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Original Research

Phytochemical Screening and Characterization of Olean-18-ene Type Triterpeniod from the Roots of *Lantana camara*

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Abstract

Lantana camara, commonly called wild or red sage or sleeper weed is the most widely spread species of the genus *Lantana* traditionally used to treat various diseases such as diarrhea, fever, and stomachache. It has also been reported to show antifungal, antiproliferative, insecticidal, antimicrobial and germicidal activity. Phytochemical screening of the methanol extract revealed the presence of flavanoids, terpenes, saponins and the absence of anthraquinones and alkaloids. Silica gel column chromatographic separation of the methanol root extract yielded an olean-18-ene type triterpene. Complete characterization of the isolated compound was done using spectroscopic techniques (UV-Vis, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-135, COSY, HMBC and HSQC).

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INTRODUCTION

Lantana camara, commonly called wild sage or sleeper weed is the most widely spread species of the genus *Lantana.* The root system is very strong with a main taproot and a mat of many shallow side roots (Sankaran, 2007; Ross, 1999) It is one of the traditional medicinal plants used in Ethiopia and other part of the world for treatment of various diseases such as diarrhea, fever (Fisseha *et al.*, 2009) and stomachache (Nayak *et al*., 2008), The plant also showed antifungal (Tripathi and Shukla, 2002 and Kumar *et al*., 2006), antiproliferative (Nagao *et al*., 2002), insecticidal, antimicrobial activities (Rajakaruna *et al*., 2002) and germicidal activities (Ghisalberti, 2005). The powdered root in milk and tea prepared from the leaves and flowers, locally called ''Yewef Kolo'' (Amharic), protects against fever and cold (Mesfin *et al*., 2009). Earlier phytochemical screening works on the leaf, flower and aerial parts of *L. camara* revealed the presence of terpenoids, alkaloids, steroids (Ganjewala *et al*., 2009; Singh *et al*., 1991; Singh *et al.*, 1990), Lantalonic acid, lantic acid, lupeol derivatives, camarinic acid, camaric acid, camarilic acid and related series of pentacyclic triterpenoids (John *et al.,* 1983a; John *et al*., 1983b). In East Africa, the genus *Lantana* is known to harbour tsetse fly (Glossina spp.), the vector of African sleeping sickness (Leak, 1999). Despite the wide use of the genus traditionally, there are no phytochemical studies reported on *L. camara* from Ethiopian flora. We hereby present a comprehensive phytochemial analysis of the methanol root extract of *L. camara* with a complete NMR characterization of one oleanene-18-type triterpene.

MATERIALS AND METHODS

Instrumentation

Melting point was recorded by Mettler Toledo apparatus, Type FP62, and it was uncorrected. Column chromatographic isolation was carried out on silica gel (230-400 mesh size, Merck). Thin layer chromatography was done on silica gel 60 F-254, 0.2 mm thick layer on aluminum sheets for detection of spots. The UV-Vis spectrum was recorded on UNICAM UV-300 double beam spectrophotometer using $CHCl_3$ as internal standard. The IR absorption spectrum was determined by Shimadzu 440 instrument using KBr disk in the range of 500-4000cm^{-1}. The ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker Avance 400MHz spectrometer using TMS as internal standard. Chemical shift values for all NMR data are reported in parts per million (ppm) relative to internal standard. All the chemicals used in this study were analytical grade.

Plant Material Collection and Preparation

The roots of *L. camara* were collected from Wonago, Ethiopia. The plant material was identified by Dr. Ensermu Kelbessa, Department of Biology, Addis Ababa University (AAU) and a voucher specimen was deposited at National Herbarium of Ethiopia, Addis Ababa University, Ethiopia. The collected root part of *L. camara* was dried and grinded in to powder using mortar and was made ready for further analysis.

Preparation of Crude Methanol Extract

The grounded (0.3Kg) root of *L. camara* was soaked in 650mL of *n*-hexane for 24x2hr with occasional shaking and filtering to remove the fat contents. After filtration, the marc left was further soaked in 650 mL of methanol (98.6 %) for (24x3hr). The methanol extract was then filtered and concentrated using Rotary Evaporator at 40^{o}C, air dried and weighted to yield 30.6g (14.3%) brownish crude extract.

Isolation and Purification of Compounds

21g of the brownish methanol extract was subjected to silica gel column chromatographic separation (150g silica gel) and eluted with increasing gradient of ethyl acetate in petroleum ether. A total of 41 fractions (each 30mL) were collected. The constituent profile of each fraction was monitored by TLC (40% ethyl acetate in petroleum ether) and visualized under UV-Vis light (λ_{max} 254 and 366nm). Fractions 17-22 were combined based on their TLC profile and was further purified by column chromatography (eluent: increasing gradient of ethyl acetate in petroleum ether). A total of 11 fractions were collected. Fractions 8-11 were combined and a pale yellow precipitate was obtained. This precipitate was further washed with *n*-hexane till it gives clear spot on TLC (M.pt: 263. 1 ^{o}C, 54.8 mg).

Preliminary phytochemical Screening

Preliminary screening tests were performed for detection of common secondary metabolites in the methanol extract of roots of *L.camara* using different reagents and analytical procedures.

Test for alkaloids

300mg of the crude extract was mixed with 2mL of concentrated hydrochloric acid. The mixture was then filtered and mixed with small amount of amyl alcohol at room temperature [Ganjewala *et al*, 2009]. The mixture was kept for observation of the color resulted from the alcoholic layer.

Test for Flavonoids

0.2g of methanol extract was dissolved in small amount of dilute NaOH and concentrated HCl (3mL) was added [Farnsworth, 1996]. A yellow solution that turns to colorless was inspected.

Test for Terpenes

0.25g of methanol extract was mixed with 2mL of $CHCl_3$ and 30mL of concentrated H_2SO_4 was added carefully to form a layer [Debjyoti, 1995]. Reddish-brown coloration of the interface was inspected.

Test for Tannins

Small quantity of the methanol extract was mixed with water and heated on water bath. The mixture was filtered and small amount of solid $FeCl_3$ was added to the filtrate [Sofowora, 1982]. Dark-green solution was inspected.

Test for Anthraquinones

0.5g of the methanol extract was boiled with concentrated hydrochloric acid for few minutes in water bath and filtered. The filtrate was allowed to cool and equal volume of $CHCl_3$ was added to it. Few drops of ammonia were added to the mixture and heated in water bath. Formation of rose-pink color was inspected [Sofowora, 1982].

Test for Saponins

0.2g of the methanol extract was shaken with 5mL of distilled water for 30 minutes and then heated to boil. Appearance of creaming miss of small bubbles (frothing) was inspected [Farnsworth, 1996].

RESULTS AND DISCUSSIONS

Preliminary Phytochemical Screening

Preliminary phytochemical screening of the methanol extract revealed the presence of terpenoids, flavonoids and saponins whereas alkaloids, tannins and anthraquinones were absent (Table 1).

Table 1: Summary of phytochemical screening tests of the methanol extract

Plant constituent	Present/absent
Alkaloids	-
Flavonoids	+
Terpenes	+
Tannins	-
Saponins	+
Anthraquinones	-

(-) Absence of plant constituent, (+) presence of plant constituent

Spectroscopic Analysis

A broad IR absorption band at 3381.95cm^{-1} indicates the existence of hydroxyl group. The absorption band at 1690cm^{-1} is attributed to carbonyl group of an ester moiety. The absorption bands observed at 1450cm^{-1} and 1270.04cm^{-1} are attributed to an olefinic system and C-O stretching vibration of an ester moiety, respectively. Furthermore, the IR absorptions at 2922.92cm^{-1} and 3010cm^{-1} suggest sp^3 C-H stretching and sp^2 C-H stretching vibrations, respectively. The UV spectrum showed characteristic absorption bands for ester carbonyl moiety and alkene double bond at λ_{max} 263.24 and 210.14, respectively.

The ^{1}H NMR spectrum revealed the existence of olefinic proton at δ5.30 (1H, *s*, H-19). This peak, being singlet, indicated the absence of proton(s) on the adjacent carbon atom(s) suggesting the presence of adjacent sp^2 quaternary carbon. A broad singlet proton at δ3.90 indicate hydroxyl group and the presence peak at δ3.20 (1H, *dd*, H-3) revealed the existence of a methine proton bearing a hydroxyl group. The ^{1}H NMR spectrum showed signals due to seven methyl protons; δ0.65, 0.70, 0.80, 0.95, 1.00, 1.05, 1.10 and 1.25 each being singlet and integrated for three hydrogens. Chemical shift values in between δ1.4-2.30 with multiplet multiplicity showed the existence of methylene protons. The presence of a triplet at δ1.75 (3H, *t*) suggest existence of methyl protons with adjacent methylene protons on the adjacent carbon atom (H-2',3', Table 2). The ^{13}C NMR spectrum revealed a total of 35 well resolved carbon peaks, of which one of them at δ79.1 (C-3) overlapped with the $CDCl_3$ peaks. The presence of ten methyl groups, five methine groups (of which one of them is olefinic (C-19), eleven methylene groups and nine quaternary carbon atoms are all evident from the ^{13}C NMR spectrum. A pair of signals at δ122.5 (C-19) and δ143.6 (C-18) belongs to olefinic carbons. Furthermore, the ^{13}C NMR supported the existence of ester carbonyl carbon at δ183.8 (C-28) and hydroxyl bearing methane carbon at δ79.0 (C-3) (Table 2).

The COSY spectrum supported correlations between $H_1 \leftrightarrow H_2$, $H_5 \leftrightarrow H_6$, $H_9 \leftrightarrow H_{11}$ and $H_{12} \leftrightarrow H_{13}$ (Table 2). The HSQC spectrum suggested significant direct connectivity

between C-5 and H-5, C-9 and H-9, C-13 and H-13, and C-27 and H-27 (Table 2). The gHMBC spectrum showed correlations between H-24 and C-5, correlations between H-6 and carbon C-5, and a long range correlation between H-6 to a quaternary carbon resonating at δ38.74 (C-8). The position of the double bond at C-18,19 was evident from the HMBC correlations of olefinic proton at δ5.30 with carbons at δ45.87 (C-13), δ143.59 (C-18) and 47.45 (C-17). The linkage of the acyl group a quaternary carbon at C-17 was further supported by the HMBC correlation between methylene protons at δ1.80 (H-16) and ester carbonyl carbon at δ183.79 (C-28). The HMBC spectrum also indicated correlations of the proton δ1.55 (H-22) with quaternary carbon at δ30.67 (C-20); methylene at δ1.45 (H-1) with carbon at δ79.04 (C-3); and methine proton at δ2.00 (H-13) with methyl carbon at δ 25.94 (C-27) (Table 2, Figure 1).

Based on the above spectral data, a triterpenoid skeleton is clearly evident having a hydroxyl group, an acyl moiety and olefinic protons constituting a total of 35 carbons. In agreement with the literature data, the spectral data of the compound is in good correlation with compounds having olean-18-ene class of triterpenoids bearing hydroxyl at C-3 and olefinic group at C-18,19. In support of this, signals from ^{13}C NMR observed at δ122.50 and δ143.59 due to olefinic carbons, at δ183.79 due to ester moiety and δ 79.04 due to an oxygenated methine carbon, combined with signals observed in the ^{1}H NMR spectrum all justify a typical structural feature of pentacyclic triterpene of oleanane type. Thus, based on the observed spectral data (Table 2) and extensive comparison with literature, the following oleane-18-ene triterpene was proposed (Figure 1). This class of triterpenes have also been a focus by several researchers and proved to have cytotoxic activity [Rios *et al*, 2001], antitumor activity [Chiang *et al*., 2003] and a range of biological and pharmacological activities as reviewed by Hua *et al.* (2006).

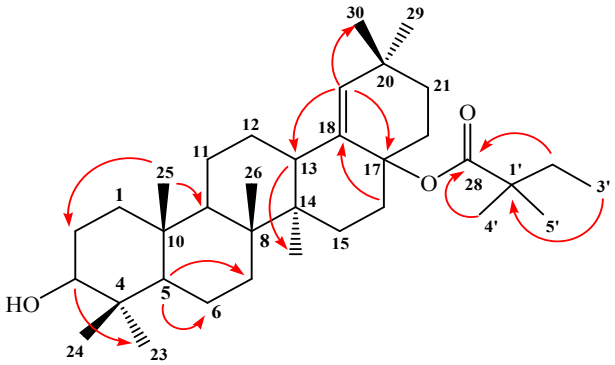

Figure 1: Proposed structure with **s**ignificant HMBC correlations

Table 2: ^{1}H, ^{13}C NMR, DEPT-135, COSY, HSQC and HMBC chemical shift data ($CDCl_3$, 400MHz)

C No.	δ_C (^{13}C-NMR)	δ_C (DEPT-35)	δ_H	COSY	HMBC	HSQC
1	38.4	38.4	1.45 (2H, *m*)	$H_1 \leftrightarrow H_2$	$H_1 \rightarrow C_3$	-
2	27.7	27.7	1.60 (2H, *m*)	-	-	-
3	79.0	79.0	3.20 (1H, *dd*)	-	-	$H_3 \rightarrow C_3$
4	39.3	-	-	-		-
5	55.2	55.2	0.87 (1H, *m*)*	$H_5 \leftrightarrow H_{6,24}$	$H_5 \rightarrow C_4$	$H_5 \rightarrow C_5$
6	21.5	21.5	1.20 (2H, *m*)	-	$H_6 \rightarrow C_5$	-
7	32.6	32.6	2.30 (2H, *m*)*	-		-
8	38.7	-	-	-	-	-
9	46.5	46.5	1.54 (1H, *m*)*	$H_9 \leftrightarrow H_{11}$	$H_9 \rightarrow C_1$	$H_9 \rightarrow C_9$
10	37.1	-	-	-	-	-
11	22.9	22.9	1.65 (2H, *m*)*	-		-
12	41.6	41.6	1.56 (2H, *m*)*	$H_{12} \leftrightarrow H_{13}$		-
13	45.9	45.9	2.00 (1H, *d*)	-		$H_{13} \rightarrow C_{13}$
14	42.1	-	-	-	-	-
15	27.1	27.1	2.15 (2H, *t*)*	-		-
16	18.3	18.3	1.80 (2H, *dt*)*	-		-
17	76.7	-	-	-	-	-
18	143.6	-	-	-	-	-
19	122.5	122.6	5.30 (1H, *s*)	-	$H_{19} \rightarrow C_{13,17}$	$H_{19} \rightarrow C_{19}$
20	30.7	-	-	-	-	-
21	32.4	32.4	1.46 (2H, *m*)*	-		-
22	33.8	33.8	1.55 (2H, *m*)*	-	$H_{22} \rightarrow C_{20}$	-
23	15.3	15.3	1.05 (3H, *s*)	-		
24	16.9	16.91	0.95 (3H, *s*)	-		-
25	28.1	28.1	0.85 (3H, *s*)	-		$H_{25} \rightarrow C_{25}$
26	17.1	17.1	0.65 (3H, *s*)	-		-
27	25.9	25.9	1.15 (3H, *s*)	-		$H_{27} \rightarrow C_{27}$
28	183.8	-	-	-	-	-
29	33.1	33.1	1.10 (3H, *s*)	-	$H_{29} \rightarrow C_{20}$	-
30	33.1	23.1	2.80 (3H, *s*)	-	$H_{30} \rightarrow C_{19}$	-
1'	47.6	-	-	-	-	-
2'	36.1	36.1	2.10 (2H, *s*)	$H_{2'} \leftrightarrow H_{3'}$	$H_{2'} \rightarrow C_{1',3'}$	-
3'	15.5	15.5	1.75 (3H, *t*)	-	$H_{3'} \rightarrow C_{2'}$	-
4'	23.4	23.4	0.79 (3H, *s*)	-	$H_{4'} \rightarrow C_{2'}$	-
5'	23.4	23.4	1.00 (3H, *s*)	-	$H_{5'} \rightarrow C_{2',1'}$	-

*assigned by HMBC and HSQC spectra, *s* = singlet, *d* = doublet. *t* = triplet, *m*= multiplate

CONCLUSIONS

The present work conducted on the methanol root extract of *Lantana camara* revealed the presence of terpenes, flavonoids, saponins and absence of anthraquinone, alkaloids and tannins. The traditional use of the plant may be attributed to its high content of terpenes, flavonoids and saponins. Despite the wide medicinal use of the genus *Lantana* in both Ethiopia and other parts of the world, to the best of our knowledge, there is no earlier phytochemical analysis work done on *L. camara* from Ethiopian flora. Thus, this work is expected to fill this gap. Apart from the phytochemical screening of the methanol root extract, isolation and NMR characterization of one olean-18-ene type triterpene was successfully achieved. As this work is one of the few attempts to phytochemically analyze the polar extracts of the plant, further work is recommended on polar fractions and extracts of the root and leaf of the plant so as to identify more novel and bioactive compounds in support of its traditional use.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgement

We are grateful to Dr. Ensermu Kelbessa and staff members of the National Herbarium of Ethiopia, Addis Ababa University, for identification of the plant material. Prof. Ermias Dagne, Department of Chemistry, Addis Ababa University is duly acknowledged for allowing us to use 400MHz NMR spectrometer. We also extend special thanks to workers of Ethiopian Pharmaceuticals Manufacturing Factory (EPHARM) for access to IR spectrophotometer.

Supplementary Data

Online version of supplementary data associated with this article can be found along with the article for further reference.

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Original Research

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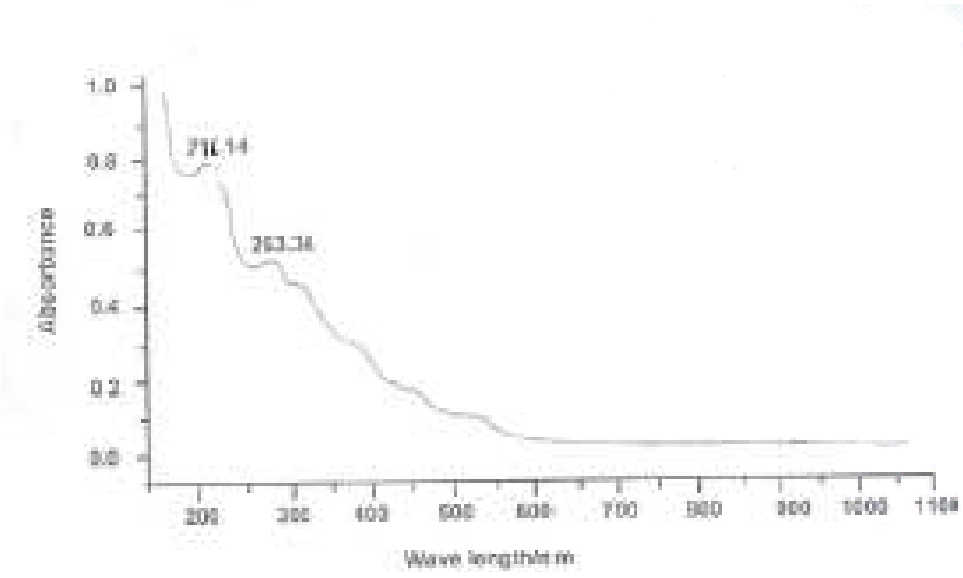
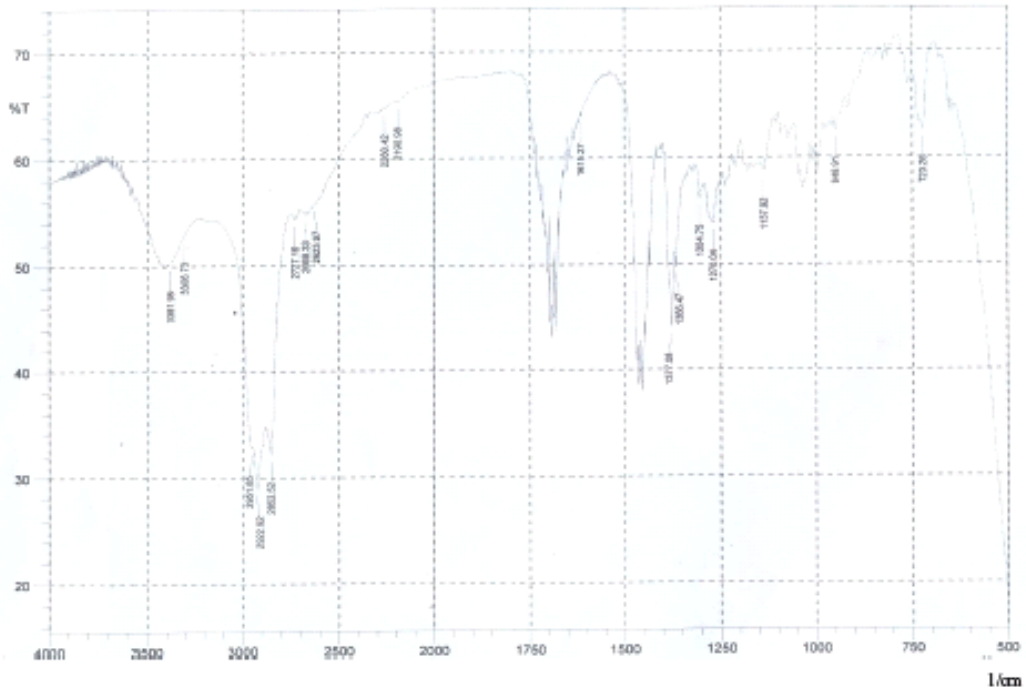
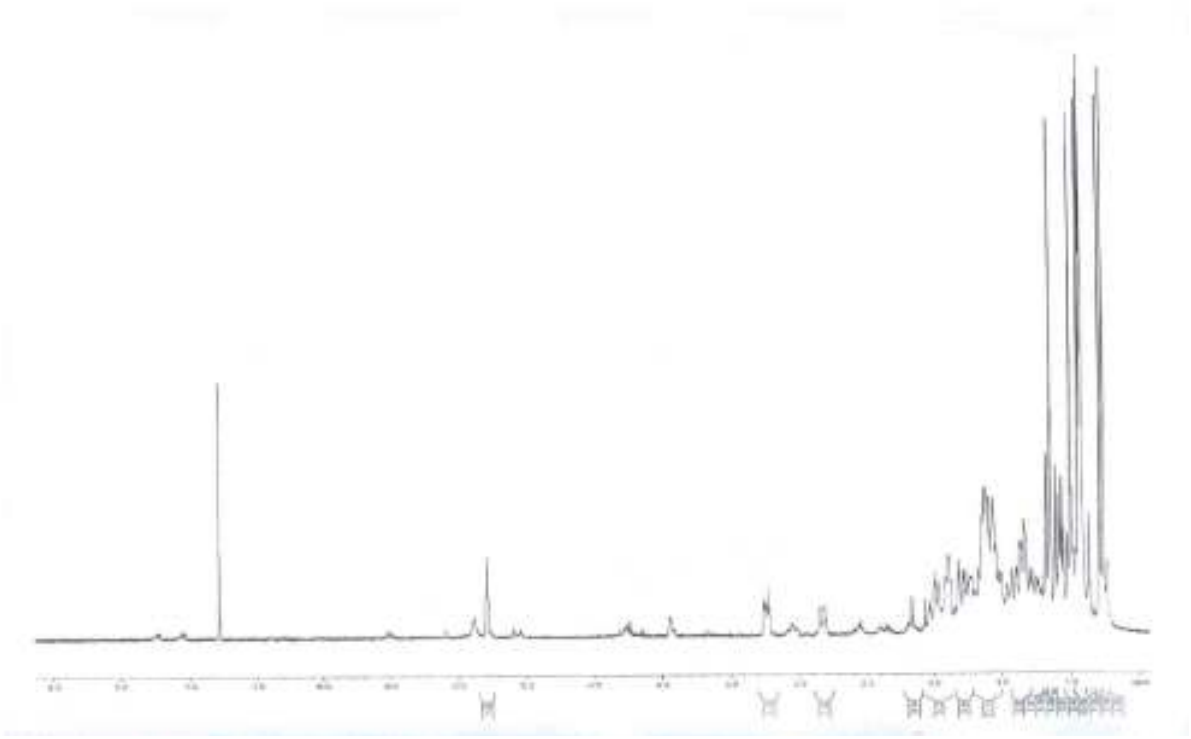
Appendix 1: UV-Vis spectrum of compound 1



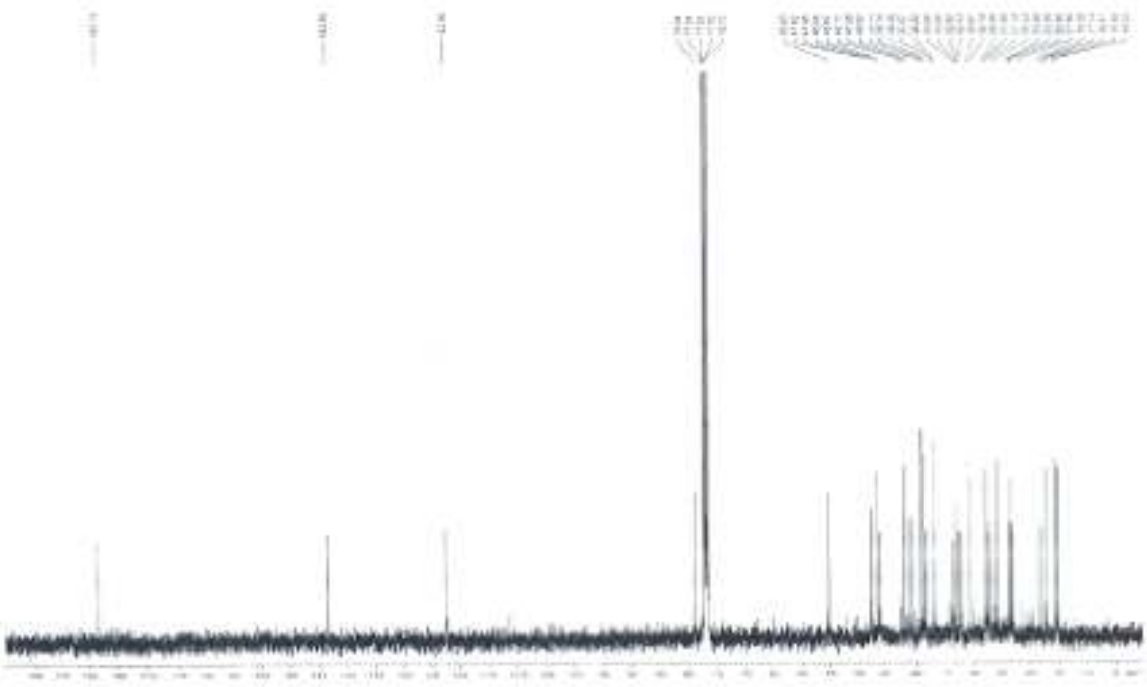
Appendix 2: IR spectrum of compound 1



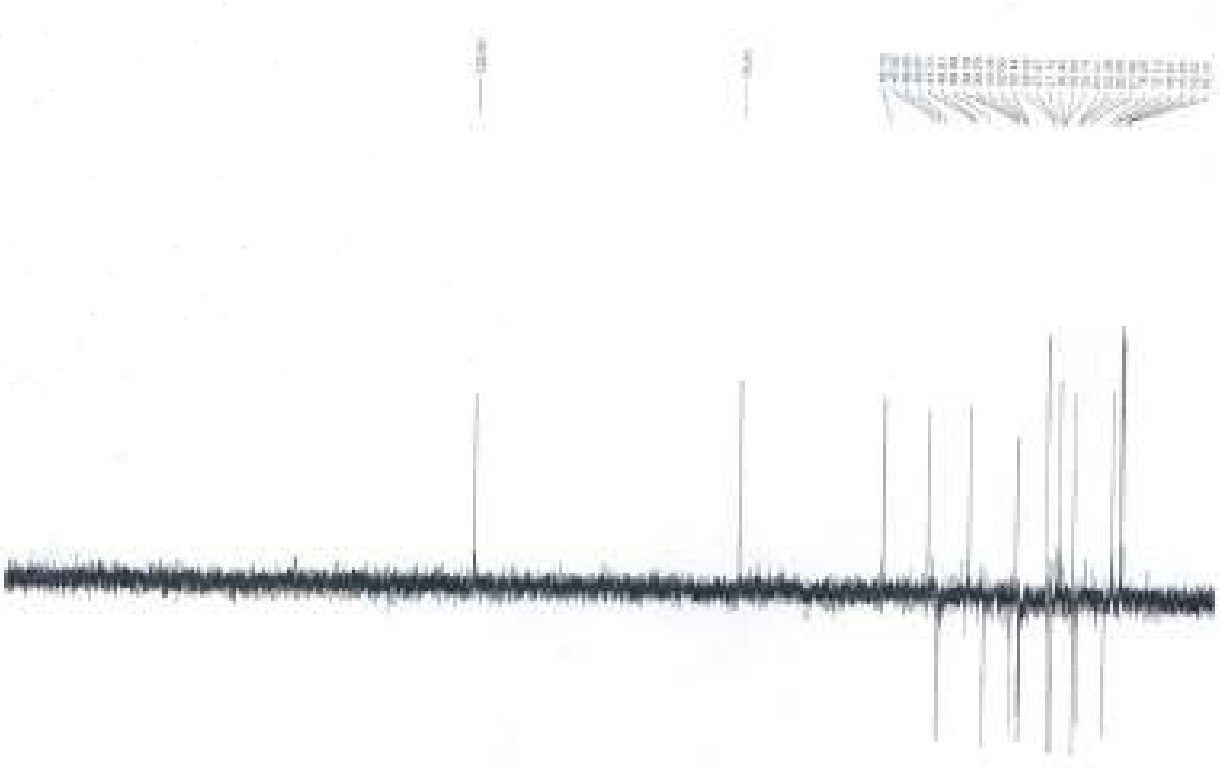
Appendix 3: ^{1}H NMR spectrum of compound 1



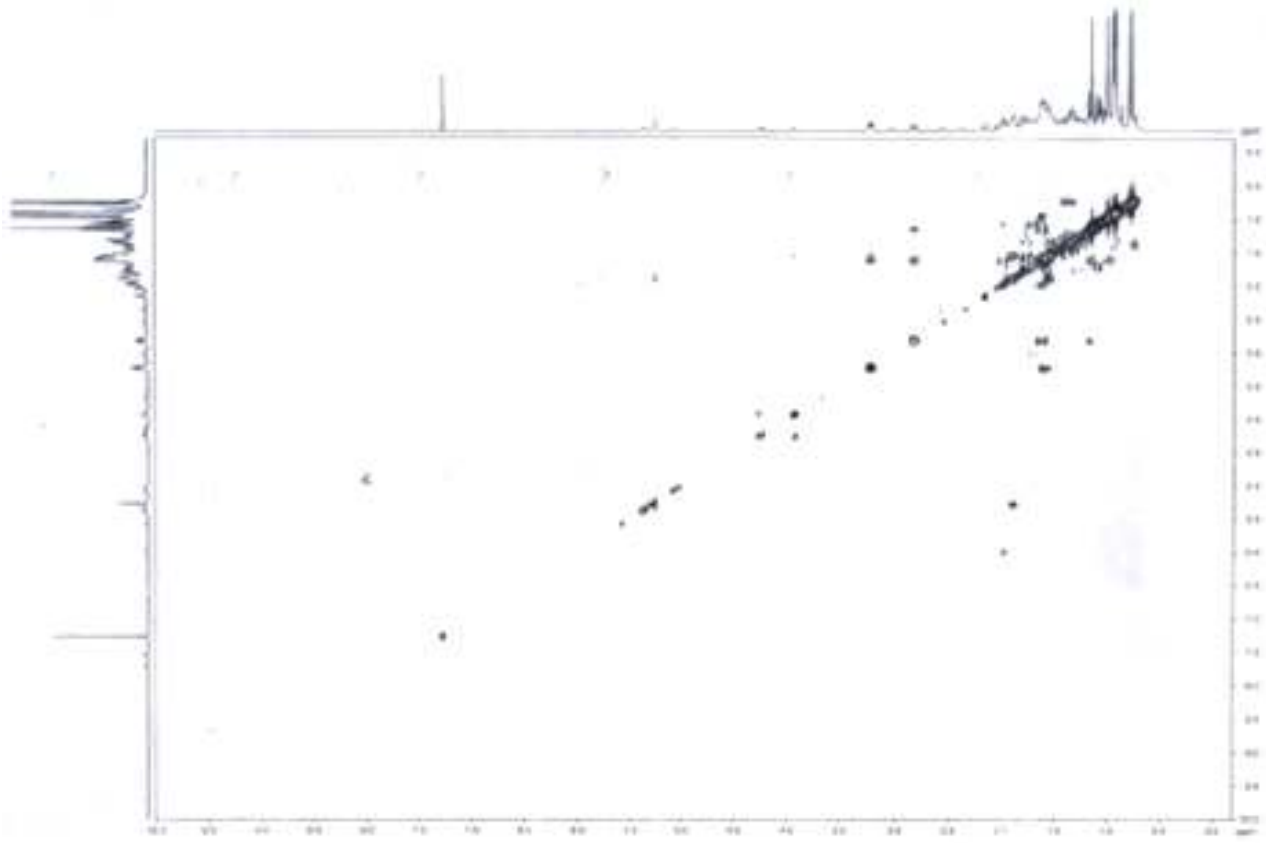
Appendix 4: ^{13}C NMR spectrum of compound 1



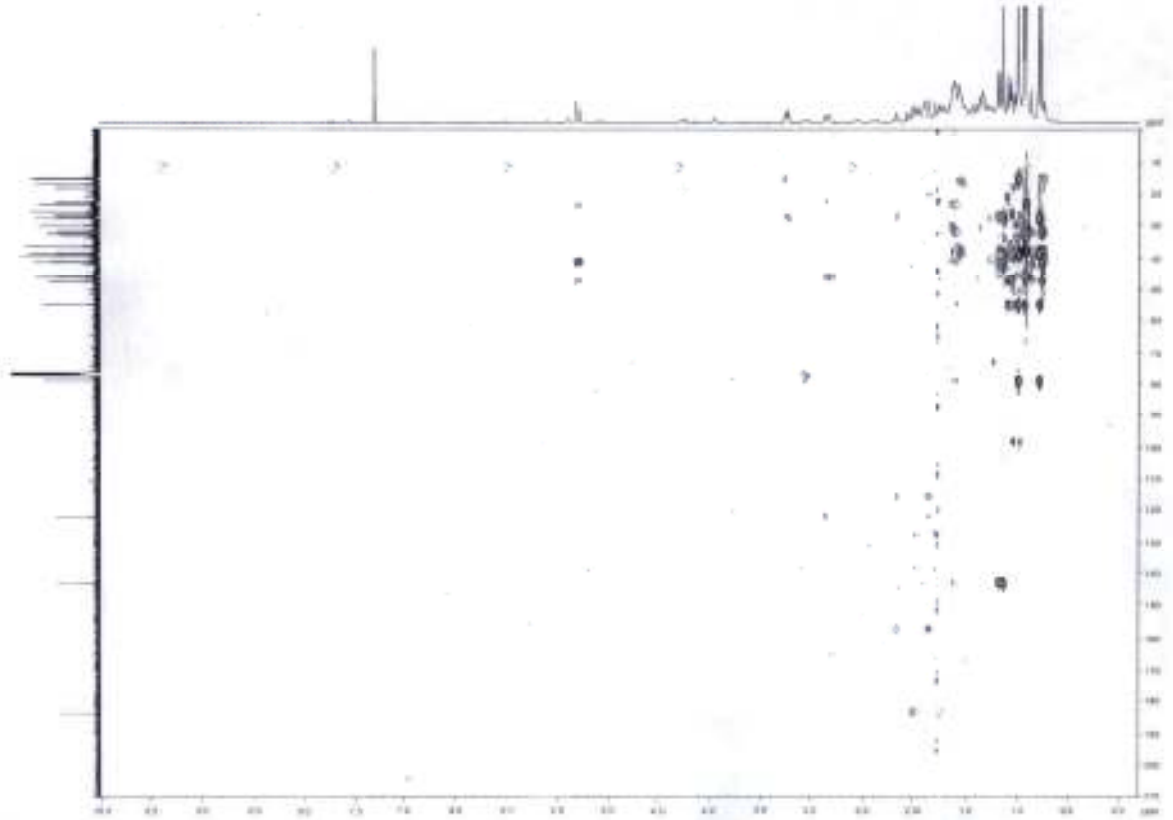
Appendix 5: DEPR-135 spectrum of Compound 1



Appendix 6: COSY spectrum of compound 1



Appendix 7: gHMBC spectrum of compound 1



Appendix 8: HSQC spectrum of compound 1