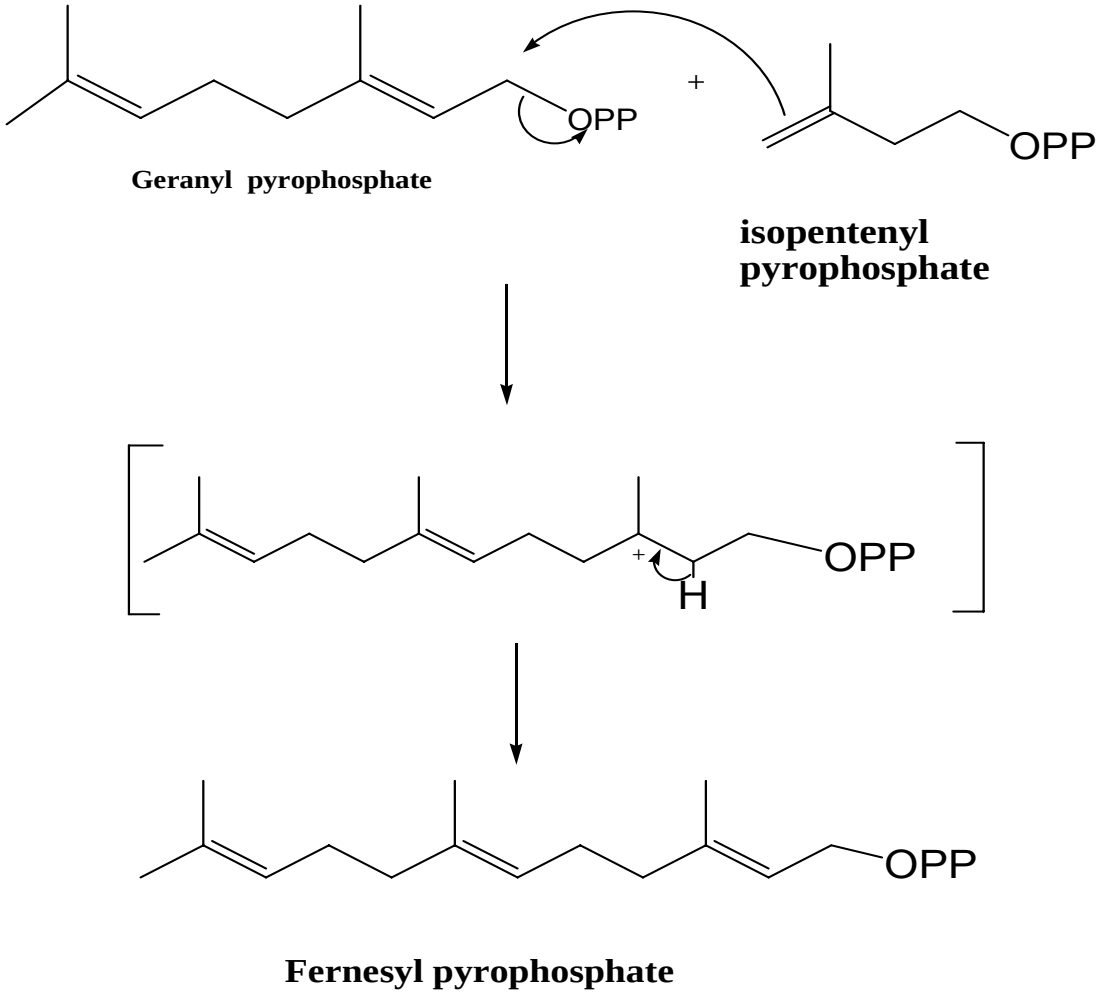
Scheme 2

Geranyl pyrophosphate is the precursor of all monoterpenes. Limonene, for instance a monoterpene fond in many citrus oils arises from geranyl pyrophosphate by a cis-to-trans double -bond isomerization to give neryl pyrophate followed by internal nucleophilic displacement of the pyrophosphate group and subsequent loss of a proton



Scheme 3

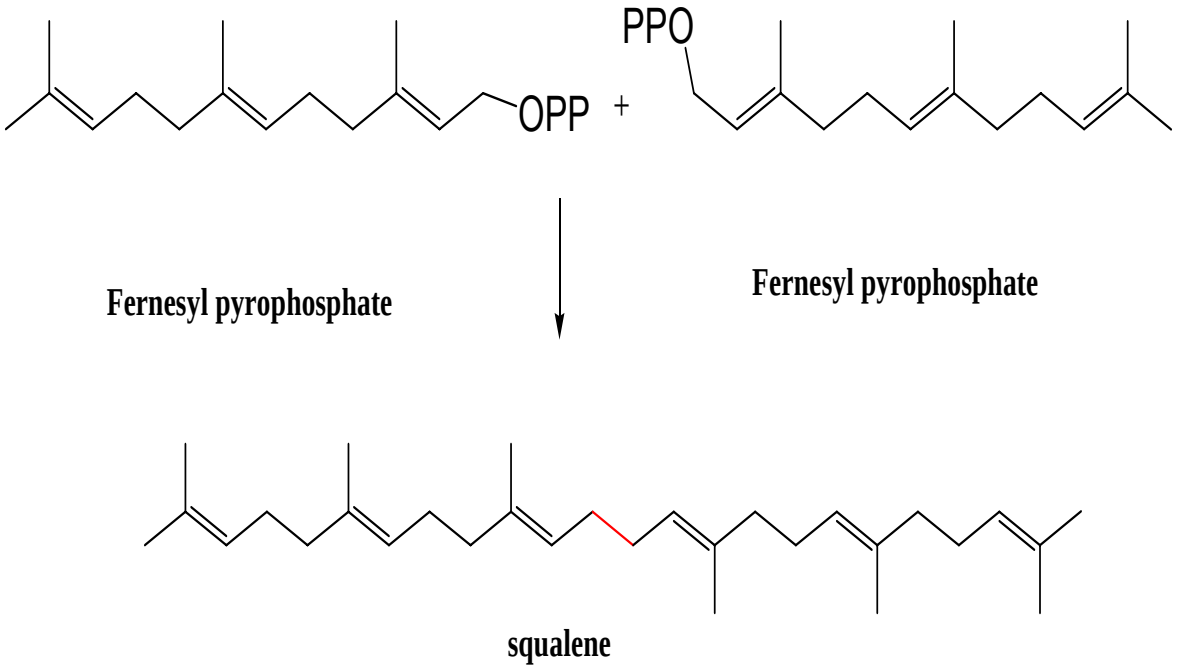
Reaction of geranyl pyrophosphate with isopentenyl yields the 15-carbon farnesyl pyrophosphate, the precursor of all sesquiterpenes. Farnesol, the corresponding alcohol, is found in citronella oil and lemon oil.



Scheme 4

Further reaction of farnesyl pyrophosphate with yet other isopentenyl pyrophosphate molecules give the 20-carbon and 25-carbons units that serve as precursors of diterpenes and sesterterpenes, respectively. Triterpenes, however, arise not by further reaction with isopentenyl pyrophosphate but by reductive tail-to-tail coupling of two 15-carbon farnesyl pyrophosphate to give squalene, a 30-carbon hexane.

Squalene, a major constituent of shark oil, is the precursor from which all triterpene and steroid arise (13).



Scheme 5

2 Objective

To isolate and characterize a compound in the seeds of Azadirachta indica.

3 Result and discussion

Compound HAI was isolated from hexane extract of Azadirachta indica

3.1 Characterization of HAI

Characterization of the compound was done using spectroscopic techniques; namely: UV, IR, and ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC and HMBC spectrum.

3.1.1 The UV, IR spectrum and ^{1}H NMR

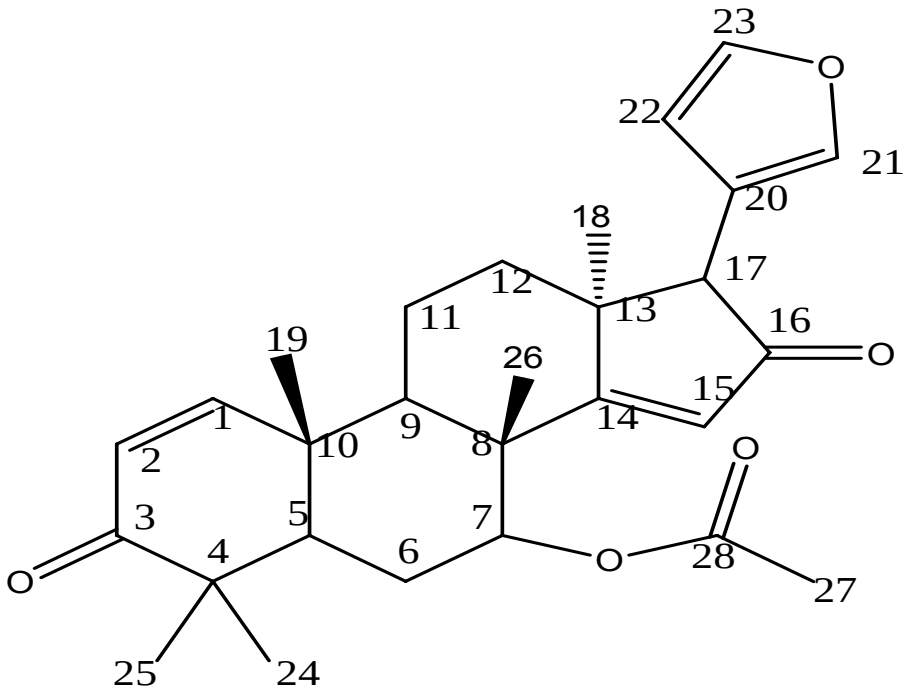
The UV spectrum (Appendix 1) revealed absorption band at 253 nm indicating the presence of α, β unsaturated carbonyl chromophore.

The IR spectrum (Appendix 2) showed an intense absorption band at (1661.72 cm^{-1}) due to carbonyl carbon (ketone) next to a double bond, absorptions at 2931.89 cm^{-1} (=C-H),and 1680 cm^{-1} indicating this molecule has double bond.

The ^{1}H NMR (Appendix 3) showed doublet at δ 7.12, 5.90 integrated for one proton each indicated olefinic protons attached to carbons which are bonded to quaternary carbons, A triplet peak at δ 2.51 integrated for one proton indicated methine proton attached to methylene, A doublet of doublet peak at δ 2,24 integrated for two protons indicated methylene protons neighboring with two methine protons, A triplet peak at δ 5.33 integrated for one proton indicated methine proton, Multiplet peak at δ 2.21 integrated for one proton indicated methine proton, Multiplet peaks at δ 2.11-1.88 integrated for two proton each indicated two adjacent methylene groups, Singlet peaks at δ 5.88 and δ 3.42 integrated for one proton each indicated methine groups attached with quaternary carbons, a singlet peak at δ 7.43 integrated for one proton indicated olefinic methine attached to quaternary carbon and oxygen, doublet peaks at δ 6.28 and δ 7.48 integrated for one proton indicated olefinic protons, singlet peaks at δ 1.03, 1.26, 1.10 , 1.10, 1.34 , 1.95 integrated for three protons each indicated the presence of six methyl groups in the molecule.

Table: 1 Proton NMR of HAI and literature value of proton NMR of Azadiradione

H	1H NMR of HAI (ppm)	Azadiradione(8)
1	7.117d	7.10s
2	5.903d	5.86d
5	2.505m	2.50s
6	2.239d	2.40m
7	5.327t	5.35s
9	2.207t	2.10m
11	2.109m	2.10m
12	1.878m	1.80m
15	5.877s	5.86s
17	3.422s	3.42s
18	1.032s	1.35s
19	1.255s	1.25s
21	7.429s	7.50s
22	6.276d	6.27s
23	7.478d	7.43s
24	1.101s	1.10s
25	1.094s	1.10s
26	1.344s	1.05s
27	1.949s	1.90s


23
O
22
21
20
18
17
12
11
13
16
O
19
26
15
1
9
14
10
8
O
2
3
5
7
4
6
O
28
27
O
25
24

HAI

Table 2. Proton decoupled 13C and DEPT spectra of HAI.

Carbon Number	**^{13}C NMR of HAI**	**DEPT**	**Remark**
1	156.695	156.695	CH
2	125.952	125.952	CH
3	203.946	--------	Quaternary carbon
4	44.104	--------	Quaternary carbon
5	38.254	38.254	CH
6	23.483	23.483	CH_2
7	73.939	73.939	CH
8	44.582	-------	Quaternary carbon
9	46.164	46.164	CH
10	40.026	-------	Quaternary carbon
11	15.845	15.845	CH_2
12	30.377	30.377	CH_2
13	47.985	-------	Quaternary carbon
14	192.256	-------	Quaternary carbon
15	123.322	123.322	CH
16	204.950	-------	Quaternary carbon
17	60.777	60.777	CH
18	26.473	26.473	CH_3
19	19.031	19.031	CH_3
20	118.456	-------	Quaternary carbon
21	142.782	142.782	CH
22	111.149	111.149	CH
23	141.669	141.669	CH
24	21.300	21.300	CH_3
25	27.016	27.016	CH_3
26	26.304	26.304	CH_3
27	20.965	20.965	CH_3
28	169.577	--------	Quaternary carbon

3.1.2 ^{13}C NMR spectrum and DEPT

Proton decoupled ^{13}C NMR spectrum (Appendix 4, Table 2) of HAI showed well resolved resonances of the 28 carbon atoms. The multiplicity of each carbon atom was determined using DEPT(Appendix 5) experiment, which showed the presence of six methyl groups, three methylene groups, ten methine groups and nine quaternary carbons, indicating the presence of 34 hydrogen atoms in the molecule. Signals displayed in the ^{13}C NMR spectrum of HAI at δ 204.950 and 203.946 are characteristic of carbonyl carbons of ketones.

Table 3. Proton decoupled 13C spectra of HAI and literature value of ^{13}C NMR of Azadiradione

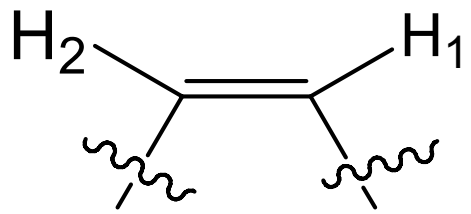
Carbon Number	**^{13}C NMR of HAI**	**^{13}C NMR of Azadiradione**
1	156.695	156.76
2	125.952	125.84
3	203.946	203.88
4	44.104	44.01
5	38.254	38.16
6	23.483	23.40
7	73.939	73.84
8	44.582	44.50
9	46.164	46.06
10	40.026	39.95
11	15.845	15.73
12	30.377	30.26
13	47.985	47.91
14	192.256	192.35
15	123.322	123.25
16	204.950	204.95
17	60.777	60.68
18	26.473	26.39
19	19.031	18.94
20	118.456	118.44
21	142.782	142.71
22	111.149	111.13
23	141.669	141.61
24	21.300	21.22
25	27.016	26.91
26	26.304	26.20
27	20.965	20.86
28	169.577	169.53

The comparison 1H NMR spectra (Table 1.) and 13C NMR spectra (Table 3) of HAI showed good agreement with Azadiradione reported in literature. Therefore HAI is most likely an Azadiradione

Using its 2D data as follows also supports the above prediction.

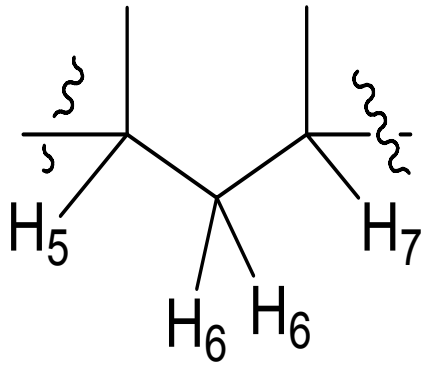
3.1.3 **C**orrelation **S**pectroscopy **(COSY)**

Correlation **S**pectroscopy **(COSY)** (Appendix 6) showed correlation between H-1 δ 7.117 and H-2 δ 5.903 indicates the following partial structure **I**, because the multiplicity of each hydrogen atom is doublet.



I

Similarly coupling is observed between H-5 δ 2.505 and H-6 δ 2.239 and also between H-6 δ 2.239 and H-7 δ 5.327 indicates the following partial structure **II**.



II

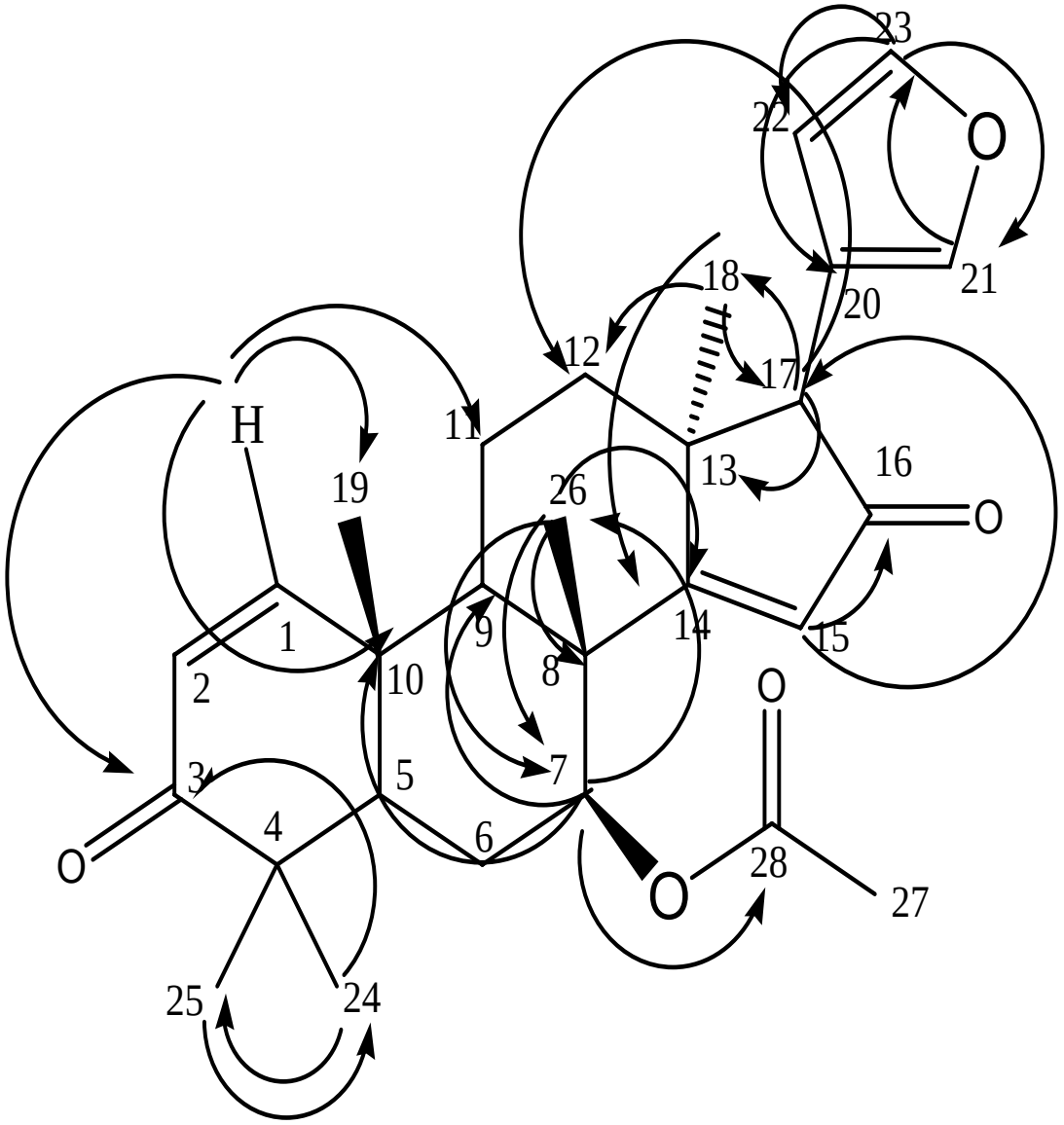
3.1.4 **H**etronuclear **S**ingle **B**ond **C**orrelation (**HSQC**)

Hetronuclear **S**ingle **B**ond **C**orrelation (**HSQC**) experiment correlates the chemical shift of proton with the chemical shift of directly bonded carbon. In the HSQC spectra (Appendix 7) showed three protons at δ 1.032 (s) connected with C-18 δ 26.473, three protons at δ 1.255(s) connected with C-19 δ 19.031, three protons at δ 1.101(s) connected with C-24 δ 21.300, three protons at δ 1.094(s) connected with C-25 δ 27.016, three protons at δ 1.344(s) connected with C-26 δ 26.304, three protons at δ 1.949(s) connected with C-27 δ 20.965, two protons at δ 2.239(m) attached with C-6 δ 23.483, two protons at δ 2.109(m) attached with C-11δ 15.845, two protons at δ 1.878(t) attached with C-7 δ 73.939, a proton at δ 7.117(d) connected with C-1 δ 156.695, a proton at δ 5.903(d) connected with C-2 δ 125.952, a proton at δ 2.505(m) connected with C-5 δ 38.254, a proton at δ 2.207(d) connected with C-9 δ 46.164, a proton at δ 5.877(s) connected with C-15 δ 123.322, a proton at δ 3.422(s) connected with C-17 δ 60.777, a proton at δ 7.429(t) connected with C-21 δ 142.782, a proton at δ 6.276(d) connected with C-22 δ 111.149, a proton at δ 7.478(d) connected with C-23 δ 141.669.

3.1.5 **H**etronuclear **M**ultiple **B**ond **C**orrelation (**HMBC**)

Hetronuclear **M**ultiple **B**ond **C**orrelation (**HMBC**) experiment gives information about coupling of hydrogens and carbons that are two or three bonds away. In the HMBC (Appendix 8), the vinylic proton at δ 7.117(d) (H-1) showed correlation with: δ 203.946 (C-3); δ 23.483(C-6); δ 46.164(C-9); δ 40.026(C-10); δ 15.845(C-11), the methine proton at δ 5.327(t) (H-7) displayed correlation with: δ 46.164(C-9); δ 40.026(C-10); δ 26.473(C-18); δ 26.304(C-26); δ 169.577(C-28), the vinylic proton at δ 5.877(s)(H-15) displayed correlation with: δ 47.985(C-13); δ 204.950(C-16); δ 60.777(C-17), the methine proton at δ 3.422(s)(H-17) showed correlation with: δ 30.377(C-12); δ 47.985(C-13); δ 204.950(C-16); δ 26.473(C-18); δ 118.456(C-20); δ 142.782(C-21); δ 111.149(C-22), the methyl proton resonate at δ 1.032(s)(H-18) correlated with carbons at: δ 192.256(C-14); δ 30.377(C-12); δ 123.322(C-15); δ 60.777(C-17), the methyl proton resonate at δ 1.255(s)(H-19) correlated with carbons at: δ 156.695(C-1); δ 40.026(C-10); δ 192.256(C-14); δ 21.300(C-24), the vinylic proton at δ 7.429(t) (H-21) showed correlation with carbons at: δ

118.456(C-20); δ 111.149(C-22); δ 141.669(C-23), the methyl proton at δ 1.344(S)(H-26) showed correlation with carbons at : δ 73.939(C-7); δ 44.104(C-8); δ 40.026(C-10); δ 192.256(C-14). These correlations are indicated below. the spectral data are consistent with the proposed structure. Therefore, HAI is most likely azadiradione.



4. Conclusion

The first attempt of this project was to isolate, characterize and compare concentration of azadiracthine of Ethiopian neem tree with that of Kenya. However; extraction of azadirachtin from the neem seeds quantitatively in pure form is quite difficult because of the following reason.

- It exists with several structurally closely related limonoids in the seeds, so it was not possible to isolate pure azadirachtin due to the lack of PTLC.

Therefore the compound HAI have been isolated by other technique that needs no PTLC; that is by solvent extraction followed by chromatographic purification.

5. Experimental

5.1 Materials

Melting point was determined on Karl kolb D-6072melt. UV spectrum was measured with GENESY'S spectrometer (200-400) in $CHCl_3$ at room temperature. IR spectrum was recorded on a Perkin-Elmer BX Infrared spectrometer. 1H NMR, 13C NMR and 2D NMR spectra were recorded on a Bruker advanced 400 MHz spectrometer with TMS as internal standard. Analytical TLC was done on 0.2mm thick layer of silica gel precoated sheets. Detection was done using vanillin/sulfuric acid and heat. Column chromatography was carried out using silica gel of particle size (0.063-0.200).

5.2 Sample collection

Fruit of neem tree were collected from Awash 7 in the region of afar about 225 Km east of Addis ababa, after collection the cover of the fruit was removed and the was air dried.

5.3 Extraction

The air dried seed was grounded using mortar and pestle, 500 g of powdered seed was extracted by soaking in 800ml of hexane for 24 hours. The extract was concentrated and gave 90ml of liquid neem oil.

5.4 Isolation

270g of silica gel was measured and mixed with 400ml of petroleum ether and packed in to a column and 90ml of concentrated sample was applied on top of the packed silica gel and then it was eluted with petroleum ether followed by petroleum ether/$CHCl_3$, $CHCl_3$, $CHCl_3$ /EtOAc. A total of 20 fractions were collected as follows.

Table 4. Ratio and volumes of solvent systems.

Fraction	Solvent	Ratio	Volume
1	Petroleum ether	pure	20ml
2	Petroleum ether	pure	20ml
3	Petroleum ether	pure	20ml
4	Petroleum ether	pure	20ml
5	Petroleum ether	pure	20ml
6	Petroleum ether/$CHCl_3$	4:1	20ml
7	Petroleum ether/$CHCl_3$	3:2	20ml
8	Petroleum ether/$CHCl_3$	1:1	20ml
9	Petroleum ether/$CHCl_3$	2:3	20ml
10	Petroleum ether/$CHCl_3$	1:4	20ml
11	$CHCl_3$	pure	20ml
12	$CHCl_3$	pure	20ml
13	$CHCl_3$	pure	20ml
14	$CHCl_3$	pure	20ml
15	$CHCl_3$	pure	20ml
16	$CHCl_3$ / EtOAc	4:1	20ml
17	$CHCl_3$ / EtOAc	3:2	20ml
18	$CHCl_3$ / EtOAc	1:1	20ml
19	$CHCl_3$ / EtOAc	2:3	20ml
20	$CHCl_3$ / EtOAc	1:4	20ml

30mg of fraction 19 was colleted and showed spot on TLC using UV lamp at 254nm.It was then characterized using spectroscopic techniques and identified as an azadiradione.

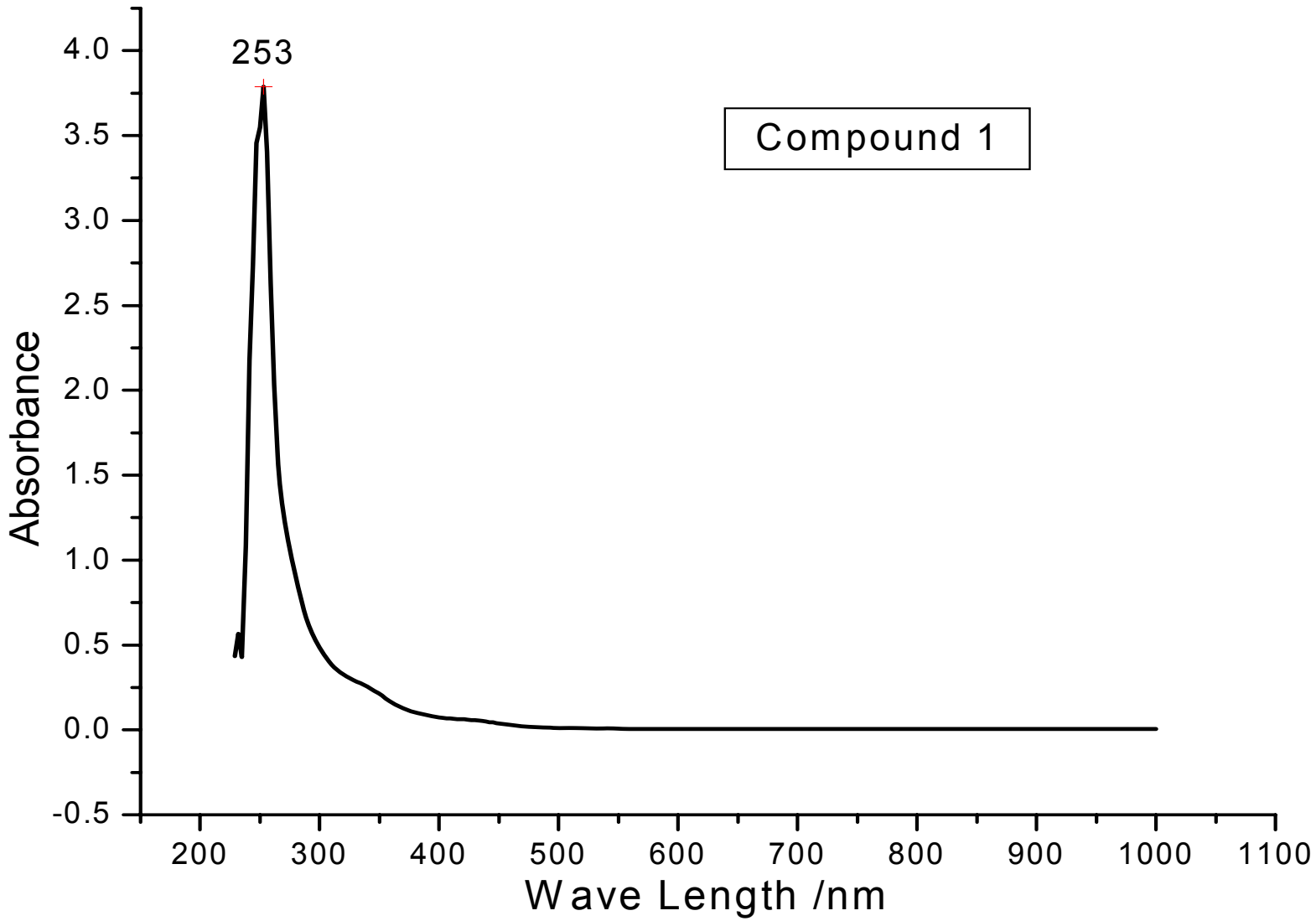
Azadiradione (HAI): is a yellowish crystal but it does not absorb in visible region, this is due to high concentration of the sample, **Rf** = 0.58 ($CHCl_3$ / EtOAc,in the ratio of 2:3), **mp** (156-164 ^{0}C).

IR ν_{max} (KCl) cm^{-1} : 3390.50 (due to moisture), 2931.89 cm^{-1} C-H (sp3), 1661.72 cm^{-1} (-CH=CH-CO-), 1382.81 cm^{-1} (CH_2 bend), 1241.55 cm^{-1}(CO single bond).

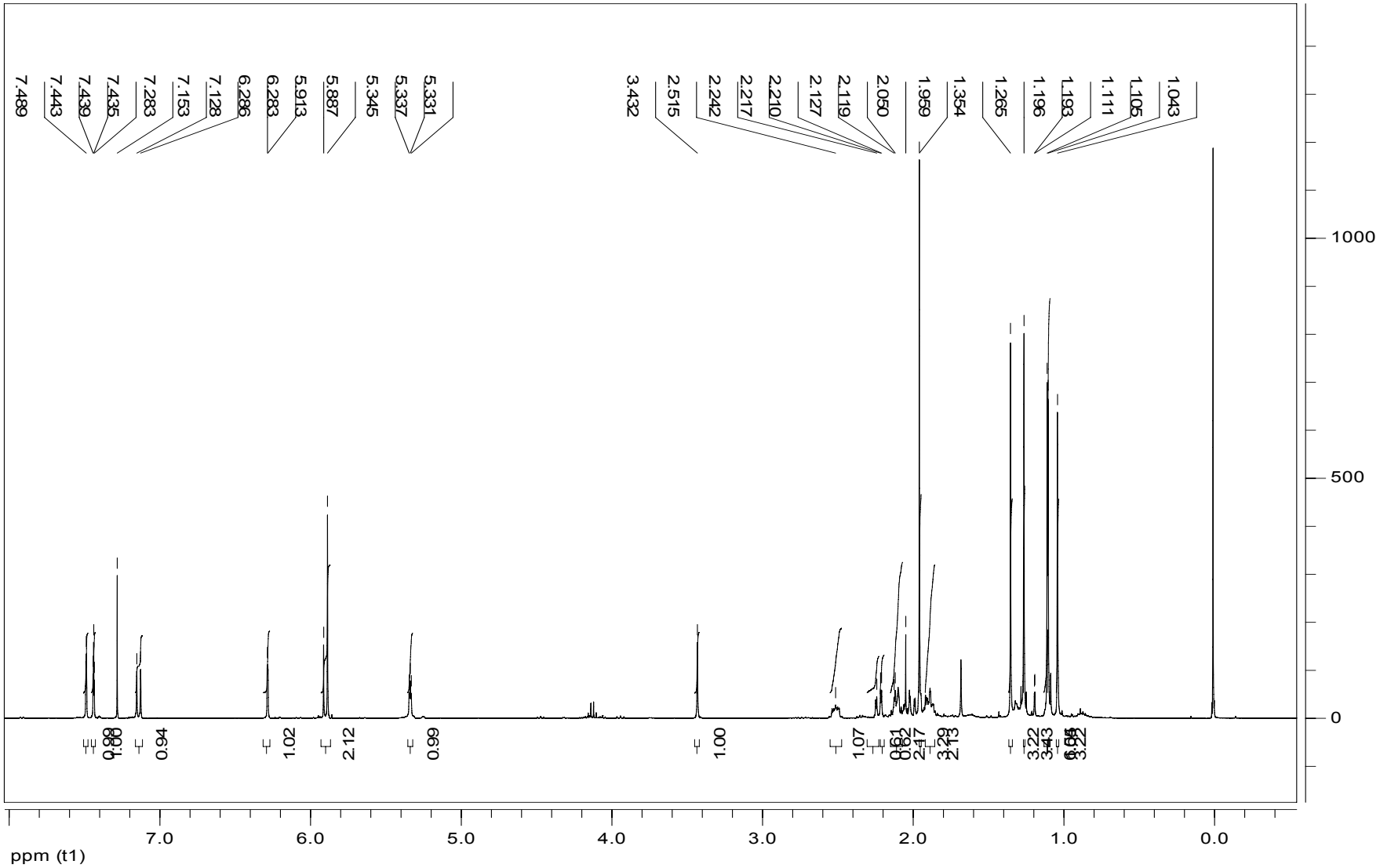
^{1}H NMR (400Hz) δ 2.505 (1H,m,H-5) δ 2.109 (2H,m,H-11), δ 1.878(2H,m,H-12), δ 5.327(1H,t,H-7), δ 2.207(1H,t,H-9), δ7.117(1H,d,H-1), δ 5.903(1H,d,H-2), δ 6.276(1H,d,H-22), δ7.478(1H,d,H-23), δ5.877(1H,s,H-15), δ3.422(1H,s,H-17), δ1.032(3H,s,H-18), δ1.255(3H,s,H-19), δ 7.429(1H,s,H-21), δ1.101(3H,s,H-24), δ 1.094(3H,s,H-25), δ 1.344(3H,s,H-26), δ1.949(3H,s,H-27).

^{13}C NMR (100H,$CDCl_3$) δ 203.946, 44.104 , 44.582, 40.026, 47.985, 192.256, 204.950, 118.456, 169.577 (9C-quaternary), δ 156.695, 125.952, 38.254, 73.939, 46.164, 123.322, 60.777, 142.782, 111.149, 141.669 (10C - methine), δ 23.483, 15.845, 30.377 (3C-methylene) δ 26.473, 19.031, 21.300, 27.016, 26.304, 20.965 (6C methyl).

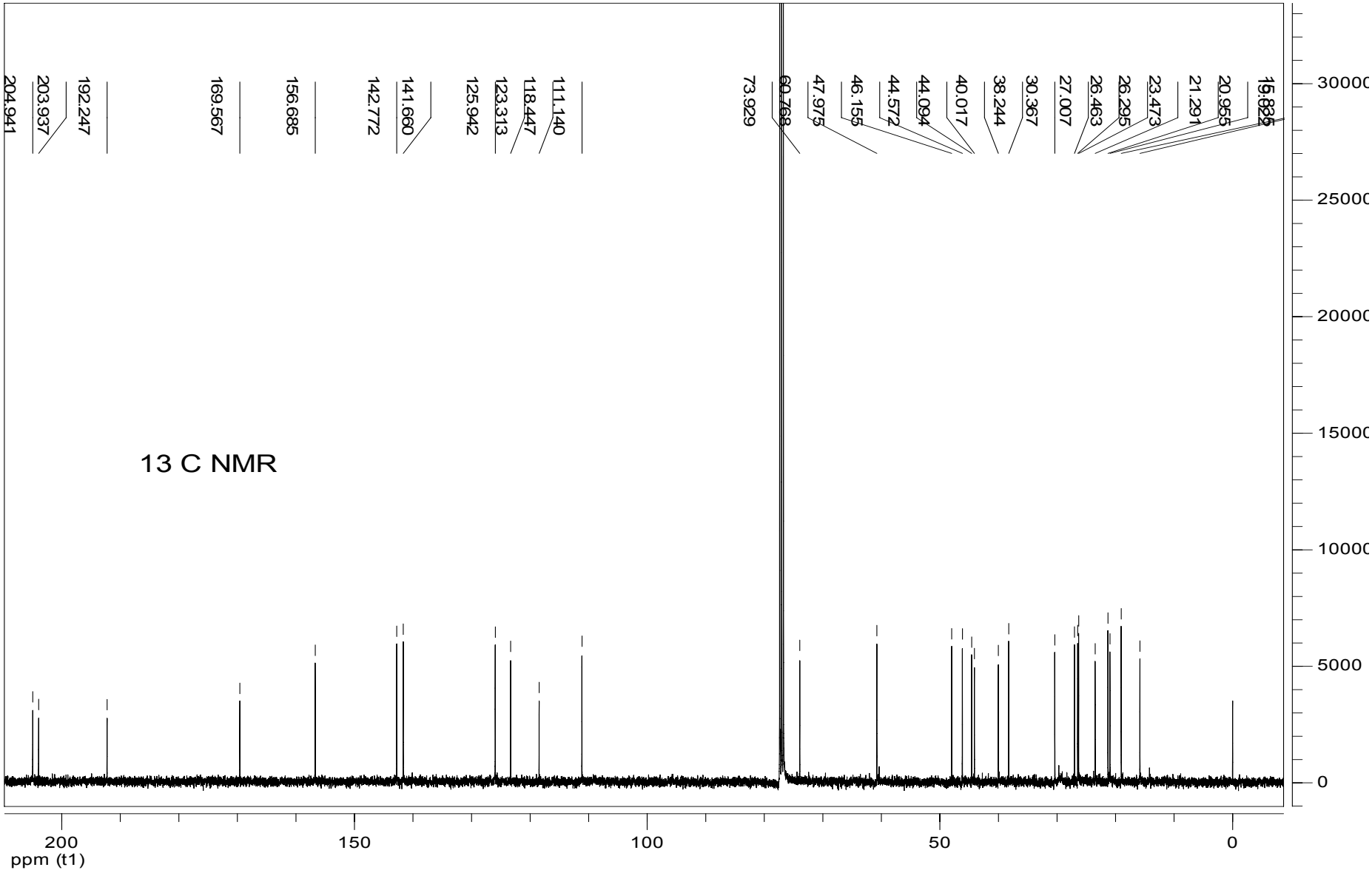
Appendix 1


253
Compound 1
Absorbance
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
200
300
400
500
600
700
800
900
1000
1100
Wave Length /nm

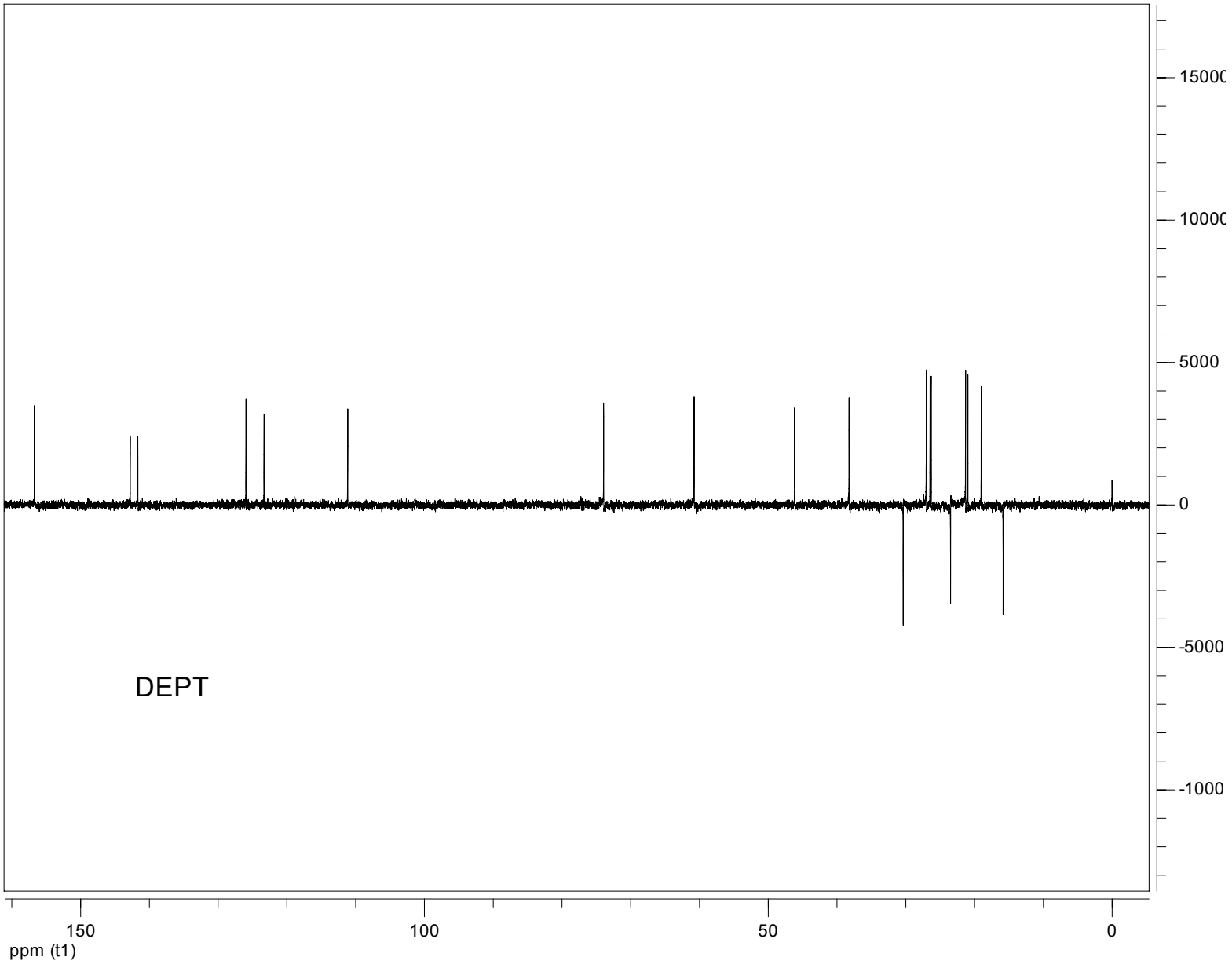
Appendix 3: 1H NMR Spectrum of HAI



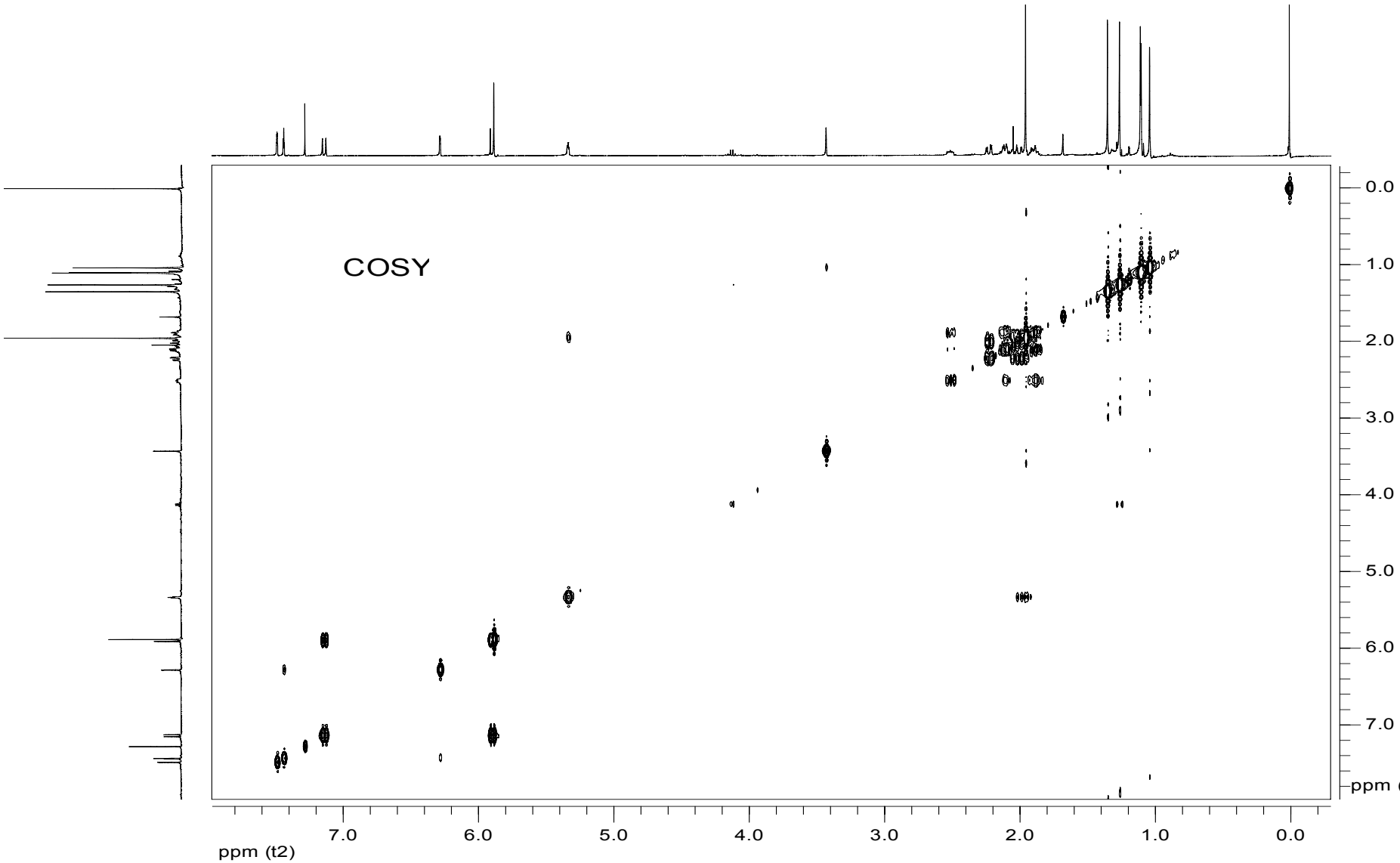
Appendix 4: 13C NMR Spectrum of HAI



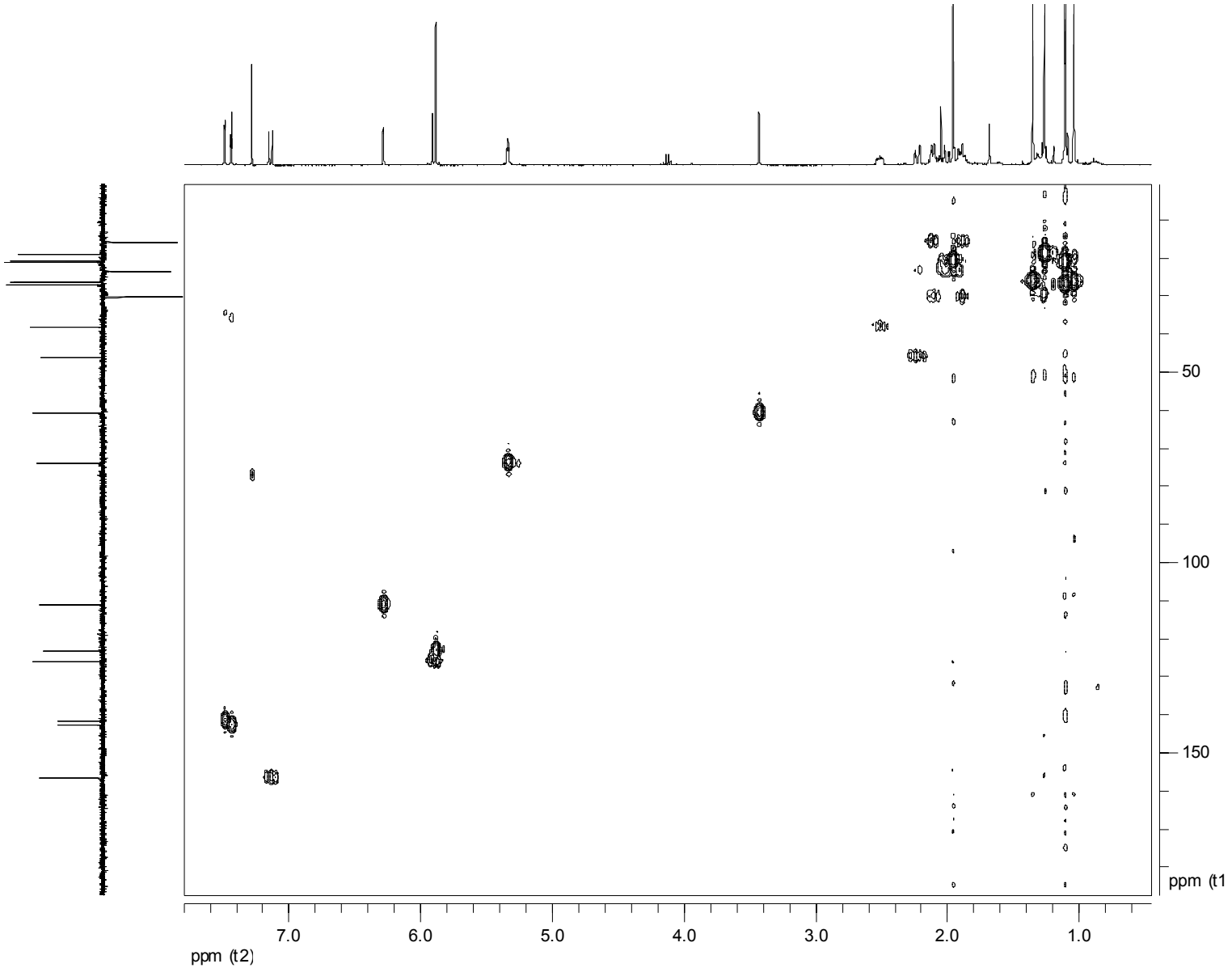
Appendix 5: DEPT Spectrum of HAI



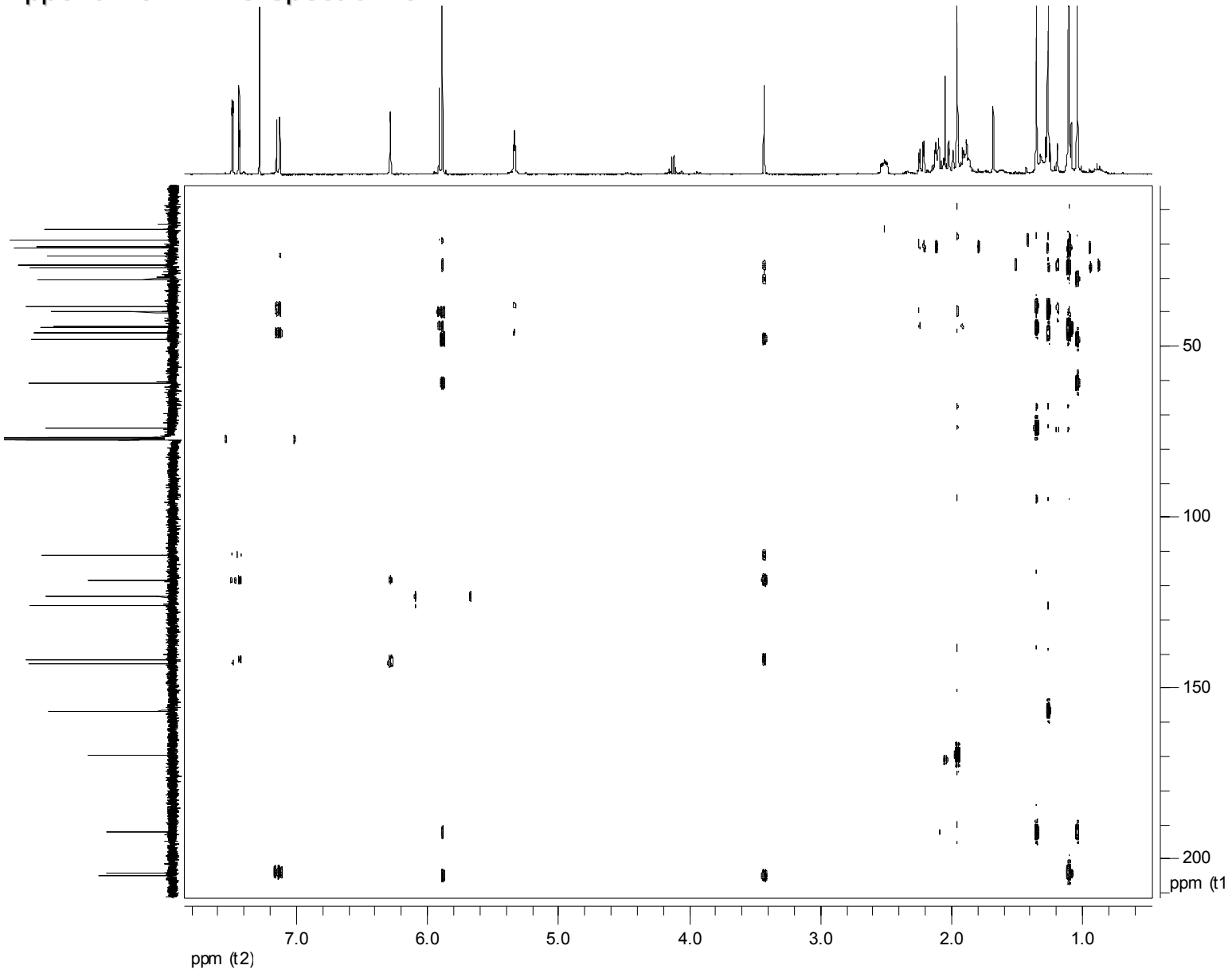
Appendix 6: COSY Spectrum of HAI



Appendix 7: HSQC Spectrum of HAI



Appendix 8: HMBC Spectrum of HAI



Reference

1. NATIONAL RESEARCH COUNCIL, Neem: a tree for solving global problems, National Academy Press, washington, DC, 1992

2. Kraus,W. et al, Tetrahedron Lett., 1978, **27**, 2395-2398.

3. Peter B.Kufman. Leland J. Cseke. Sara Warber. James A. Duke. Harry L. Brielmann *Natural products from plants*, 1999, 9-18.

4. Moron, J., Merrien, M.-A., and Polonsky,J. *Phytochemistry* , 1971, **30**,585.

5. Rosalyn Rappaport, Controlling Crop Pests and Diseases, 1992, 47-48.

6. McMurry, Organic chemistry, 2000, 1131-1132.

7. Francis a. Carey, Organic chemistry, 2000, 1028-1034.

8. Lee, S.M., Olsen, J.I., *Phytochemistry* , 1988, **27**, 2773-2775.

9. Bina Shaheen Siddiqui, *Phytochemistry* ,1998, **47**, 1631-1636.

10. Vijaya Kumar,N.M.Mohamed Niyaz, *Phytochemistry* , 1998, **49**, 215-218.

11. Niranjan Ramji, Madyastha.*Phytochemistry* , 1998, **49**, 265-267.

12. Lanza, E. and Palmer, J. K.*Phytochemistry*, 1977, **16**, 1555.

13. Banthorpe, D.V., Ekundayo,O. and Njar, V. C. O. *Phytochemistry* , 1984, **23**, 291.

14. Nozoe, S., Morisaki, M., Tsuda, K.,Iitaki. J.Am.chem.Soc.1965, 87, 4968.

15. Bornemann, N., Patterson, G. M. L. and Moore, R. E. J. Am. Chem. Soc.1988, 110, 2339.