ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

PHYTOCHEMICAL INVESTIGATION ON AERIAL PARTS OF VERBENA OFFICINALIS (ATUCH)

A GRADUATE PROJECT
(CHEM. 774)

ADDISU ALEMAYEHU

JULY, 2006

PHYTOCHEMICAL INVESTIGATION

ON

AERIAL PARTS OF VERBENA OFFICINALIS

A GRADUATE PROJECT SUBMITED

TO THE SCHOOL OF GRADUATE STUDIES

ADDIS ABABA UNIVERSITY

IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE IN CHEMISTRY

BY

ADDISU ALEMAYEHU

JULY, 2006

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

PHYTOCHEMICAL INVESTIGATION ON AERIAL PARTS OF VERBENA OFFICINALIS

BY
Addisu Alemayehu

Department of Chemistry

Faculty of Science

Approved by Examining Board:

Prof. Ermias Dagne _____
Dr. Wendimagegn Mammo _____
Dr. Gizachew Alemayehu _____
Dr. Ashebir Fiseha _____
Dr. Nigist Asefaw _____

Acknowledgement

My special and endless thanks go to Almighty God in giving me patient and strength. Second but the most profound gratitude goes to my advisor Dr.Ashebir Fiseha for his unreserved and constructive guidance while doing my project. And I also thank Dr. Wendimagegn Mammo for his invaluable comments, guidance and literature support.

All the NMR spectra reported in this work were measured on Bruker 400 MHz NMR spectrometer purchased by grant to the National Products Chemistry project. Running cost of the instrument is covered by budget from the Faculty of Science. I am deeply grateful for this service. I also thank Ato Yosef Atelaw for running the spectra.
I would like to express my deepest gratitude to Dr. Yonas Chebude and W/t Medhanit for running IR spectra of the sample.

My deepest gratitude goes to Ato Mekonnen Workeneh who informed me the medicinal value of the plant traditionally and helped me in collecting the plant material.

I am grateful to acknowledge my friends Temesgen G., Abebaw D. and my sister Yalemwork who encouraged me unreservedly during my study.

Table of contents **Page**

List of figures **page**

List of schemes **page**

List of Tables **page**

List of Appendices **Page**

Abstract

PHYTOCHEMICAL INVESTIGATION ON AERIAL PARTS OF VERBENA OFFCINALIS

Advisor: Dr. Ashebir Fiseha

By: Addisu Alemayehu

Chloroform extract of aerial parts of Verbena Officinalis afforded ursolic acid methyl ester (VOD) whereas methanol extract gave Iridoid glycoside; Vebenaline (VOG). The structural elucidation of these compounds were conducted using ^{1}H NMR, ^{13}C NMR, 2D NMR (COSY, HSQC, & HMBC), and UV & IR spectra. Moreover, ^{1}H NMR & proton decupled ^{13}C NMR spectrum of these two compounds matched with the literature values of previously reported.

1. INTRODUCTION

1.0 General

Phytochemical studies of plants, especially of medicinal plants, are of great importance in developing drugs. They are useful in the study of chemotaxonomy and plant biodiversity as well as in documenting knowledge. Enhancing the knowledge of biological and pharmacological effects of plant constituents, and determination of the structures of the active principals, may help in sustaining the use of these products offers challenging problems of stereo specific synthesis with a fascinating diversity of structures to synthetic organic chemists. These molecules of life have attracted the attention and efforts of the foremost organic chemists because of their economic importance in industry, their value in medicine, and their importance in regulating the interactions between plants and animals in nature. The contribution of natural products to the development of medicine could be demonstrated by the amount of plant-derived drugs being used. In general 40% of modern drugs are said to be of natural origin. Many antecedents, plant growth regulating hormones, fungicides and agricultural chemicals are developed from natural products from time to time [1].

Natural products in fact do have a function in the organism from which they originate. Many of them are now considered to have vital roles as mediators of ecological interactions, thereby ensuring the continual survival of a particular organism. The ability to synthesize an array of secondary metabolites, which may repel or attract other organisms, is one facet of the survival strategy [2]. Flower color, pollination, and deterrent properties in plants and chemical defense in termite may explain some of the importance of secondary metabolites for the survival of an organism [3].

1.1 Verbenaceae

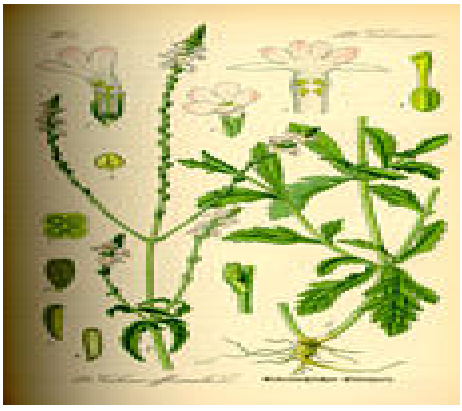
Verbenaceae is represented by 31 genera and 45 species in Ethiopia and 250 species in America. *Verbena Officinalis*, subkingdom is vascular plant, division flowering plants, class dicotyledons, subclass asteridae, order lamiales, family *verbenaceae* (verbena family), genus verbena L. vervain and species verbena officinalis L-herb of the cross. The

duration of the plant is annual [3, 4]. Verbena Officinalis (Fam.Verbenaceae) is a perennial herb with several stiffly erect stems, which can be found in West Asia, North Africa and throughout Europe [4]. The species has been introduced too many others part of the world [5] and are commonly known as vervain. It is known for its antidepressant and anticonvulsant effect as well as its use for the treatment of jaundice, cough, cold and digestive problems [6]. Mabey has also recorded its use for healing liver and gallbladder disease and nervous exhaustion [7].

1.2 Physical characteristics of the plant

The plant vervain has perennial growing to 0.6m to 0.5m. It is not frost tender. It is in leaf from April to October, in flower from July to September, and the seeds ripen from August to September. The flowers are hermaphrodite (have both male and female organs) and are pollinated by bees, flies and lepidopetra (moths and Butterflies). The plant is self-fertile. It is noted for attracting wild life. The plant prefers light (sandy), medium (loamy) and heavy (clay) soils and requires well-drained soil. The plant prefers acid, neutral and basic (alkaline) soils. It cannot grow in the shade. It requires moist soil. The plant can tolerate strong winds but not maritime exposure.

The plant habitat and possible locations are cultivated beds, and flowers and leaves of the plant used in making tea [6, 7].



Verbena officinalis.http://www.liberherbarium II.

1.3 Medicinal uses of vervain

Vervain, which has tonic and restorative properties, is sometimes used as domestic herbal remedy. It is useful when taken internally in the treatment of headaches, fevers, nervous exhaustion, depression, and gallbladder problems, insufficient lactation etc. [7, 8]. It should not be given to pregnant women, though can be used to assist contractions during labour. Externally, it is used to treat minor injuries, sores neuralgia and gum disease [8].

The leaves and flowering stems are analgestic, antibacterial, anticoagulant, antispasmodic, astringent, depurative, diaphoretic, mildly diuretic, emmenagogue, galactogogue, stimulant, tonic and vulnerary [7, 8, 9]. The plant is harvested as flowering begins in the summer and dried for later use [7, 8] some remarkable results have been obtained when using this plant in the treatment of certain tumours [7].

The root is astringent; it is used in the treatment of dysentery [8].

The plant is a very easily grown plant; it succeeds in any moderately fertile well drained but moisture retentive soil in a sunny position. Plants are very tolerant of neglect and will maintain themselves for a number of years even growing in dense weed competition [4, 10]. It is used traditionally in Gojjam, to treat dysentery, impotency and counter acting effect of snake poison.

The plant *V. Officinalis* studied before in China and Brazil showed the presence of monoterpenoids, iridoids glycosides: hastatoside, verbenaline (verbenaloside), verbenin. Phenylproponide glycosides: acetoside (verbascoside), eukovoside and triterpenoids: Ursolic acid, β-Sistosterol and Olenoic acid [10].

1.4 Monoterpenes

The fundamental skeletons of monoterpenes can be grouped into four categories

(a) Open chain (b) cyclohexanes (mono-and polycyclic), (c) cyclopentane (the iridoids and secoiridoids), (d) irregular systems [11].

1.4.1 Iridoids and secoiridoids

The iridane skeleton found in iridoids in monoterpenoid in origin and contains a cyclopentane ring which is usually fused to a six membered oxygen heterolcyle, e.g

heptalactone from catmint Nepeta cataria (Libiatae/Lamiaceae), a power attractant and stimulant for cats. The iridoid system arises from geraniol by a type of folding which is different from that already encountered with monoterpenoids, and also different is the lack of phosphorylated intermidates and subsequent carbocation mechanism in its formation [12].

The sub group of iridoids comprises of several hundreds of monoterpenes, all with a cyclopentane skeleton of the type (**24)**. In most cases a hemiacetal bridge links position 1 and 9, forming an hydroxytetrahydro-, or dihydro-, pyrane ring, condensed to the cyclopentane ring, whilst the hydoxy group at position 1 is conjugated to a glucose molecule, e.g. **(25).**

Thus iridoids are invariably found in nature as 1-D-glucosides, soluble in water. The cyclohexane monoterpenes, being insoluble in water, are accumulated in special oleipher glands of leaves, whilst iridoids are largely diffused in plant tissues (leaves, seeds, barks and roots etc.), especially in the dicotyledons. This difference in distribution is accompanied by much higher incorporation yields of MVA into the iridoids compared to that into the cyclohexyl monoterpenes. The name iridoid originates from the fact that the first members (iridomyrmecin and anisomorphal) were first isolated from the secretion of ants of the genes *iridomyrmex* [11].

1.4.2 Biosynthesis of iridoids

The biosynthesis of the iridoids has attracted the attention of many research teams for two main reasons: first, because good results can be obtained on incorporating labeled precursors second, because of the close biogeneric relationship between the iridoids and many indole and isoquinoline alkaloids, in which 10 (or 9) carbon atoms of the iridoid skeleton are bound [12,13]. Iridoids represent a large group of monoterpenoid compounds that apparently seem to be formed in plants by an alternative cyclization of gernyl diphosphate. The structures of most of these compounds are established based on a cyclopentane-pyran skeleton, carboxylic functionalities and oxidative cleavage at the 7, 8- or 8, 9-bond of the cyclopentane moiety [14, 15]. The number and nature of iridoids occurring in plants is a measure of the complexity of the routs involved in their biosynthesis [15, 16, and 17]. Previously people assumed that the compounds possessed a

common structural feature: they contained an integral number of C_5 units. Furthermore, isoprene (2-methylbutane-1, 3-diene) was often obtained on pyrolysis of these C_{10} compounds, and it was suggested that isoprene was the building block for terpene biosynthesis. Condensation of successive isoprene units in a head-to-tail fashion would produce compounds of formula $(C_5)_n$ [15].

An important place amongst the iridoids is held by loganine, which is the commonst precursor of the so-called secoiridoids, for instance of sweroside, and of the terpene portion of the above-mentioned alkaloids. Biosynthetic studies carried out up to the present indicate that the most probable sequence from nerol to loganine is as shown in figure.1.

The identification in loganine of the hydrogen atoms originally carried by geraniol and MVA has been performed using (2R)-, (2S)-, (4R)-, (4S) - and (5R)-[^{3}H] MVA as labeled precursors. Nerol has been hypothesized as an intermediate, since 10-hydroxynerol **(27)** is a more efficient precursor than 10-hydroxygeraniol. D. Arigoni postulated the formation of the trialdehyde **(28)** to explain, amongst other facts, the randomization of the label between position 9 and 10 of loganin, obtained from vinca rosea after feeding experiments with [2-^{14} C] mevalonic acid. A label localized on carbon 9 has been found in some iridoids, or in compounds strictly correlated to them, as in verbenaline or skytanthine.

The distribution of labeling in verbenaline and skythanthine resulting from incorporation experiments with [2-^{14}C] mevalonic acid in plants of different age. Two points arise from such data. The first is that, in mature plants, the five- membered ring is probably formed from an acyclic monoterpene, which has different functions at the two terminal carbons, e.g., C-9 as an aldehyde and C-10 as an acid in **(28)**. An enzyme might also be distinguish between two aldehyde functions in the non-equivalent positions at 9 and 10. The second point is that, in the iridoids which have incorporated [2-^{14}C] mevalonic acid, there is no asymmetric labeling observed between positions of the molecule deriving from TPP and DMAPP, whilst such an asymmetry is generally observed for the cyclohexane-type monoterpenes.

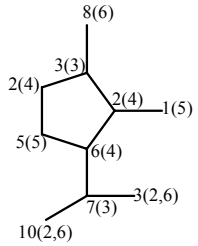
The fundamental cyclization to iridoidal is formulated as attack of hydride on the dialdehyde, produced by a series of hydroxylation and oxidation reactions on geraniol.

Further oxidation gives iridotrial, in which hemiacetal formation then leads to production of the hetrocyclic ring. In iridotrial, there is an equal chance that the original methyl from the geraniol ends up as the aldehyde or in the hetrocyclic ring. A large number of iridoids are found as glycoside, e.g. Loganin, glycosylation effectively transforming the hemiacetal linkage into an acetal. The path way to loganin involves, in addition, a sequence of reactions in which the remaining aldehyde group is oxidized to the acid and methylated, giving deoxyloganin, and the final step is a hydroxylation reaction. Loganin is a key intermidte in the biosynthesis of many other iridoid structures, and also features in the pathway to a range of complex terpenoid indole alkaloids and tetrahydroiso quinoline alkaloids [15].

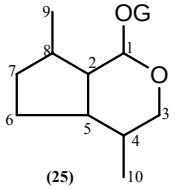
In loganic acid **(32),** isolated after feeing Swertia caroliniensis with [2-^{14}C, 2-$^{3}H_2$] MVA, a slightly smaller ^{3}H/^{14}C ratio was found at C-4 than one should expect according to the scheme of fig.1. This deviation, of the same order of magnitude as that found in the biosynthesis of steroids, can be attributed to use of DMAPP by the prenyl transferases, which competes kinetically with the dynamic equilibration between DMAPP and IPP isomerase.

The skeleton of the secoiridoids **(26)** is formally derived from the iridoids **(24)** by breaking the **C-3-C-4** bond. The exact mechanism by which loganine is converted into secologanine **(35)** is not known; Fig.2 illustrates one possibility.

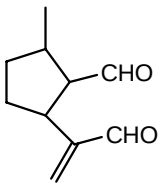
(24)



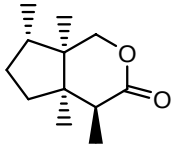
IRIDANE



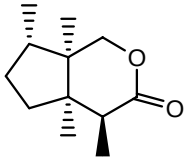
G=B-D-Glucose



ANTISOMORPHAL



IRIDOMYRMECIN



LOGANIN

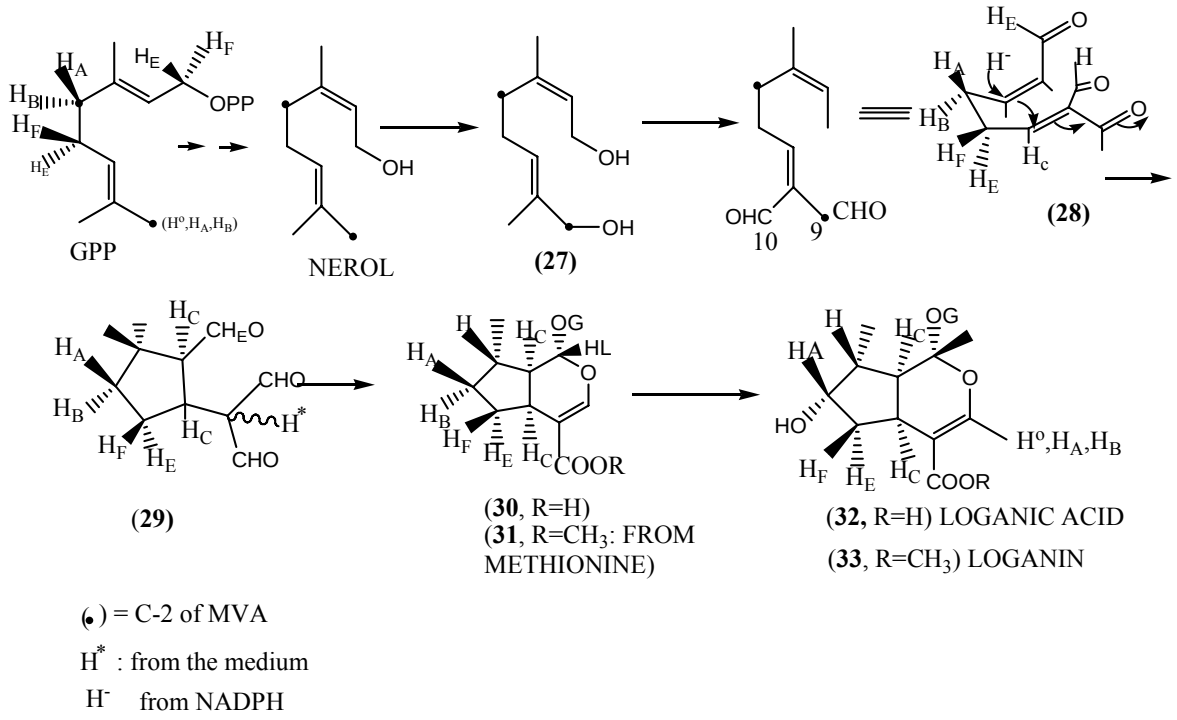


Figure 1. Biosynthesis of Loganin.

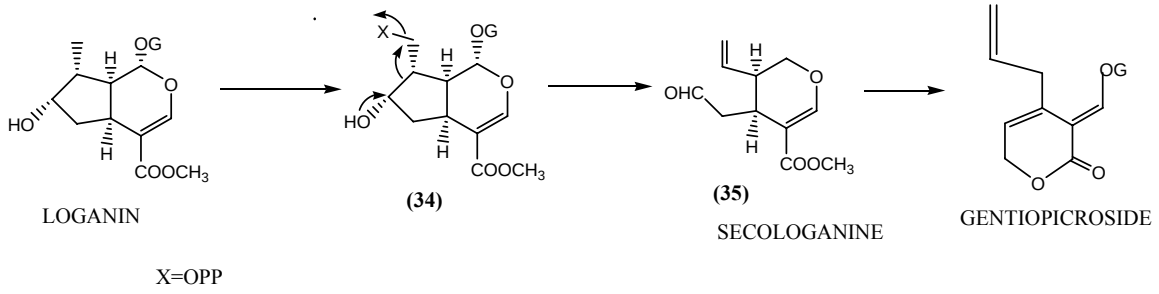


Figure 2. Probable biosynthesis of gentiopicroside and related secoiridoids.

1.5 Triterpenes (C_{30})

Squalene as a conceivable progenitor of the higher terpenoids. Squalene was first isolated from shark liver, squalus spp., but was later found to be unbiquitously distributed. Squalene consists of two all-trans farnesyl groups joined tail to tail. The mechanism of this puzzling coupling remained unsolved for a long period. It was first noted one proton

of the central unit was derived from NADPH by using an isotopic labeling technique. The solution to the problem came eventually with the isolation of an intermediate, presqualene pyrophosphate, having a cyclopropane structure [14].

The two main groups of triterpenes, the tetra cyclic triterpenes including the sterols, and the pentacyclic triterpenes. The conformation of squalene oxide on the enzyme surface and the extent of backbone rearrangement, which follows upon cyclization, give rise to a number of subgroups [15].

1.5.1 Biosynthesis of triterpenoids

Triterpenes are not formed by extension of the now familiar process of adding IPP to the growing chain. Instead, two molecules of farnesyl PP are joined tail to tail to yield the hydrocarbon squalene (Figure 3) originally isolated from the liver oil of shark (squalus sp.). Squalene was subsequently found in rat liver and yeast, and these systems were used to study its biosynthetic role as a precursor of triterpenes and steroids; several seed oils are now recognized as quite rich sources of squalene, e.g. *Amaranthus cruents* (Amarantaceae). During the coupling process, which on paper merely requires removal of the two phosphate groups, a proton from a C-1 position of one molecule of FPP is lost, and a proton from a NADPH is inserted. Difficulties with formulating a plausible mechanism for this unlikely reaction were resolved when an intermediate in the process, **presqualene diphosphate**, was isolated from rat liver. Its characterization as a cyclopropane derivative immidately ruled out all the hypotheses current at the time.

The formation of presqualene PP is represented in figure 3 as attack of the 2, 3-double bond of FPP on to the farnesyl cation, analogous to the chain extension using IPP. The resultant tertiary cation is discharged by loss of a proton and formation of the cyclopropane rings giving presqualene PP. Obviously, to form squalene, carbons-1 of the two FPP units must eventually be coupled, whilst presqualene PP formation has actually joined C-1 of one molecule to C-2 of the other. To account for the subsequent change in bonding of the two FPP units, a further cyclopropane cationic intermidatee is proposed. Loss of diphosphate from presqualene PP would give the unfavourable primary cation, which via Wagner-Meerwein rearrangement can generate a tertiary carbocation and achieve the required C-1-C-1' bond. Breaking the original but now redundant C-1-C-2'

bond can give an allylic cation, and the generation of **squalene** is completely by supply of hydride from NADPH [20].

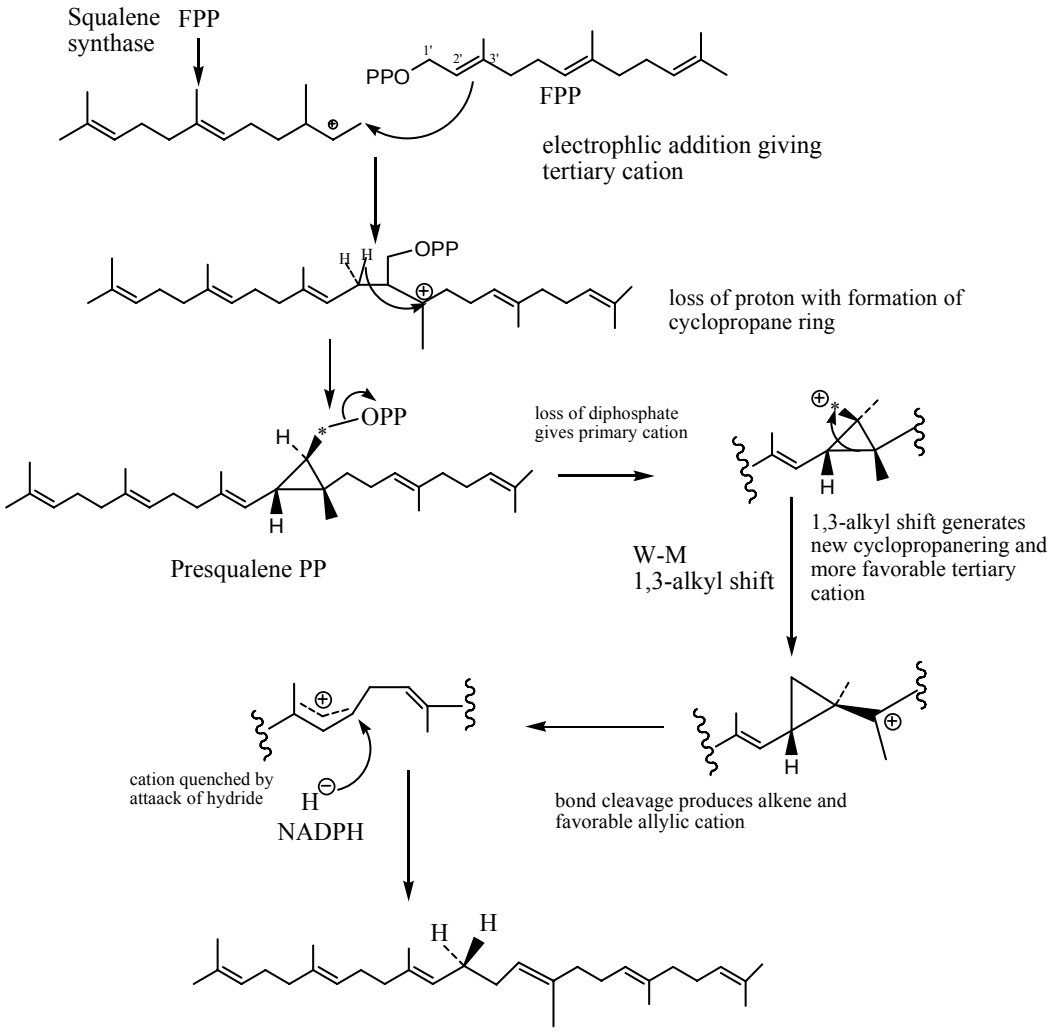


Figure 3. Biosynthesis of squalene.

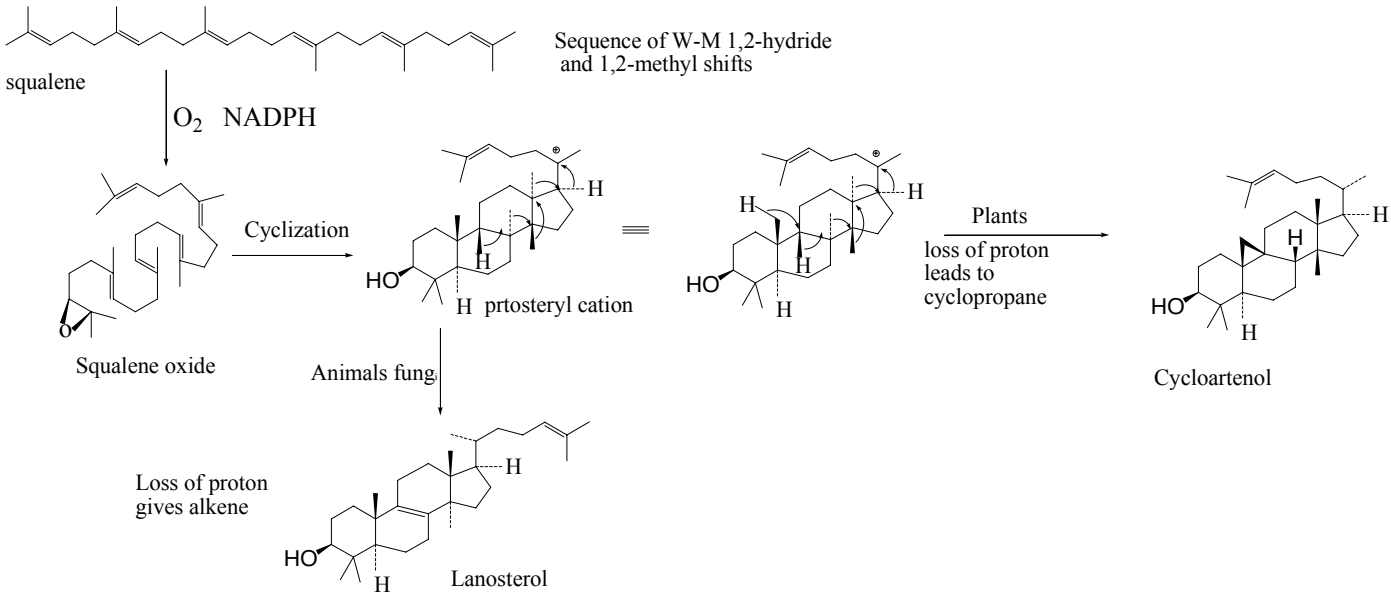


Figure 4. Biosynthesis of Lanosterol.

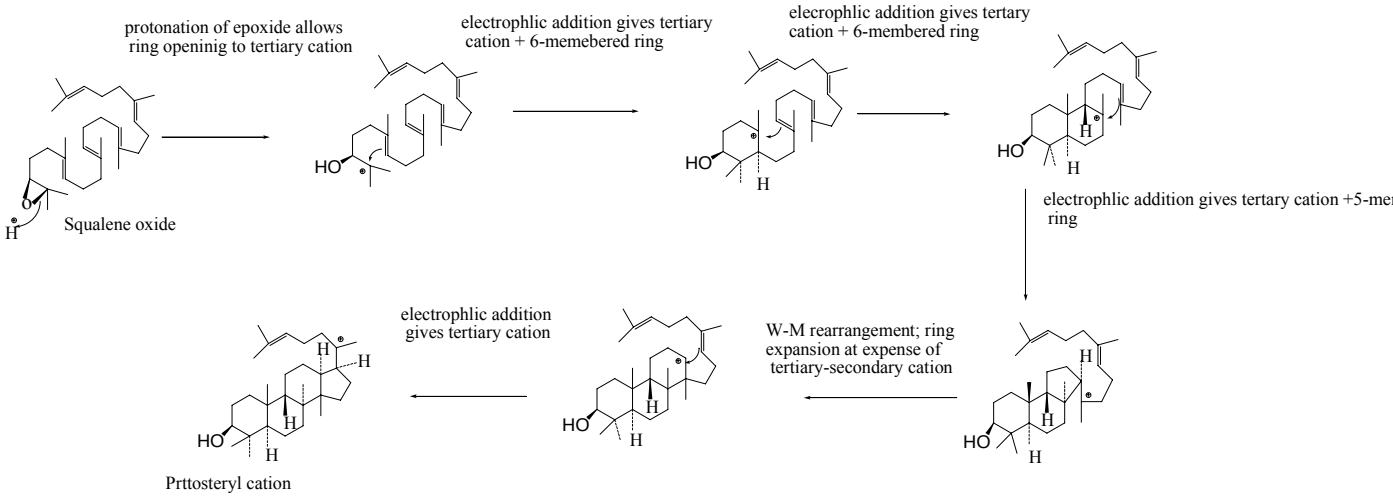


Figure 5. Biosynthesis of prosteryl cation.

Cyclization of squalene is via the intermediate **squalene-2, 3-oxide** (Figure 4), produced in a reaction catalysed by a flavoprotin requiring O_2 and NADPH cofactors. If squalene oxide is suitably positioned and folded on the enzyme surface, the polycyclic triterpene

structures formed can be rationalized in terms of a series of cyclizations, followed by a sequence of concerted Wagner-Meerwein migrations of methyl's and hydrides (Figure 4) the cyclizations are carbocation mediated and proceed in a step-wise sequence (Figure 5). Thus, protonation of the epoxide group will allow opening of this ring and generation of the preferred tertiary carbocation, suitably placed to allow electrophilic addition to a double bond, formation of six–membered ring and production of a new tertiary carbocation [21]. This process continues twice more, generating the preferred tertiary carbocation (Markovnikov addition) after each ring formation, though the third ring formed is consequently a five-membered one. This is expanded to a six-membered ring via a Wagner-Meerwein 1, 2-alkyl shift, resulting in some relief of ring strain, through sacrificing a tertiary carbocation for a secondary one. A further electrophilic addition generates the tertiary protosteryl cation (Figure 6) [22]. The stereo chemistries in this cation are controlled by the type of folding achieved on the enzyme surface, and this propably also limits the extent of the cyclization process. Thus, if the folded squalene oxide approximates to a chair-boat-chair-boat conformation (Figure 6), the transient **protosteryl cation** will be produced with these conformational characteristics**.** This cation then undergoes a series of Wagner-Meerwein 1,2-shifts, firstly migrating a hydride and generating a new cation, migrating the next hydride, then a methyl and so on until a proton is lost forming a double bond and thus creating **lanosterol** (Figure 6). The stereochemistry of the protosteryl cation in Figure 6 shows how favorable this sequence will be, and emphasizes that in the ring system, the migrating groups are positioned anti to each other, one group entering whilst the other leaves from the opposite side of the sterocenter [23]. This, of course, inverts configuration at each appropriate center. No anti group is available to migrate to C-9 (steroid numbering), and the reaction terminates by loss of proton H-9 [24]. Lannosetrol is a typical animal triterpenoid, and the precursor for cholesterol and other sterols in animals and fungi. In plants, its intermediate role is taken by cycloartenol (Figure6), which contains a cyclopropane ring, generated by inclusion of carbon from the methyl at C-10. For cycloartenol, H-9 is not lost, but migrates to C-8, and the carbocation so formed is quenched by cyclopropane ring has then to be reopened. Most natural triterpenoids and steroids contain a 3-hydroxyl group, the original epoxide oxygen from squalene oxide [25].

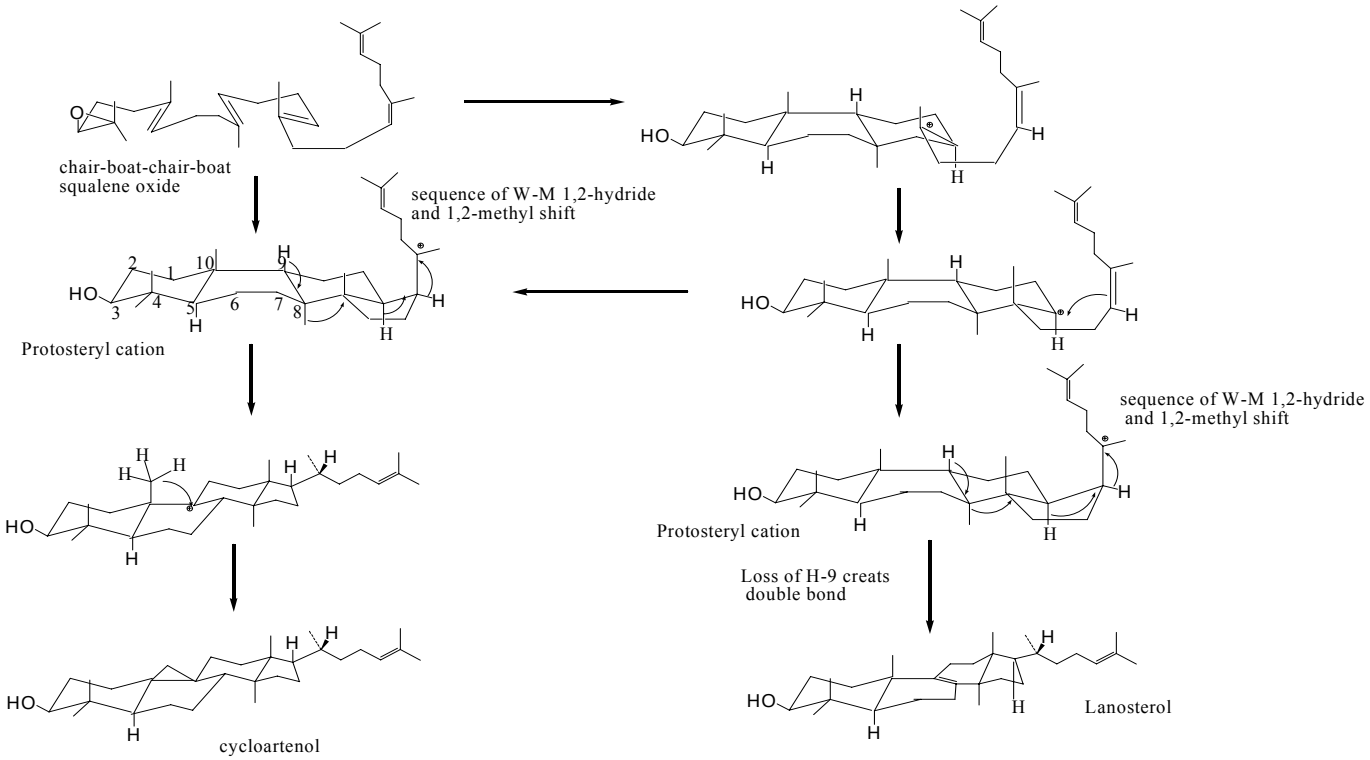


Figure 6. Conformational characteristic in biosynthesis of cycloartenol and lonasterol [14, 15].

Should squalene oxide be folded on to another type of cyclase enzyme, this time in a roughly chair-chair-chair-boat conformation then an identical carbocation mechanism ensues, and the transient dammarenyl cation formed now has different stereochemical features to the protosteryl cation [27]. Whilst a series of Waggner-Meerwein migrations can occur, there is relatively little to be gained on purely chemical grounds, since these would invert stereochemistry and destroy the already favourable conformation. Instead, the dammarenyl cation typically undergoes further carbocation promoted cyclizations, without any major changes to the ring system already formed. There are occasions in which the migrations do occur, however, and euphol from Euphorbia species (Euphorbiaceae) is a stereoisomer of lanosterol (Figure 4) [18].

Should the Waggner-Meerwein rearrangements not occur, the dammarenyl cation could be quenched with water, giving the epimeric dammarenediols, as found in Dammar resin

from balanocarpus hemi (Dipterocarpaceae) and ginseng (panax ginseng; Araliaceae) (Figure 7). Alternatevely, the migration shown to give the baccharenyl cation relives some ring strain by creating a six-membered ring, despite sacrifing a tertiary carbocation for a secondary one. A pentacyclic ring system can now be formed by cyclization on to the double bond, giving a new five-membered ring and the tertiary lupenyl cation. Although this appears to contradict the reasoning used above for the dammarenyl-baccharenyl transformation, the contribution of the enzyme involved must also be considered in each case. A five-membered ring is not highly strained as evidenced by all the natural examples encountered. Loss of a proton from the lupenyl cation gives **lupeol**, found in lupin (*Lupinus luteus*; Leguminosae/Fabaceae) [19]. Ring expansion in the lupenyl cation gives the oleanylsystem, and labeling studies have demonstrated this ion is discharged by hydride migrations and loss of a proton, giving the widely distributed **β-amyrin.** Formation of the isomeric α-amyrin involves first the migration of a methyl in the oleanyl cation, then discharge of the new taraxasteryl cation by three hydride migrations and loss of a proton. Loss of a proton from the non-migrated methyl in the taraxasteryl cation is an alternative way of achieving a neutral molecule, and yields taraxasterol found in dandelion. Comparison with α-amyrin shows the subtly different stereochemistry present because the inversions of configurationcaused by hydride migrations have not occurred. Where evidence is available, these extensive series of cyclizations and Wagner-Meerwein rearrangements appear to be catalysed by a single enzyme, which converts squalene oxide into the final product, e.g. lanosterol, cycloartenol, α-amyrin, or β-amyrin [27, 28].

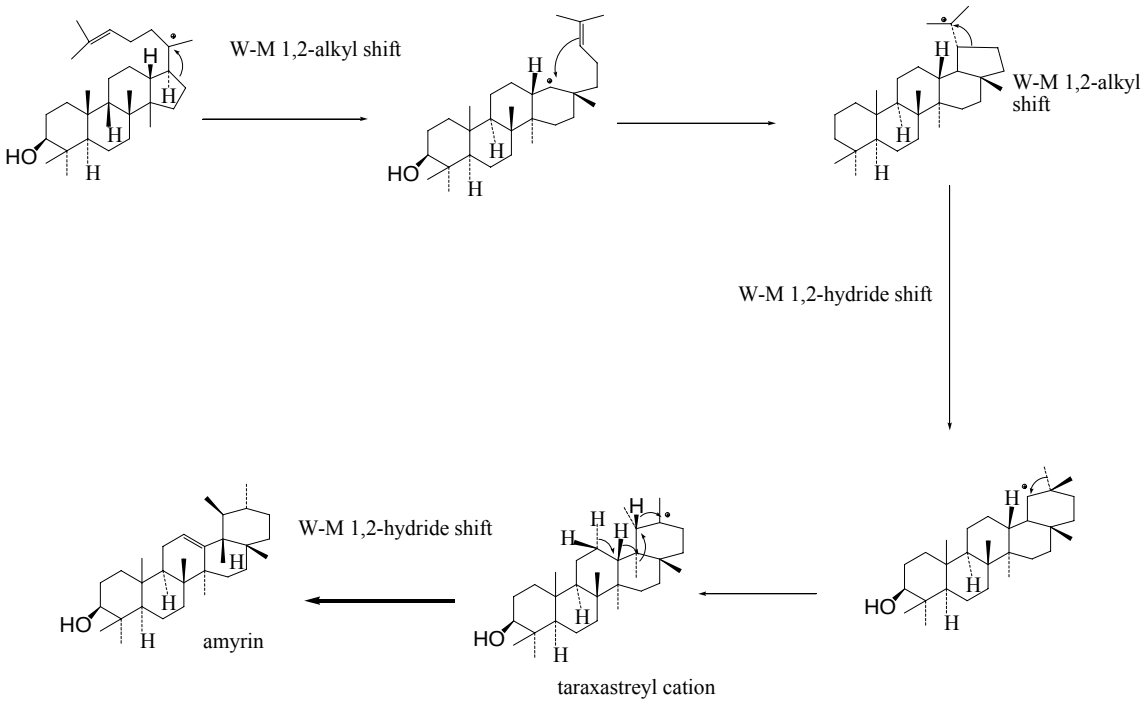
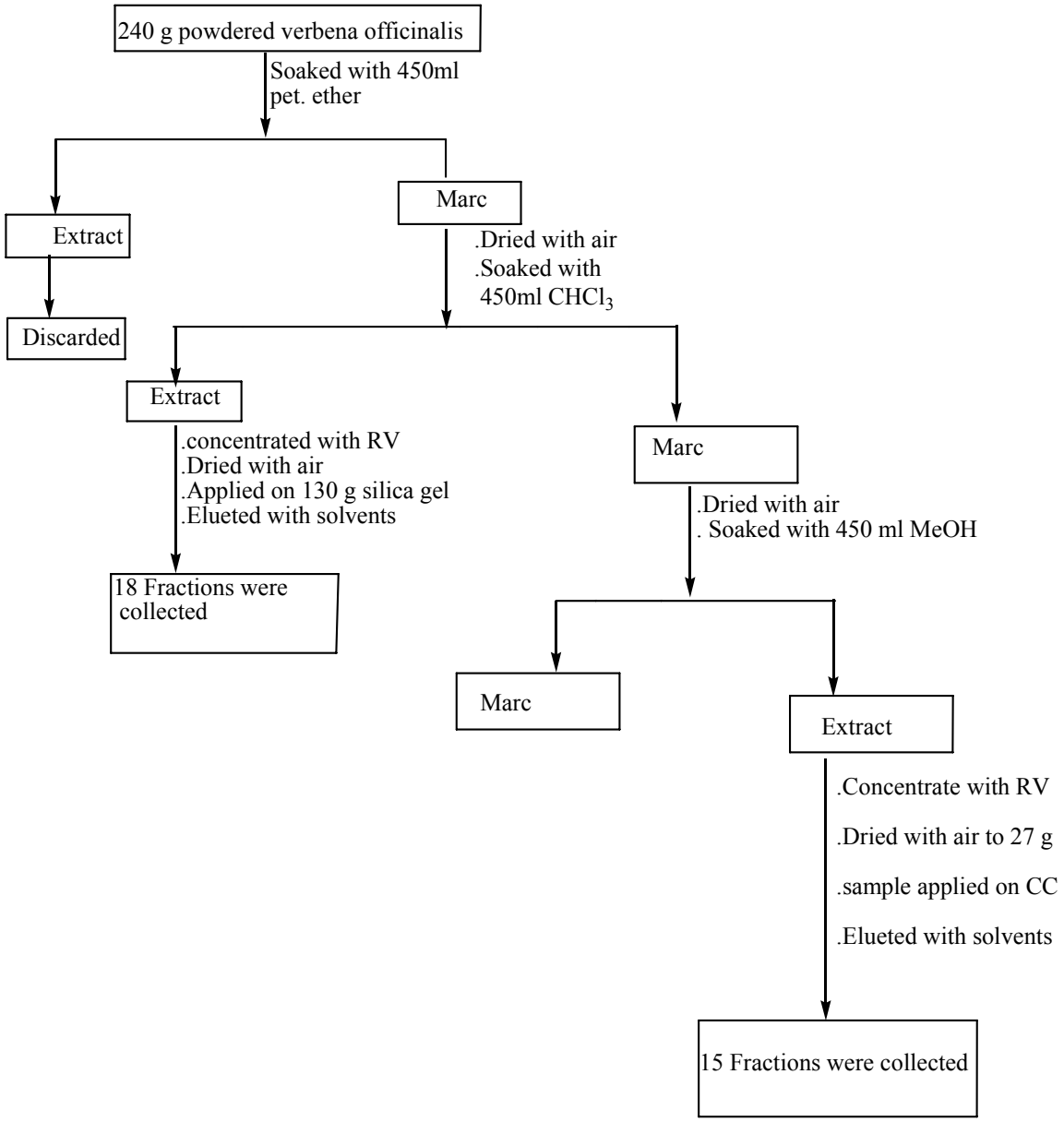


Figure 7. Biosynthesis of amyrin.

2. OBJECTIVE OF THE STUDY

The main objective of this project is to isolate compounds from verbena officinalis (verbenacea), herb that are grown in Ethiopia and to elucidate there structures using chemical and spectroscopic methods (IR,1D & 2D NMR and UV / VIS).

3. METHOD OF EXTRACTION



Scheme1. Extraction outline for the plant material.

4. RESULTS AND DISCUSSION

Ground aerial parts (240 g) of verbena officinalis were subjected to exhaustive extraction successively with petrol ether, chloroform and methanol. The solvent from each extract was recovered under reduced pressure using rotavapor to obtain a petrol extract (PE,5 g), a chloroform extract (CE, 20 g) and a methanol extract (ME, 27 g) respectively.

Chromatographic purification of the chloroform extract gave a compound coded; VOD whereas the methanol extract yielded a compound coded; VOG. The structures of these compounds have been elucidated on the basis of spectroscopic evidences.

4.1 Characterizations of compound VOD

Compound VOD is a pale yellow crystalline solid obtained from chloroform extract and its characterization was determined using spectroscopic techniques.

UVλ_{max} MeOH: 469 (0.12), 440 (0.12), 422 (0.27), 393 (0.30) see Appendix 1 this absorption is due to bathochromic shift in n- π^*, molecule-molecule and molecule-solvent aggregation.

The **IR v cm^{-1}**(KBr disk) spectrum (Appendix 2) showed absorption bands assigned to hydroxyl group (3436, broad), to carbonyl system of an ester group (1690), to an olefinic system (1458), to saturated groups (2928), and to C-O stretching at 1029. It also revealed the presence of a week alkene C-H stretching at 3010 see Appendix 2.

^{1}H NMR δ (400MHz, $CDCl_3$ +CD_3OD): δ 5.21multiplet and integrated for one proton indicate olefinic proton. δ 3.65 broad singlet indicate OH proton, δ 3.25 (sharp singlet the solvent CD_3OD), δ 3.14 with multiplet multiplicity indicate a proton attached with a chairal carbon, δ 2.20 with doublet multiplicity and integrated for one proton indicate methine, protons in δ 2.02-1.15 multiplet and integrated for 22H showed methylene protons, a δ 1.10 singlet and integrated for 3H, showed methyl protons, δ 0.96 singlet and integrated to 3H,methyl protons, δ 0.95 singlet & integrated to 3H showed methyl protons, δ 0.93 singlet & integrated to 3H showed methyl protons, 0.87 doublet & integrated to 3H showed methyl protons, and δ 0.83 doublet integrated to 3Hshowed methyl protons ,0.76singlet & integrated to 3H showed methyl protons.

Table 1. Proton decoupled ^{13}C NMR and DEPT spectra of VOD.

Position	^{13}C NMR for compound VOD	DEPT	Remark
1	39.05	39.05	CH_2
2	26.78	26.78	CH_2
3	78.78	78.78	CH
4	39.45	-	Quaternary C
5	55.24	55.24	CH
6	18.27	18.27	CH_2
7	33.02	33.02	CH_2
8	39.45	-	Quaternary C
9	47.77	47.77	CH
10	38.65	-	Quaternary C
11	23.43	23.43	CH_2
12	125.46	125.46	CH
13	138.15	-	Quaternary C
14	42.03	-	Quaternary C
15	29.59	29.59	CH_2
16	24.17	24.17	CH_2
17	47.54	-	Quaternary C
18	55.24	55.24	CH
19	39.05	39.05	CH
20	39.05	39.05	CH
21	30.67	30.67	CH_2
22	38.65	38.67	CH_2
23	27.96	27.96	CH_3
24	15.31	15.31	CH_3
25	15.90	15.90	CH_3
26	16.81	16.81	CH_3
27	23.22	23.22	CH_3
28	180.50	-	Quaternary C
29	16.89	16.89	CH_3
30	21.02	21.02	CH_3
OMe	52.8	52.80	CH_3

The proton decoupled ^{13}C NMR spectrum (Appendix 4, Table 1) of VOD showed well-resolved resonances of the 31 carbon atoms even though two carbons at δ 55.24 and two carbons at δ 39.05 are overlapped.

The multiplicity of each carbon atoms was determined using DEPT-135 experiment, which revealed the presence of eight methyl groups, seven methine groups, nine methylene groups, and seven quaternary carbon atoms, indicating 49 proton connected

with 31 carbon atoms. Since, IR and NMR spectrum showed the presence of ester and alcohol functionalities there should exist at least three oxygen atoms. The ^{13}C NMR spectrum of VOD displayed signals characteristic of one double bond (δ 138.15 & 125.46) and an ester carbonyl (δ 180.05). It showed the carbon attached with hetro atom (O), which is methine at δ 78.78. It also revealed 27 saturated aliphatic carbons between δ 15.31 and 55.24.

Table 2. Comparison of ^{13}C NMR of compound VOD with literature value of ursolic acid.

Position	^{13} C NMR for compound VOD	Literature value of Ursolic acid [21,22,23]
1	39.05	39.8
2	26.78	27.8
3	78.78	79.6
4	39.45	39.9
5	55.24	56.7
6	18.27	19.4
7	33.02	34.3
8	39.45	40.7
9	47.77	47.6
10	38.65	38.1
11	23.43	24.3
12	125.46	126.8
13	138.15	139.6
14	42.03	42.8
15	29.59	29.2
16	24.17	25.3
17	47.54	47.6
18	55.24	54.3
19	39.05	40.4
20	39.05	40.4
21	30.67	31.7
22	38.65	38.1
23	27.96	28.7
24	15.31	16.0
25	15.90	16.3
26	16.81	17.6
27	23.22	24.0
28	180.50	181.6
29	16.89	17.8
30 OMe	21.02 52.8	21.5 -

The comparison of the ^{1}H NMR spectra and ^{13}C NMR spectra (see Appendix 3,4,5) of compound VOD with Ursolic acid reported in the literature showed a good agreement except that the acid proton is replaced by methyl. Therefore VOD is most likely Ursolic acid methyl ester (methyl ursolate).

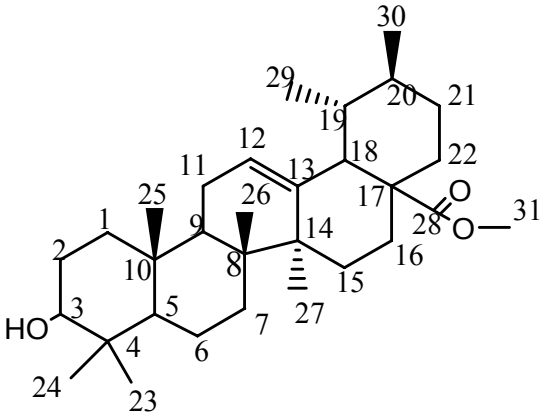


Figure 8. Structure of Ursolic acid methyl ester (VOD)

4.2 Characterization of compound VOG

Characterization of VOG was determined using spectroscopic techniques.

UVλ_{max} (log ε) MeOH: Showed absorption at 294 (0.695) showed bathochromic shift indicating the presence of n-π* transition and possibly conjugation see Appendix 5.

The **IR** spectrum (Appendix 6) showed absorption bands assigned to hydroxyl group (3358 cm^{-1}, broad), to saturated groups (2911 cm^{-1}). It also revealed the presence of C-H stretching of alkene at 3030 cm^{-1}. The carbonyl stretch for ketone indicated by 1737 cm^{-1} and sharp peak at 1693 cm^{-1} indicate an ester carbonyl. A peak at 1645.35cm^{-1} and 1443.93cm^{-1} indicate a carbon to carbon double bond stretch. Carbons to oxygen stretch shown by 1296.64cm^{-1} peak see Appendix 6.

The melting point of compound VOG, is in the range of 180-182^{0}C (with literature value of 182.5 ^{0}C).

The **^{1}H NMR** (Appendix 7) showed singlet at δ 7.45 integrated to one proton, which is olefinic proton with no hydrogen atom in the adjacent carbon atoms .A doublet at δ 5.06 showed anomeric methine proton. A doublet at δ 1.16 integrated to three protons showed methyl group attached with methine. A doublet at δ 4.49 integrated to one proton showed methine proton. Five glucose methine protons ranging from δ 3.03-3.70 with different multiplicities and two protons each overlapping at δ 3.03 and 3.16. A doublet at δ 2.46 showed methine proton. Diastereotopic methylene protons indicated by δ 1.97 (dd) and δ 2.46 (m). A proton at δ 2.42 (m) showed methine proton. A proton at δ 3.46 (d) showed more deshielded proton which is methine proton.

Table3. Proton decoupled ^{13} C & DEPT spectra of VOG.

position	^{13} C NMR	DEPT	Remark
1	100.03	100.03	CH
3	152.64	152.64	CH
4	104.35	104.35	Quaternary C
5	42.34	42.34	CH
6	214.03	214.03	Quaternary C
7	42.73	42.73	CH_2
8	28.35	28.35	CH
9	43.53	43.53	CH
10	20.71	20.71	CH_3
11	166.80	166.80	Quaternary C
OMe	51.66	51.66	CH_3
1’	96.68	96.68	CH
2’	73.53	73.53	CH
3’	77.69	77.69	CH
4’	70.67	70.67	CH
5’	77.15	77.15	CH
6’	61.80	61.80	CH_2

The proton decoupled **^{13}C NMR** spectrum (Appendix 8, Table 3) of VOG showed well resonance of the 17 carbon atoms. These include one carbonyl carbon (214.03), one ester carbonyl (166.80), one double bond (152.64 & 104.35) which is conjugated with an ester carbonyl group. It also showed six carbon atoms with chemical shift (δ 61.80, 70.67, 73.53, 77.15, 77.69, and 96.68), which are glucose carbons. It also revealed the presence of saturated aliphatic carbons between δ 20.71 and 43.53 and the carbon attached with oxygen of an ester, which is methyl at δ 51.66. A chemical shift (δ 100.03) indicates

methine, which is attached with two oxygen atoms. The multiplicity of each carbon atoms was determined using **DEPT-135** experiment (Appendix 8, Table 4), which revealed two methyl groups, two methylene groups, ten methine groups and three quaternary carbons, indicating 20 hydrogen atoms connected to carbon atoms.

Table 4. Comparison of Proton decoupled ^{13} C NMR spectra of VOG and literature value of ^{13}C NMR of verbenaloside.

Position	^{13} C NMR of VOG **δ (ppm)**	Literature value of ^{13} C NMR for verbenaloside [29]
1	100.03	99.90
3	152.64	154.30
4	104.35	104.60
5	42.34	43.70
6	214.03	215.78
7	42.73	43.80
8	28.35	29.60
9	43.53	44.90
10	20.71	19.90
11	166.80	169.00
OMe	51.66	52.90
1’	96.68	97.00
2’	73.53	73.50
3’	77.69	77.10
4’	70.67	70.40
5’	77.15	76.60
6’	61.80	61.50

The comparison of the ^{1}H NMR spectra and ^{13} C spectra (Appendix 7&8) of VOG with verbenaloside reported in the literature showed a good agreement. Therefore VOG is most likely verbenaloside. Moreover, the above prediction also supported by using its 2D NMR spectra data as follows.

Table 5. ^{1}H-^{1}H Correlation Spectroscopy (COSY) for verbenaline (VOG).

Position	δ H (ppm)	δ correlated proton
1	4.49(d, J=8Hz)	H-9, H-6',H-5, H-2'
3	7.45(s)	H-5, 3.46
5	3.46(d, J=8Hz)	H-9, 2.09
7	1.97(dd, J=2.8Hz), 2.46(m)	H-$7_{a, b}$, H-8
8	2.42(m)	H-10, H-$7_{a,b}$, H-9
9	2.09 (dt, J=2.8Hz)	H-5, H-8,H-1,H-1'
10	1.15(d, J=6.8Hz)	H-8
OMe	3.65(s)	-
1'	5.06(d, J=8Hz)	H-9, H-2'
2'	3.03(m)	H-3', H-1'
3'	3.16(m)	H-2',H-4'
4'	3.03(m)	H-3', H-5'
5'	3.16 (m)	H-6', H-4',H-3'
6'	3.42(m), 3.70(m)	H-$6_{a,b}$, H-1, H-5'

Heteronuclear single quantum correlation (**HMQC**) experiment correlates the chemical shift of proton with the chemical shift of directly bonded carbon. In the **HMQC** spectral data (Appendix 10) showed three protons at δ 1.15 (d) connected with C-10 δ 20.71,one proton at δ 1.97(dd) connected with C-7 δ 42.34, one proton at δ 3.40 (m) connected with C-6' δ 61.80, one proton at δ 2.42(m) connected with C-7 δ 42.34, one proton at δ 2.09 (dt) connected with C-9 δ 43.80, one proton at δ 2.46 (m) connected with C-8 δ 28.34, two proton overlapping at δ 3.03 (m) connected with C-2', 4' δ 73.53 & 70.67, two protons overlapping at δ 3.16 (m) connected with C-3',5' δ 77.15 & 77.68, one proton at δ 3.45 (m) connected with C-6' δ 61.80 , one proton at δ 3.70 (m) connected with C-6' δ 61.80, three proton at δ 3.65 (s) connected with OMe δ 51.68, one proton at δ 3.46 (d) connected with C-5 δ 42.73,one proton at δ 5.06 (d) connected with C-1' δ 96.68, one proton at δ 4.49 (d) connected with C-1 δ 100.03, one proton at δ 7.45 (s) connected with C-3 δ 152.64, one proton at δ 2.49 (s) overlapped with other protons shows OH proton with no carbon attachment.

Hetronuclear multiple bond correlation **(HMBC**) experiment gives information about coupling of hydrogen and carbons that are two or more bonds away. In the HMBC (Appendix 11), the methyl protons at δ 1.15 (d) (H-10) showed correlation with: δ 28.35

(C-8); δ 42.53 (C-9); δ 42.73 (C-7). The methine proton at δ 3.46 (d) (H-5) showed correlation with: δ 214.03 (C-6); δ 104.35 (C-4); δ 42.34 (C-7). δ 3.67 (s) (H-OMe); δ 104.35 (C-4); δ 166.80 (C-11); δ 152.64 (C-3). δ 1.97 (dd) displayed correlation with: δ 20.71 (C-10); δ 28.34 (C-8); δ 214.03 (C-6). δ 4.49 (d) (H-1); correlated with: δ 96.68 (C-1'). The methine proton at δ 2.09 (H-9) correlated with: δ 42.73 (C-5), δ 28.34 (C-8); δ 20.71 (C-10); δ 96.68 (C-1') and δ 214.03 (C-6). Olefinic proton at δ7.45 (H-3) showed strong correlation with: δ 42.73 (C-5); δ 96.68 (C-1'); δ 100.03 (C-1). In addition the rest correlated protons are shown in Table 6.

From COSY, HSQC and HMBC the following partial structures I, II & III are deduced and their combination give the final structure.

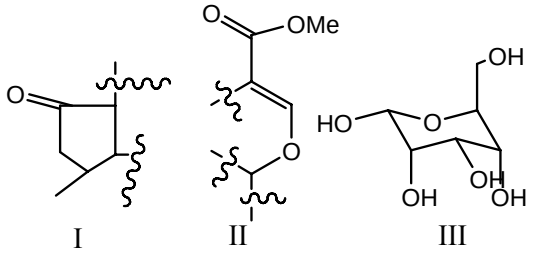


Figure 9. Partial structures of VOG based on 2D NMR

Table 6. **^{1}H NMR** and **HMBC** spectral data of VOG verbenaloside in DMSO-d_6.

H/C	δ H(ppm)	δ C (ppm)	HMBC
1	4.49(1H, d, J=8Hz)	100.03	C-1',6',9
3	7.45(1H,s)	152.64	C-6, 11,4,1',5,9
4	-	104.35	-
7	1.97(1H,dd,J=2.8Hz,2.8Hz)	42.34	C-, 10,8,6
	2.42(1H,m)	42.34	C-10, 8,6
6	-	214.03	-
5	3.46(1H,d,J=8Hz)	42.73	C-6, 11,3,4
8	2.42(1H,m)	28.34	C-10, 7,9
9	2.09(1H,dt,J=2.8Hz)	43.53	C-6,5,8,10,1'
10	1.15(3H,d,J=6.8Hz)	20.71	C-8, 7,9
11	-	166.80	
OMe	3.67(3H,s)	51.66	C-4, 11,3
1'	5.06(1H,d,J=8Hz)	96.68	C-1, ,2'
2'	3.03(1H,m)	73.53	C-1',6'
3'	3.16(1H,m)	77.68	C-2',4'
4'	3.03(1H,m)	70.67	C-3',5',6'
5'	3.16(1H,m)	77.15	C-1, 2', 4'
6'	3.42(1H,m)	61.80	C-3', 5'
	3.70(1H,m)		

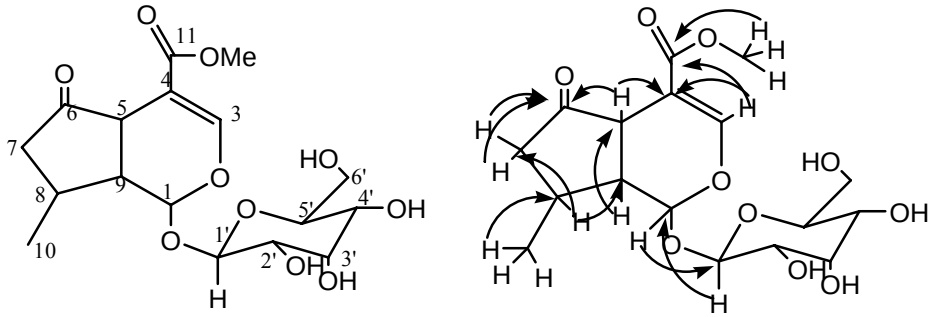


Figure 10. Major HMBC correlation in verbenaloside (VOG)

Conclusion

Quantitative determination of verbena officinalis using HPLC, and HPTLC showed the presence of ursolic acid from chloroform extract but in this project ursolic acid methyl ester has been isolated. Further, from the methanol extract iridoid glycoside, verbenaline were obtained. Any one who would like to continue his study on this plant better to do on methanol extract since these extract have more compounds. This conclusion is made upon TLC analysis of spots on the rest fractions collected.

5. EXPERIMENTAL

5.1 General

IR spectrum was obtained as pellets on Perkin-Elmer Bx infrared spectrometer in the range 4000-400 cm^{-1}.

^{1}H NMR, **^{13}C**, and **2D NMR** spectra were recorded on a Bruker advance 400 MHz spectrometer with TMS as internal standard.

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

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

The melting point determined by Electrothermal IA 9200, digital melting point apparatus.

5.2 Sample collection

The plant verbena Officinalis (called Atuch in Amhara region) was collected from Amhara region Gojjam, in the local district of Motta and identified by Prof. Sebsibie Demissew of the National herbarium, department of Biology, Addis Ababa University, and a specimen collected from the same locality is available in the National herbarium, AAU.

5.3 Extraction

The verbena officinalis was dried with air and ground using Mortar and pestle. 240 g of powdered verbena oficinalis were consecutively extracted with 450 ml petroleum ether. Then the residue dried with air and soaked with 450 ml of chloroform for 48 hr, the filtrate was concentrated with rotavapor to 20 g solid. The residue of chloroform extract after dried with air soaked with 450 ml methanol for 48 hr, and the filtrate (extract) was concentrated with rotavapour to 27 g solid.

5.4 Isolation of VOD

About 140 g of silica gel (for flash chromatography) was measured and mixed with 200 ml hexane and packed in to a column. The dried 20 g crude chloroform extract of verbena

Officinalis was subjected to column chromatography using Hexane / Chloroform and Chloroform / Ethyl acetate (90:10) as eluent. Ten 30ml fractions were collected in the chloroform / ethyl acetate; fractions 4-7 were mixed upon analysis with TLC and contained the desired compound (15 mg) after further purification with 10 g silica gel column with the same solvent Fractions collected using Hexane / Chloroform different rations were discarded because their TLC result did not show spots. The compound was labeled as VOD and it was then characterized using spectroscopic methods and identified as ursolic acid methyl ester. The compound obtained, a pale yellow powder with melting point of 220-223 ^{0}C (with literature value 230 ^{0}C [28]).

UVλ_{max} MeOH: 469 (0.12), 440 (0.12), 422 (0.27), 393 (0.30) see Appendix 1.

IR v $_{max}$ (KBr) cm^{-1}: 3436 (OH); 2928 (CH_3, CH_2 &CH), C-H stretch for saturated groups; 1690 (C=O for ester group); 1458 (C=C); 1384 (C=C); 1037 (C-O) see appendix 2.

^{1}H NMR δ (400MHz, $CDCl_3$&CD_3OD): 5.21(t, 1H,H-12), 3.14(m, 1H, H-3), 2.20(d, 1H, H-18), 2.02-1.15(m, 22H), 1.10(s, 3H,C-23Me), 0.96(s, 3H, C-27Me), 0.95(s, 3H,C-26Me), 0.93(s, 3H,C-24Me), 0.87(d, 3H,C-29), 0.83(d, 3H,C-30), 0.76(s, 3H,C-25) see Appendix 3.

^{13}C NMR (100 Hz, $CDCl_3$ + CD_3OD): δ 180.50, 138.15, 47.54, 42.03, 39.45, and 38.65 (seven quaternary carbons); δ 125.46, 78.78, 55.24, 47.77 and 39.05 (seven methine carbons); δ 39.05, 38.67,33.02, 30.67, 29.59, 26.78, 24.17, 23.43,and 18.27 (nine methylene carbons); δ 52.80, 27.96, 23.22, 21.02, 16.89, 16.81, 15.90, and 15.31 (eight methyl carbons) see appendix 4.

5.5 Isolation of VOG

120 g silica gel was measured and mixed with 200 ml chloroform and packed into a column. The dried 27 g crude methanol extract of verbena officinalis was subjected to column chromatography using Chloroform / Ethyl acetate and then Ethyl acetate /

Methanol as eluent. About 15 fractions each 50 ml were collected. Fractions eluted with Ethyl acetate/ Methanol (30:70) was found to have silvery crystal. The crystal formed were dried with air after a few weeks and further applied on a 15 g silica gel using Chloroform / Methanol as eluent, starting with 100 % Chloroform. The polarity of eluent was increased successively and 10 fractions each 25 ml were collected. Fraction 3, eluted with 30 % Methanol: 70 % Chloroform was found to show two spots with tail and impurities were further purified by 10 g silica gel column. Upon TLC (4 ml CF: 1 ml Methanol) analysis with an Rf value of 0.32 which have been sprayed with valinine in H_2SO_4 and showed gray color with heating which is characteristics for the presence of terpene. Fraction 3 was dried with air after it was concentrated using rotavapour and it was called VOG. The compound obtained was a white gray crystal with melting point of 180-182 ^{0}C (literature value of 182.5 ^{0}C [29]).

UVλ_{max} MeOH: 293 (0.695) see Appendix 5.

IR υ_{max} (KBr) cm $^{-1}$: 3358 (OH), 3030 (C-H, olefinic stretch), 2911 (saturated C-H stretch), 1737 (C=O), 1693 (C=O, for ester), 1645 (C=C), 1296 (C-O) see Appendix 6.

^{1}H NMR (400MHz, DMSO-d_6): δ 7.45 (1H, s, H-3), δ 5.03 (1H, d, H-1'), δ 4.48 (1H, d, H-1), δ 3.70 (1H, m, H-6$_\beta$), δ 3.67 (3H, s, OMe), δ 3.46 (1H,m H-5),δ 3.42 (1H, m, H-6$_\alpha$), δ 3.16 (1H, m, H-3'), δ 3.03 (2H, m, H-2'& H-4'), δ 2.46(1H, d, H-5),δ 2.42 (1H, m, H-7$_b$), 2.40 (1H, m, H-8), δ 2.09 (1H, m, H-9), δ 1.95 (1H, dd, H-7$_a$), δ 1.15 (3H, d, H-10) see Appendix 7.

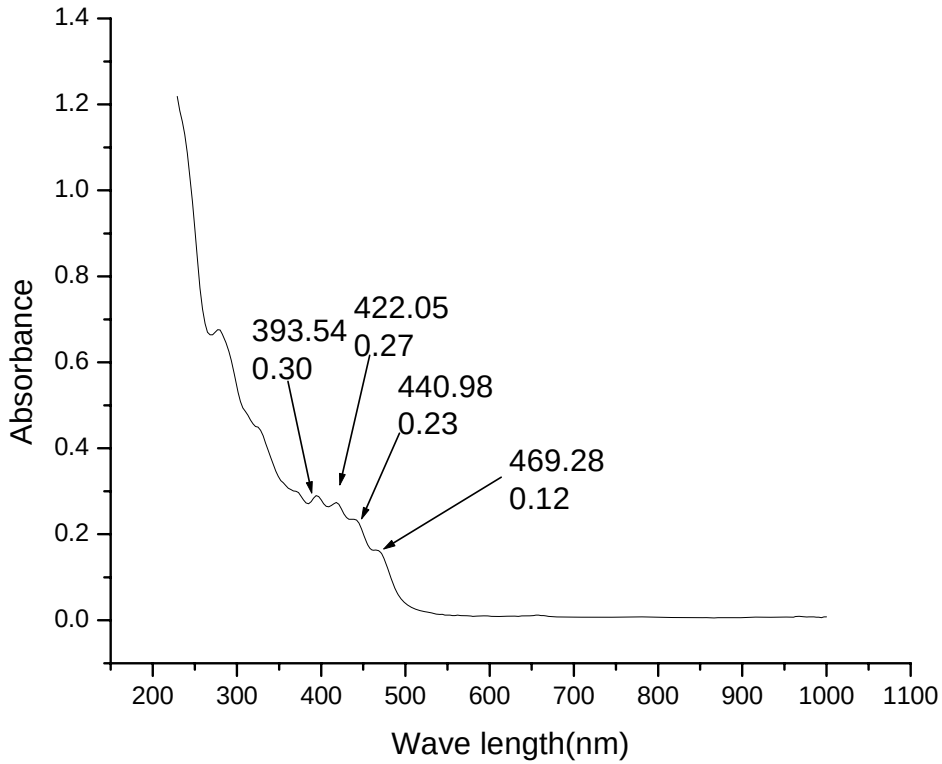
^{13}C NMR (100Hz, DMSO-d_6): δ 104.35, 214.03, and 166.80 (three quaternary carbons); δ 42.34, 61.80 (2 C, methylene); δ 20.71, 51.66 (2C, methyl); δ 100.03, 152.64, 42.73, 28.35, and 43.53 (5 C, methine aliphatic); δ 96.68, 73.53, 77.69, 70.67, 77.15, and 61.80 (5 C, glucose methine): see Appendix 8.

6. REFERENCE

1. Le Quesene, P.W. In New Trends in Natural product chemistry, proceeding of the 2nd international symposium. Pakistan-U S. Binational work shop, (eds. Atta-Ur-Rahman and Quesene, P.W.), Shamin printing press, Karachi; **1986**, p.271.
2. Hagos, M. Ph.D. Dissertation, Uppsala University, Uppsala; **1989**, P.11.
3. Harborne, J.B. *Natural Product Reports*; **1986,** p.323.
4. http://www.Liber Herbarium Verbena Officinalis Plant profile.
5. Mills. S. Y. The dictionary of modern Herbalism.
6. Thulin, M. Flora of Ethiopia. (Hedberg, I. and Edwards), National Herbarium, Addis Ababa University; **1989**, 3.
7. Duke. J. A and Ayensu. E.S- Medicinal plants of China reference publications; **1985** ISBN 0-917256-20-4.
8. Facciola. S. Cornucopia- A Source book edible plants. Kampong publications; **1990** ISBN 0-9628087.
9. Grieve. A modern Herbal. Penguin; **1984** ISBN-0-14-046-440.9
10. Bown. D. Encyclopedia of Herbs and their uses. Dorling Kindersley, London;. **1995** ISBN 0-7513-020-31.
11. Claphman, Tootin and Warburg. Flora of the British Isles. Cambridge University press; **1962**. A Very Compressive Flora, the standard reference book.
12. Chiej.R. Encyclopaedia of Medicinal Plants. Mac Donald; **1984** ISBN 0-356-10541-5 covers plant growing in Europe.
13. Huxley. A. The New RHS Dictionary of Gardening. MacMillan Press; **1992** ISBN 0-333-347494-5.
14. Biosynthesis of Natural Products. Paolo Mnitto / Ellis Horwood Ltd., Publishers; **1981**.
15. Medicinal Natural Products. A Biosynthetic Approach. Second edition, Paul M Dewick ; **2001**.
16. Yeung. Him-Che. Handbook of Chinese Herbs and formulas. Institute of Chinese medicine, Los Angeles; **1985**.
17. Santos, M.I.S. and Kaplan, M.A.C., *J. Braz. Chem. Soc;* **2001**, 12,144.
18. Mann, J., Secondary Metabolism, 2nd ed, Clarendon Press, Oxford; **1987**.

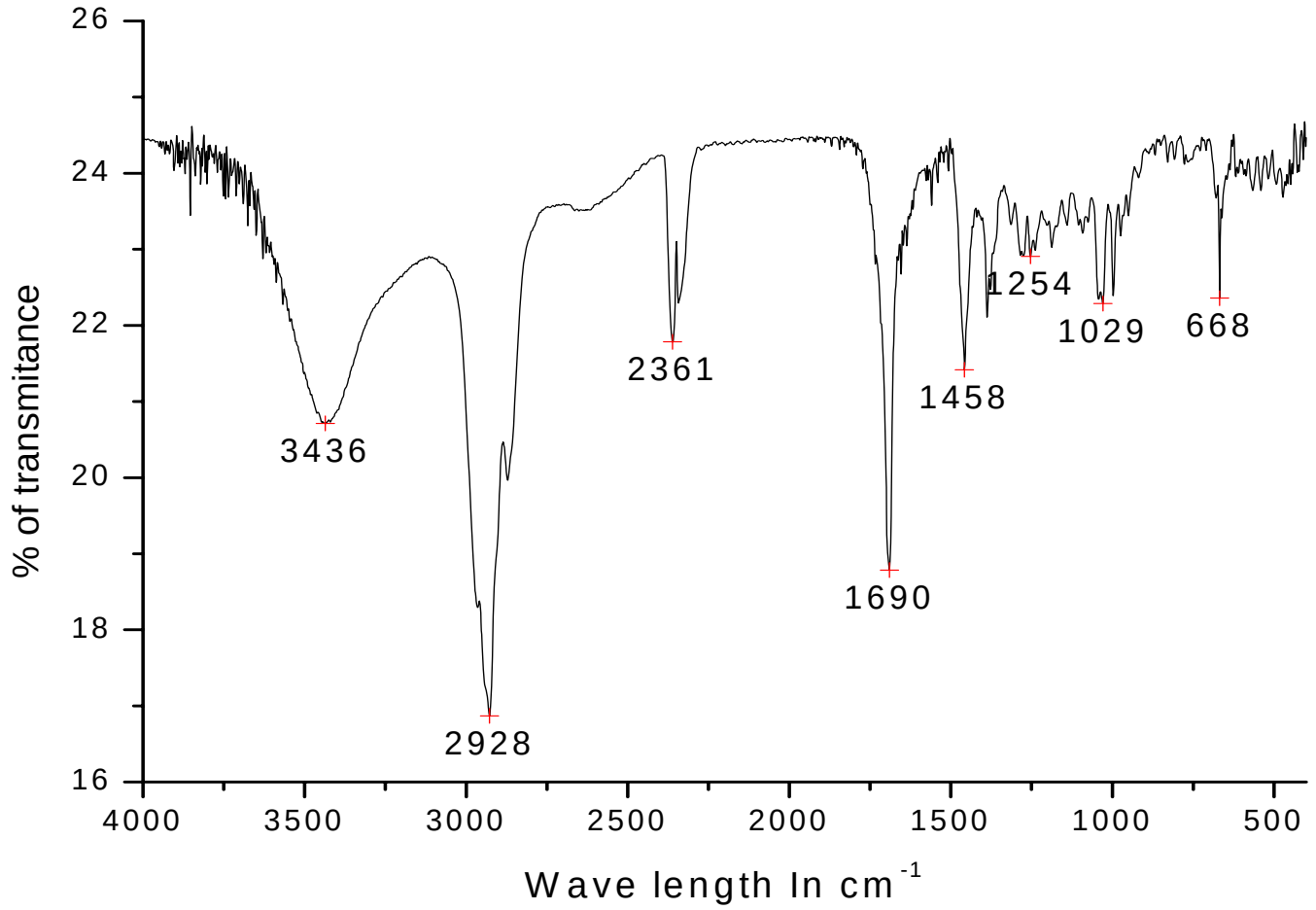
19. pure Appl.; **2003**, 75, (2), P. 369.
20. http://www.Quimica Nova-Dirhamnosyl Flavonoid and other constituents from Brillantasia Palisatii, 5/6/2006.
21. http://www.chemical constituents of Hydyotis Herbacea.
22. Mukherjee. S.K. *et al, Phytochemistry* ; **1982**, 21, (9), 2416-2417.
23. Santos, M.I.S. and Kaplan, M.A.C., *J. Braz.Chem.Soc*. ; **2001**, 12,144.
24. Jensen, S.R.,Damtoft,S. and Weiergang, I., phytochemistry; **1994**,35, 621.
25. Bailleal, F. *Phytochemistry*; **1977**, 16, 723.
26. Damtoft, S. *Phytochemistry*; 1983, 22, 695; 2614.
27. Jensen, S.R. *phytochemistry*; **1989**, 28, 97
28. Chapman and Hall, Chemical data base, Dictionary of Natural Products; **1994**, 1st ed, 5, 6027.
29. Mundikinajeddu Deepak and Sukhdev Swami Handa: *Phytochem. Anal*.; **2000,** 11. 351.

APPENDICES

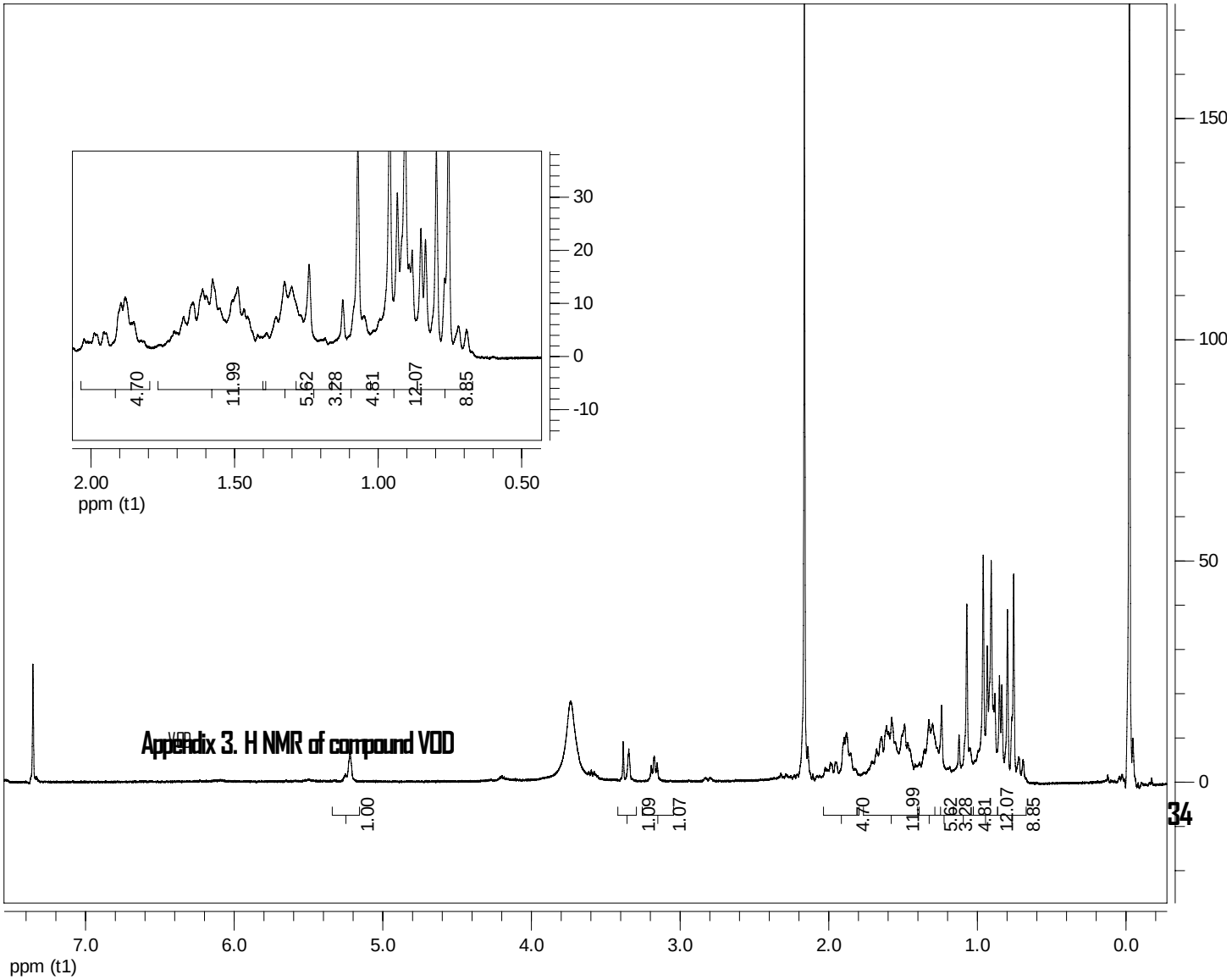


plot of wave length vs absorbance for compound VOD

Appendix1. Uv/vis spectrum of compound VOD.

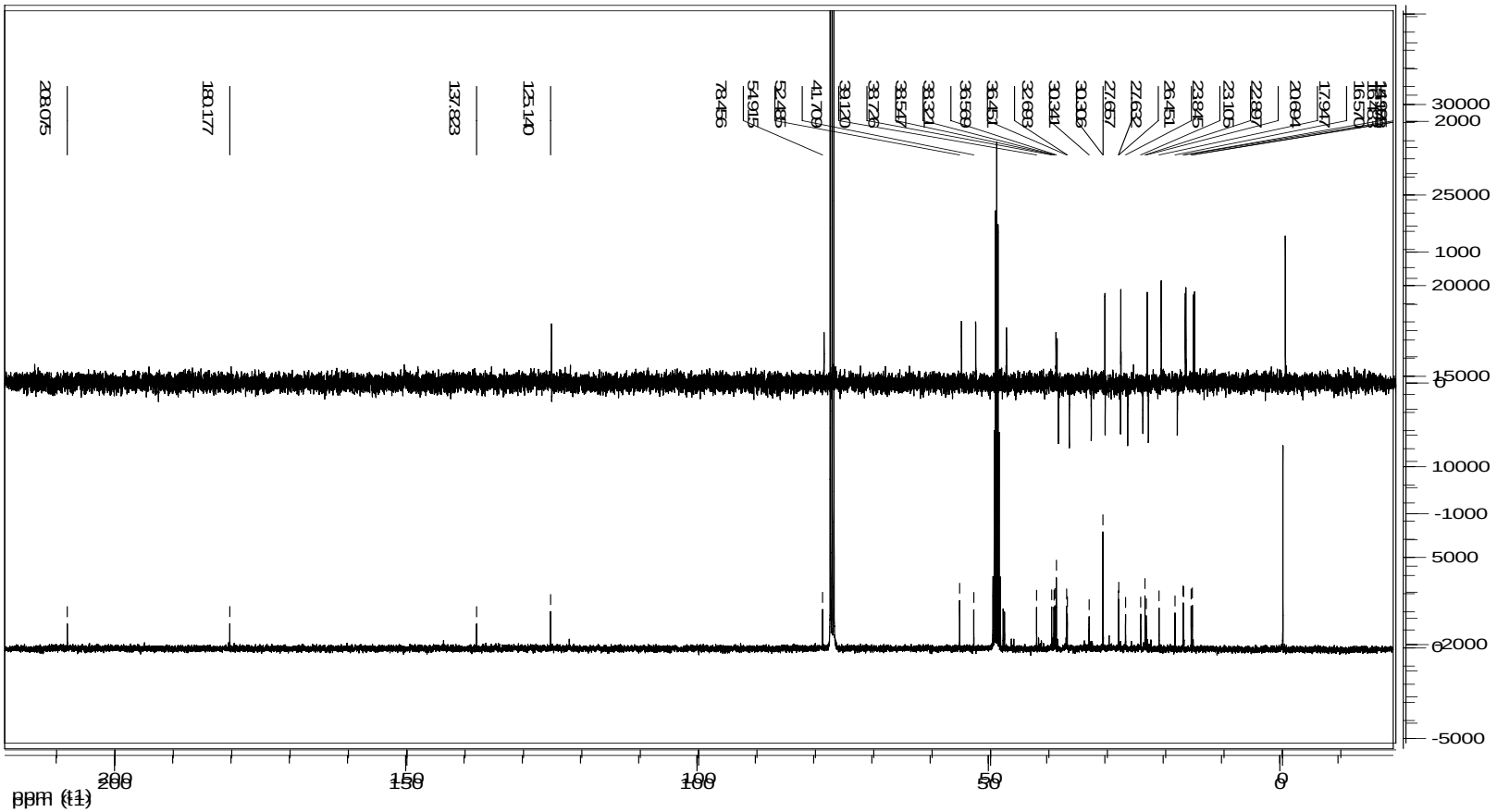


Appendix2. IR Spectrum of Compound VOD.

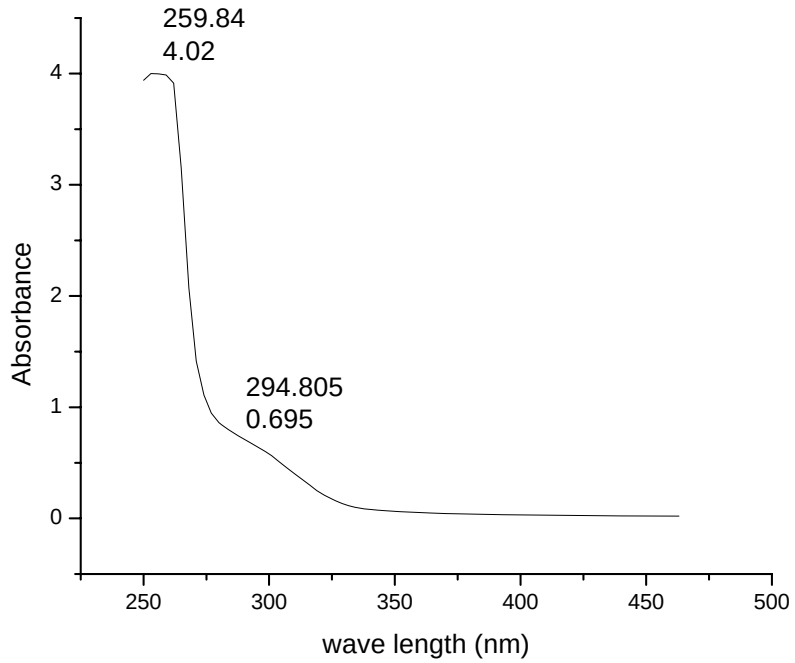


Appendix 3. H NMR of compound VOD

34

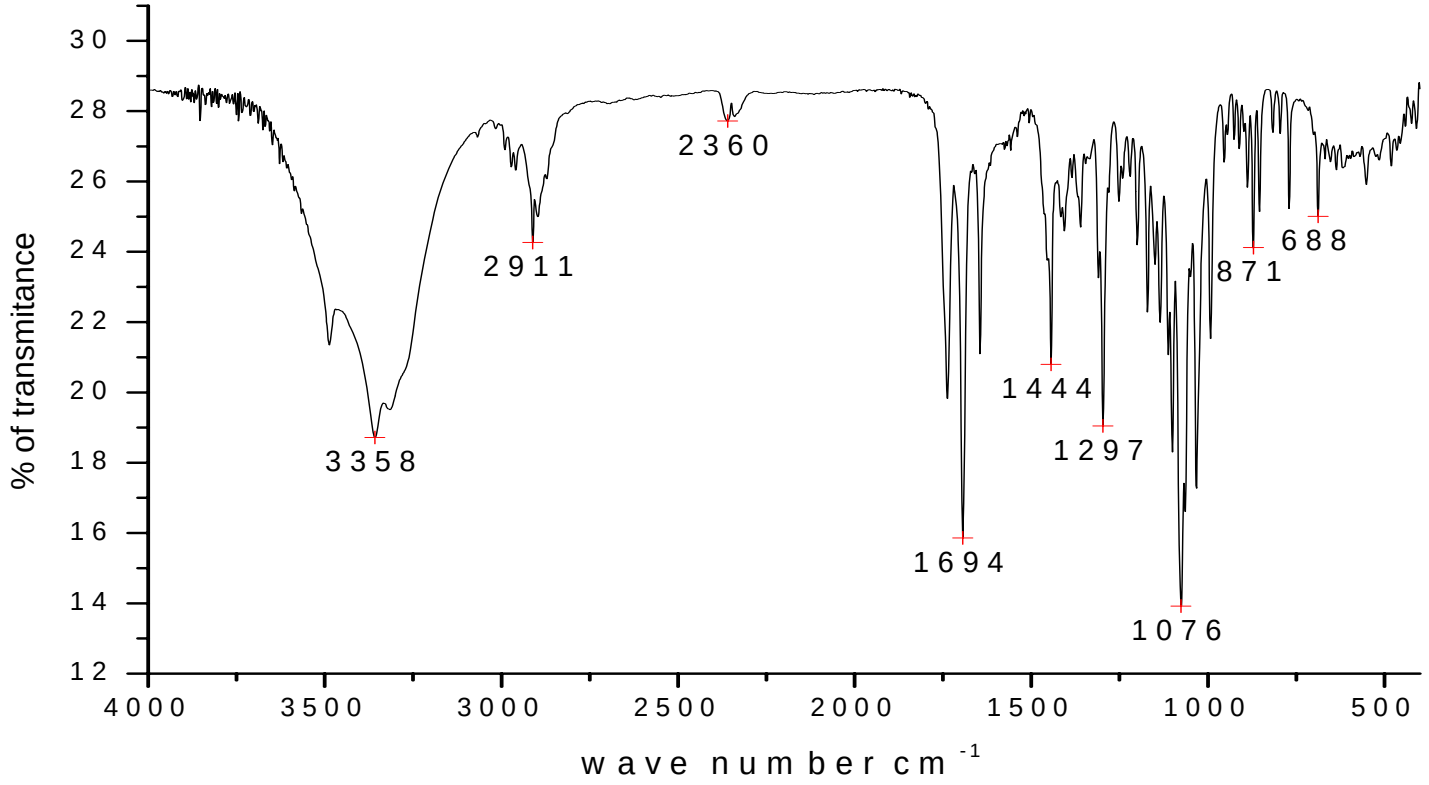


Appendix4. ^{13}C NMR & DEPT spectrum for compound VOD.

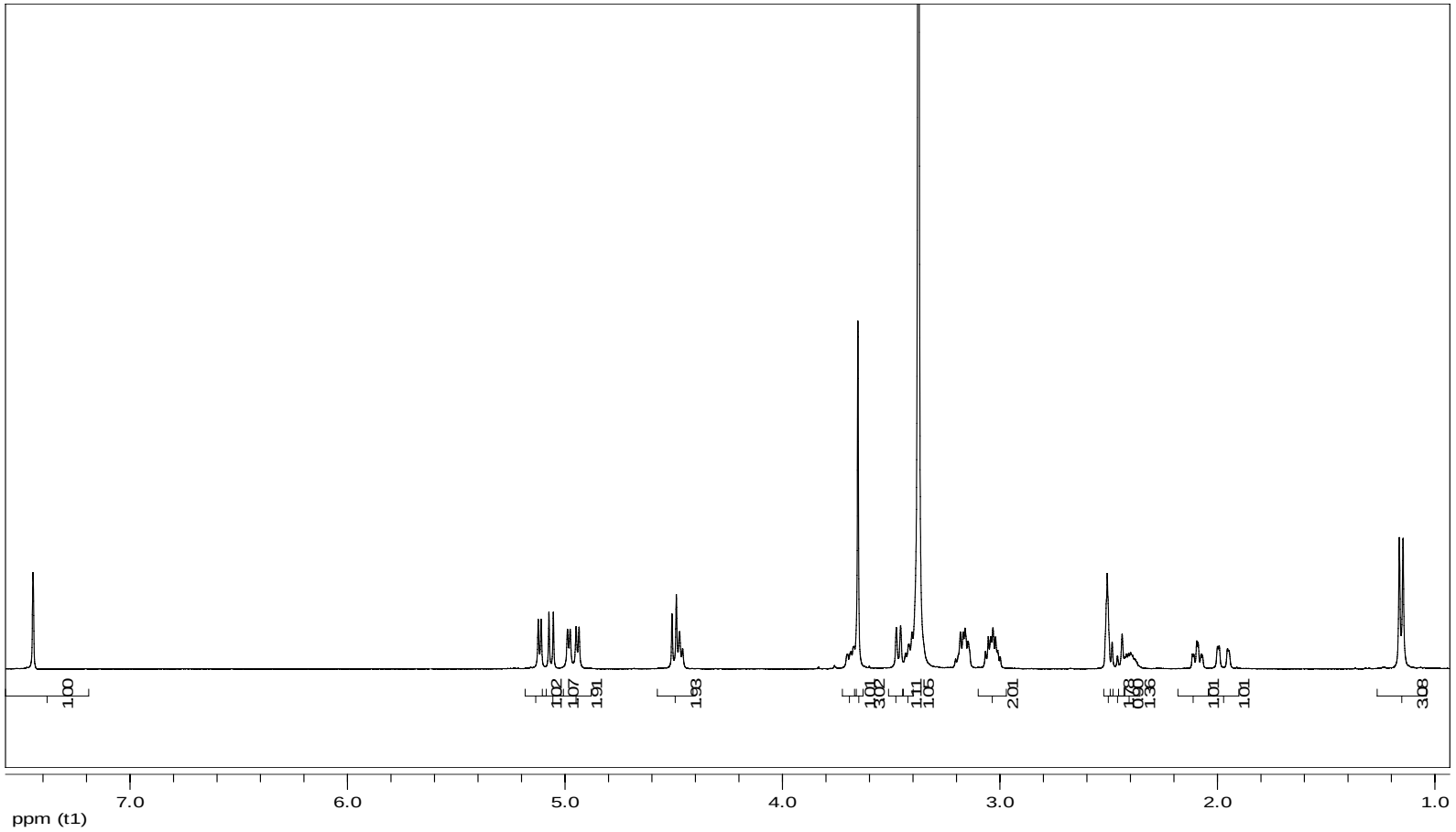


plot of Absorbance vs wavelength for compound VOG

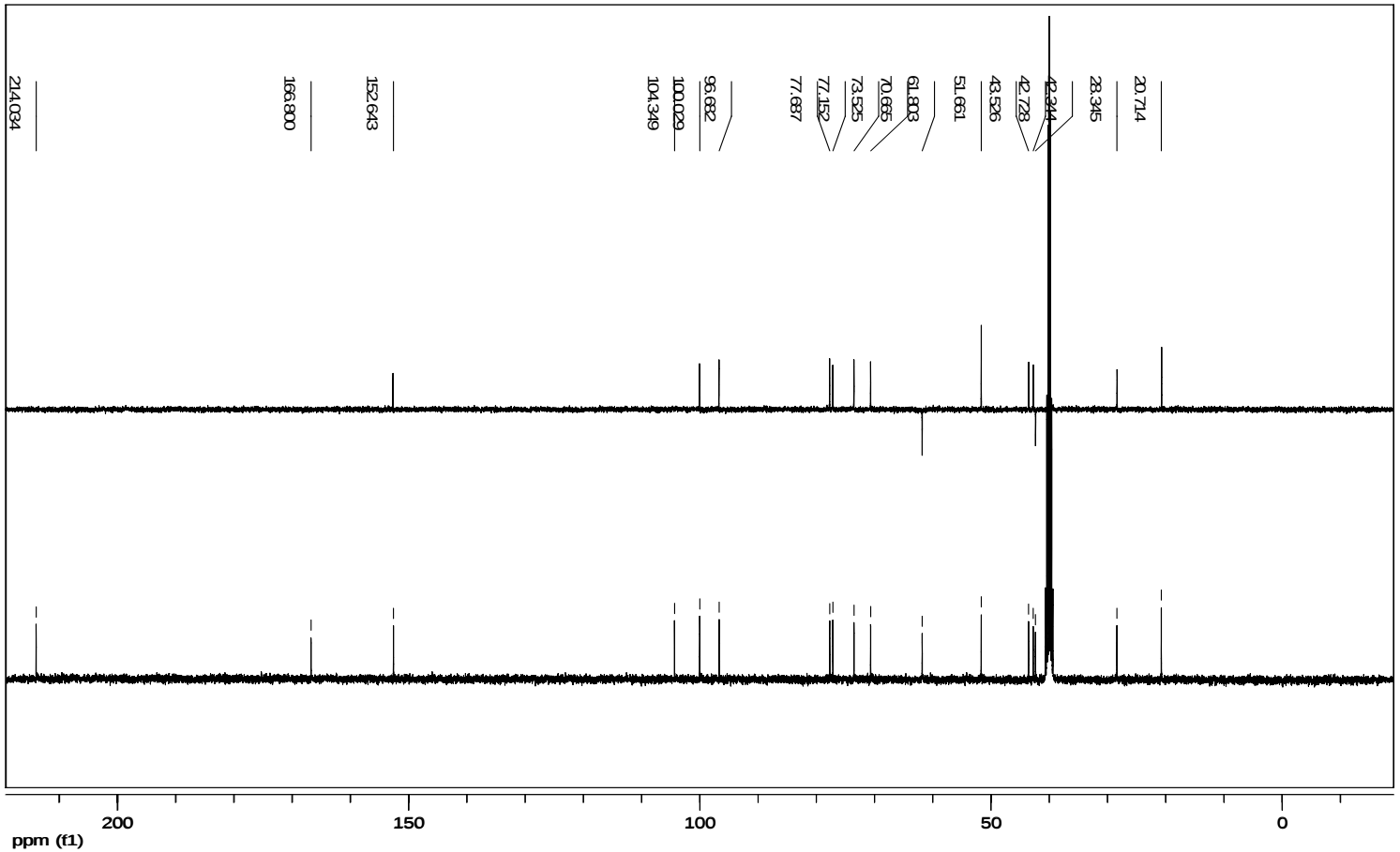
Appendix 5. UV/Vis spectrum of VOG.



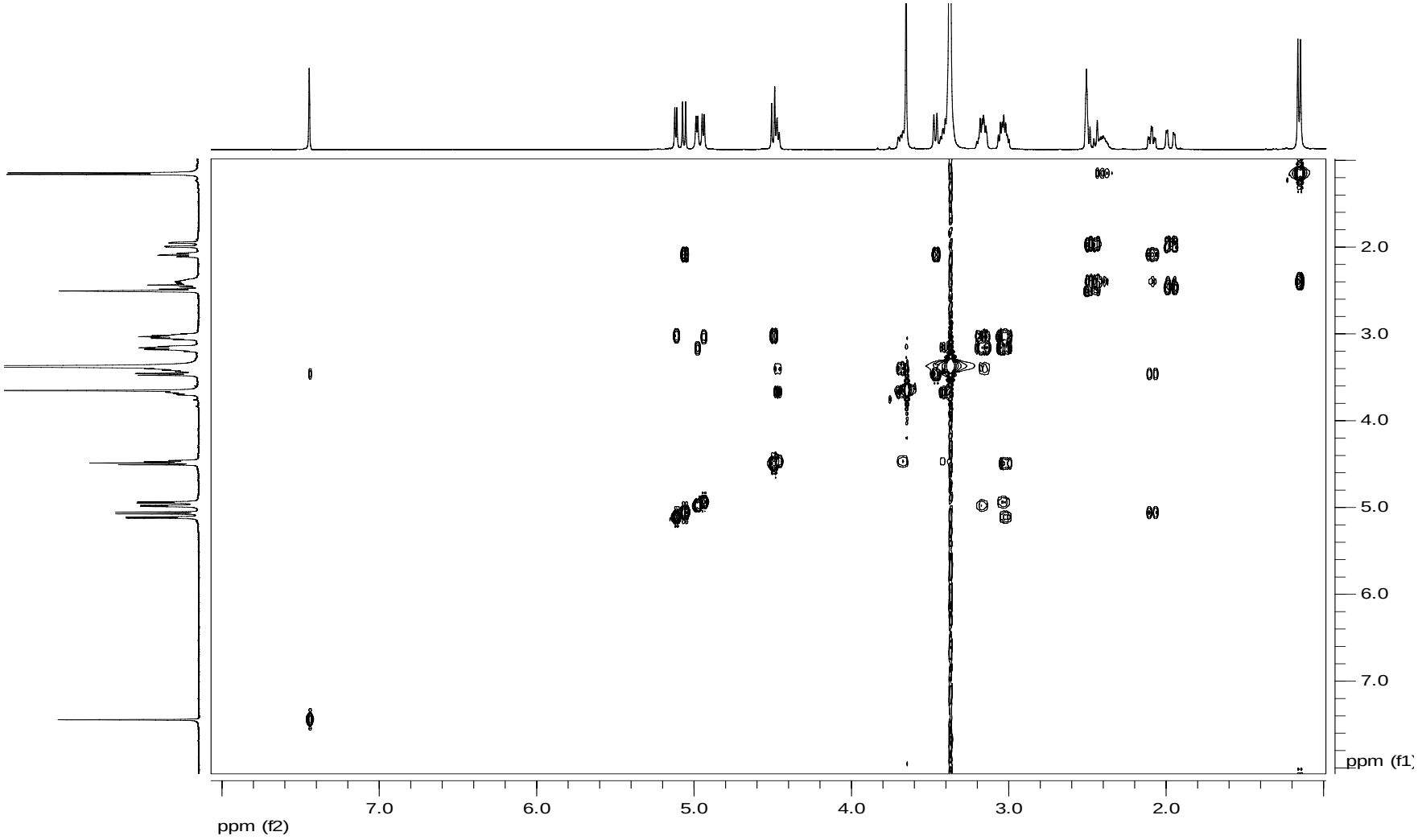
Appendix 6. IR Spectrum of compound VOG.



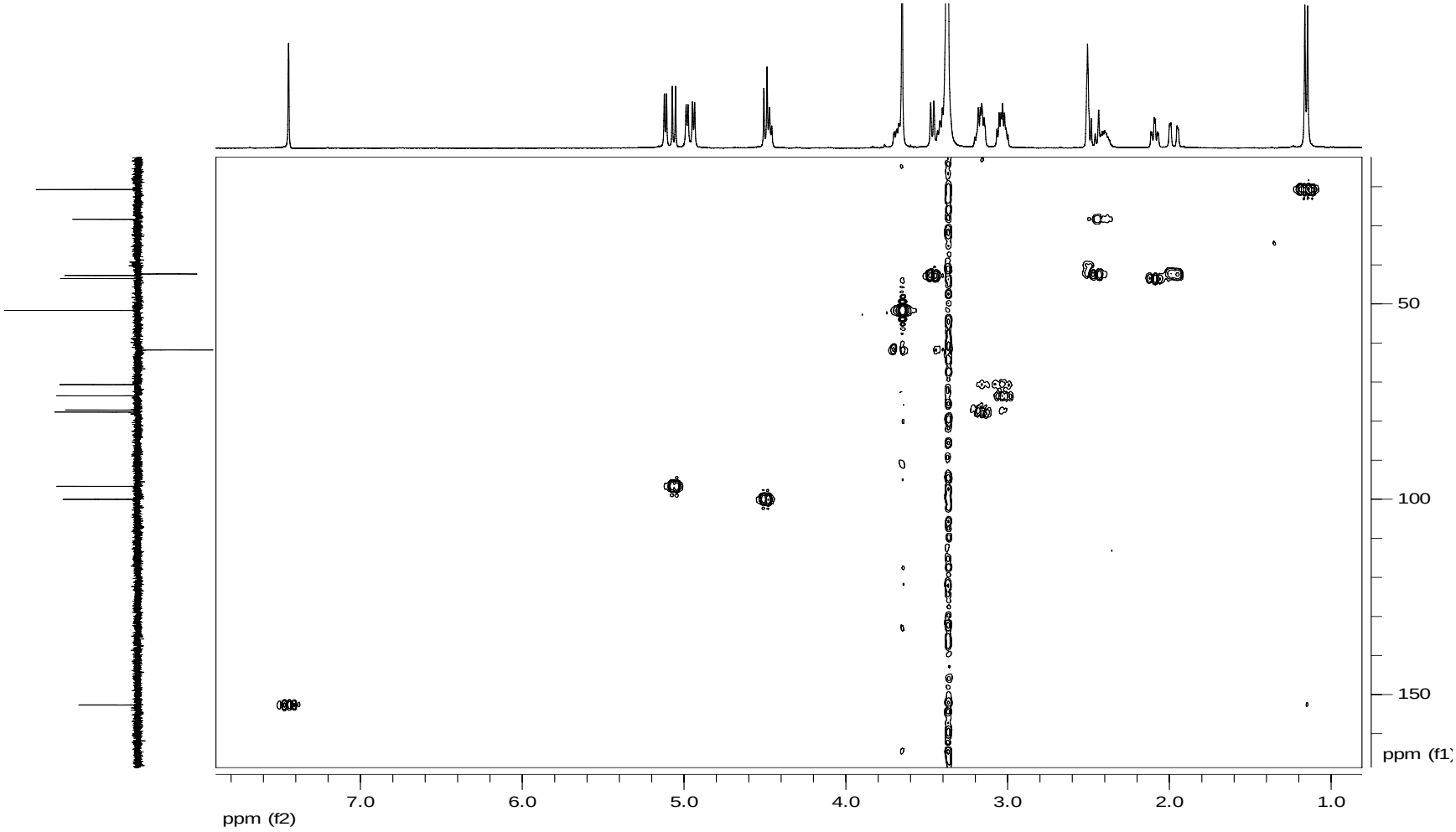
Appendix 7. ^{1}H NMR of compound VOG.



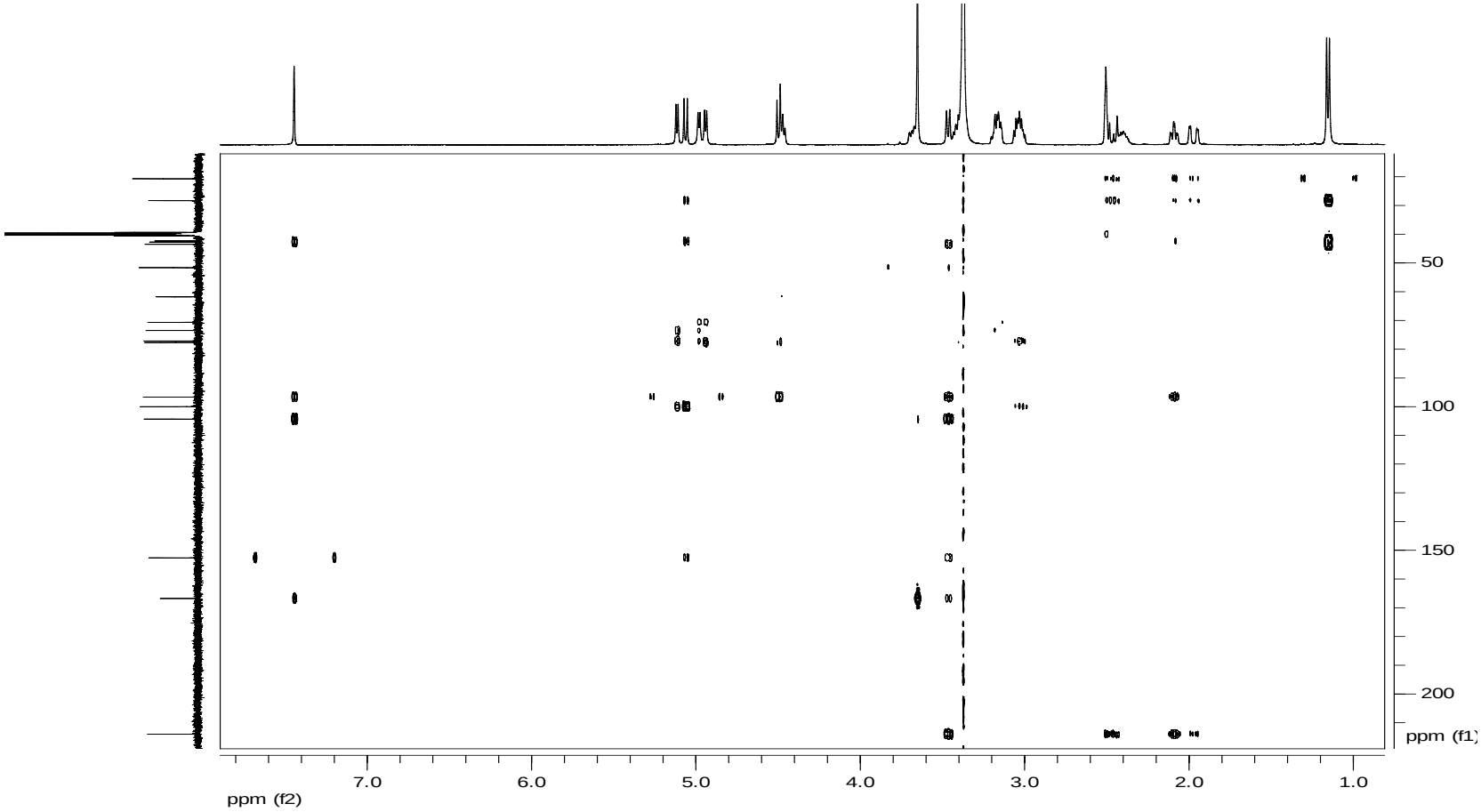
Appendix 8. ^{13}C NMR & DEPT Spectrum of compound VOG.



Appendix 9. H-H COSY spectrum of compound VOG.



Appendix 10. HMQC spectrum of compound VOG.



Appendix 11. HMBC spectrum of compound VOG.