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Isolation and characterization of *stigmasterol* and *β-sitosterol* from *Anacardium occidentale* stem bark *(Anacadeceae):* Cashew stem bark

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ABSTRACT: The phytochemical screening of the stem bark extract of *Anacardium occidentale* revealed the presence of alkaloids, carbohydrates, reducing sugars, deoxysugars, saponins, phenolics, tannins, terpenoids, flavonoids, steroids, and proteins. This study aims to identify and characterize the bioactive principle from the stem bark of *A. occidentale* which is widely used in folk medicine. Column chromatography of the crude extracts led to several fractions. Thin Layer Chromatography (TLC) spotting and the spraying reagent (Concentrated H_2SO_4 and vanillin in methanol) were used to identify the fraction containing phytosterols. The isolation and purification yielded white crystalline powder which was subjected to physical, chemical, and spectral identification by IR, 1H-NMR, and 13C-NMR. The compound obtained was identified as a mixture of stigmasterol and β-sitosterol.

Keywords: *Anacardium occidentale,* phytosterols, stigmasterol, β-sitosterol.

INTRODUCTION

Natural products especially from plants have historically been an extremely productive source for new medicines in all cultures and continue to deliver a great variety of structural templates for drug discovery and development. Although products derived from natural sources may not necessarily represent active ingredients in their final form, most drugs in the market have their origin in nature (Chin *et al.,* 2006; Newman *et al.,* 2012). Drugs like; artemisinin from *Artemisia annua*, Quinine from the cinchona tree (*Cinchona officinalis*), vincristine from Madagascar periwinkle (*Catharanthus roseus*), morphine isolated from *Papaver somniferum*, Taxol obtained from *Taxus brevifolia*, and as reported by (Pujol *et al*., 2007). have their sources from nature. Given this, a natural product is a pharmacologically or biologically active chemical compound or substance, which is found in nature and produced by a living organism. The lengthy process of natural product evaluation has resulted in optimal interactions with biological macromolecules and targets.

Natural products exist as primary or secondary metabolites. Primary metabolites comprise of amino acids, sugars, and nucleic acids commonly found in all cells and are usually tagged as essential in catabolism, anabolism, and replication of cells. Secondary metabolites are organic compounds in the correct chiral configuration to exert biological activity but have no “primary” function directly involved in the normal growth, development or reproduction of an organism. Natural products are usually relatively small molecules with a molecular weight below 3,000 Daltons and exhibit considerable structural diversity

(Kinghorn *et al.,* 2009). The product categories in which natural compounds can be found as active ingredients include prescription and non-prescription drugs (pharmaceuticals), cosmetic ingredients (cosmeceuticals), dietary supplements, and natural health product ingredients (nutriceuticals) (Chernyak, 2012). Natural products continue to provide a valuable and rich component for the discovery of varieties of drug candidates and templates that are suitable for further optimization by synthetic means (Ertl *et al.,* 2008).

The discovery of valuable therapeutic agents from natural sources has continued into the 21st century by extending into new and untapped terrestrial and marine source organisms as the chemical novelty associated with natural products. This natural source is higher than that of structures from any other source. There is growing awareness of the limited structural diversity in existing compound collections and the extreme chemical diversity, the high biological potency, and the potential to frequently discover drug-like characteristics in natural products. Therefore, they constitute a valuable platform for the development of new therapeutics for a variety of indications, although they may still not contain enough versatility to yield suitable treatments for all heritable human diseases.

Anacardium occidentale is a plant belonging to the family Anacardacea commonly called cashew in English and known in the major Nigerian languages: Hausa, Igbo, and Yoruba, as 'Kashu', 'Okpokpo' and 'Kaju,' respectively. It is widely cultivated in Asia and Africa. The cashew tree, *Anacardium occidentale*, is a botanical species native to Eastern Brazil and was introduced into other tropical regions such as India, Africa, Indonesia, and Southeast Asia in the 16th and 17th centuries by the Portuguese (Marcionilia *et al.,* 2009). It is a small evergreen tree that grows 10 - 12 m tall, with a short, often irregularly shaped trunk. The true fruit of the cashew tree is the nut, a kidney-shaped structure approximately 2 - 3 cm in length. The nut is attached to the end of a fleshy pulp called the cashew apple (De Lima *et al.,* 2008).

Several studies have been carried out to isolate natural products from various parts of *A. occcidentale.* 2-hydroxy-6-pentadecylbenzoic acid and 2, 6-dihydroxybenzoic acid have been isolated from the cashew apple (Assunção and Mercadante, 2003). Other compounds isolated include myricetin, quercetin, kaempferol, and rhamnetin (De Brito *et al.,* 2007). cyanidin, peonidin, and delphinidin were also isolated (Paramashivappa *et al.,* 2001). 2-hydroxy6-pentadecylbenzoic acid, cardanol, and salicyclic acid were isolated from the hydro-ethanolic extract of *A. occidentale* nuts (Tedong *et al.,* 2010).

Also isolated from the ethanol extract of *A. occidentale* flower were ethyl gallate, and quercetin 3-galactoside. From the ethanol extract of the tender leaves, *β*-sitosterol was isolated (Subramanian *et al.,* 1969). The cracked bark of *A. occidentale* was found to contain fatty acid esters, the notable one being 5-methylbut-2-en-1-yl 3-hydroxy5-methoxy cyclohexane carboxylate. Also noteworthy is a unique new androstane steroid derivative being reported for the first time in *Anacardium occidentale* (Fadeyi *et al.*, 2015). These are compounds which could serve as new lead compounds with promising biological activities (Fadeyi *et al.*, 2015). The structures of some of the compounds isolated from *A. occientale* are seen in Figure 1.

Therefore, the objective of this study is to identify and characterize the bioactive principle from the stem bark of *A. occidentale* which is widely used in folk medicine

MATERIALS AND METHODS

Collection, identification and preparation of plant materials

The fresh stem barks of *A. occidentale* were collected from the Faculty of Agricultural Science farm, University of Benin, Ugbowo Campus, Benin City, Nigeria. The plant stem bark sample was authenticated by Prof. MacDonald Idu of the Department of Plant Biology and Biotechnology University of Benin and Voucher number UBH-A389 was issued. The fresh samples were rinsed with running water, dried at ambient temperature (30ºC) and ground to powder using a mechanical grinder. The powdered stem bark was stored in an air-tight sample bottle and kept for further analysis.

Extraction, fractionation, and isolation procedures

The pulverized stem bark of *A. occidentale* (3.1 kg) was extracted with methanol (10.5 L) by cold maceration for four days. The crude extract was obtained after filtration and concentration, using a rotary evaporator under reduced pressure at 45ºC. The extract was weighed and the percentage yield was calculated using the formula in Equation 1.0 based on the initial weight of the crude powdered sample. The extract was stored in a refrigerator at 4ºC for further analysis.

$$\%\ yield\ = \frac{weight\ of\ crude\ extract}{weight\ of\ powdered\ sample} \times 100 \text{ ---------- Eqn. 1}$$

Chromatographic fractionation-Vacuum Liquid Chromatography (VLC)

The crude extract (220 g) was subjected to fractionation by silica gel ($SiO_{2,}$) column chromatography (13 length x 150 width cm). The column was eluted using solvents and solvent mixtures in order of increasing polarity; n-hexane (100%; 2 litres), n-hexane: ethyl acetate (1:1; 2 litres), ethyl

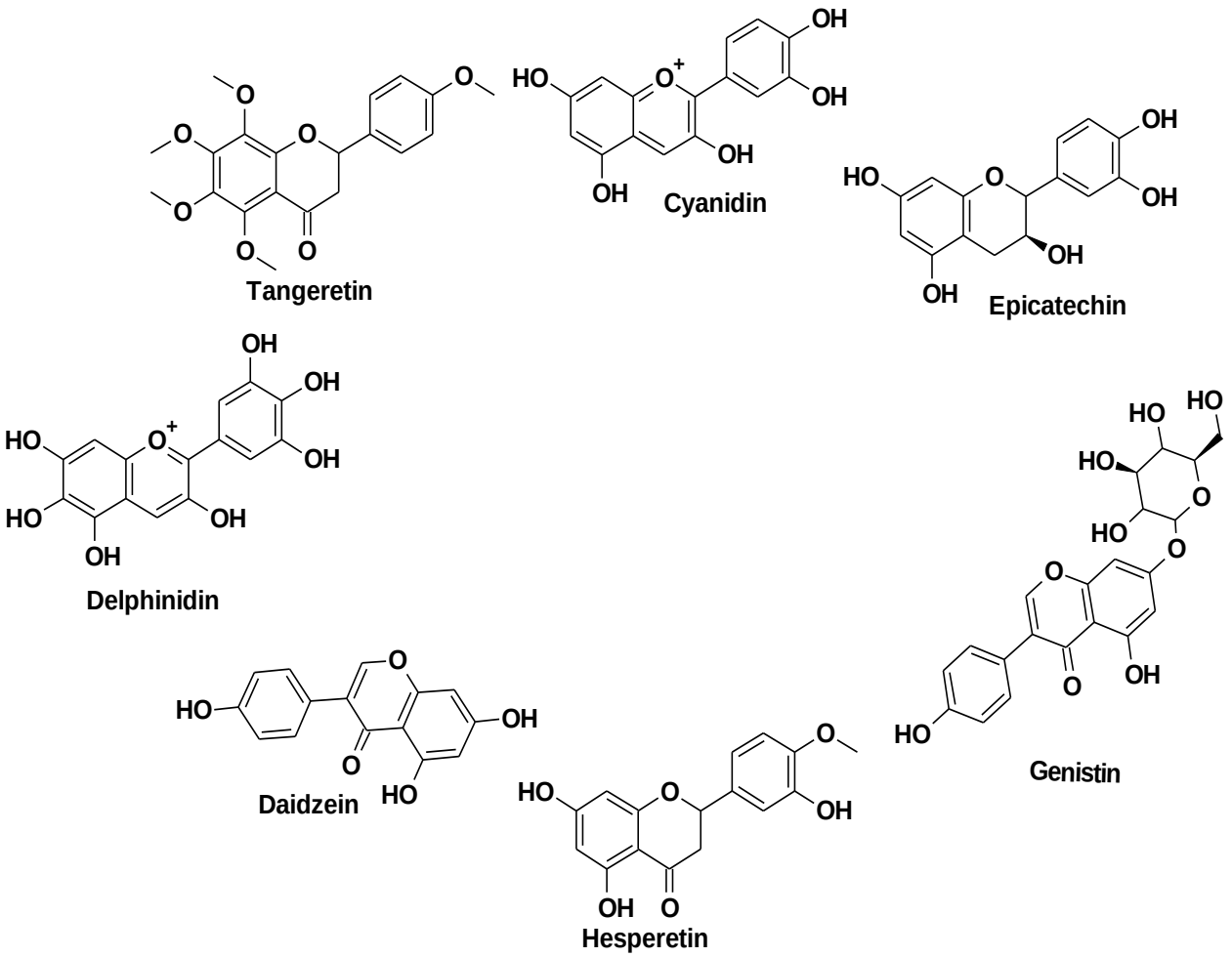

Figure 1. Chemical structures of some compounds isolated from *A. occidentale*

acetate (100%; 3 litres), ethyl acetate: methanol (1:1; 3 litres), and methanol (100%; 1.5 litres). The five fractions obtained were concentrated using the rotary evaporator at reduced pressure and their percentage yields were calculated. The fractions together with the crude extract were kept in labelled sample bottles and stored in the refrigerator at 4^0C for further analysis.

Isolation and purification

A slurry of silica gel in Hexane was added into a gravity column (75 cm x 8 cm), and the solvent was allowed to gently flow through continuously with mild tapping until the column was well equilibrated. The n-hexane/ethyl acetate fraction (4 g) was adsorbed onto silica gel (200-400 mesh) and applied as a concentrated band to the top of the column. The column was eluted using different solvent combinations (Eluent ratio; n-hexane/EtOAc 98:2, n-hexane/EtOAc 96:4, n-hexane/EtOAc 94:6, n-hexane/EtOAc 92:8, n-hexane/EtOAc 90:1 to n-hexane/EtOAc 50:50). Fractions obtained totalling 55 fractions were collected in 250 mL conical flasks.

The Thin Layer Chromatography (TLC) analysis was done using a suitable solvent system of hexane/ethyl acetate (9.5:0.5, 9.1:4.1, 7.5:2.5, 1:1), hexane/acetone (9:1,4:1,7:3) and dichloromethane/methanol (9.5:0.5, 9:1, 4:1). The plates were viewed under the UV light at 254 and 366 nm and were later sprayed with vanillin sulphuric acid and iodine vapour and then visualized by drying with a hot air.

The column fractions were monitored by thin layer chromatography (TLC) and fractions with similar TLC profiles were bulked to give rise to 4 sub-fractions (F1-F4). Sub-fraction F3 of n-hexane/ethyl acetate was subjected to repeated column chromatography leading to the isolation of a white crystalline solid which was further purified by recrystallization with n-hexane. The structure of

the isolated compound was elucidated by a combination of spectroscopic analyses Mass spectrometry (MS), Infrared spectroscopy, (IR), Proton Nuclear Magnetic resonance (^{1}H-NMR), and Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy in comparison of NMR data with literature.

The melting points of the isolated compound were determined using the capillary melting point apparatus.

Spectroscopic characterization

Mass spectrometry (MS), IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopic methods were used in the structure elucidation of the isolated compound. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded in deuterated chloroform ($CDCl_3$) on Bruker Advance II 600 NMR spectrometer at National Centre for Natural Products Research, University of Mississippi, United States, while the infrared spectrum was recorded by FTIR Agilent Technologies. The result from IR spectroscopic analysis displayed absorption bands at 3425.40 cm^{-1} which is a characteristic feature of an O-H stretch. Again, the absorption band at 2933.4 cm^{-1} and 2866.83 cm^{-1} is assigned to the CH and CH_3 stretch, respectively. Absorption at 1461.10 cm^{-1} is a bending frequency for cyclic (CH_2)n and 1379 cm^{-1} for $–CH_2$ $(CH_3)2\gamma$. At 1051.10 cm^{-1} absorption frequency, it suggests a cycloalkane hence its resemblance i.e. absorption frequency is commonly observed in Stigmasterol (Grasselli, 1973).

The ^{1}H-NMR spectrum of the isolated compound displayed signals between 0.70 to 5.38 ppm, with six intense peaks signifying the presence of six methyl groups at δ 0.70, 0.72, 0.85, 0.85, 0.87 and 0.87 ppm. The proton (H-3) of a sterol moiety was displayed as a triplet of doublet at δ 3.55 ppm. Again, at δ 5.19 ppm and at δ 5.39 ppm, the signals appeared as singlets in the region of the ethylene protons which integrated for four protons. The result of the proton NMR shows that the H-3 proton appeared as a multiplet at δ 3.55 ppm and the existence of signals for olefinic proton at δ5.07 (m), 5.19 (m), 5.38 (m). Angular methyl protons at 1.04 (s) and 0.70 (s), correspond to C_{18} and C_{19} protons, respectively.

Analysis of the ^{13}C-NMR spectrum of the isolated compound displayed signals at 140.8 and 121.7 ppm for the C_5=C_6 double bond, respectively, 71.8 for C_3 *β*-hydroxyl group, 19.0 and 12.2 for angular methyl carbon atoms for C_{19} and C_{18}, respectively. 138.3 ppm for C-22 and 129.3 ppm for C-$_{23}$. The C_5, C_6, C_{22} and C_{23} appeared to be alkene carbons.

In addition, twenty-nine carbon signals including six methyl, nine methylene, eleven methine and three quaternary carbons were seen on the spectra. Literature review shows that *β*-sitosterol ($C_{29}H_{50}O$) and stigmasterol ($C_{29}H_{48}O$) are always in a mixture form hence it is usually very difficult to obtain stigmasterol in pure state. The only identifiable difference between the two compounds is the presence of a C_{22}=C_{23} double bond in stigmasterol and a C_{22}-C_{23} single bond in *β*-sitosterol which can only be differentiated spectroscopically with ^{13}C-NMR. Furthermore, the Rf value of 0.57 (nhex/Acetone 90:10) is the same for stigmasterol and beta-sitosterol despite the use of several solvent systems.

Statistical analysis

The results obtained were expressed as mean ± standard error of the mean (SEM) of six replicates. The data were subjected to one-way analysis of variance (ANOVA) and the difference between means was determined by Duncan's multiple range tests using the Statistical Analysis System (SPSS Statistics 17.0) where applicable. P values <0.05 were considered as significant.

RESULTS AND DISCUSSION

From the positive phytochemical tests for steroids given by compound 1, it is assumed to be a compound containing a steroidal nucleus. Compound (1) is a white crystalline substance with a melting point of 134-136°C and an Rf value of 0.55 (EtAc/Hex: 1/3). According to (Luhata and Munkombwe, 2015; Khan and Hossain, 2015; Ododo *et al.,* 2016), β-sitosterol and Stigmasterol have the same Rf value of 0.55 and are always in a mixture form.

The IR spectroscopic analysis was observed to have an absorption band of 3547.41cm^{-1} which is characteristic of O-H stretching. Absorption at 3232.75 cm^{-1} is due to cyclic olefinic –HC= CH- structure, 3025 cm^{-1} due to =CH structure, and 2857.75 cm^{1} assigned to C-H structure. 1462 cm^{-1} is a bending frequency for cyclic (CH2) n and 1382cm^{-1} for –CH2 (CH3)2γ. The absorption frequency at 1071.28cm^{-1} signifies cycloalkane. These absorption frequencies resemble the absorption frequencies observed for Stigmasterol. The proton NMR showed the proton of H-3 appeared as a multiplet at δ 3.529 ppm and revealed the existence of signals for Olefinic proton at δ5.067(m), 5.197 (m), 5.378 (m), and 2.323(m). Angular methyl proton at 0.69(s), 0.80(s), and 1.02(s) corresponds to C18 and C19 proton respectively.

The ^{13}C-NMR has shown recognizable signals at 140.943 ppm and 1211.321 ppm which are assigned C5 and C6 double bonds respectively (Ododo *et al*., 2016, Luhata and Munkombwe, 2015, Yinusa *et al.,* 2014). The value at 19.064ppm corresponds to angular carbon atom (C19) 138.404 ppm for C-20 and 129.341ppm for C-21. Spectra shows twenty-nine carbon signals including six methyls, nine methylenes, eleven methane, and three quaternary carbons. The alkene carbons appeared at 140.943, 138.404, 129.341, and 1211.321 ppm (Prakash *et al.,* 2012; Luhata and Munkombwe, 2015).

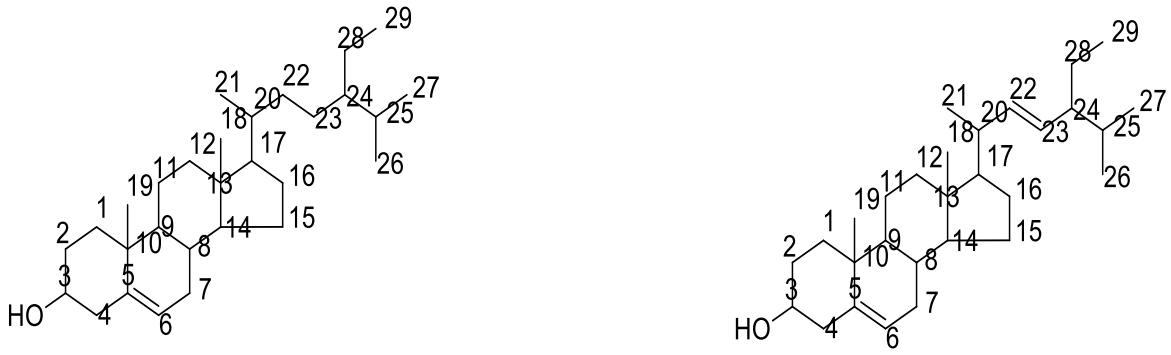

Figure 2. Chemical structure of β-sitosterol and stigmasterol.

Table 1. Phytochemical screening of anacardium occidentale stem bark extract

Phytochemical	Inference
Alkaloids	+
Carbohydrates	+
Reducing Sugars	+
Deoxysugars	+
Saponins	+
Phenolics	+
Flavonoids	+
Tannins	+
Terpenoids	+
Steroids	+
Proteins	+

+ indicates the presence of the phytochemical.

According to the literature β-sitosterol and Stigmasterol are always in a mixture form which may have a maximum portion of stigmasterol. It is very difficult to obtain Stigmasterol in a pure state (Luhata *et al.,* 2015). The only difference between the two compounds is the presence of a C22=C23 double bond in Stigmasterol and a C22-C23 single bond in β-sitosterol. Stigmasterol and beta-sitosterol have the same Rf value of 0.55 (EtAc/Hex: 1/3) despite the use of several solvent systems.

The alkene carbons appeared at 140.9, 121.755, 132.50 and 124.448 ppm. (Prakash et al., 2012; Luhata and Munkombwe, 2015) According to Prakash *et al.* (2012), Bulama *et al*. (2015), Luhata and Munkombwe (2015), Khan and Hossain (2015) and Ododo *et al.* (2016), β-sitosterol and Stigmasterol have the same Rf value of 0.55 and are always in a mixture form. It is very difficult to obtain Stigmasterol in a pure state. The only difference between the two compounds is the presence of a C=C double bond at C22 and C23 in Stigmasterol and a C-C single bond at C22-C23 in β-sitosterol. Furthermore, literature have shown that sitosterol is difficult to obtain in a pure state (Luhata and Munkombwe, 2015). Therefore, compound (X1) is a mixture of β-sitosterol and Stigmasterol. β-sitosterol is a colourless solid with a melting point of 147-149°C. The structures of stigmasterol and phytosterol are given in Figure 2.

The ^{1}H and ^{13}C NMR values for all the protons and carbons were assigned based on COSY, HMQC and HMBC correlations and were given in Table 2. The results of the phytochemical compositions of *Anacardium occidentale* based on experimental colour changes are presented in Table 1. From the phytochemical study. the + indicates that alkaloids, carbohydrates, reducing sugars, deoxysugars, saponins, phenolics, flavonoids, tannins, terpenoids, steroids, and proteins are present in the stem bark extract of *Anacardium occidentale*.

Conclusion

From the obtained results, compound (1) isolated from the

Table 2. 1H and 13C NMR chemical shift values for compound 1 recorded in $CDCl_3$ *(400 MHz)* a-b.

Carbon atom	^{13}C-NMR Experimental	^{13}C-NMR literature	^{1}H-NMR Experimental	^{1}H-NMR Literature	DEPT
C-1	37.2	37.1			CH_2
C-2	31.6	31.5			CH_2
C-3	71.8	71.7	3.51 (td,1H)	3.51(tdd,1H)	CH
C-4	42.3	42.1			CH2
C-5	140.7	140.8			C=C
C-6	121.7	121.6	5.36 (t,1H)	5.31(t.1H)	C=CH
C-7	31.6	31.5			CH_2
C-8	31.9	31.7			CH
C-9	50.1	50.0			CH
C-10	36.5	36.1			C
C-11	21.0	21.1			CH_2
C-12	39.2	39.5			CH_2
C-13	42.3	42.1			C
C-14	56.8	56.7			CH
C-15	24.1	24.2			CH_2
C-16	28.6	28.8			CH_2
C-17	55.9	55.8			CH
C-18	12.0	12.1	1.03 (s,3H)	1.03 (s,3H)	CH_3
C-19	19.0	19.8	0.70 (s.3H)	0.71 (s.3H)	CH_3
C-20	40.4	40.4-40.5			CH
C-21	21.0	20.9	0.86 (d,3H)	0.91 (d,3H)	CH_3
C-22	138.3	138.2	5.01 (m,1H)	4.98 (m,1H)	C=C
C-23	129.2	129.1-129.6	5.14 (m,1H)	5.14 (m,1H)	C=C
C-24	51.2	51.1-51.3			CH
C-25	31.8	31.9			CH
C-26	21.2	21.2	0.84 (d,3H)	0.80 (d,3H)	CH_3
C-27	19.0	19.0	0.85 (d,3H)	0.82 (d,3H)	CH_3
C-28	25.3	25.4-25.5			CH_2
C-29	12.0	12.2-25.3	0.86 (t,3H)	0.83 (t,3H)	CH_3

From the spectroscopic values obtained of the isolated compound, we report that the compound could be *β*-Sitosterol and Stigmasterol.

stem bark extract of *Anacardium occidentale* yielded a mixture of stigmasterol and beta-sitosterol. The structure of the isolated compounds was elucidated and identified on the premise of spectroscopic methods and by comparing their physical properties with that of the previous literature. The complete 1H and 13C NMR spectral assignments of the two isolated compounds were made based on COSY, HSQC, and HMBC spectroscopic data.

The basic economic importance of these two metabolites (secondary) is their acceptance as the health-supporting constituents of natural foods which are made up of them. Interestingly, both compounds showed anti-inflammatory, and anti-malaria effects, stigmasterol and Beta-sitosterol are mainly known and used for their cholesterol management and stabilising properties, reducing the risk of heart disease.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Appendix

FTIR spectrum of the isolated compound(s). The spectra data for the compounds with ^{1}H- and ^{13}C-NMR are shown below.

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Background Scans:16
Resolution:8
System Status:Good
Method Name: Transmittance Method
User:Admin
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Range:4000 - 650
Apodization:Happ-Genzel
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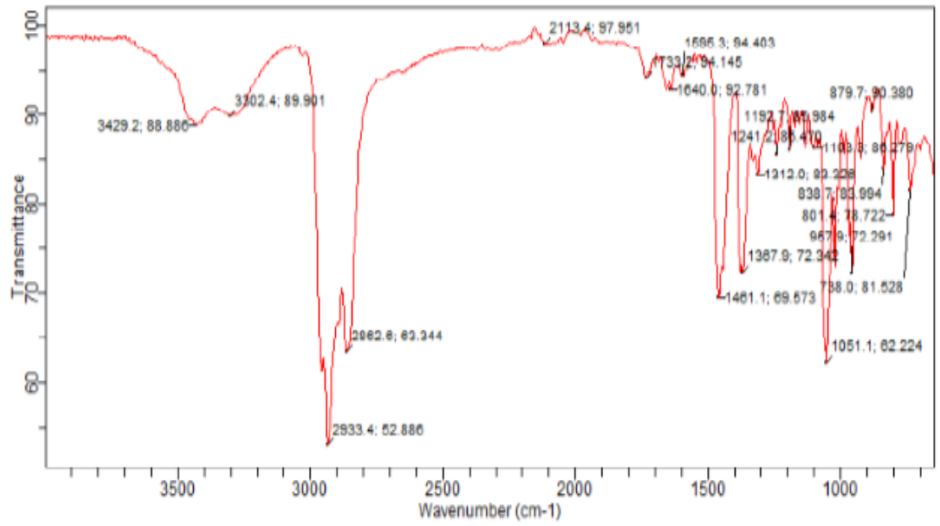
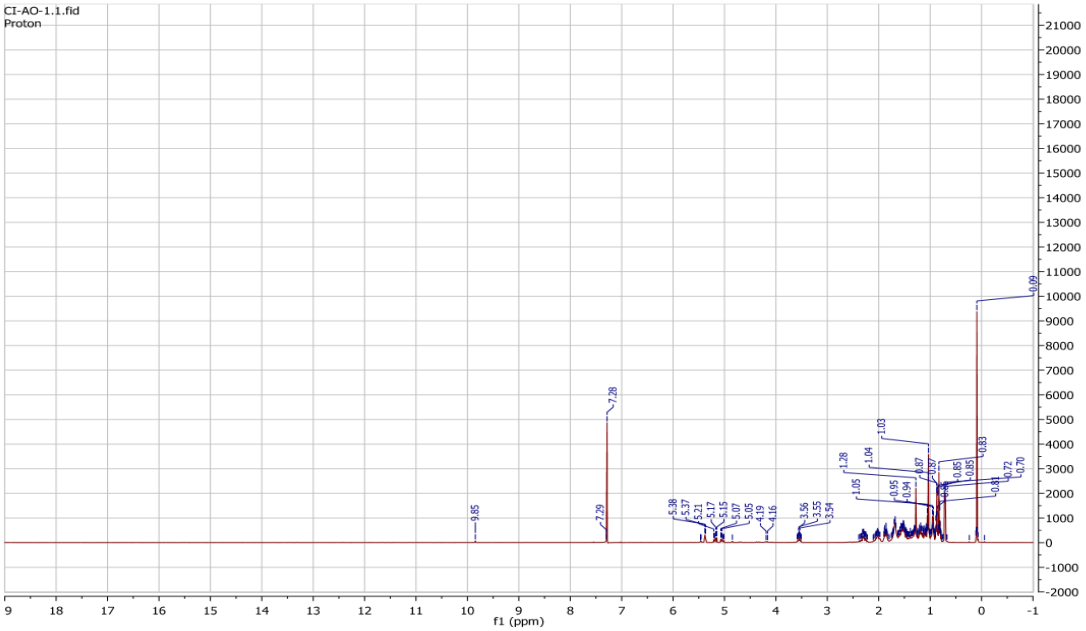
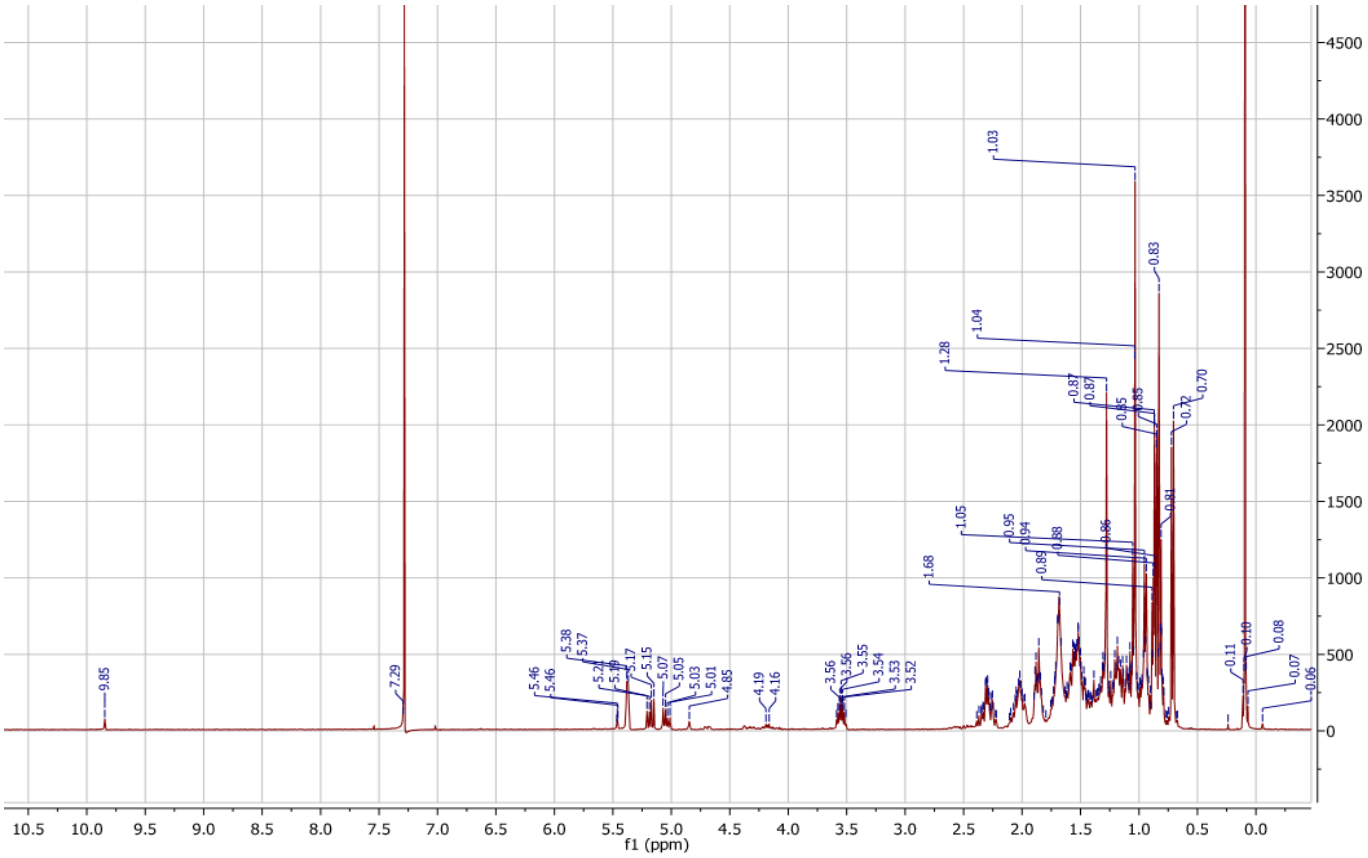


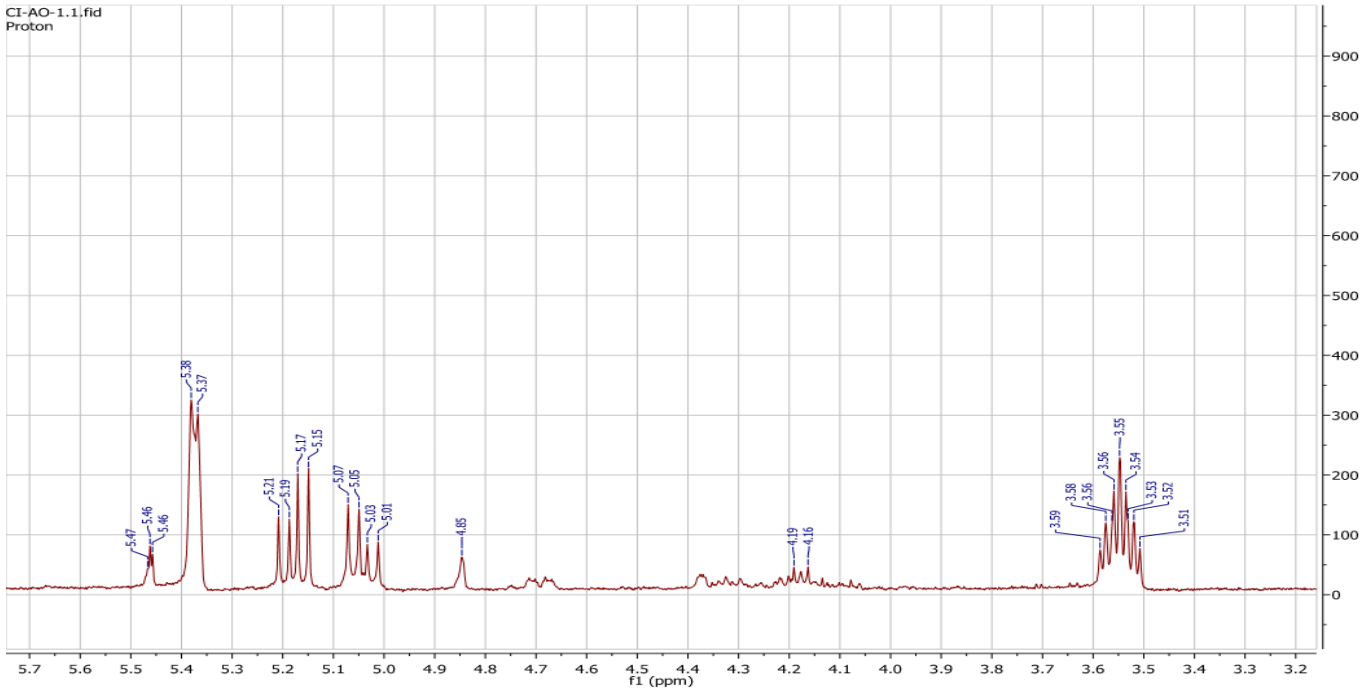
Appendix a. FTIR spectrum of the isolated compound(s).



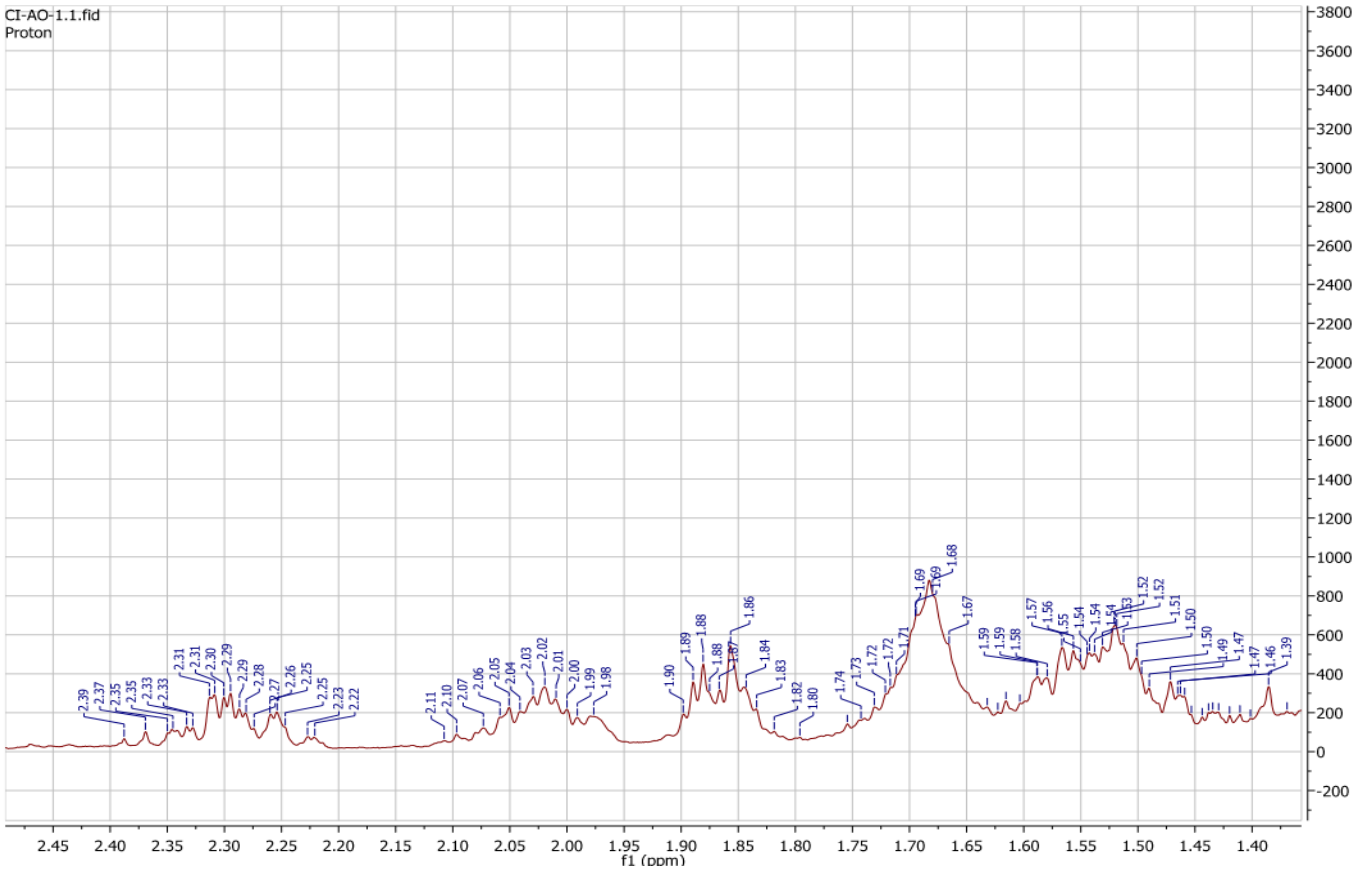
Appendix b. ^{1}H-NMR spectrum of the isolated compound(s).



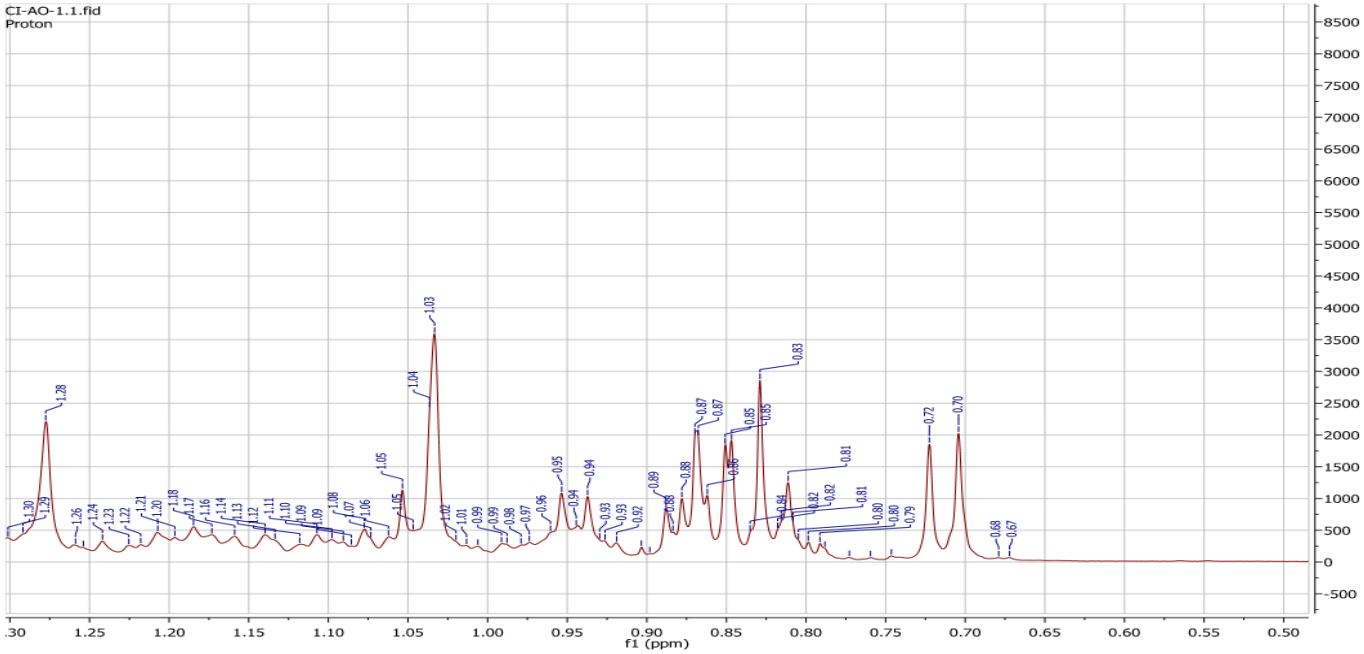
Appendix c. [1]H-NMR spectrum of the isolated compound(s).



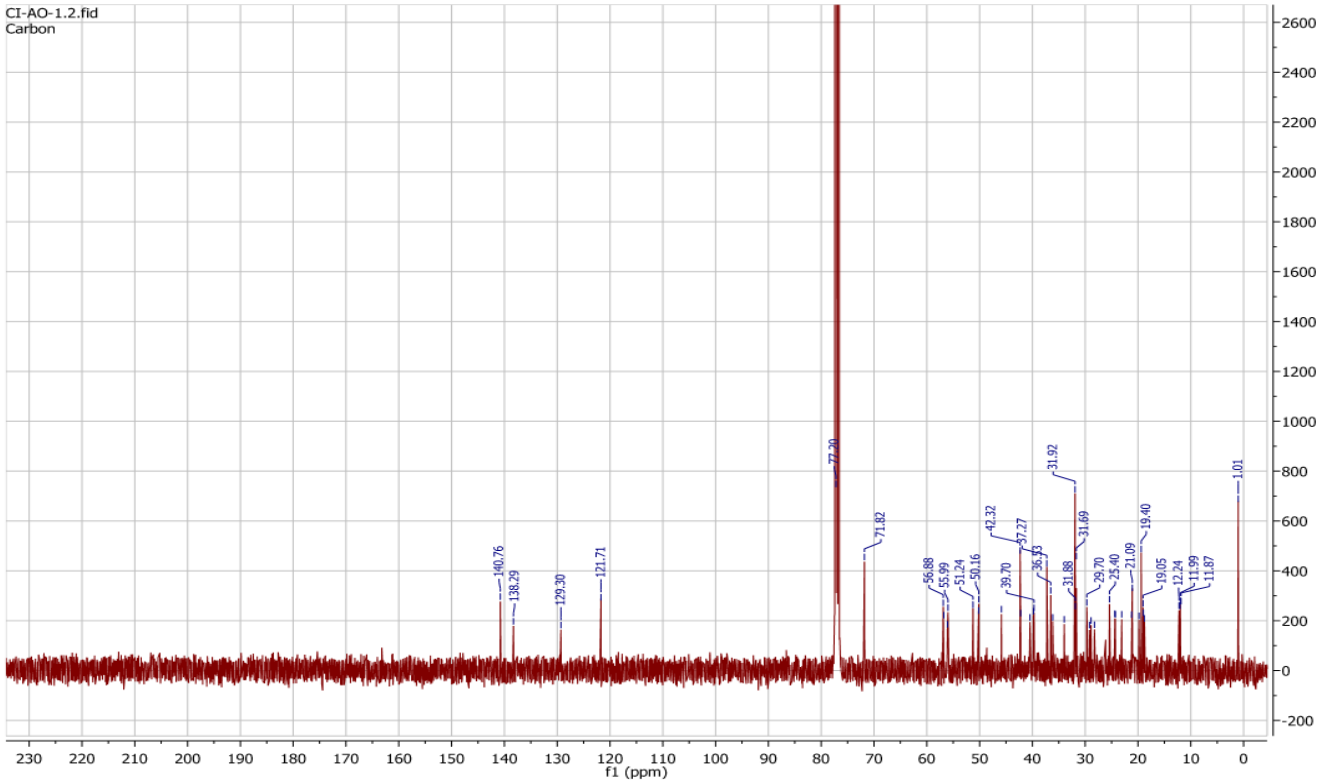
Appendix d: spectrum of the isolated compound(s).



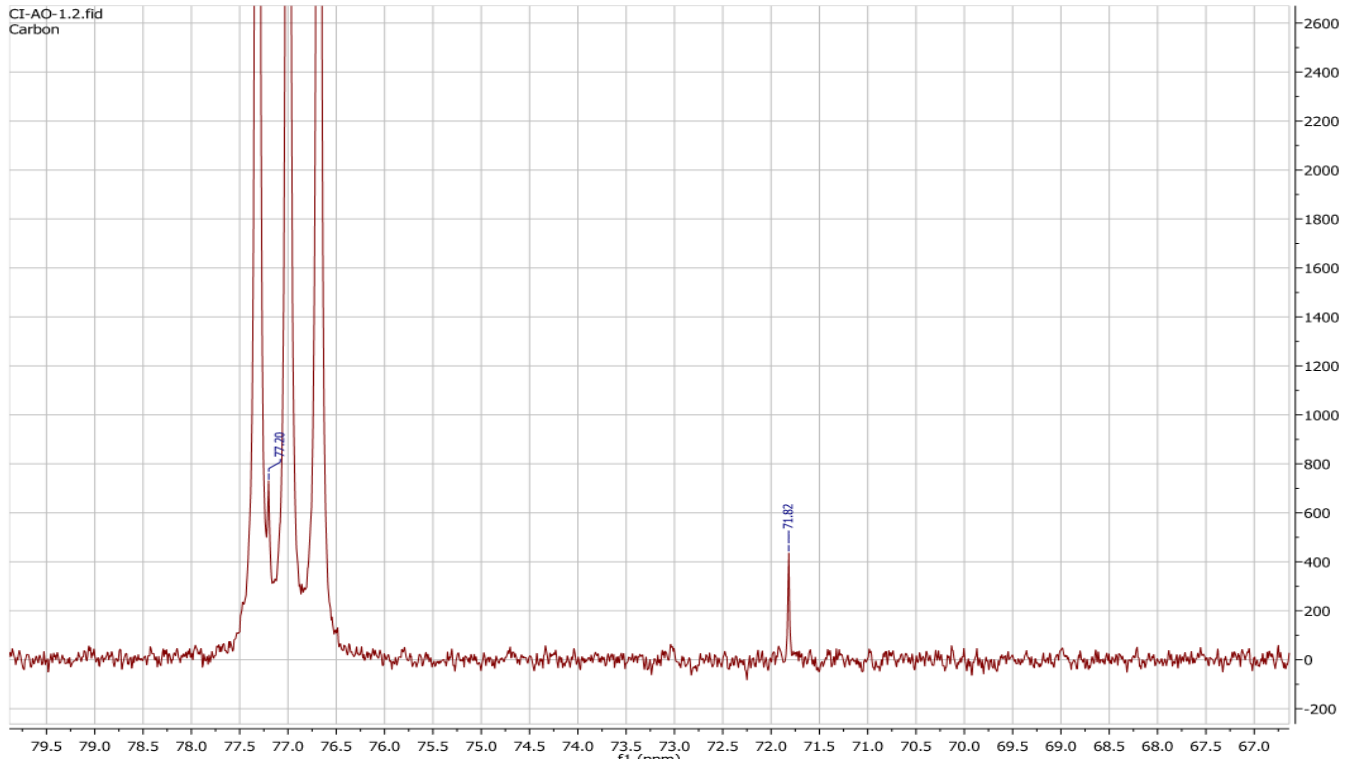
Appendix e. ^{1}H-NMR spectrum of the isolated compound(s).



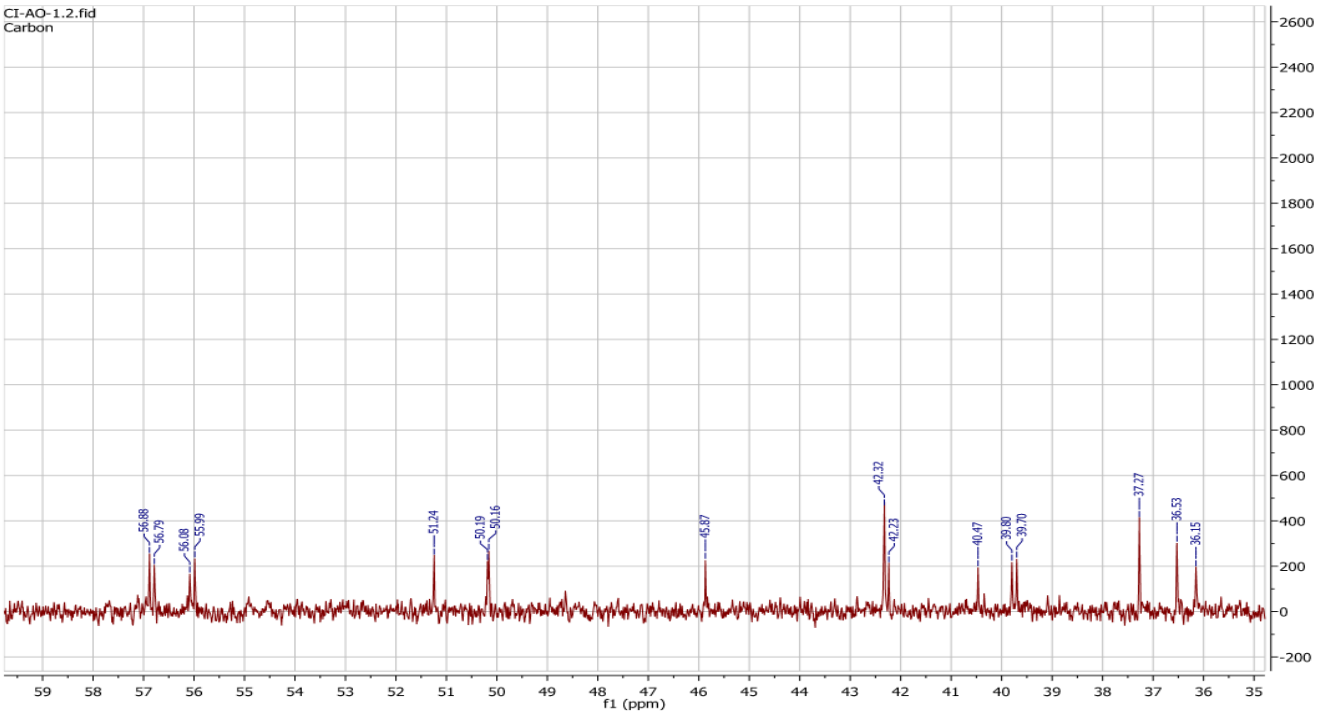
Appendix f. ^{1}H-NMR spectrum of the isolated compound(s).



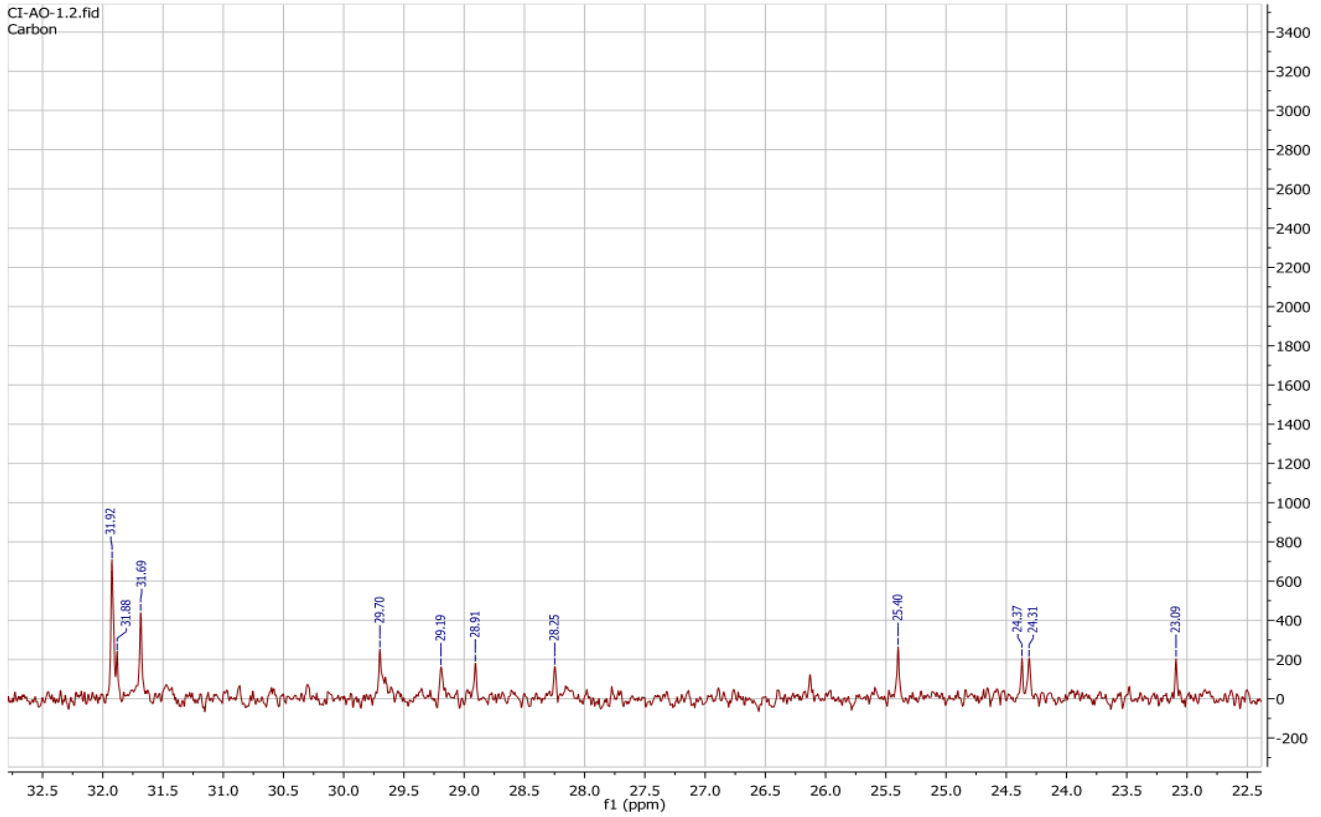
Appendix g. ^{13}C-NMR spectrum of the isolated compound(s).



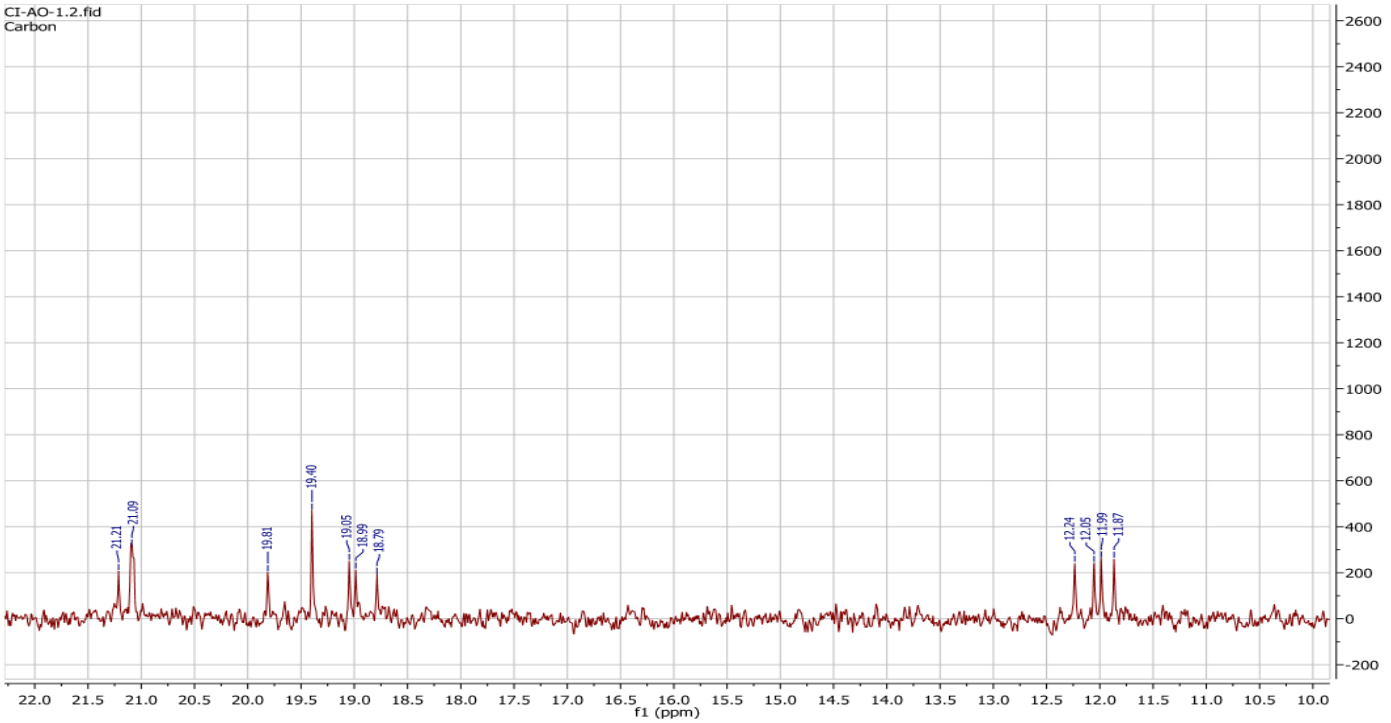
Appendix h. ^{13}C-NMR spectrum of the isolated compound(s).



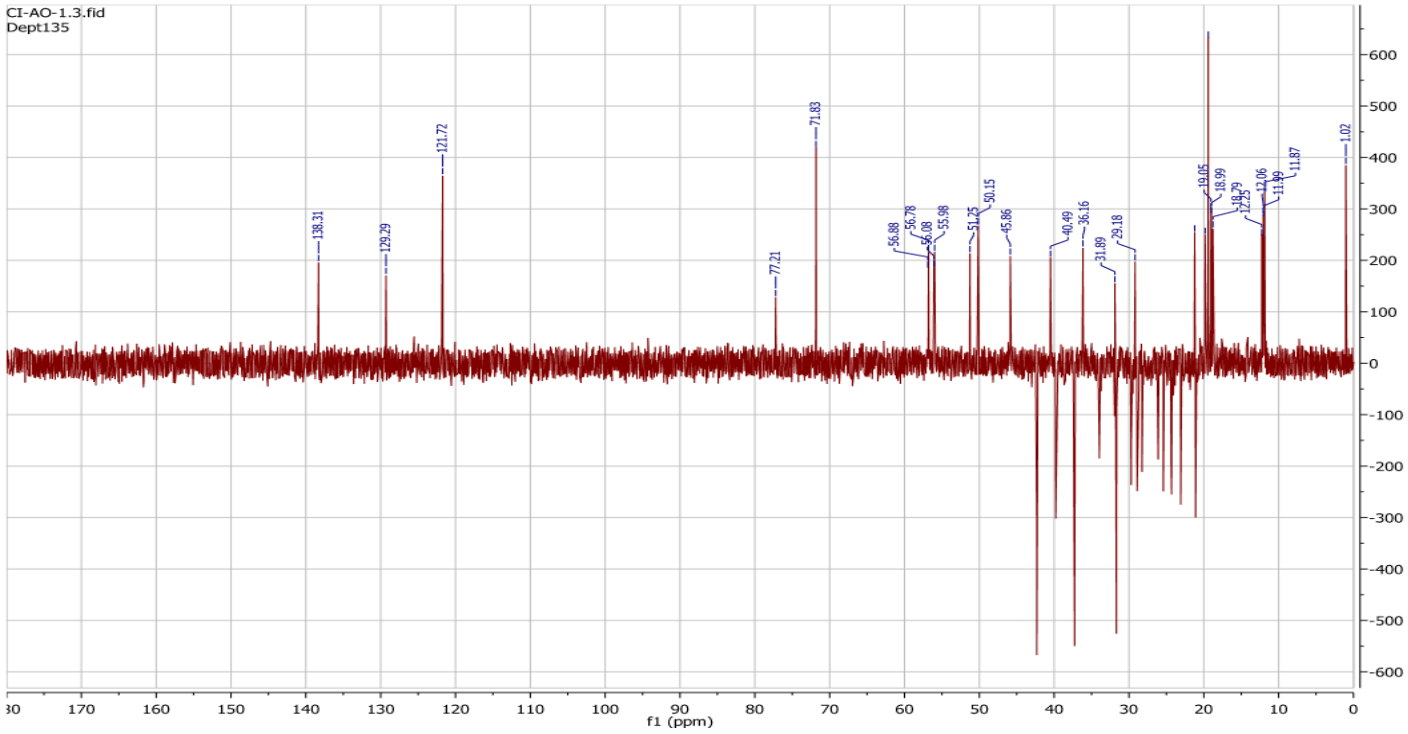
Appendix i. ^{13}C-NMR spectrum of the isolated compound(s).



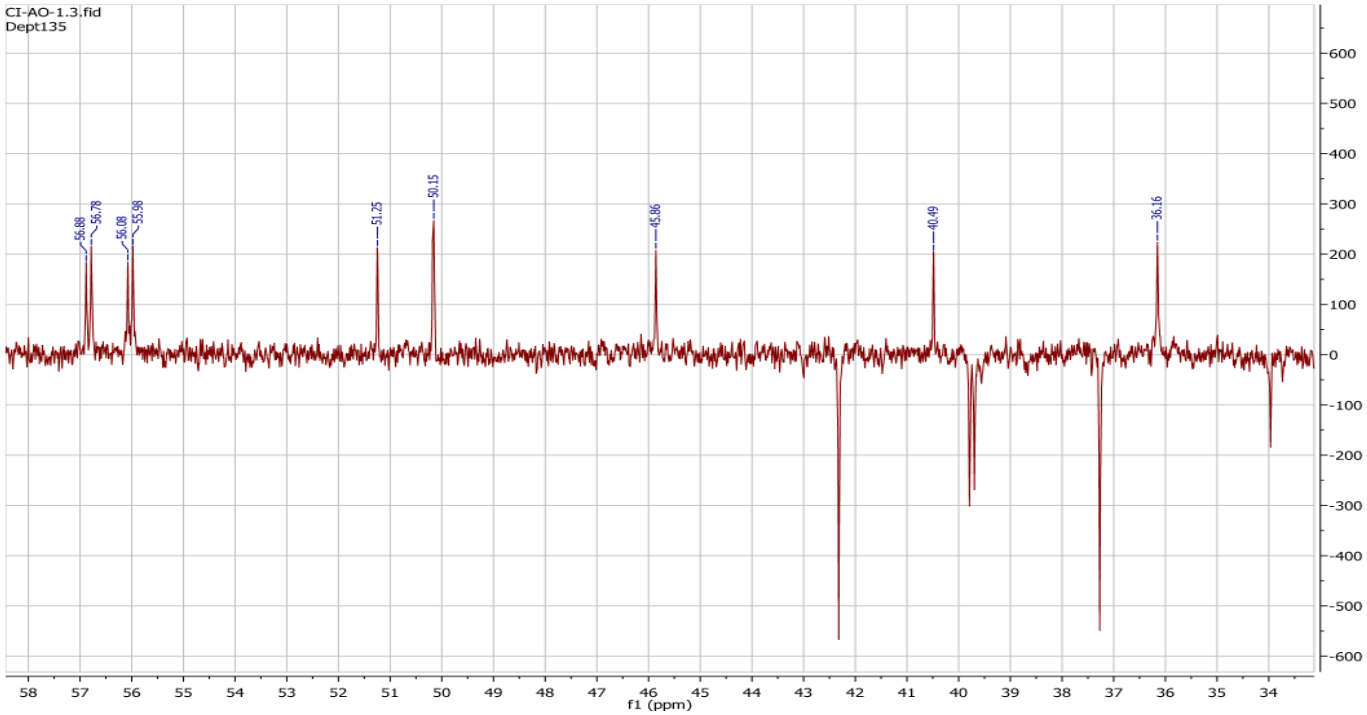
Appendix j. ^{13}C-NMR spectrum of the isolated compound(s).



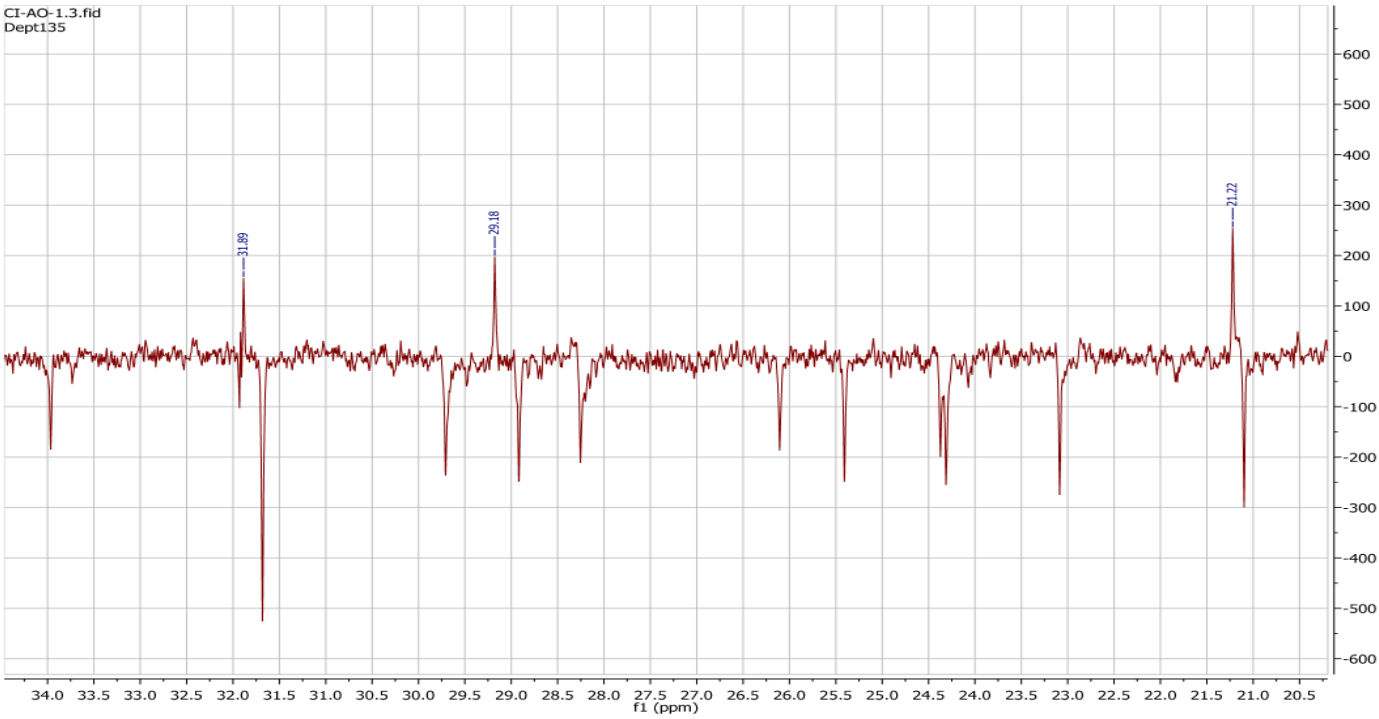
Appendix k. ^{13}C-NMR spectrum of the isolated compound(s).



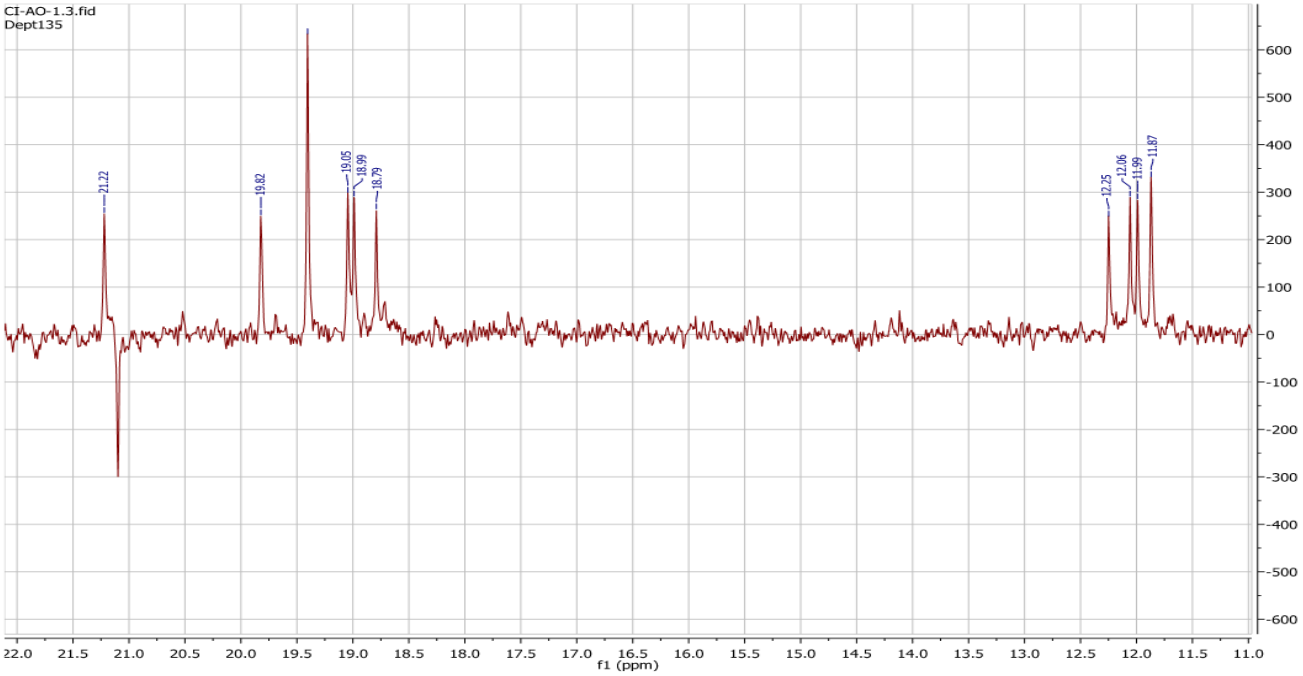
Appendix l. Dept 135 spectrum of the isolated compound(s).



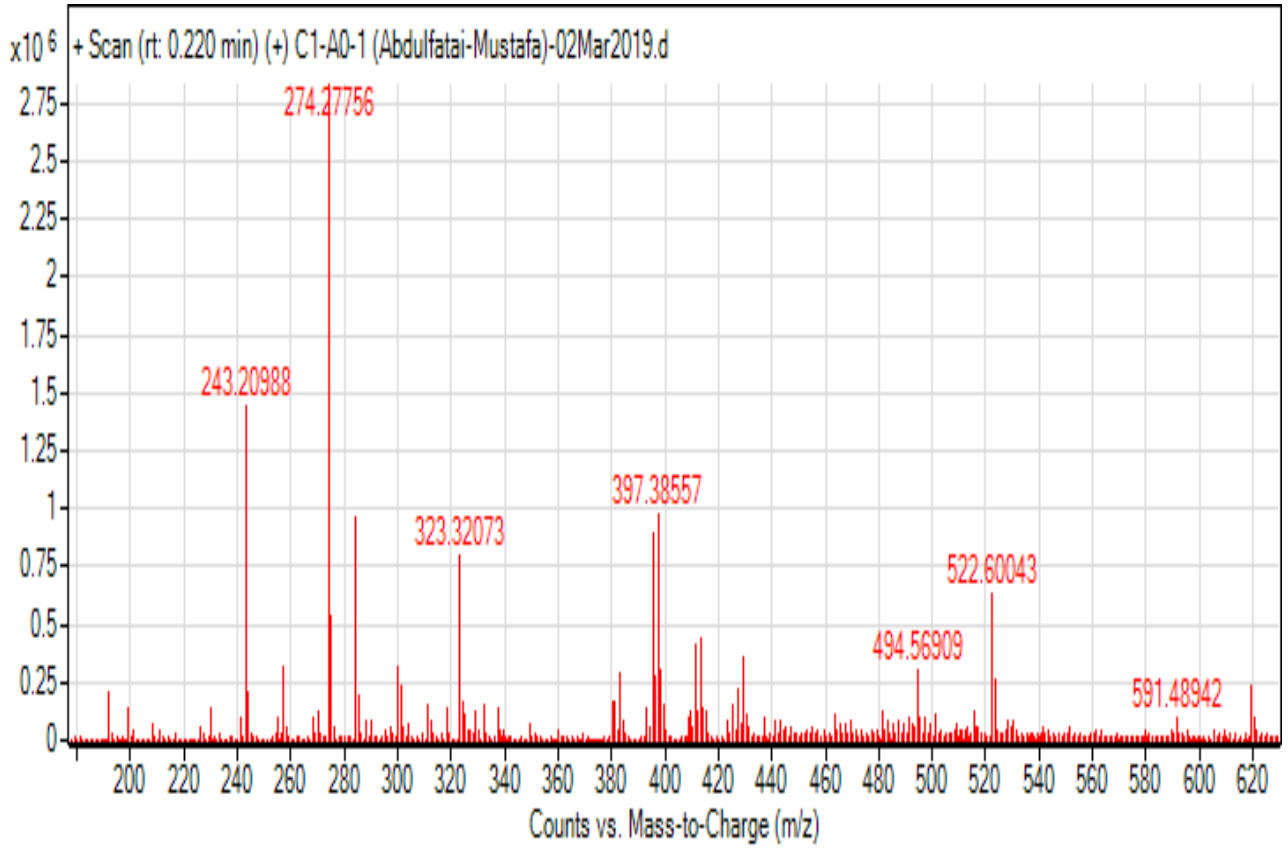
Appendix m. Dept 135 spectrum of the isolated compound(s).



Appendix n: Dept spectrum of the isolated compound(s).



Appendix o: Dept spectrum of the isolated compound(s).



Appendix p: Mass Spectrum of the isolated compound(s).