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Full Length Research Paper

Triterpenes from the stem bark of *Protorhus longifolia* exhibit anti-platelet aggregation activity

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Two triterpenes were isolated from the chloroform extract of *Protorhus longifolia.* Their structures were established through spectral analysis (nuclear magnetic resonance (NMR), infrared (IR), liquid chromatography mass spectrometry (LC-MS)) as 3-oxo-5α-lanosta-8,24-dien-21-oic acid (1) and 3β-hydroxylanosta-9,24-dien-24-oic acid (2). The two triterpenes were screened for their antioxidant, cytotoxicity, anti-platelet aggregation and anti-inflammatory activity. The antioxidant activity of the compounds was measured using 1,1′-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) free radicals scavenging and reduction potential assays. The cytotoxic effects of the compounds was determined against human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cell lines, while the acute anti-inflammatory activity was determined using the carrageenan-induced rat paw edema model. The anti-platelet aggregation activity of the compounds was separately investigated on thrombin, adenosine diphosphate (ADP), epinephrine and arachidonic acid induced rat platelet aggregation. Although, both compounds exhibited poor antioxidant activities, they showed good concentration dependent anti-platelet aggregation activity. The highest activity by compound 1 (IC_{50} of 0.99 mg/ml) was observed on the thrombin-induced platelet aggregation. Compound 2 (3 mg/ml) exhibited anticoagulant activity on whole blood and significantly ($P < 0.05$) inhibited the acute inflammation of rat paw. The compound also weakly inhibited the growth of HEK293 and HepG2 cells. The triterpenes could be potential pharmacologically active anti-platelet aggregation agents.

Key words: Triterpenes, antioxidant, anti-inflammatory, anti-platelet aggregation, cytotoxicity.

INTRODUCTION

Platelet aggregation is the key event in the process of blood clotting under both physiological and pathophysiological conditions. Platelet dysfunctions significantly contribute to pathogenesis of cardiovascular diseases. Despite the availability of the current therapies to prevent platelet dysfunctions, atherothrombotic diseases continue to pose a threat to human health. The search and discovery of new and more effective anti-platelet aggregation agents of natural origin is encouraged (Amrani et al., 2009). Medicinal plant-based traditional healing has not only been the medicine of all ages, but it also provides a lead to discovery of new pharmacologically active drugs.

Protorhus longifolia (Benrh.) Engl. (Anacardiaceae) also known as *uNhlangothi* (Zulu) is an ever green indigenous tall tree. It is the only species in the genus *Protorhus* that is found in Southern Africa, while about 20 of the other species are predominantly found in Madagascar (Archer, 2000). It grows in forests, on river banks and in woodlands. It is one of the top 10 most

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important and frequently traded medicinal plants in Eastern Cape province of South Africa (Dold and Cocks, 2002; Keirungi and Fabricius, 2005). The bark has been traditionally used to cure various diseases, such as, heartwater and diarrhea in cows (Dold and Cocks, 2001), hemiplegic paralysis, heart burn and bleeding from the stomach; unspecified parts being used to strengthen the heart (Gerstner et al., 1939; 1941; Pujol, 1990). The leaf extracts of *P. longifolia* have been reported to possess antimicrobial activity (Suleiman et al., 2009, 2010). The anti-platelet aggregation activity of the stem-bark extracts of this plant has also been recently reported (Mosa et al., 2011).

In this study, we report the anti-platelet aggregation activity of the two lanostane-type triterpenes isolated from the chloroform extract of *P. longifolia*. To the best of the authors' knowledge, this is the first time these compounds are isolated from this plant.

MATERIALS AND METHODS

Plant collection

Plant material (stem bark) of *P. longifolia* (Benrh.) Engl. was purchased from Empangeni *muthi* market. The plant was identified and confirmed by Mrs. N.R. Ntuli, of Botany Department, University of Zululand and voucher specimen (RA01UZ) was prepared and deposited in the University herbarium. The plant material was thoroughly washed with tap water, and then was air dried. The air dried plant material was ground into powder (2 mm mesh) and was stored in a sterile brown bottle at 4°C until required.

Animals

Adult rats (Sprague-Dawley) of either sex were collected from the animal house in the Department of Biochemistry and Microbiology, University of Zululand. The animals were maintained under standard conditions (temperature 23 ± 2°C and 12 h light dark cycle); had free access to standard pellet feed and enough drinking water. Approval for experimental procedures was obtained from Research Animal Ethics Committee, University of Zululand.

Chemicals and reagents

Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). All chemicals and solvents used were of analytical grade.

Extraction and isolation

The powdered plant material (1 kg) was extracted (1:5 w/v) sequentially with hexane and chloroform (24 h on platform shaker per solvent, 150 rpm, room temperature) to give hexane and chloroform extracts. The extracts were concentrated under reduced pressure using rotary evaporator (40 ± 2°C). The chloroform extract (8.0 g) was subjected to silica gel column chromatography (20 × 500 mm; Silica gel 60; 0.063 to 0.2 mm; 70 to 230 mesh ASTM; Merck chemical supplies, Darmstadt, Germany), with a hexane-ethyl acetate gradient (9:1, 8:2, 7:3, 6:4, 5:5, 4:6 and 3:7). Thin layer chromatography (TLC) (silica gel 60 TLC aluminum sheets 20× 20 cm, F_{254}) was used to analyze the collected fractions. The spots were first viewed under ultraviolet (UV) light, developed using a 10% H_2SO_4 spray reagent, and then were heated. The collected fractions were combined based on their TLC profile to yield 16 combined fractions (Fraction A to P). The seventh and ninth fractions were separately re-crystallized in methanol and hexane, respectively to obtain compounds 1 (0.23 g) and 2 (0.37 g).

Structure elucidation

The structure of compounds 1 and 2 were established using 1 and 2D nuclear magnetic resonance (NMR) techniques (^{1}H-^{1}H, ^{13}C-^{13}C, DEPT, COSY, HMQC, HMBC and NOESY) (in $CDCl_3$, Bruker 600 MHz), infrared (IR) (Perkin-Elmer 100 FTIR) and liquid chromatography mass spectrometry (LC-MS) and the molecular formula was identified by ESI-MS (positive mode), $[M + H]^+$ (in DCM, Waters API Q-TOF Ultima). Melting point (Reichert Thermovar) of the compounds was also determined. The compounds were identified mainly by comparing their spectra (^{1}H and ^{13}C-NMR) to similar compounds reported in literature.

Compounds characterization

3-Oxo-5α-lanosta-8,24-dien-21-oic acid (1): white amorphous powder, mp192 to 194°C, IR (KBr) ν_{max}= 3431 cm^{-1}, ^{1}H and ^{13}C NMR (Table 1), ESI-MS (positive mode) *m/z* % 455 $[M+H]^+$, protonated ion in LC-MS at *m/z* 455.3524 (calculated for $C_{30}H_{46}O_3$, 455.3525). 3β-Hydroxylanosta-9,24-dien-24-oic acid (2): white flakes (paper-like solids), mp134 to 136°C, IR (KBr) ν_{max}= 3360 cm^{-1}, ^{1}H and ^{13}C NMR (Table 1), molecular formula $C_{30}H_{47}O_3$, calculated 455.77.

Antioxidant activity

Antioxidant activity of the triterpenes was measured in terms of their ability to scavenge 1,1′-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) free radicals, and to chelate Fe^{2+} ions (Opoku et al., 2002, 2007; Simelane et al., 2010). Percentage inhibition of each parameter measured was calculated from the formula:

$$\text{Inhibition (\%)} = (1 - A_t/A_0)100,$$

where A_t is the absorbance of test sample and A_0 is the absorbance of the fully oxidized system sample. Each experiment was carried out in triplicate. Half maximal inhibition (IC_{50}) values were determined using statistical package Origin 6.1.

Anti-platelet aggregation activity

The method of Tomita et al. (1983) was followed to obtain platelets. A rat was killed by a blow to the head and blood was immediately collected from the abdominal aorta. The blood was mixed (5:1 v/v) with an anticoagulant (acid-dextrose-anticoagulant of 0.085 M trisodium citrate, 0.065 M citric acid and 2% dextrose). The platelets were obtained by a series of centrifugation and washing of the blood. The platelets were finally suspended in a resuspending buffer (pH 7.4; containing 0.14 M NaCl, 15 mM Tris-HCl and 5 mM glucose).

Anti-platelet aggregation activity was determined by the method described by Mekhfi et al., (2004) with some modifications. The compounds were separately solubilized in dimethyl sulfoxide (DMSO) before making up the volume with 50 mM Tris-HCl buffer (pH 7.4; containing 7.5 mM ethylenediaminetetra-acetic acid (EDTA) and 175 mM NaCl) to a final 1% DMSO concentration.

Different concentrations (1, 3 and 10 mg/ml) were used in the assay. The anti-platelet aggregation activity of the compounds was separately tested on thrombin (5 U/ml), adenosine diphosphate (ADP) (5 mM), epinephrine (10 mM) and arachidonic acid (10 mM) induced platelet aggregation. The platelets (100 µl) were incubated for 5 min with different concentrations of the compounds and an aggregation inducer (20 µl) was introduced to the mixtures. Aggregation was determined with the Biotek plate reader (ELx 808 UI, Biotek Instrument Supplies) using Gen5 software by following change in absorbance at 415 nm. DMSO (1%) was used as negative control.

All assays were done in triplicate and the mean slope (A) ± standard error of mean (SEM) reported. The inhibitory effect of the compounds on each parameter was calculated as:

$$\text{Inhibition (\%)} = \{(A_0 - A_1)/A_0 \times 100\}$$

where A_0 is the mean slope of control and A_1 is the mean slope of the compound. IC_{50} values were determined using statistical package Origin 6.1.

Anticoagulant activity

The anticoagulant activity of the compound was investigated on rat whole blood. Concentrations of the compound were prepared as in the anti-platelet aggregation experiment. The anticoagulant activity of the compound (1, 3 and 10 mg/ml) was tested against thrombin (5 U/ml), ADP (5 mM), epinephrine (10 mM) and arachidonic acid (10 mM) as controls. DMSO (1%) was used as blank. The compound (50 µl) and the clotting agonist (20 µl) were added separately into the corresponding wells. Blood was drawn from the abdominal aorta of a rat and was immediately mixed with either the clotting agonist or the compound in the corresponding wells. The reaction was monitored by visualization to record the time it took the blood to clot.

Anti-inflammatory activity

The anti-acute inflammatory activity of the compound was determined using the model of carrageenan-induced rat paw oedema as described by Carvalho et al. (1999) with some modifications. Two groups of four rats (200 ± 20 g) each, were orally administered (50 and 500 mg/kg body weight, respectively) daily for four days before the experiment with the compound suspended in 0.5% carboxymethyl cellulose (CMC). Negative and positive control animals were administered with 0.5% CMC and indomethacin (10 mg/kg), respectively. One hour after the last administration, acute inflammation was produced in all the rats by subcutaneous injection of 0.1 ml (1% w/v) carrageenan solution into the plantar surface of the right hind paw; the left paws were injected with normal saline for comparison. The paw volume until knee joint was measured by a water displacement method using a digital plethysmometer (EL 7500, Panlab, Spain) immediately and at 1 h interval for 4 h following the carrageenan injection. The anti-inflammatory activity was calculated by measuring the volume difference between the right and left paws in comparison with the control group.

Cytotoxicity

The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium- bromide (MTT) cell proliferation assay (Mosman, 1983) was used to determine the cytotoxicity of compound. The cytotoxicity of the compound was tested in human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells. The cells were seeded in a 48-well plate at a density of 2.5×10^4 cells per well. Following an overnight incubation at 37°C, the cells were incubated with the compound at varying concentrations (50, 100, 150, 200, 250, 300 and 350 µg/200 µl) in medium (modified eagles medium + Gutamax + antibiotics + 10% fetal bovine serum) for 48 h. Thereafter, the medium was removed from the cells and 200 µl MTT solution (5 mg/ml phosphate buffer saline) as well as 200 µl of cell culture medium was added to the corresponding wells. The cells were incubated at 37°C for 4 h and the reaction was terminated by addition of DMSO (200 µl). The cell viability was determined using Mindray microplate reader at 570 nm. The experiment was done in triplicate and the results were expressed as mean ± standard deviation (SD). Lethal concentration of the compound that results in 50% cell death (LC_{50}) was determined by regression analysis using QED statistics program.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. The results were presented as mean ± standard error of the mean (SEM). Statistical difference was accepted at $P < 0.05$. Student's t-test was used to analyze statistical difference between control and treated groups.

RESULTS AND DISCUSSION

Platelet aggregation is pivotal in blood-clotting under both physiological and pathophysiological conditions. While clotting could be beneficial to stop bleeding, unchecked internal formation of blood clots could be fatal. Despite the availability of the current therapies to prevent platelet dysfunctions, atherothrombotic diseases continue to pose a threat to human health.

The consistent anti-platelet aggregation activity exhibited by the chloroform extract in the preliminary screening led to the fractionation of this extract over silica gel column chromatography to obtain the active compounds. The structures of the isolated compounds 1 and 2 (Figure 1) were confirmed through ^{1}H and ^{13}C NMR spectra and by comparison with similar compounds in literature.

The ^{1}H-NMR spectra of compound 1 (Figure 1) suggested a triterpenoid proton pattern with large clusters of signals of CH_3, CH_2 and CH between the δ 2.5 and 0.8. However, one olefinic proton was identified at δ 5.33. The ^{13}C-NMR did help to further analyze the compound. The presence of carbonyl ketone at 217.7 ppm, four olefinic carbons between 134.4 and 123.5 ppm, a carboxylic carbon at 181.3 ppm and four quaternary carbons assisted in suggesting a lanosteryl skeletal structure (Vincken et al., 2007). The ^{13}C-NMR distortion-less enhancement by polarisation transfer (DEPT) further indicated the presence of 11 CH_2 which also helped in elucidating the structure along with the 2D NMR and mass spectra. Detailed assignment of the ^{13}C- NMR and significant ^{1}H-NMR is presented in Table 1. These data are in correlation with those of 3-oxo-5α-lanosta-8,24-dien-21-oic acids reported in the literature (Keller et al., 1996; Ko et al., 2007).

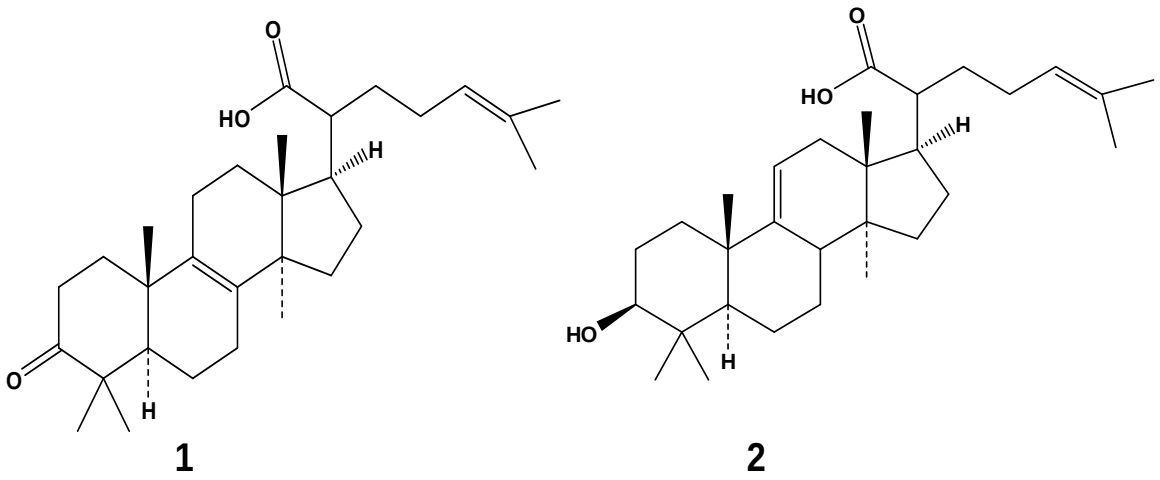

Figure 1. Chemical structures of compounds 1 and 2.

Table 1. ^{13}C-NMR data and significant ^{1}H-NMR data of compounds 1 and 2. Chemical shifts are expressed in δ (ppm).

Position	1		2	
	δ_C	δ_H	δ_C	δ_H
1	35.6	-	30.3	-
2	34.5	-	23.9	-
3	217.7	-	77.2	3.5 (1H, s, OH)
4	47.3	-	37.3	-
5	51.5	-	44.5	-
6	20.1	-	17.7	-
7	25.9	-	26.0	-
8	134.4	-	49.7	-
9	132.2	-	145.7	-
10	37.2	-	34.9	-
11	21.1	-	118.2	5.2 (1H, t)
12	29.3	-	29.3	-
13	44.0	-	43.3	-
14	49.7	-	51.0	-
15	29.7	-	31.2	-
16	27.4	-	27.3	-
17	47.0	-	47.2	-
18	15.8	0.98 (3H, s)	16.5	-
19	19.6	1.04 (3H, s)	18.5	-
20	47.4	-	48.2	-
21	181.3	-	181.5	-
22	32.4	-	32.4	-
23	26.9	-	25.3	-
24	123.5	5.33 (1H, t)	123.6	5.33 (1H, m)
25	132.7	-	132.2	-
26	17.6	1.62 (3H, s)	17.6	1.62 (3H, s)
27	25.7	1.67 (3H, s)	25.7	1.65 (3H, s)
28	21.3	1.14 (3H, s)	21.8	1.21 (3H, s)
29	26.6	0.99 (3H, s)	27.7	0.92 (3H, s)
30	24.5	1.05 (3H, s)	22.7	1.02 (3H, s)

Table 2. Inhibitory activity (IC_{50} mg/ml) of the isolated compounds on platelet aggregation induced by thrombin, ADP and epinephrine.

Platelet agonist	IC_{50} (mg/ml)			
	1	2	1 and 2	CE
Thrombin	0.99 ± 0.25	1.04 ± 0.31	0.88 ± 0.53	0.67 ± 0.11
ADP	> 10 ± 1.23	8.54 ± 0.75	4.53 ± 0.94	0.84 ± 0.29
Epinephrine	1.08 ± 0.10	2.70 ± 0.58	>10 ± 0.73	0.72 ± 0.44

Data were expressed as mean ± SEM, n = 3. The chloroform extract (CE) and a 1:1 mixture of the two compounds (1 and 2) were used for comparison.

The ^{1}H-NMR of the isolated compound 2 (Figure 1) followed by the triterpenoid pattern with a large clusters of signals of CH_3, CH_2 and CH between the δ 2.5 and 0.8. The ^{13}C-NMR did help to further analyze the compound. The presence of four olefinic carbons between 145 and 118 ppm, a carboxylic carbon and five quaternary carbons assisted in suggesting a lanosteryl skeletal structure (Vincken et al., 2007). Detailed assignment of the ^{13}C-NMR and significant ^{1}H-NMR is presented in Table 1. These data were compared to those in literature of a 3α-hydroxylanosta-8,24-dien-21-oic acids (Keller et al., 1996) (Appendix 1 to 9).

Despite the fact that the activity of the isolated compounds was relatively not as high as that of the crude extract, the isolated triterpenes showed good anti-platelet aggregation activity (Table 2) induced by the three platelet agonists (thrombin, ADP and epinephrine). The highest activity by compound 1 (IC_{50} of 0.99 mg/ml) was observed on the thrombin-induced platelet aggregation. Compound 2 also showed a weak inhibition (33.6%) only at the highest concentration (10 mg/ml) on arachidonic acid induced platelet aggregation. The lower activity relative to the crude extract could indicate the synergistic effect with other compounds, decomposition during fractionation, or removal of protective matrix. A number of triterpenoids from various plants have been reported to have anti-platelet aggregation (Jin et al., 2004; Yang et al., 2009; Sankaranarayanan et al., 2010).

Free radicals are implicated in various human diseases including cardiovascular diseases (Atawodi, 2005). They stimulate platelet aggregation by interfering with several key steps of platelet functions (Ambrosio et al., 1997; Bakdash and Williams, 2008). Thus, compounds with antioxidant activity may help reduce the risks of atherothrombotic diseases. The beneficial effects of antioxidants on the inhibition of platelet activation and aggregation have previously been reported (Krotz et al., 2004; Sobotková et al., 2009). Some triterpenes have been reported to possess antioxidant properties (Sekiya et al., 2003; Russel and Paterson, 2006). The antioxidant activity of the isolated triterpenes was also investigated. Both compounds (1 and 2) exhibited poor (<20%) antioxidant activity as they weakly scavenged DPPH and ABTS radicals, and had low reduction potentials. Zhou et al. (2008) also reported a weak free radical scavenging activity exhibited by the lanostane-type triterpenes isolated from *Poria cocos.* The results suggest that the anti-platelet aggregation activity of the compounds could be mediated through mechanisms other than antioxidant activity.

Inflammation also significantly contributes to platelet hyperaggregation and consequent blood coagulation. Owing to the link between blood coagulation and inflammation, compounds with anti-inflammatory activity may significantly prevent platelet hyperaggregation. Some lanostane-type-triterpenoids are known to have anti-inflammatory activities (Ríos et al., 2000; Biswas et al., 2009). The compound 2 exhibited anti-inflammatory activity in a concentration and time dependent fashion as it reduced the rat paw oedema volume (Figure 2). The most significant activity ($P < 0.05$) relative to the control group was observed after 2 to 3 h with the 500 mg/kg sample. The carrageenan-induced paw oedema model is suitable for the evaluation of acute anti-inflammatory agents acting by inhibition of cyclooxygenase (COX) which is involved in the synthesis of prostaglandins (Seibert et al., 1994; Mossa et al., 1995; Sawadogo et al., 2006). Carrageenan-induced paw oedema is believed to involve two phases in which, the first phase (1 h) involves the release of inflammatory mediators, histamine and serotonin while the second phase (over 1 h) is mediated by prostaglandins (Vinegar et al., 1969). The significant anti-inflammatory activity exhibited by compound 2 at 2 to 3 h (Figure 2) is typical of inhibitors of arachidonic acid metabolites synthesis. Since the compound inhibited the inflammatory process in a way similar to that of the non-steroidal anti-inflammatory drug (NSAID) (indomethacin) known to be COX-1 and 2 inhibitor, the compound could possibly be exerting its therapeutic activity through the inhibition of COX-1 and/or COX-2 (Esteves et al., 2005). It is apparent that the anti-platelet aggregation activity of the compound could partly contribute to its anti-inflammatory activity.

The ability of compound 2 to inhibit inflammation and arachidonic acid-induced platelet aggregation which is mediated by thromboxane A_2 and prostaglandin H_2 (Parise et al., 1984), indicates its potential to be developed into a good pharmacological anti-platelet and

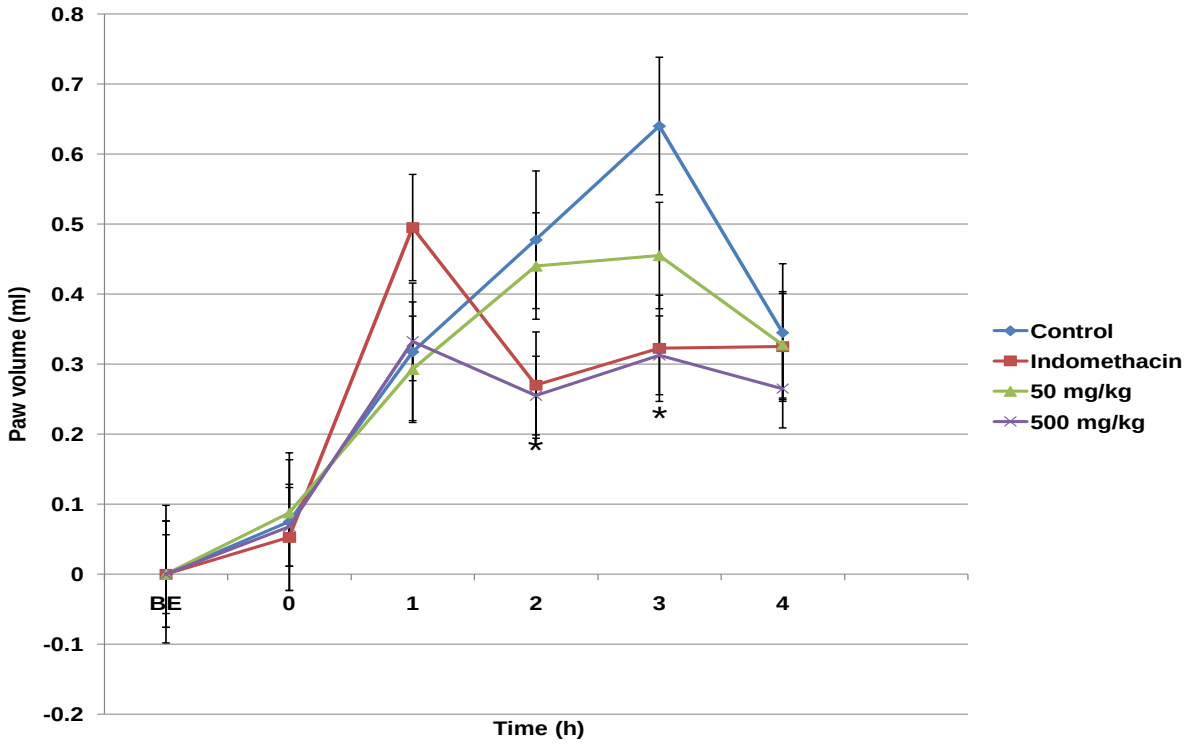

Figure 2. Effects of compound 2 from the chloroform extract of *P. longifolia* on carrageenan-induced paw edema in rats. Paw oedema volume was measured immediately before and at 1 h interval for 4 h following carrageenan injection. Data was expressed as mean ± SEM, n= 4. *****P < 0.05 compared to the control group. (*BE*- before experiment).

Table 3. Effect of compound 2 on *in vitro* clotting time (min) of rat's whole blood.

Well	Clotting time (min)
A (1% DMSO + blood)	< 1
B (thrombin + blood)	< 1
C (ADP + blood)	< 1
D (epinephrine + blood)	< 1
E (arachidonic acid + blood)	< 1
F: (compound (1 mg/ml) + blood)	< 1
(compound (3 mg/ml) + blood)	> 5
(compound (10 mg/ml) + blood)	> 5

The results are presented as time (min) it took the blood to clot in the presence and absence of the compound. The experiment was carried out in duplicate using a 96-well microtitre plate.

anti-inflammatory drug. The efficiency of compound 2 to prevent blood-clotting is further evidenced by its ability to considerably delay the *in vitro* clotting time of the rat's whole blood (Table 3).

A number of reports indicate a considerable high cytotoxicity of triterpenes (Lee et al., 2007; Peteros and Uy, 2010). However, compound 2 exhibited weak cytotoxic effects on HEK293 and HepG2 (IC_{50} 8520 and 7960 µg/ml, respectively). According to the American National Cancer Institute guidelines, a compound is only considered significantly active with IC_{50} value less than 30 µg/ml (Suffness and Pezzuto, 1990). While triterpenes are considered potential anticancer agents (Su et al., 2000; Gonzalez et al., 2002; Lin et al., 2003; Zhou et al., 2008; Cheng et al., 2010), compound 2's weak inhibition of hepatocellular carcinoma growth may indicate lack of anticancer properties. An insignificant cytotoxicity of some lanostane triterpenoids against tumour cell lines has also previously been reported (Kima et al., 2004; Shao et al., 2005). The cytotoxicity level of the isolated compound encourages the development of this compound into a pharmacological anti-platelet aggregation drug.

It is apparent that the triterpenes (3-Oxo-5α-lanosta-8,24-dien-21-oic acid (1) and 3β-Hydroxylanosta-9,24-dien-24-oic acid (2)) from the chloroform extract of *P. longifolia* possess anti-platelet aggregation activity. The anti-platelet aggregation activity of compound 2 could be linked to its anti-inflammatory activity which is indicative of a cyclooxygenase inhibitor. The two triterpenes could be potential pharmacologically active anti-platelet aggregation agents. The results give a rationale for the use of *P. longifolia* in folk medicine to manage blood-clotting related diseases. Further work is required to elucidate the

possible mechanism in which these compounds exert their therapeutic activities.

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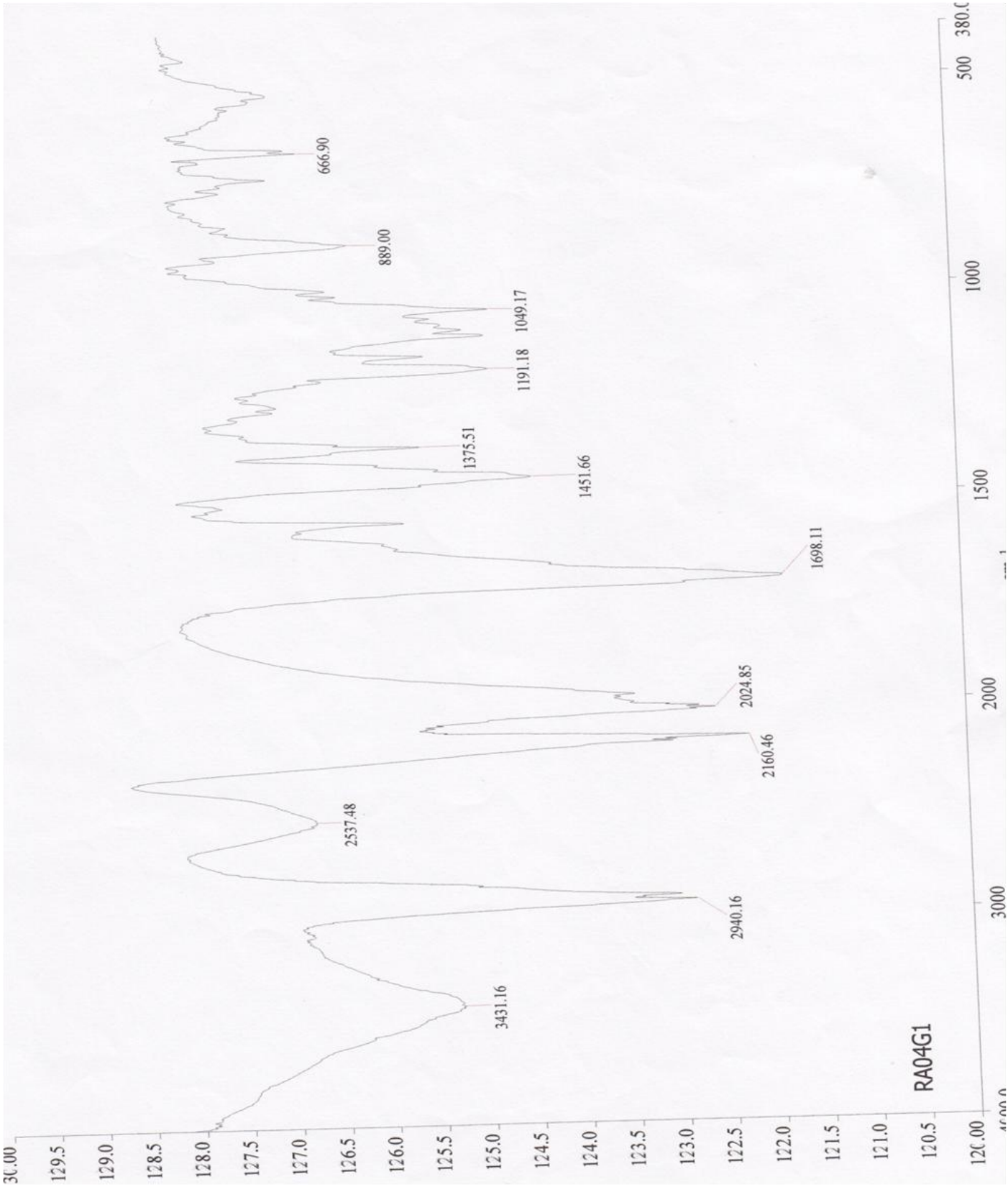
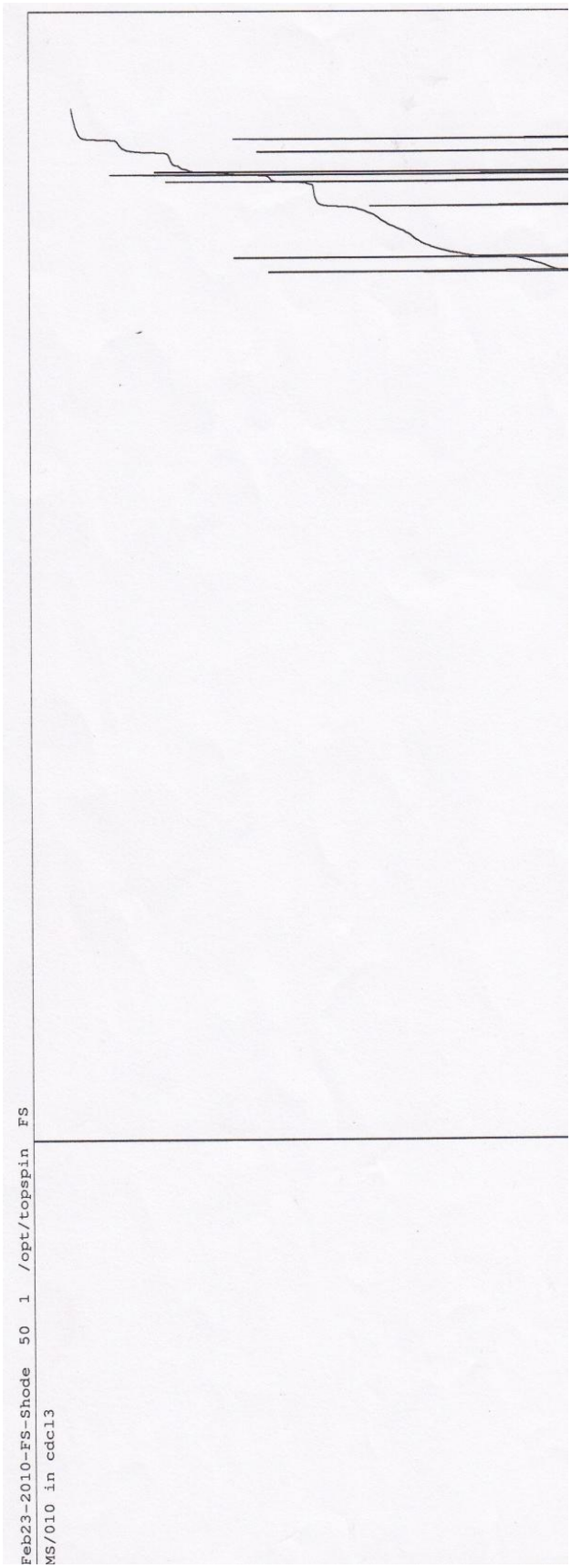
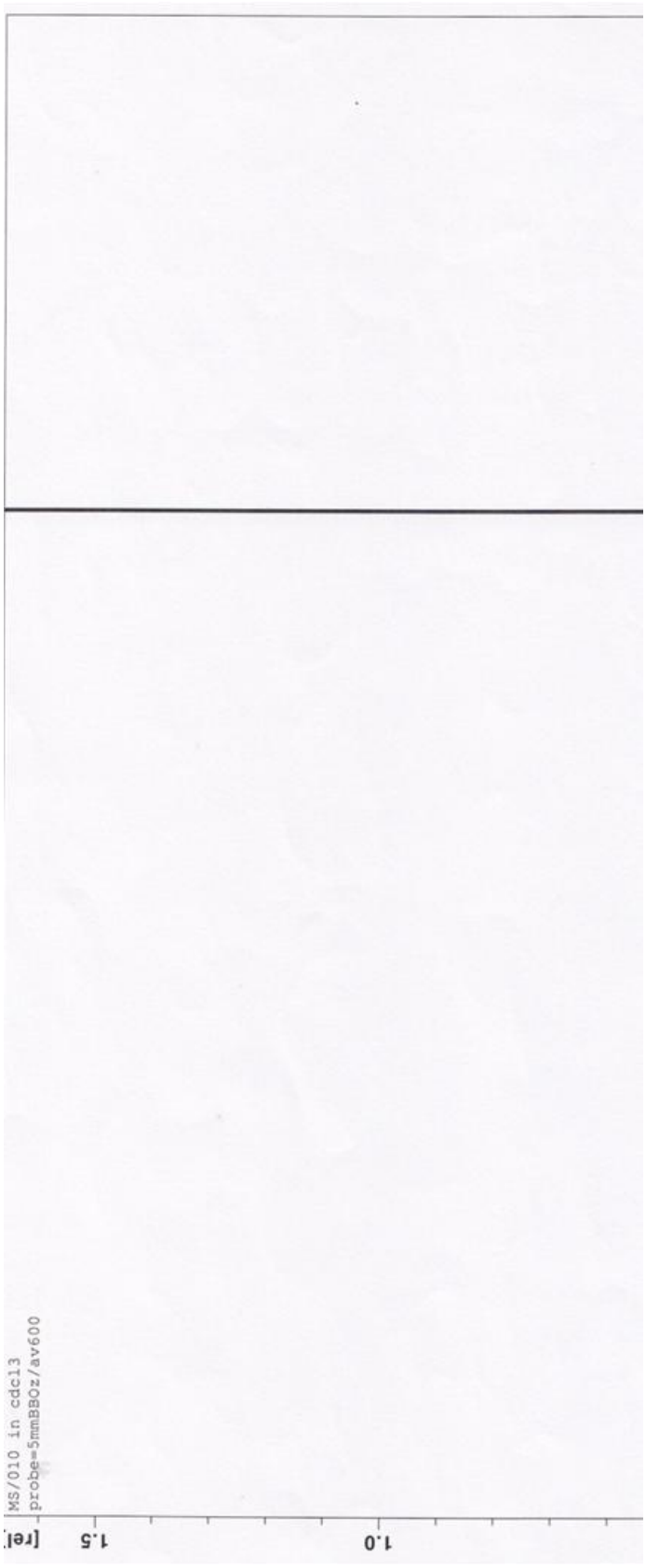
SUPPORTING DOCUMENTS (APPENDICES)



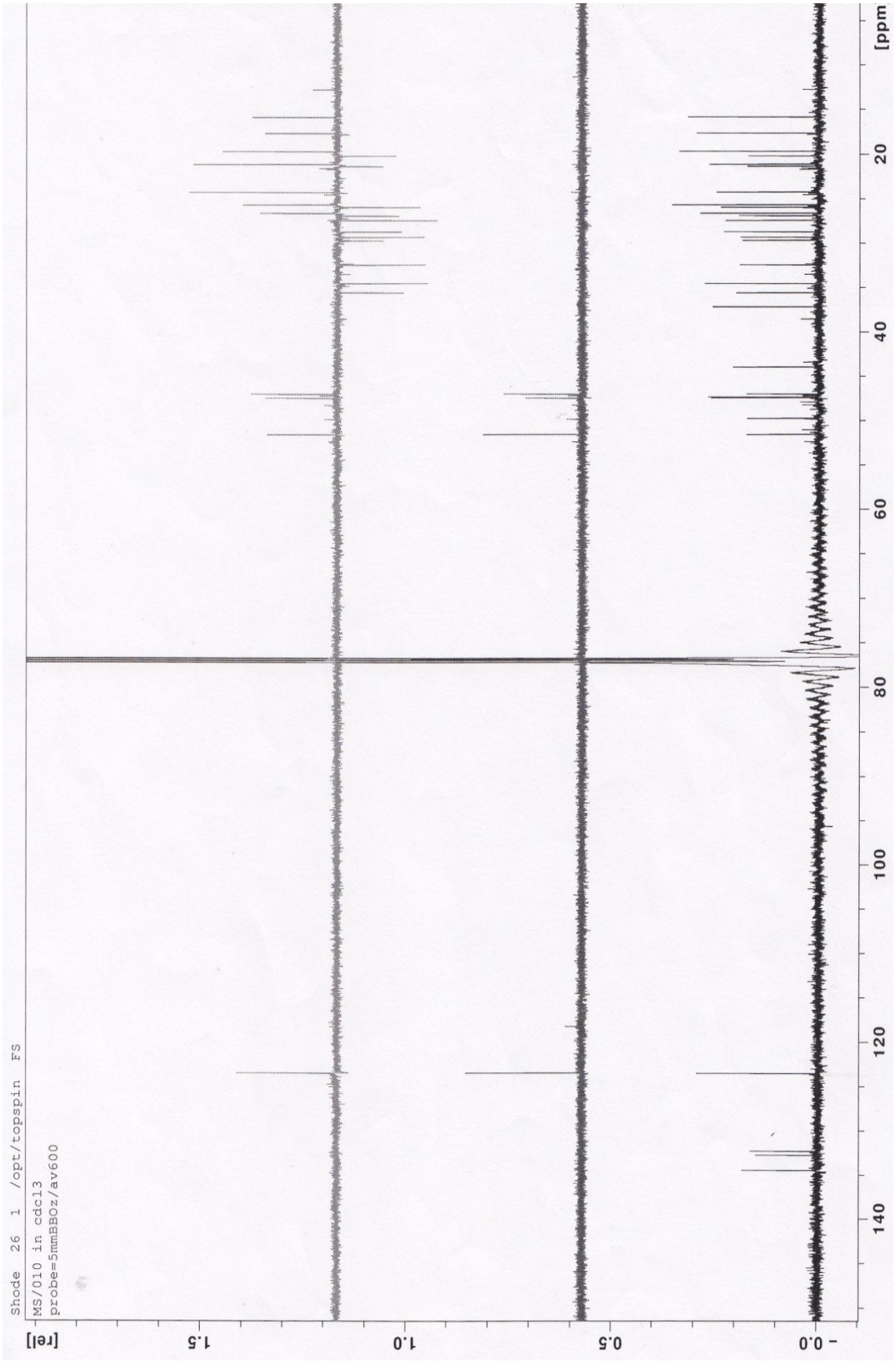
Appendix 1 IR spectrum of compound 1.



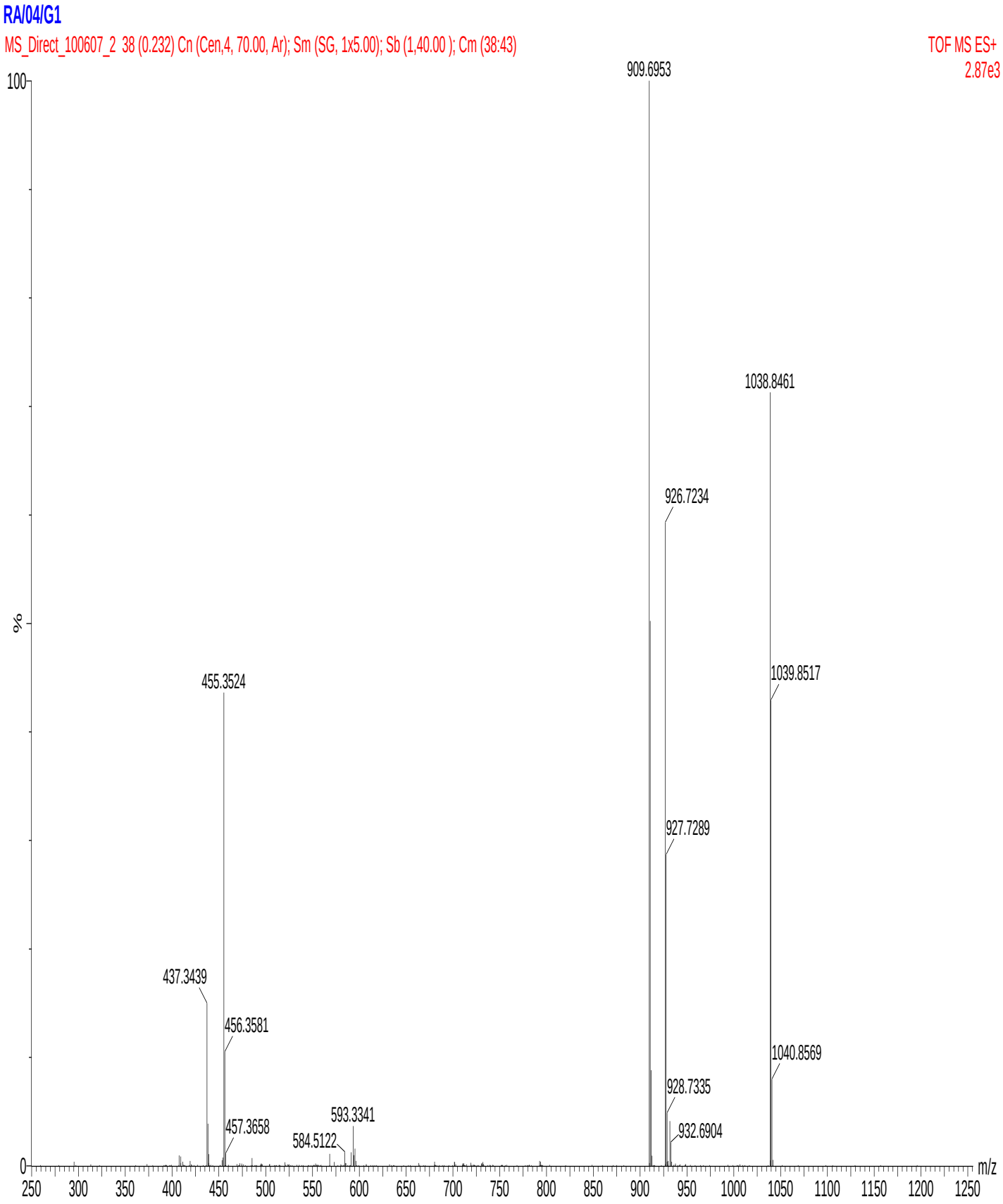
Appendix 2. ^{1}H-NMR spectrum of compound 1.



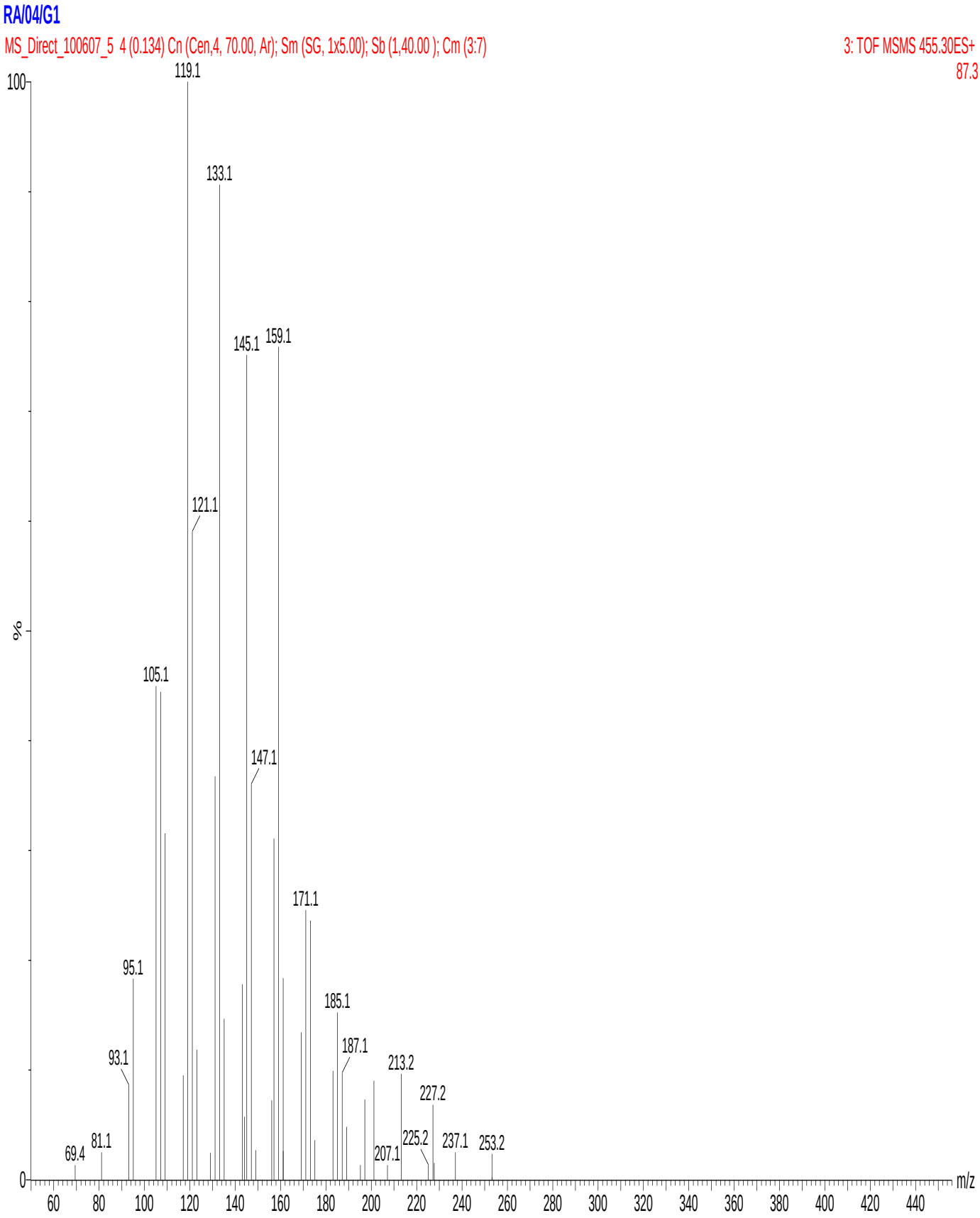
Appendix 3. ^{13}C-NMR spectrum of compound 1.



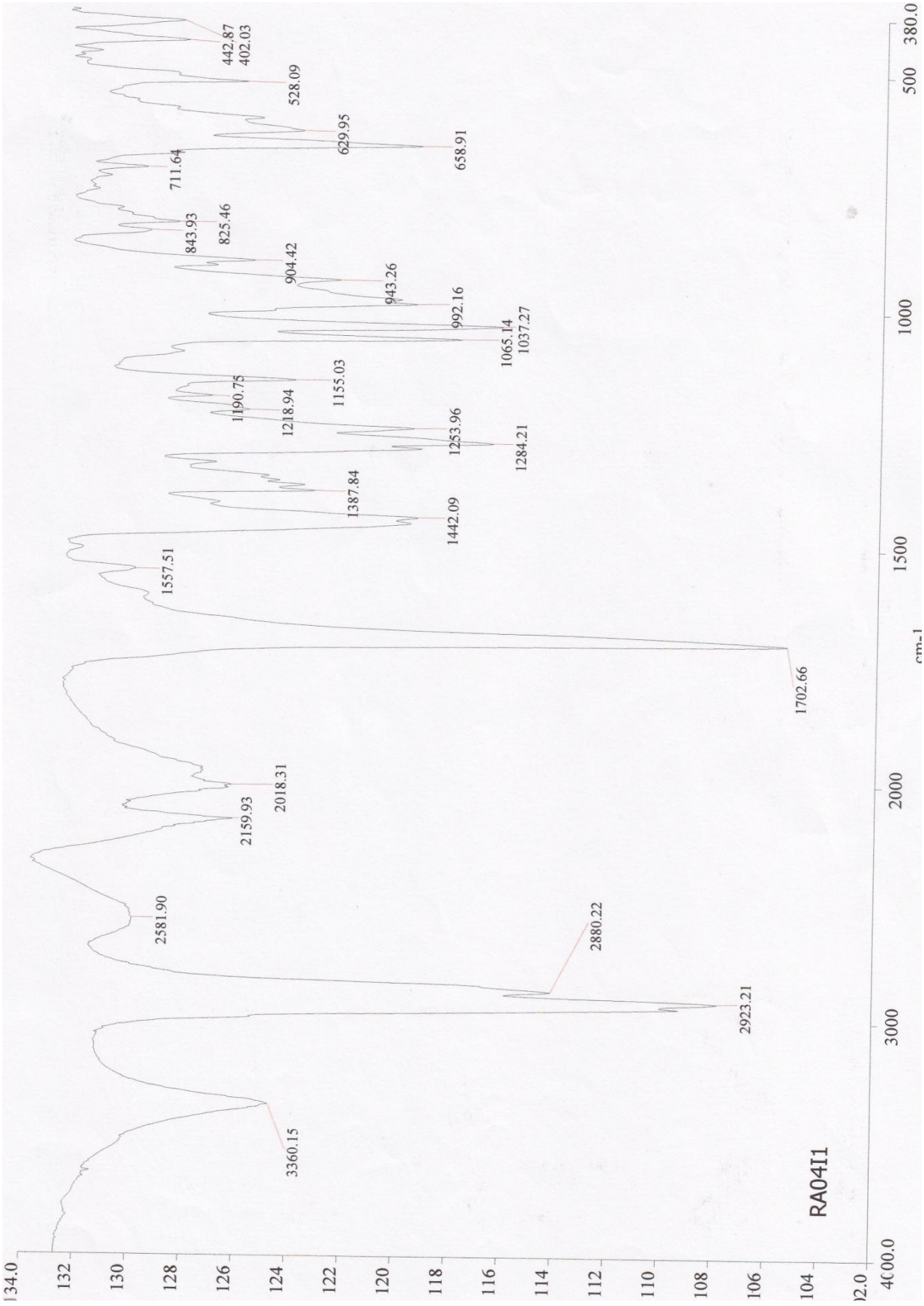
Appendix 4. ^{13}C-NMR, DEPT 90 and 135 spectra of compound 1.



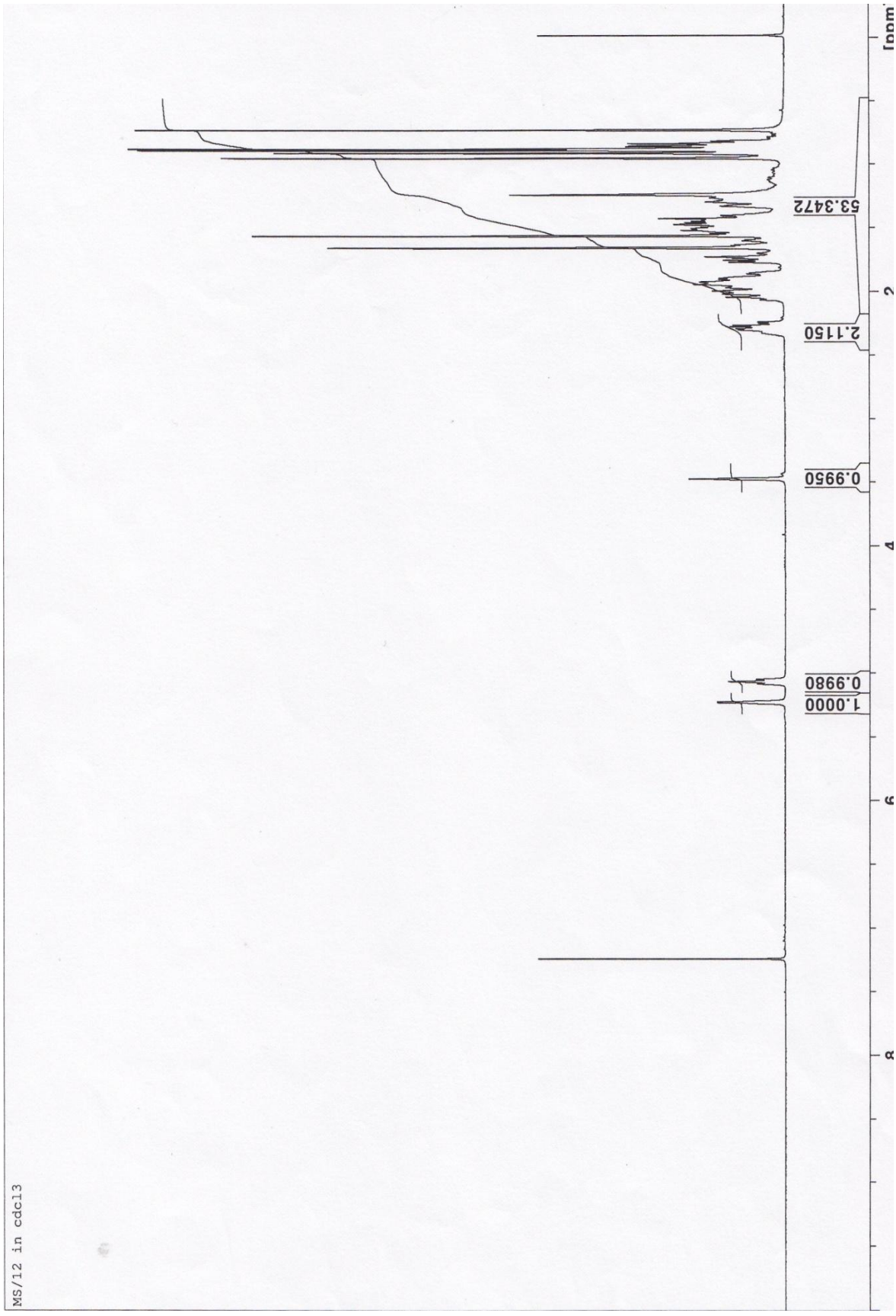
Appendix 5a. MS spectrum of compound 1.



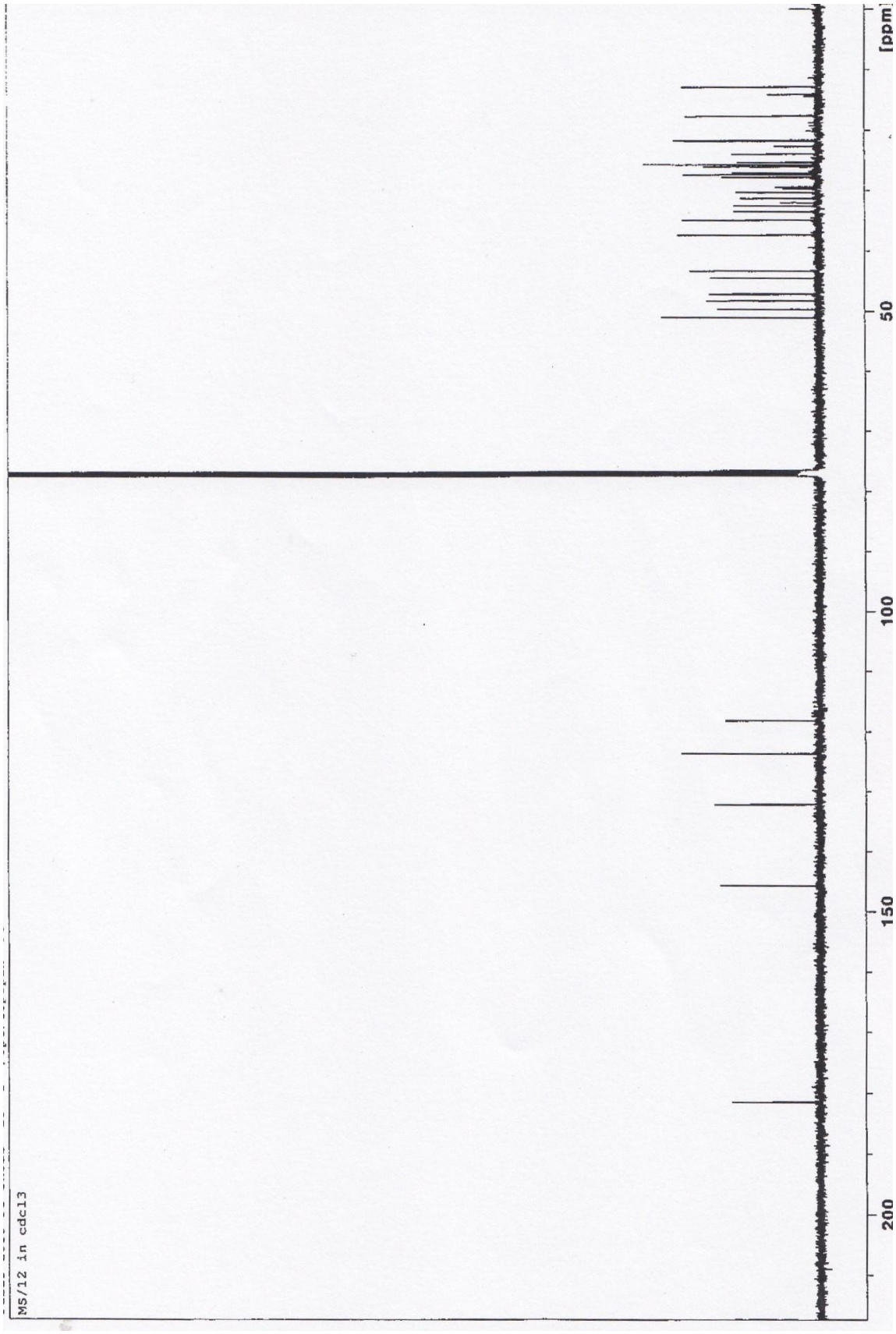
Appendix 5b. MS spectrum of compound 1.



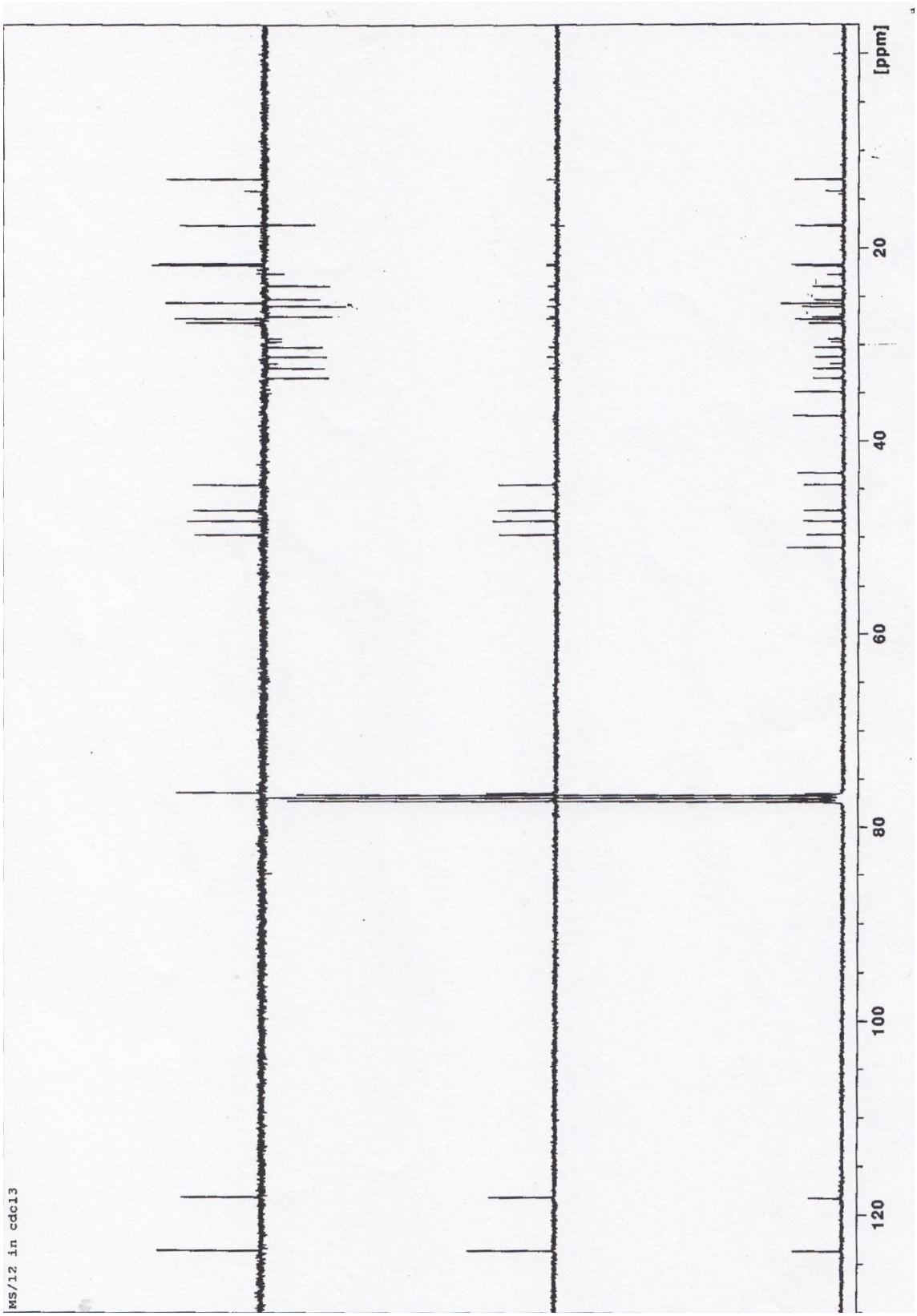
Appendix 6. IR spectrum of compound 2.



Appendix 7. ^{1}H-NMR spectrum of compound 2.



Appendix 8. ^{13}C-NMR spectrum of compound 2.



Appendix 9. ^{13}C-NMR, DEPT 90 and DEPT 135 spectra of compound 2.