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RESEARCH PAPER

The Main Bioactive Constituents of Traditional Kurdish Plant *Achellia oligocephala* DC.; their Antiproliferative and Antioxidant Activities.

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A B S T R A C T:
Achellia oligocephala DC. , which grows in the mountains, is very popular as wound healing and gastrointestinal complaints. This research article reports the first study of the phytochemical investigation and biological properties of bioactive secondary metabolites from the traditional Kurdish plant *Achellia oligocephala*. (+) - Luteolin-6-C-glucoside (AO1) and Lupeol (AO2) were isolated from aerial parts for the first time from this plant. The antiproliferative activity was measured against three human tumour cell lines, MCF7, SkBr3, and BG-1 cancer cells by using the MTT assay. Notably, the (+) - Luteolin-6-C-glucoside showed significant antiproliferative activity against MCF7 cancer cell line, IC_{50} value (10 µg/mL). The antioxidant activity of AO1 and AO2 were evaluated on total antioxidant capacity (TOAC) test. Interestingly, its showed a remarkable antioxidant activity compared to standard antioxidant. This study confirms that (+) - Luteolin-6-C-glucoside can be considered a natural anticancer and antioxidant compound.

KEY WORDS: *Achellia oligocephala* DC., (+) - Luteolin-6-C-glucoside (isoorientin), Traditional medicinal plant, Antiproliferative and Antioxidant activity.
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1. INTRODUCTION

A large number of higher plants have been used by local inhabitants all over the world as ethnobotanical healings; many of them have yielded important pharmaceutical lead compounds after recent scientific investigations. The Middle East region of the Asian continent is particularly rich in medicinal plants, and most of them have not been investigated yet for their metabolite contents (Mükemre et al., 2015).

The region of Iraqi Kurdistan is a particularly little studied zone as concerns ethnobotanical and phytochemical investigation. Several plants are currently used by local people for their medicinal properties. (Braiem et al., 2017).

Approximately 140 species in the world represent *Achillea* species (Asteraceae). In folk medicine, these species are used as herbal remedies due to their anti-inflammatory, analgesic, antispasmodic, digestive, wound healing, hemostatic and cholagogue effects (Şabanoğlu et al., 2017).

Achellia oligocephala DC. which grows on mountains around choman in Erbil city of Iraq, especially on Halgurd Mountain (Amin et al.,

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2016); It is commonly used by local people for treating wound healing and gastrointestinal complaints: yet the phytochemical constituents and the evaluation of biological properties of *Achellia oligocephala* have not been investigated.

To this aim, In this research article, I report the main bioactive constituents of the non-volatile fractions from the aerial parts, and the evaluation of their cytotoxic activity on three human cancer cell lines, as well as their antioxidant in vitro activity for the first time.

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Figure 1: *Achellia Oligocephala DC.*

2. MATERIALS AND METHODS

2.1. General

For most general experimental techniques and procedures see (Gilardoni et al., 2015), all solvents that have been used in this research were of HPLC and the analytical grade was purchased from Carlo Erba (Milano, Italy). Thin-layer chromatography was performed with both reversed phase: TLC on Merck 60 RP-18 on aluminum plates with 0.2 mm, and direct phase: TLC on silica gel Fluka on aluminum plates with 0.2 mm, (Germany), and stained with 5 % H_2SO_4 in MeOH before heating. Ultraviolet (UV) lamp: fluorescent lamp at 254 and 366 nm; cammag (Italy). Rotavapor: IKA rotation instrument. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with Bruker instrument (300 and 400 MHz), chemical shifts (δ, ppm) of AO1 were reare relative to deuterated MeOH signals at δ 3.27 (central line of a quintuplet) and at d(C) 49.0 (central line of a septuplet), respectively. Moreover, AO2 referenced against solvent signals (^{1}H-NMR: $CDCl_3$ δ=7.26; ^{13}C-NMR: $CDCl_3$ δ=77.02). Preparative middle pleasure liquid chromatography (MPLC) separations were carried out on a Biotage one Isolera instrument (Sweden).

2.2. Plant Material Collection

Aerial parts of *Achellia oligocephala* DC. for this study were collected on Halgurd Mountain-Erbil on the beginning of April 2018. The plant was identified and classified (accession number 7236) by Professor Abdulhussain Al-Khayyat in Erbil, Iraq (Amina et al., 2016).

2.3. Extraction, Purification and Isolation

The collected aerial parts of *A. oligocephala* were allowed to dry for three weeks in the well-ventilated shade. Then, the air-dried samples were grinding into fine particles. *Achellia oligocephala* powdered aerial parts (500 g) was extracted exhaustively at ventilated room temperature by maceration with 1 L of MeOH for 10 days with occasional shaking and followed by filtration. The methanol filtrate was concentrated to yield the methanol extract (8.9 g). This was partitioned with liquid-liquid extraction between distilled water and ethyl acetate (1:1) to afford an EtOAc fraction (2.55 g) as the organic phase. The aqueous layer (non-organic) was further partitioned against n-butanol to give a soluble fraction in n-BuOH (2.3 g). For this work, only EtOAc soluble fraction was used because of the antioxidant activity of this fraction (Gilardoni et al., 2015) . 1 g of ethyl acetate fraction was separated by a medium pressure liquid chromatography (MPLC) “Isolera ONE” (Biotage) instrument on a direct phase column. A linear gradient was applied from an 80:20 A/B mixture (A = n-hexane and B = ethyl acetate), to 100% solvent B (ethyl acetate), over 40 minutes, at a flow rate of 30 mL/minute, and the wavelength has been detected at UV 254-366 nm. Repeated Coloum chromatography and MPLC on both direct and reversed-phase columns of the chlorophyll-free ethyl acetate extract of aerial parts, afforded AO1 (42.6 mg) and AO2 (29.1 mg).

2.4. Identification of Bioactive Compound (AO1)

Yellow powder; $[\alpha]^{D}_{20}$+1.90 (c=0.021, Methanol); ESI-MS (negative ion mode, m/z): 447 [M-H]- for $C_{21}H_{20}O_{11}$; and its melting point is 244–245°C (Kumazawa et al., 2000). ^{1}H-NMR Spectral Data (300 MHz, CD_3OD): δ 6.49 (1H, s, H-8), 6.55 (1H, s, H-3), 6.91 (1H, d, J=8.5 Hz, H-5′), 7.37 (1H, dd, 1.5 Hz, J=8.5, H-6′), 7.38 (1H, m, H-2′). Sugar moiety; 3.46 (1H, m, H-3″), 3.49 (2H, m, H-4″), 3.50 (2H, m, H-5″), 3.75 (1H, m, H-6a″), 3.90 (1H, dd, J=12.0, 2.0 Hz, H-6b″), 4.19 (1H, t, J=9.0 Hz, H-2″), 4.91 (1H, d, J=9.0 Hz, H-1″). Moreover, ^{13}C-NMR (75 MHz, CD_3OD) δ: 95.47 (C-8), 104.20 (C-3), 105.50 (C-10), 109.46 (C-6), 114.44 (C-2′), 117.08 (C-5′), 120.62 (C-6′), 123.83 (C-1′), 147.34 (C-3′), 151.35 (C-4′), 158.99 (C-9), 162.34 (C-5), 165.16 (C-7), 166.55 (C-2), 184.30 (C-4). Sugar moiety; 63.18 (C-6″), 72.09 (C-4″), 72.87 (C-2″), 75.59 (C-1″), 80.42 (C-3″), 82.94 (C-5″).

2.5. Identification of Bioactive Compound (AO2)

White amorphous powder; ESI-MS [M]+ m/z 426 (Jash et al., 2013), for $C_{30}H_{50}O$; IR (NaCl) λmax 3350, 2946, 1455, 1380, 1265, 739 cm^{-1} and its melting point is 214°C (Gallo and Sarachine, 2009a); ^{1}H-NMR Spectral Data (200 MHz, $CDCl_3$): δ 0.70 (1H, m, H-5), 0.77 (1H, s, H-24), 0.80 (1H, s, H-28), 0.84 (1H, s, H-25), 0.93 (1H, m, H-1b), 0.94 (1H, m, H-15b), 0.95 (1H, s, H-27), 0.98 (1H, s, H 23),1.04 (1H, s, H-26), 1.11 (1H, m, H-12b), 1.18 (1H, m, H-22), 1.26 (1H, m, H-21), 1.30 (1H, m, H-11b), 1.31 (1H, m, H-9), 1.36 (1H, m, H-7), 1.40 (1H, m, H-6b), 1.42 (1H, m, H-18), 1.44 (1H, m, H-11a), 1.48 (1H, m, H-16), 1.57 (1H, m, H-6a), 1.60 (1H, m, H-15a), 1.61 (1H, m, H-2), 1.62 (1H, m, H-13), 1.64 (1H, m, H-1a), 1.68 (1H, m, H-12a), 1.69 (1H, s, H-30), 2.38 (1H, m, H-19), 3.19 (1H, dd, J = 10.1; 5.2 Hz, H-3), 4.57 (1H, br s, H-29b), 4.70 (1H, br s, H-29a). Moreover, ^{13}C-NMR (100 MHz, CD_3OD) δ: 38.7 (C-1), 27.5 (C-2), 79.0 (C-3), 38.9 (C-4), 55.3(C-5), 18.3(C-6), 34.3(C-7), 40.8(C-8), 50.5(C-9), 37.1(C-10), 21.0(C-11), 25.2(C-12), 38.1(C-13), 43.0(C-14), 27.4(C-15), 35.6(C-16), 42.8(C17), 48.0(C-18), 48.3(C-19), 151.0(C-20), 29.9(C-21), 40.0(C-22), 28.0(C-23), 15.4(C-24), 16.1(C-25), 16.0(C-26), 14.6(C-27), 18.0(C-28), 109.3(C-29), 19.3(C-30).

2.6. Anti-Proliferative Activity

2.6.1. Cell Culture

The lung cancer cell line 549 and MCF-7 breast cancer cell line has been kept in DMEM/F-12 medium which augmented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 mg/mL streptomycin/penicillin. Moreover, BG-1 ovarian cancer was cultured in RPMI-1640 medium supplemented with 100 mg/mL streptomycin/penicillin, 10% FBS, and 2 mM L-glutamine, all the materials that mentioned above have been ordered from Invitrogen, Gibco, Milan, Italy. Then the cells were switched to medium without FBS the day before experiments and subsequently they treated in medium supplemented with less percentage of FBS, which was 2.5%.

2.6.2. Cell Proliferation Assay

The isolated compounds (AO1 and AO2) has been tested on the three cancer cell lines proliferation ability. The cell viability was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, the base of this assay depends on the conversion of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to MTT-formazan the special mitochondrial enzyme. In regular growth condition, the tumour cells were seeded in 96-well plates as a quadruplicate and grown until they reach 70% confluency. Subsequently, the gown cells were washed by a physiological buffer solution (PBS) twice once they had attached. After that, they attend to with decimally increasing in concentrations (1–100 μM) of each isolated compound and incubated under humidifying condition 5% CO_2, 37ºC for 48hrs in cell culture medium augmented with 2% FBS. Cancer cell viability was fixed by MTT assay according to the manufacture's protocol (Sigma-Aldrich, Milan-Italy). For each AO1 and AO2 dose exposure, the mean absorbance was determined as a % of the cells treated with plotted sample drug concentration and vehicle absorbance. The drug concentrations was represented by IC_{50} values, that reduced the mean absorbance at 570 nm to 50% of those in the control condition or untreated wells (Mosmann, 1983).

2.7. Antioxidant Activity (Total Antioxidant Power)

The suitable concentration of the isolated compound standards (1, 3, 5, 7 and 10 mg/mL), have been prepared by dissolving the powder of each AO1 and AO2 in distilled water. 0.35 mL of each aliquots were mixed with 3.5 mL of the Antioxidant assay reagent solutions including; (28 mM of sodium phosphate, 0.6 M of H_2SO_4, and 4 mM ammonium molybdate). The solution containing tubes were incubated at 95 ^{0}C for 1 hr and 30 mints as described in detail in (Gülçin et al., 2010, Büyükokuroğlu et al., 2001). Then, the heated tubes were cooled down to room temperature, then the absorbance of each sample was measured at 695 nm against the blank. Total antioxidant capacity was expressed as equivalents of ascorbic acid as descibed else were (Sun et al., 2011, Umamaheswari and Chatterjee, 2008).

2.8. Statistical Analysis

The IC_{50} for all the values in each experiment was determined by linear regression analysis through equation (y=(value)x+(value); where y is 50% inhibition, and X is the value of IC_{50} for each given y value). The data were shown as mean±SD; all the data on both types of antioxidant property tests are the average of three independent experiments. The data has been analysed statically by GrapghPad Prism software program.

3. RESULTS AND DISCUSSION

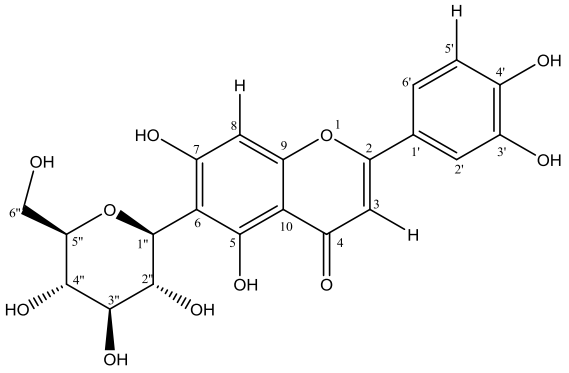
3.1. Phytochemical Investigation

Achellia oligocephala aerial parts have been collected on Halgurd Mountain in Kurdistan region of Iraq. The chlorophyll-free methanol extract of aerial parts have been partitioned between distilled water and ethyl acetate. Repeated normal coloum chromatography and MPLC of the residue on both reversed (C-18) and silica gel phases afforded two main bioactive compounds; (+) - Luteolin-6-C-glucoside (isoorientin) (AO1) and lupeol (AO2) (see figure 2), and structures were established on the basis of their melting point, IR, ESI-MS, ^{1}HNMR, ^{13}CNMR, 2DNMR data and comparison with literature data (Oliveira et al., 2013, Jash et al., 2013) (figures 5-12 in supplementary material). However, this is the first report of these constituents in traditionally used plant *A. oligocephala* DC.

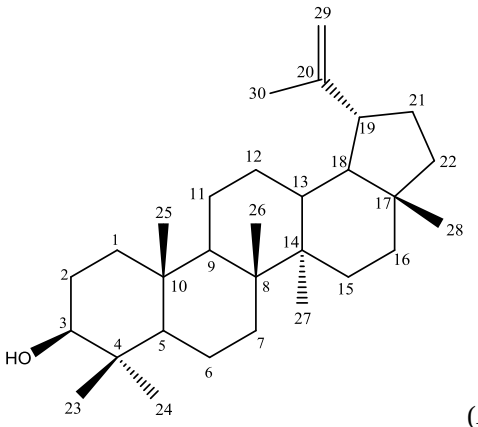
The homogeneity of compound AO1, was proved by a single spot in TLC using R-C18 as adsorbent, TLC (MeOH/H_2O 65:35): R_f= 0.64. AO1 got it as a yellow powder, the ESI-MS spectrum (negative ion mode) showed the molecular ion peak [M]- at 447 *m/z* (Jash et al., 2013), suggesting the formula $C_{21}H_{20}O_{11}$. Moreover, In the ^{1}H-NMR spectrum, the singlet at δ 6.49 (1H, H-8) and the signal at δ 109.46 (quaternary carbon) were attributed to H-6 and C-6 of the 5,6,7- trisubstituted A-ring system of a flavonoid compounds. Moreover, (8H, 4 (OH) of flavone at position 5, 7, 3′, 4′ and 4 (OH) of sugar moiety at position 2″, 3″, 4″, 6″). Thus, the C-7 OH at 7.39 ppm shows cross peaks with carbons C-7 (165.2 ppm), C-8 (95.5 ppm) and C-6 (109.5 ppm), while the other OH group cannot be distinguished from the baseline due to rapid chemical exchange between hydroxyl groups and protic solvents especially in case if you have used MeOH-d as a solvent but Proton exchange rates in alcohol –OH groups can be reduced by dissolving in DMSO-d6 or by supercooling aqueous solutions or by using organic co-solvents. Kontogianni and co-workers investigated in detail correlations between hydrogen bonds and solvent effects of phenol –OH chemical shifts for numerous phenolic acids, flavonoids and oleuropein derivatives (Kontogianni et al., 2013). The C-5 OH resonance in DMSO-d6 is more deshielded in the presence of a C-2–C-3 double bond: luteolin, and apigenin, compared to those molecules without a C-2–C-3 double bond e.g., eriodictyol, and naringenin. This was attributed to the extensive conjugation of the C-2–C-3 double bond and the ring C with the OC-4 carbonyl group which results in a more polarizable CO bond and, thus, a stronger intramolecular hydrogen bond (Charisiadis et al., 2014). On the basis of the melting point, ESI-MS, ^{1}H-NMR,^{13}C-NMR and 2DNMR spectral data (figures 5-9 in supplementary material) and comparison with the literature data (Oliveira et al., 2013, Çalış et al., 2006), component AO1 was identified as luteolin-6-*C*-glycoside, as known as (isoorientin) (figure 2).

Compound 2 was obtained as a white amorphous powder. ESI showed the molecular ion peak [M]+ at *m/z* 426 (Jash et al., 2013),

corresponding to the $C_{30}H_{50}O$ as a molecular formula, [21H ($7CH_3$ group at position C23, C24, C25, C26, C27, C28, C30)], [22H ($11CH_2$ group at position C1, C2, C6, C7, C11, C12, C15, C16, C21, C22, C29)], [6H (6CH group at position C3, C5, C9, C13, C18, C19)], and IH (OH group at position 3). The ^{1}H-NMR spectrum indicated the presence of characteristic protons of seven methyl groups [δH 0.78, 0.81, 0.85, 0.97, 0.99, 1.05 and 1.70], all of them on quaternary carbons. The double doublet at δH 3.21 (1H, *dd*, *J* = 5.2 Hz and 10.1) in the spectrum of AO2 was typical for a triterpenoid compound with a 3-hydroxy substituent. The exocyclic double bond protons resonances were observed at δH 4.59 and 4.71 (1H each, br, singlets). Thus, the structure of compound AO2 was identified as lup-20(29)-en-3β-ol (Lupeol) was definitely confirmed by the close similarity of the melting point with (Gallo and Sarachine, 2009a); IR, ^{1}H-NMR and ^{13}C-NMR with the literature (Garcia et al., 2015, Jash et al., 2013, Adzu et al., 2015) (figures 10, 11 and 12 in supplementary material).



(AO1)



(AO1)

Figure 2: Isolated compounds from *Achellia oligocephala* DC.: (AO1) = - (+) - Luteolin-6-C-glucoside; (AO2) = lupeol.

To the best of our knowledge we determined, for the first time, the structures of two secondary metabolites from *A. oligocephala*: (+) - Luteolin-6-C-glucoside (isoorientin) and lupeol. Metabolites of those classes occur in nature in several families of plants. In addition to many other plants, according to the data reported previously, isoorientin and lupeol has been isolated from the other *Achillea* species; isoorientin isolated from *Achelia nobilis* (Marchart et al., 2003, Krenn et al., 2003). On the other hand, lupeol isolated from *Achillea tenuifolia* Lam (Moradkhani et al., 2014), and *Achillea santolina* (Al-Snafi, 2013). Interestingly, isoorientin is one of the two bioactive compounds isolated from *A. oligocephala,* it is the component of many natural plant extracts, and many other researchers have proved its anti-inflammatory, anti-cancer and antioxidant effects (Kim et al., 2018, Kim et al., 2016, An et al., 2015).

3.2. Biological Activity

3.2.1. Antiproliferative Activity (MTT assay) and Antioxidant Activity (Total Antioxidant Power)

The effects of AO1 and AO2 on the proliferation of three human tumour cell lines, SkBr3, MCF7 breast and ovarian BG-1 cancer cells were evaluated in comparison with the well-known antitumor drug cis-diamminedichloroplatinum (II) (cisplatin) by the MTT assay. AO1 showed a novel cytotoxic activity due to its inhibition activity significantly higher than cisplatin against MCF7 cell line (Table 1). In addition, AO1 compound is more activite than AO2 against SkBr3, MCF7 breast cancer cells, in which the IC_{50} of AO1 was 10 ± (3) , and 24 ± (1) respectively (Table 1).

Table 1: Antiproliferative activity (MTT assay) of AO1 and AO2 from *Achellia Oligocephala*.

	IC_{50}(μM) ± S.D		
Compounds	**MCF7**	**SkBr3**	**TG-1**
AO1	10 (±3)	24 (±1)	>50
AO2	23 (±3)	>50	>50
Cisplatin	17 (±2)	10 (±2)	12 (±3)

The antioxidant properties of the AO1 and AO2 were evaluated by total antioxidant capacity in vitro (TOAC) test compared to the reference ascorbic acid, according to the procedures described in the kind of literature

(Gülçin et al., 2010, Umamaheswari and Chatterjee, 2008). As shown in figures 3 and 4, both bioactive compounds (AO1 and AO2) have antioxidant power and the IC_{50} values against Ascorbic acid standard. Interestingly, the higher activity was exhibited by AO1 were: (AO1, IC_{50} 2.565± 0.001, AO2, IC_{50} 3.72± 0.001 and Ascorbic acid, IC_{50} 0.869± 0.001).

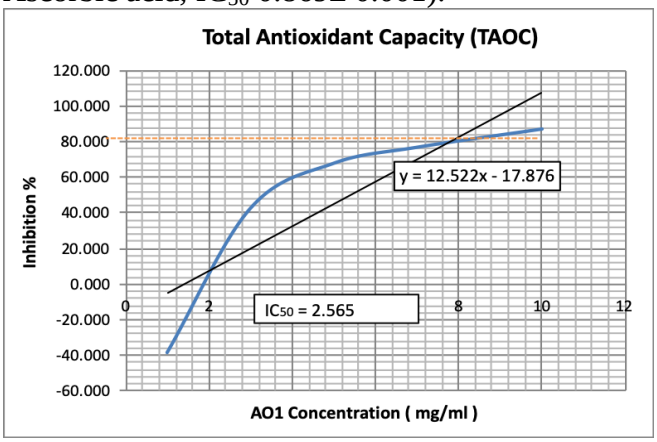


Figure 3: Total antioxidant capacity of AO1, IC_{50} value expressed in mg/mL.

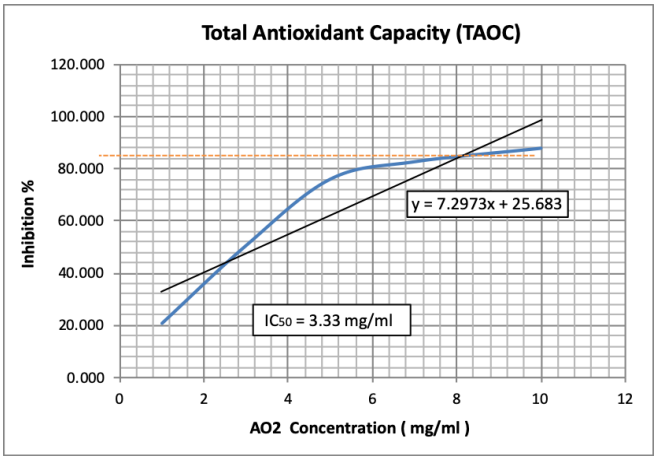


Figure 4: Total antioxidant capacity of AO2, IC_{50} value expressed in mg/mL.

It has been reported that isoorientin showed anti-inflammatory effects by treatment significantly reduces the expression of TNF-α and IL-1β in a concentration dependent manner. NF-$\kappa\beta$ is known to control the expression of cell survival genes, cytokines and proinflammatory markers. Moreover, isoorientin inhibited the LPS induced translocation of NF-$\kappa\beta$/p65 as evidenced by Western blotting (Anilkumar et al., 2017). In addition, Nam and co-workers have reported that isoorientin in KIOM-2015EW may contribute to its anti-inflammatory properties (Nam et al., 2017). According to the literature data, also lupeol was found to possess significant anti-inflammatory activity; Lupeol has been shown to exhibit various pharmacological activities under in vitro and in vivo conditions. These include its beneficial activity against inflammation, cancer, arthritis, diabetes, heart diseases, renal toxicity and hepatic toxicity (Saleem, 2009, Geetha and Varalakshmi, 2001, Agarwal and Rangari, 2003, Gallo and Sarachine, 2009b). Thus, the biological activities of the two isolated pure compounds (AO1 and AO2) corroborated the biological activities of this plant and validate the traditional use of *Achillea oligochephala* in Kurdistan.

1. CONCLUSIONS

This is the first scientific investigation study on the secondary metabolites of *Achellia oligocephala* DC., both from phytochemical and pharmacological points of view. The plant was collected in Kurdistan Region, where it is used as a herbal remedy, especially against wound healing and gastrointestinal complaints. Two bioactive compounds have been isolated from *A. oligocephala*: (+) - isiorientin for the first time from this plant. AO1 showed remarkable cytotoxic activity due to inhibition effects higher than cisplatin against MCF7 human tumour cell line. In addition, AO1 and AO2, showed significant total antioxidant activity compared to standard antioxidant. In conclusion, the current investigation confirms that AO1 can be considered as a potential natural chemotherapeutic agent. Moreover, the biological properties determined for the isolated compounds from aerial parts of *A. oligocephala*, have validated the traditional uses of this plant in Kurdistan.

Acknowledgements

Thanks to Prof. Abdul Hussain Al Khayyat for the botanical classification and identification.

Conflict of Interest (Nothing)

SUPPLEMENTARY MATERIALS:

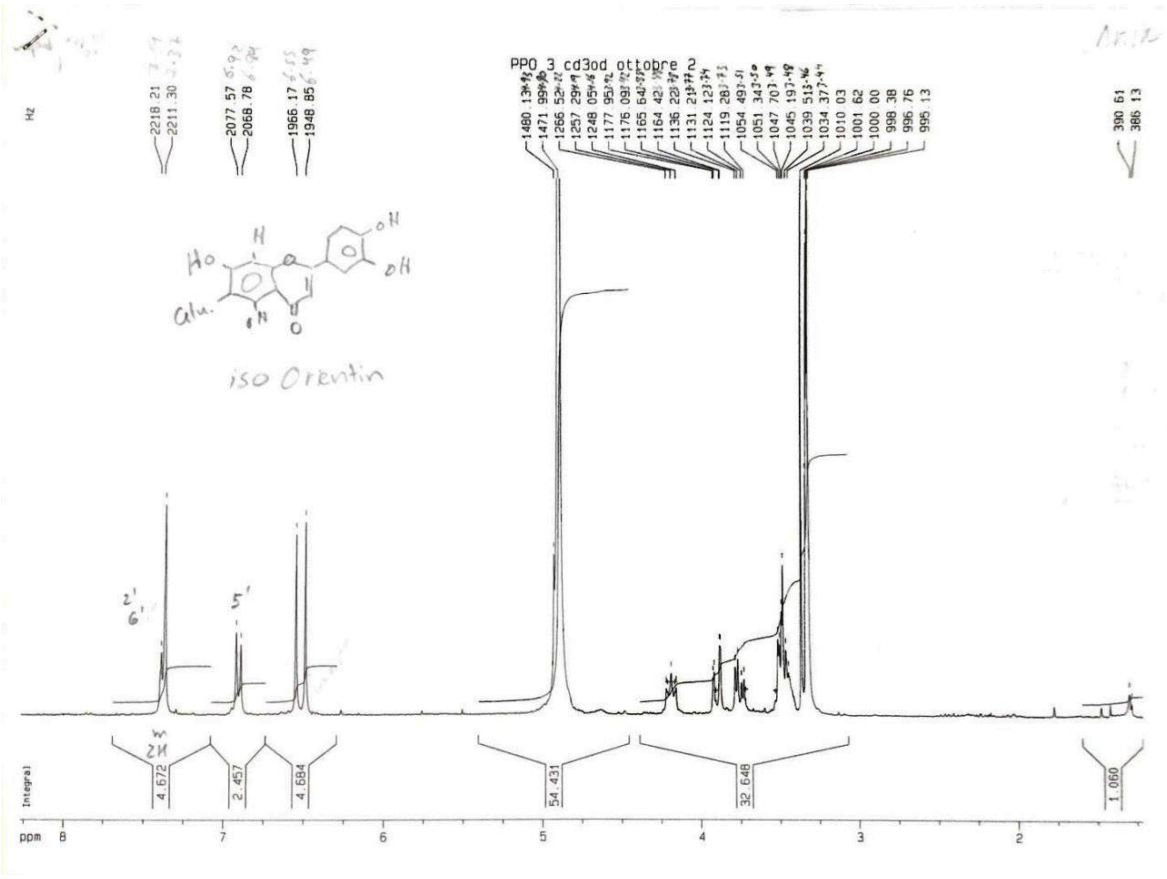


Figure 5: ^{1}H-NMR spectrum (300 MHz) of compound AO1 (in CD_3OD).

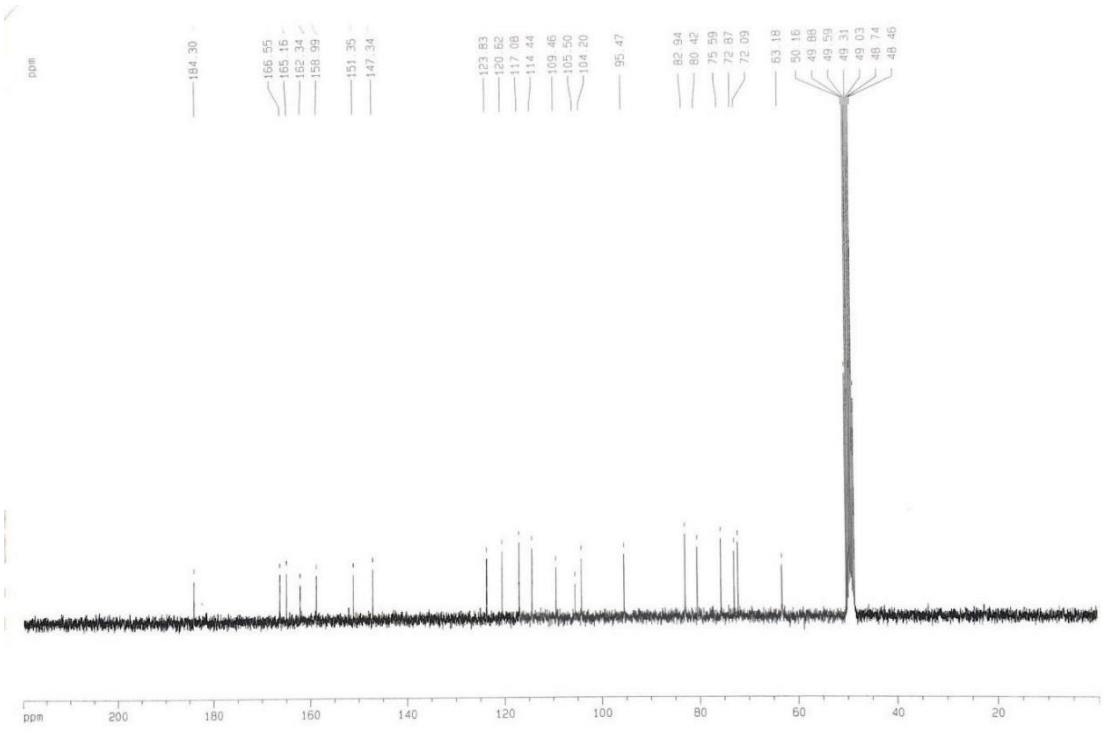


Figure 6: ^{13}C-NMR spectrum (75 MHz) of compound AO1 (in CD_3OD).

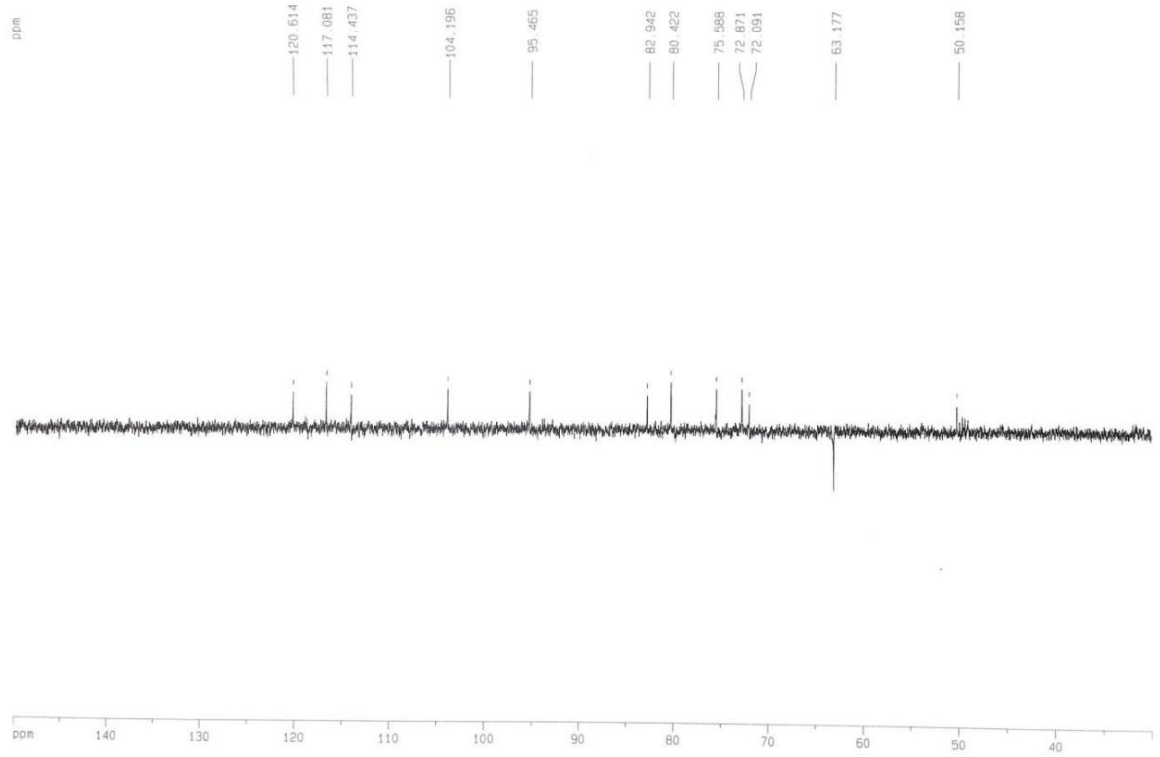


Figure 7: DEPT spectrum (75 MHz) of compound AO1 (in CD_3OD).

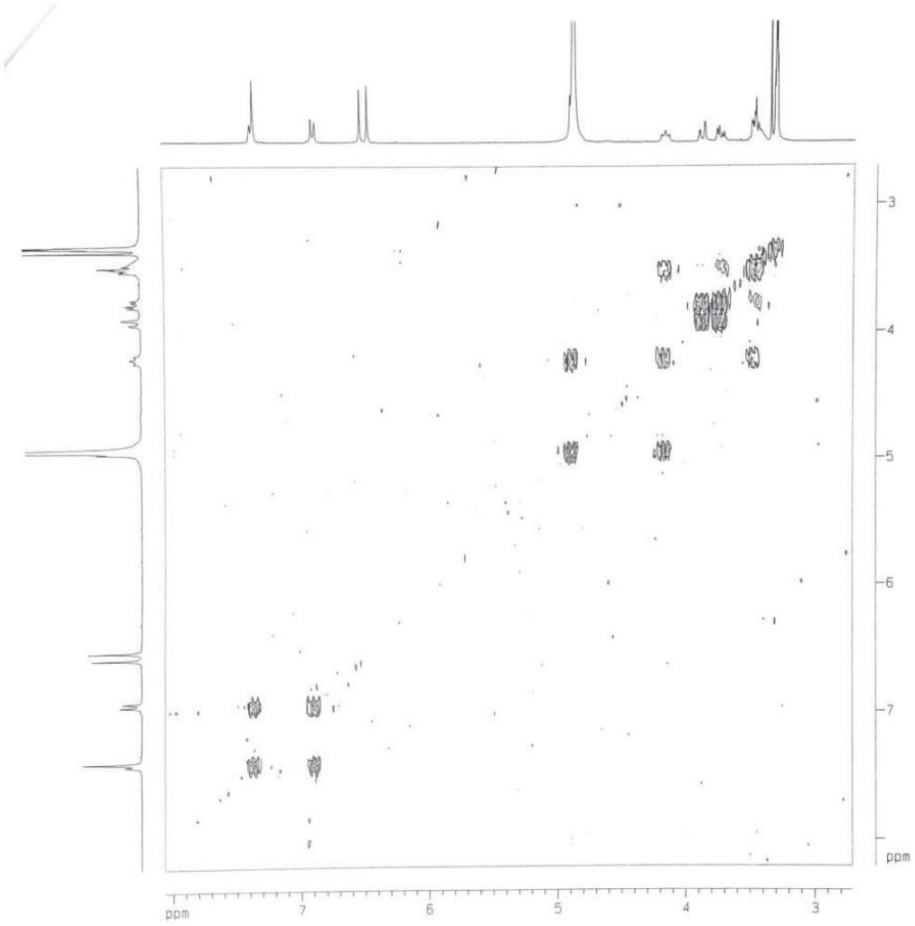


Figure 8: COSY spectrum (300 MHz) of compound AO1 (in CD_3OD).

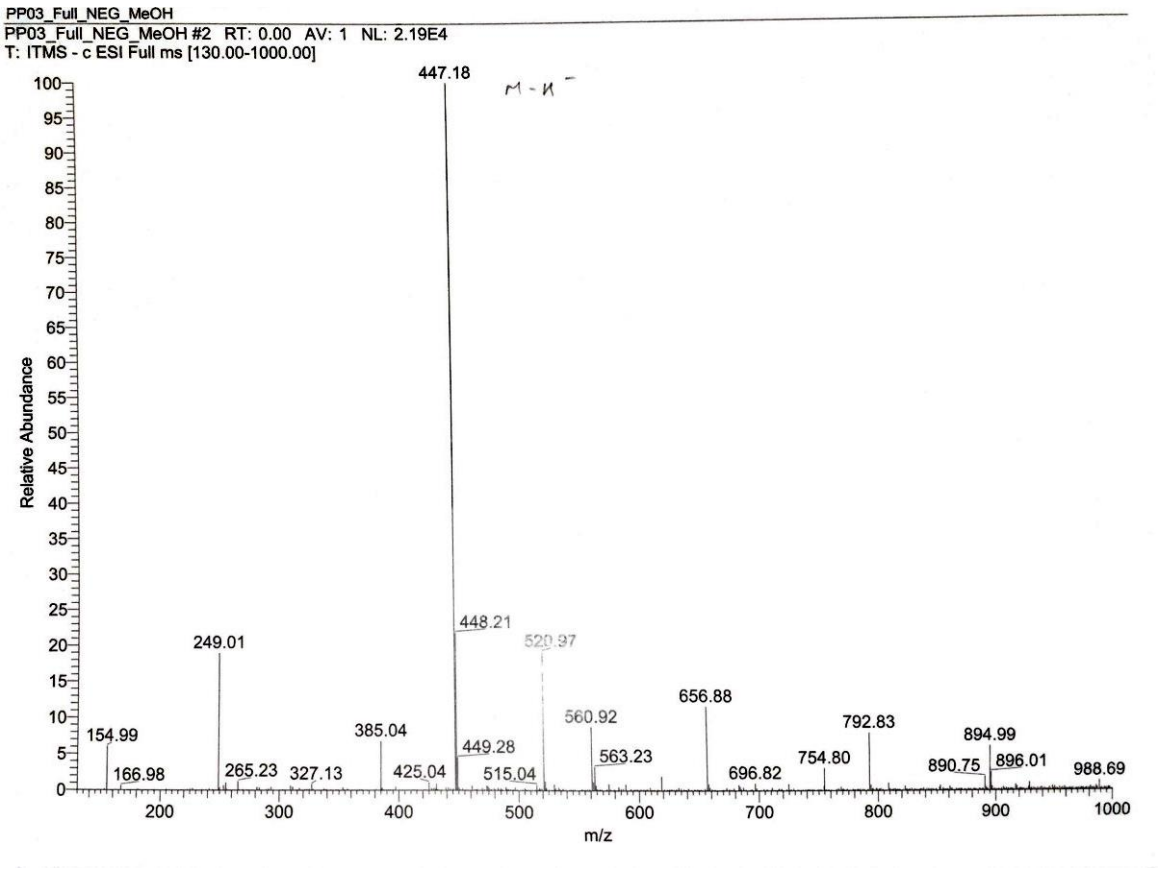


Figure 9: LC-ESI-MS spectrum of compound AO1 measured in negative ion mode.

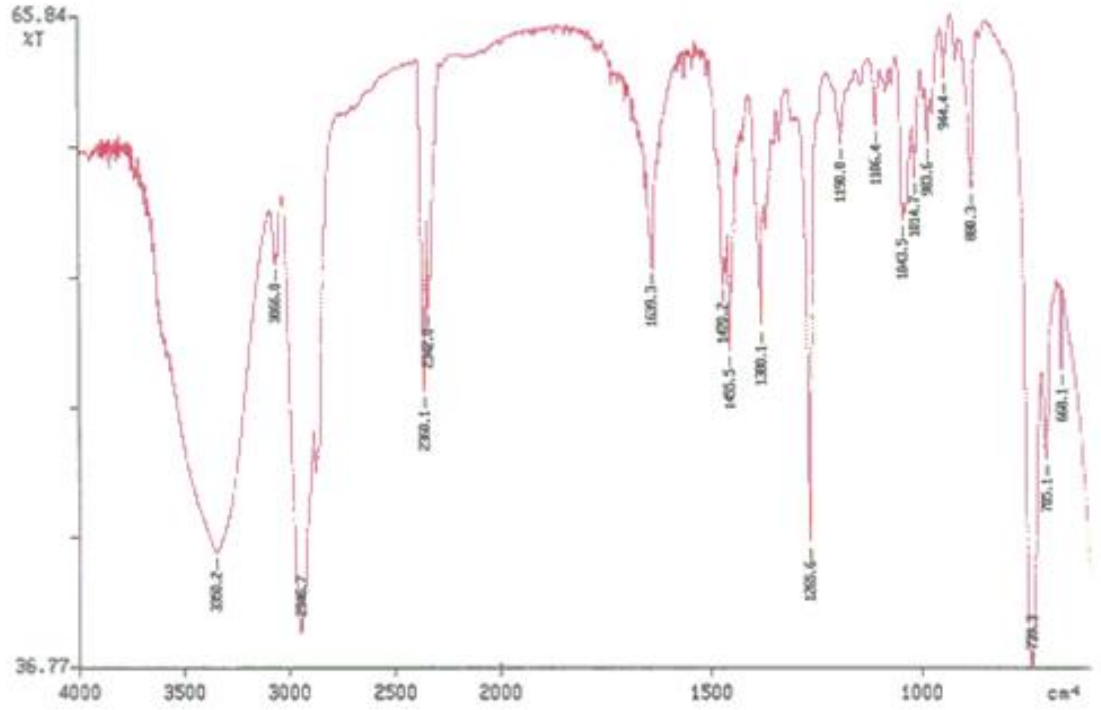


Figure 10: FT-IR spectrum of compound AO2.

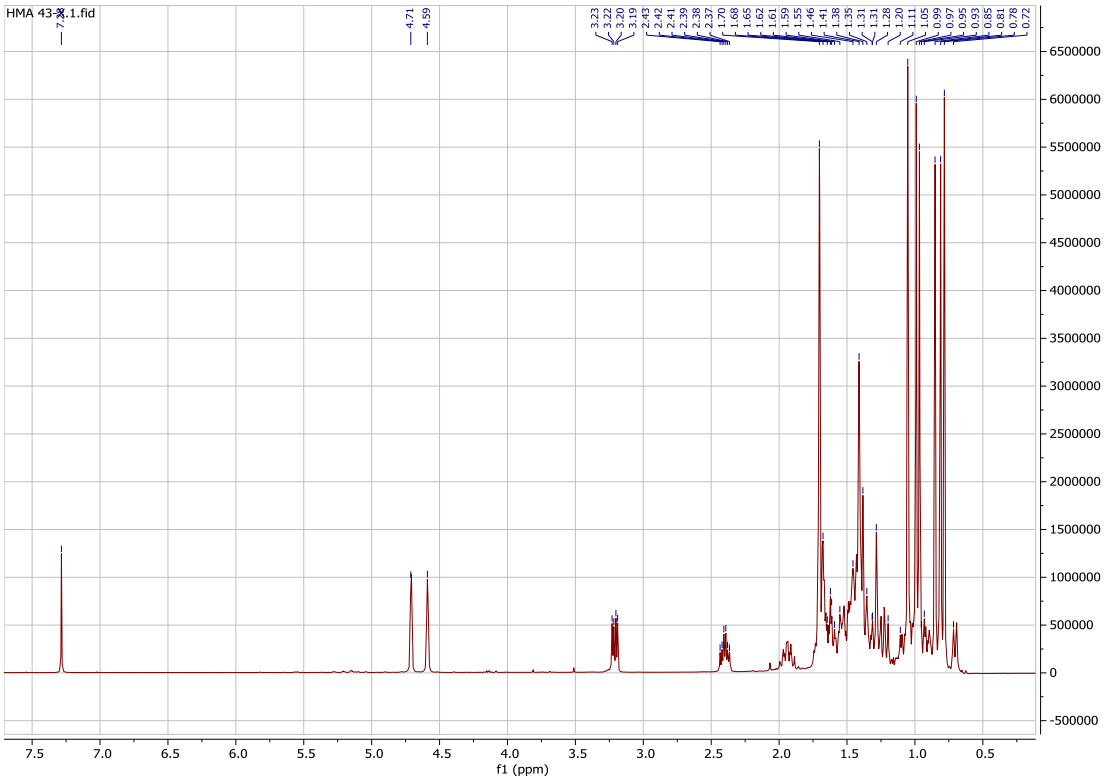


Figure 11: ^{1}H-NMR spectrum (400 MHz) of compound AO2 (in $CDCl_3$).

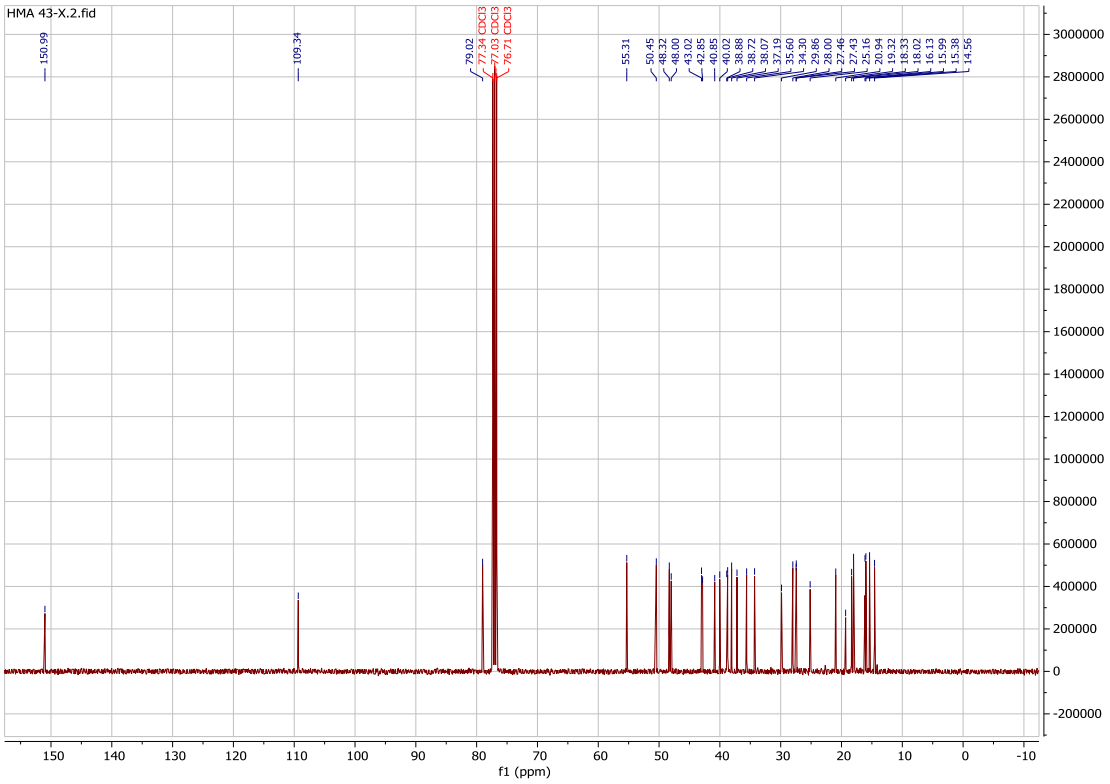


Figure 12: ^{13}C-NMR spectrum (100 MHz) of compound AO2 (in CD_3OD).

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