

Comprehensive Lichenometabolomic Exploration of *Ramalina conduplicans* Vain using UPLC-Q-ToF-MS/MS: An Identification of Free Radical Scavenging and Anti-Hyperglycemic Constituents

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Materials and Methods (with additional details)

General

The NMR spectra were recorded on a Bruker Evans III HD 400 MHz spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C respectively, using 1 pulse programme at 250 °C. Samples were dissolved in Acetone- d_6 and Trimethylsilane (TMS) was used as internal standard. ^1H NMR experiment of each sample recorded with 32 scans and ^{13}C NMR experiment with 68 scans. The chemical shifts are expressed as δ values in parts per million (ppm) and the coupling constants (J) are given in hertz (Hz). The 2D experiments (1H-1H COSY, HSQC and NOESY) were performed using standard Bruker microprograms. Mass data were acquired on Xevo™ G2 XS-ESI-QToF mass spectrometer (Waters Corp., Manchester, UK). For thin layer chromatography (TLC) analysis, precoated Merck plates (silica gel 60 F₂₅₄) were utilized. Silica gel (100-200 mesh) (Qing-dao Marine Chemical, Inc., Qingdao, China) was chosen for column chromatographic separation. Semi-preparative chromatography was performed on Gilson HPLC (Middleton, WI, USA) instrument equipped with 321 binary pump, GX-281 liquid handler and UV-155 detector with X Select HSS T3 (250 mm × 100 mm, 5 μm) (Waters Corp., Ireland) as stationary phase using Trilution LC v2.1 platform. Formic acid (Optima™ Mass spec grade) from Thermo Fisher Scientific (Geel, Belgium), HPLC grade acetonitrile and (LiChrosolv) from Merck (Darmstadt, Germany) and Ultra-pure water (Millipore system, Massachusetts, USA) were used.

Instrumental UPLC conditions

Instrumental conditions were setup as per our recent report (Reddy et al., 2019) with slight modifications. Chromatographic separation was performed on Acquity H Class UPLC system (Waters, Milford, MA, USA) with a conditioned auto sampler, using an ACQUITY UPLC CSH Phenyl-Hexyl column (100 mm × 2.1 mm id., 1.7 μm particle size) (Waters, Milford, MA, USA). Column temperature was maintained at 40°C. High-resolution masses of secondary metabolites were measured after UPLC separation. Mobile phase consisting of water with 0.1% formic acid in water (solvent A) and acetonitrile with 0.1% formic acid (solvent B) was pumped at flow rate of 0.4 mL/min. Gradient elution program was as follows: 0 min, 5% B; 3.00 min, 20% B; 5.00 min, 35% B; 7.50 min, 50% B; 10.00 min, 70% B; 12.50 min, 95% B; 17.00 min 95% B; 21.00 min 5% B. Equilibration time was 4.0 min and injection volume was 2 μL . LC-QToF-MS^E mode was applied to analyze the samples in both TIC as well as MS/MS mode where collision energy was ramped at 15 eV – 45 eV. Eluted compounds were detected from m/z 50 to 1200 using Xevo G2-XS Q-ToF mass spectrometer (Waters, Manchester, UK), which was connected to Electro spray ionization (ESI) interface with negative ion mode using the following instrument settings, capillary voltage, 2.0 KV; sample cone, 40 V; source temperature, 120 °C; desolvation temperature 350 °C; cone gas flow rate 50 L/h; desolvation gas (N_2) flow rate 850 L/h, argon as CID gas for MS/MS experiments. All analyses were performed using the lock spray, which ensured accuracy and reproducibility. Leucine - Enkephalin (5 ng/mL) was used as lock mass, generating a reference ion in negative mode at m/z 554.2615 introduced by a lock spray at 10 $\mu\text{L}/\text{min}$ for accurate mass acquisition. Data acquisition was achieved using MassLynx v4.1. Acquiring data in this manner provided information of intact precursor ions as well as fragment ions.

Lichen Sample collection and identification

The lichen, *Ramalina conduplicans* was collected from tree bark in Bichpuri Range, Bijrani Zone of Corbett National Park, alt. N 29°26'40" E79°04'06 (1283 m.) in the month of May 2019. The morphological features of lichen thallus and ascomata were observed under Magnüs MS 24/13, spot tests for color reaction were carried out by 10% aqueous solution of potassium hydroxide (K), Steiner's stable *p*-phenylene diamine solution (PD) and calcium hypochlorite solution (C). For anatomical investigation of fruiting bodies light microscope of ZEISS Axiostar was used. The lichen substances were identified with thin Layer Chromatography in solvent system 'A' following White and James, 1985. The standard literatures [1] were referred for identification of lichen samples. Voucher specimen (Satish Mohabe & A.Madhusudhana Reddy 7658YVUH) of species was deposited at the Herbarium, Department of Botany, Yogi Vemana University, Kadapa, Andhra Pradesh. Corresponding data are shown in supporting information.

Extraction and isolation

The sorted-out lichen *Ramalina conduplicans* (300 g) was shade dried, powdered and extracted with acetone (6L) at room temperature for 48 h. Resulting acetone extract was evaporated to dryness under reduced pressure affording syrupy residue (20 g). This crude extract was subjected to gradient column chromatography (SiO₂, 60–120 mesh), eluting with hexane/EtOAc mixture of increasing polarity with 10% intervals yielded 8 fractions. These 8 fractions were reconstituted in acetonitrile and subjected to UPLC Q-ToF MS^E. Based on TIC profile, we selected fractions 3–6 for purification (mass profile shown in supporting information, discussion in results section). All these fractions were subjected to semi-preparative HPLC (X Select HSST3 OBD Prep Column 5 µm, 10 mm × 250 mm, 0.1% formic acid with water (solvent A) and acetonitrile (solvent B) as mobile phase at flow rate 4 mL/min, detected at 254 nm. Since the mobile phase composition aligns with diligent composition, the interference of the solvent UV spectra with compound UV spectra is minimal. We also used control for avoiding the solvent matrix in the analysis. The method uses MS as additional tool to confirm the eluted compounds from UV. Semi-preparative HPLC were conducted by gradient elution programs to obtain compounds as follows: Fraction 3 (quantity 70 mg, loop volume 250 µL was eluted by 0 min, 5%B; 5 min, 5%B; 10.00 min, 35% B; 16.00 min, 60% B; 25 min, 95% B; 30 min, 95% B; 5% B; 30.50 min, 5% B; 35.00 min. to yield **3** (homosekikaic acid, 7 mg), **7** (divaricatic acid, 4 mg), **11** (divaricatinic acid, 3 mg), **12** (olivetolic acid, 5 mg), and **14** (atranol, 2 mg). Fraction 4 (quantity 50 mg, loop volume 250 µL was eluted by 0 min, 30%B; 5 min, 30%B; 10.00 min, 50% B; 23.00 min, 95%B; 27.00 min, 95% B; 27.50 min, 30% B; 30% B; 30.00 min. at flow rate 4 mL/min, detected at 254 nm to yield **10** (methyldivaricatinic acid, 3 mg), **8** (decarboxydivaricatic acid, 5 mg), **9** (decarboxystenosporic acid, 2 mg), and **4** (hyperhomosekikaic acid, 1 mg). Fraction 5 (quantity 25 mg, loop volume 250 µL was eluted by 0 min, 5% B; 8.50 min, 30% B; 15.00 min, 50% B; 22.00 min, 95% B; 28.00 min, 95% B; 29.0 min, 5% B; 5% B; 35.00 min at flow rate 4 mL/min, detected at 254 nm) to yield **13** (divarinolmonomethylether, 3 mg) and compound **5** (2 mg). Fraction 6 (quantity 40 mg, loop volume 250 µL was eluted by 0 min, 10% B; 8.50 min, 40% B; 18.00 min, 55% B; 25.00 min, 75% B; 32.00 min, 95% B; 36.0 min, 95% B; 10% B; 37.00 min, 10% B; 42.00 min at flow rate 4 mL/min, detected at 254 nm) to yield **1** (sekikaic acid, 5 mg), **2** (4-O-methylnorhomosekikaic acid, 7 mg) and 2,4-di-O-methyldivaric acid **6** (2 mg).

In-vitro Antihyperglycemic and antioxidant assay

DPPH radical scavenging activity

DPPH radical scavenging assay was carried out as previously reported [2]. Scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals by the Acetone extract (AE) (50 µg of 2 mg/mL solution dissolved in DMSO) and compounds (**1-14**) (50 µg of 2 mg/mL solution dissolved in DMSO) was measured in 100 mM Tris-HCl buffer (pH 7.4) by recording absorbance at 517 nm spectrophotometrically. Ascorbic acid (50 µg of 2 mg/mL solution dissolved in DMSO) served as standard. Results were expressed as % scavenging and calculated by using formula: $(A_c - A_t)/100 * A_c$, where A_c was the absorbance of control and A_t , the absorbance of test sample. Different concentrations of compounds were evaluated to obtain 50% scavenging activity (SC_{50}). The SC_{50} was calculated based on equation obtained from regression analysis.

ABTS radical scavenging activity

Scavenging of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^+$) was performed as per earlier method [3]. Acetone extract (AE) (20 µg of 2 mg/mL solution dissolved in DMSO) and compounds (**1-14**) (20 µg of 2 mg/mL solution dissolved in DMSO) were incubated with $ABTS^+$ solution in 6.8 mM phosphate buffer (pH 8.0) as described earlier. Decoloration of $ABTS^+$ solution was determined by measuring absorbance at 734 nm spectrophotometrically. Ascorbic acid (20 µg of 2 mg/mL solution dissolved in DMSO) served as standard. The activity was expressed as % scavenging and calculated as follows: $(A_c - A_t)/100 * A_c$, where A_c was the absorbance of control and A_t , the absorbance of test sample. SC_{50} of compounds was calculated as per above formula.

Free radical induced DNA damage

Protective effect of acetone extract (AE) and Compounds (**1-14**) on oxidative DNA damage was evaluated as per the previous method [4]. 2 µL calf-thymus DNA mixed with 5 µL of 39 mM Tris buffer (pH 7.4) and 5 µL (10 µg) acetone extract and compounds (**1-14**) (10 µg of 2 mg/mL solution dissolved in DMSO) mixture was incubated at room temperature for 20 min. Reaction was initiated by adding 5 µL $FeCl_3$ (500 µM) and 10 µL H_2O_2 (0.8 M) and incubated for 10 min at 37 °C. Reaction was stopped by adding 3 µL DNA loading dye. Finally, mixture was subjected to 0.8% agarose gel electrophoresis in TAE (40 mM Tris, 20 mM acetic acid and 0.5 M EDTA) buffer (pH 7.2). 3 µL of Ethidium bromide was added to agarose solution to stain DNA bands. Image was viewed under trans illuminating UV light and photographed (Bio-Rad ChemiDoc™ XRS with Image Lab™ Software). Band intensity of DNA was measured by using ImageJ software.

Intestinal α -glucosidase inhibition

Intestinal α -glucosidase enzyme inhibition assay was performed as per previous method [2]. 20 µL (40 µg) of acetone extract and compounds (**1-14**) (40 µg of 2 mg/mL solution dissolved in DMSO) were incubated with 50 µL of rat intestinal α -glucosidase enzyme (89.93 mM, prepared in 0.9% NaCl) in 100 mM Phosphate buffer (pH 6.8) for 10 min. After incubation period, 50 µL of substrate (4-nitrophenyl α -D-glucopyranoside) solution was added. Release of *p*-nitrophenol from substrate was measured by recording absorbance at 405 nm spectrophotometrically. Acarbose (40 µg of 2 mg/mL solution dissolved in DMSO) was taken as standard. The activity was expressed and calculated as follows: $(A_c - A_t)/100 * A_c$, where A_c was the absorbance of control and A_t , the absorbance of test sample.

Statistical analysis

Comparisons within the groups were done applying one way ANOVA followed by post-test; Tukey's Multiple comparison test Statistical significance was set at $p < 0.05$. Data analysis was performed by using GraphPad PRISM Version 5.01 (GraphPad Software Inc. California, USA).

Table S1: LC-QToF-MS^E data of compounds from acetone extract of *R. conduplicans*

Ret time	Mol. Wt. with Adduct	Formula	Error (App m)	Fragment ions	Compound name
	<i>m/z</i> Adduct				
7.05	387.0358 [M – H] [–]	C ₁₈ H ₁₁ O ₁₀	1.6	343.0457 (C ₁₇ H ₁₁ O ₈), 325.0363 (C ₁₇ H ₉ O ₇), 313.0357 (C ₁₆ H ₉ O ₇), 299.0560 (C ₁₆ H ₁₁ O ₆), 269.0454 (C ₁₅ H ₉ O ₅), 243.0301 (C ₁₃ H ₇ O ₅), 241.0507 (C ₁₄ H ₉ O ₄), 227.0350 (C ₁₃ H ₇ O ₄), 185.0608 (C ₁₂ H ₉ O ₂), 121.0292 (C ₇ H ₅ O ₂).	Salazinic acid ^{&}
7.50	195.0657 [M – H] [–]	C ₁₀ H ₁₁ O ₄	0.0	151.0756 (C ₉ H ₁₁ O ₂).	Divaric acid ^{&}
8.00	151.0396	C ₈ H ₇ O ₃	0.7	123.0445 (C ₇ H ₇ O ₂), 105.0339 (C ₇ H ₅ O) 81.0331 (C ₅ H ₅ O).	Atranol (14)*
8.53	309.1704 [M – H] [–]	C ₁₇ H ₂₅ O ₅	0.6	265.1805 (C ₁₆ H ₂₅ O ₃), 209.1540(C ₁₃ H ₂₁ O ₂), 152.0835 (C ₉ H ₁₂ O ₂).	ND
9.13	223.0970 [M – H] [–]	C ₁₂ H ₁₅ O ₄	0.0	179.1070 (C ₁₁ H ₁₅ O ₂), 137.0967 (C ₉ H ₁₃ O).	Olivetolic acid (12)*
9.22	223.0969 [M – H] [–]	C ₁₂ H ₁₅ O ₄	–0.4	179.1071 (C ₁₁ H ₁₅ O ₂), 165.0915 (C ₉ H ₁₃ O), 151.0764 (C ₉ H ₁₁ O ₂), 137.0968 (C ₉ H ₁₃ O).	Methyldivaric atinate (10)
9.26	209.0813 [M – H] [–]	C ₁₁ H ₁₃ O ₄	–0.5	165.0913(C ₁₀ H ₁₃ O ₂), 150.0679 (C ₉ H ₁₀ O ₂), 122.0363 (C ₇ H ₆ O ₂).	Divaricatinic acid (11)

9.36	165.0914 [M – H] [–]	C ₁₀ H ₁₃ O ₂	–1.2	150.0678 (C ₉ H ₁₀ O ₂).	Divarinolmon omethylether (13)*
10.29	295.2263 [M – H] [–]	C ₁₈ H ₃₁ O ₃	–3.4	277.2149(C ₁₈ H ₂₉ O ₂), 233.1534(C ₁₅ H ₂₁ O ₂), 195.1378(C ₁₂ H ₁₉ O ₂), 171.1055(C ₉ H ₁₅ O ₃), 167.0712(C ₉ H ₁₁ O ₃), 138.0320 (C ₇ H ₆ O ₃).	ND
11.07	343.1554 [M – H] [–]	C ₂₀ H ₂₃ O ₅	2.6	299.1650(C ₁₉ H ₂₃ O ₃), 283.1335(C ₁₈ H ₁₉ O ₃), 177.0549(C ₁₀ H ₉ O ₃), 165.0915(C ₁₀ H ₁₃ O ₂), 151.0761(C ₉ H ₁₁ O ₂).	Decarboxy divaricatic acid (8)*
11.10	373.0934 [M – H] [–]	C ₁₉ H ₁₇ O ₈	2.9	195.1020(C ₁₁ H ₁₅ O ₃), 177.0163(C ₉ H ₅ O ₄), 163.0386(C ₉ H ₇ O ₃), 133.0263(C ₈ H ₅ O ₂), 119.0502 (C ₈ H ₇ O).	Atranorin ^{&}
11.25	343.0824 [M – H] [–]	C ₁₈ H ₁₅ O ₇	1.7	328.0587 (C ₁₇ H ₁₂ O ₇), 259.0608(C ₁₄ H ₁₁ O ₅), 231.0657 (C ₁₃ H ₁₁ O ₄)	Usnic acid ^{&}
11.81	371.1965 [M – H] [–]	C ₂₁ H ₂₇ O ₃	1.5	205.0864(C ₁₂ H ₁₃ O ₃), 179.1073 (C ₁₁ H ₁₅ O ₂), 137.0966 (C ₉ H ₁₃ O).	Decarboxyste nosporic acid (9)*
11.88	401.1983 [M – H] [–]	C ₂₃ H ₂₉ O ₆	2.0	209.1175(C ₁₂ H ₁₇ O ₃), 194.0952(C ₁₁ H ₁₄ O ₃), 166.0912 (C ₁₀ H ₁₃ O ₂).	Compound -5 [#]
11.95	223.0966 [M – H] [–]	C ₁₂ H ₁₅ O ₄	–1.8	209.0809 (C ₁₁ H ₁₃ O ₄), 165.0915 (C ₁₀ H ₁₃ O ₂), 150.0677(C ₉ H ₁₁ O ₂), 122.0365(C ₇ H ₆ O ₂).	2,4-di- <i>O</i> - methyldivaric acid (6)*
12.43	387.1443 [M – H] [–]	C ₂₁ H ₂₃ O ₇	–0.3	195.0656(C ₁₀ H ₁₁ O ₄), 177.0550 (C ₁₀ H ₉ O ₃), 151.0756(C ₉ H ₁₁ O ₂),	Divaricatic acid (7) *

				133.0649(C ₉ H ₉ O).	
12.51	431.1686 [M – H] [–]	C ₂₃ H ₂₇ O ₈	–2.3	253.1070(C ₁₃ H ₁₇ O ₅), 195.0647 (C ₁₀ H ₁₁ O ₄), 177.0528(C ₁₀ H ₉ O ₃), 151.0754 (C ₉ H ₁₁ O ₂).	4'- <i>O</i> -Methyl norhomosekikaic acid (2)*
12.71	417.1547 [M – H] [–]	C ₂₂ H ₂₅ O ₈	–0.5	225.0761(C ₁₁ H ₁₃ O ₅), 209.0813(C ₁₁ H ₁₃ O ₄), 181.0861(C ₁₀ H ₁₃ O ₃), 166.0628 (C ₉ H ₁₀ O ₃), 165.0913(C ₁₀ H ₁₃ O ₂), 150.0678(C ₉ H ₁₀ O ₂), 137.0235(C ₇ H ₅ O ₃), 122.0365 (C ₇ H ₆ O ₂).	Sekikaic acid (1)*
13.17	415.1759 [M – H] [–]	C ₂₃ H ₂₇ O ₇	0.5	223.0971(C ₁₂ H ₁₅ O ₄), 205.0865 (C ₁₂ H ₁₃ O ₃), 179.1071(C ₁₁ H ₁₅ O ₂), 161.0965 (C ₁₁ H ₁₃ O)	ND
13.42	445.1860 [M – H] [–]	C ₂₄ H ₂₉ O ₈	–0.4	253.1075(C ₁₃ H ₁₇ O ₅), 209.0813 (C ₁₁ H ₁₃ O ₄), 194.0941(C ₁₁ H ₁₄ O ₃), 165.0913(C ₁₀ H ₁₃ O ₂), 150.0679 (C ₉ H ₁₀ O ₂), 137.0237(C ₇ H ₅ O ₃), 122.0367(C ₇ H ₆ O ₂).	Homosekikaic acid (3)*
14.10	473.2178 [M – H] [–]	C ₂₆ H ₃₃ O ₈	1.5	253.1075(C ₁₃ H ₁₇ O ₅), 237.1127 (C ₁₃ H ₁₇ O ₄), 209.0816 (C ₁₂ H ₁₇ O ₃)	Hyper homosekikaic acid (4)*

ND = Not determined, * = Isolated compounds # = tentatively assigned new compound, & = identified in the extraction concluded through ms/ms and compare with the in-house sample.

Physicochemical data of isolated compounds:

4.4.1. Sekikaic acid (1): White amorphous powder; IR (KBr) ν_{\max} : 3538, 3489, 1710, 1426, 1360, 1221 cm^{-1} ; ^1H NMR (400 MHz, Acetone- d_6): δ 6.70 (1H, s), 6.47 (1H, d, J = 2.69 Hz), 6.41 (1H, d, J = 2.69 Hz), 3.94 (3H, s), 3.87 (3H, s), 3.06 – 2.98 (4H, m), 1.80 – 1.64 (4H, m), 1.00 (3H, t, J = 7.33, 14.67 Hz), 0.93 (3H, t, J = 7.21, 14.67 Hz); ^{13}C NMR (100 MHz, Acetone- d_6): δ 174.60, 170.24, 166.79, 166.43, 157.78, 157.44, 150.11, 147.87, 126.57, 112.45, 108.04, 107.43, 106.31, 100.71, 57.57, 56.85, 40.28, 40.26, 27.06, 26.71, 15.58, 15.53 ppm. HRESIMS (m/z): 417.1553 [$\text{M} - \text{H}$] $^-$ (calcd for $\text{C}_{22}\text{H}_{25}\text{O}_8$, 417.1549).

4.4.2. 4'-O-methylnorhomosekikaic acid (2): White amorphous powder; IR (KBr) ν_{\max} : 3626, 3598, 3010, 2253, 1713, 1435, 1370, 1224, 1040, 918 cm^{-1} ; ^1H NMR (400 MHz, Acetone- d_6): δ 6.71 (1H, s), 6.41 (1H, d, J = 2.44 Hz), 6.31 (1H, J = 2.44 Hz), 3.94 (3H, s), 3.08 – 3.04 (2H, m), 2.99 – 2.95 (2H, m), 1.79 – 1.72 (2H, m), 1.70 – 1.63 (2H, m), 1.42 – 1.37 (4H, m), 1.30 – 1.25 (2H, m), 0.95 – 0.89 (6H, m); ^{13}C NMR (100 MHz, Acetone- d_6): δ 174.99, 170.37, 166.91, 164.61, 158.14, 157.52, 150.67, 148.11, 126.63, 113.07, 107.99, 107.41, 105.58, 102.75, 57.57, 40.32, 38.32, 33.81, 33.73, 26.68, 24.13, 15.60, 15.33 ppm. HRESIMS (m/z): 431.1706 [$\text{M} - \text{H}$] $^-$ (calcd for $\text{C}_{23}\text{H}_{27}\text{O}_8$, 431.1706).

2.4.3. Homosekikaic acid (3): White amorphous powder; IR (KBr) ν_{\max} : 3781, 3634, 3595, 3008, 2946, 2295, 2253, 1714, 1435, 1371, 1223, 1040, 919, 748 cm^{-1} ; ^1H NMR (400 MHz, Acetone- d_6): δ 6.69 (1H, s), 6.46 (1H, d, J = 2.59 Hz), 6.40 (1H, d, J = 2.59 Hz), 3.93 (3H, s), 3.86 (3H, s), 3.06 – 3.03 (2H, m), 3.01 – 2.99 (2H, m), 1.78 – 1.72 (2H, m), 1.71 – 1.63 (2H, m), 1.41 – 1.36 (4H, m), 0.94 – 0.89 (6H, m); ^{13}C NMR (100 MHz, Acetone- d_6): δ 174.65, 170.23, 166.74, 166.40, 158.06, 157.41, 150.08, 148.15, 126.56, 112.42, 107.93, 107.48, 106.36, 100.71, 57.55, 56.84, 40.23, 38.28, 33.79, 33.70, 26.71, 24.12, 15.57, 15.33 ppm. HRESIMS (m/z): 445.1859 [$\text{M} - \text{H}$] $^-$ (calcd for $\text{C}_{24}\text{H}_{29}\text{O}_8$, 445.1862).

2.4.4. Hyperhomosekikaic acid (4): Pale yellow amorphous powder, ^1H NMR (400 MHz, Acetone- d_6): δ 6.72 (1H, d, J = 1.8 Hz), 6.47 (1H, d, J = 2.5 Hz), 6.41 (1H, d, J = 2.5 Hz), 3.87 (3H, s), 3.83 (3H, s), 3.01 – 2.96 (2H, m), 2.64 – 2.59 (2H, m), 1.76 – 1.62 (6H, m), 0.95 (3H, t, J = 7.5), 0.95 (3H, t, J = 7.3); HRESIMS (m/z): 473.2176 [$\text{M} - \text{H}$] $^-$ (calcd for $\text{C}_{26}\text{H}_{33}\text{O}_8$, 473.2175).

2.4.5. Compound 5: White gummy liquid, ^1H NMR (400 MHz, Acetone- d_6) and ^{13}C NMR (100 MHz, Acetone- d_6): see in table-1. HRESIMS (m/z): 401.1961 [$\text{M} - \text{H}$] $^-$ (calcd for $\text{C}_{23}\text{H}_{29}\text{O}_6$, 401.1964).

2.4.6. 2,4-di-O-methyldivaric acid (6): yellow gum; IR (KBr) ν_{\max} : 3537, 3003, 1711, 1426, 1360, 1221, 1093 cm^{-1} ; ^1H NMR (400 MHz, Acetone- d_6): δ 6.46 (1H, d, J = 2.56 Hz), 6.40 (1H, d, J = 2.56 Hz), 3.93 (3H, s), 3.86 (3H, s), 3.01 – 2.97 (2H, m), 1.77 – 1.72 (2H, m), 0.91 (3H, t, J = 7.33 Hz); ^{13}C NMR (100 MHz, Acetone- d_6): δ 150.08, 112.49, 100.76, 57.85, 56.88, 40.29, 26.74, 15.30 ppm. HRESIMS (m/z): 223.0975 [$\text{M} - \text{H}$] $^-$ (calcd for $\text{C}_{12}\text{H}_{15}\text{O}_4$, 223.0970).

2.4.7. Divaricatic acid (7): White amorphous powder; IR (KBr) ν_{\max} : 3673, 3638, 3597, 3005, 2943, 2293, 2253, 1714, 1436, 1370, 1223, 1040, 918, 748 cm^{-1} ; ^1H NMR (400 MHz, Acetone- d_6): δ 6.78 (1H, s), 6.75 (1H, s), 6.46 (1H, s), 6.41 (1H, s), 3.86 (3H, s), 3.00 (2H, t, J = 7.78, 15.41 Hz), 2.95 (2H, t, J = 7.78, 15.56 Hz), 1.72 – 1.64 (4H, m), 0.98 – 0.93 (6H, m); ^{13}C NMR (100 MHz, Acetone- d_6): δ 174.08, 170.74, 166.93, 166.50, 165.76, 155.90, 149.96, 149.46, 117.49, 112.46, 112.40, 110.19, 106.55, 100.80, 56.89, 40.27, 39.65, 26.91, 26.68, 15.48, 15.36 ppm. HRESIMS (m/z): 387.1451 [$\text{M} - \text{H}$] $^-$ (calcd for $\text{C}_{21}\text{H}_{23}\text{O}_7$, 387.1444).

2.4.8. Decarboxydivaricatic acid (8): White amorphous powder; ^1H NMR (400 MHz, Acetone- d_6): δ 11.24 (1H, s), 6.78 (1H, s), 6.75 (1H, s), 6.46 (1H, s), 6.41 (1H, s), 3.86 (3H, s), 3.00 (2H, t, J = 7.78, 15.41 Hz), 2.56 (2H, t, J = 7.78, 15.56 Hz), 1.72 – 1.64 (4H, m), 0.98 – 0.93 (6H, m); ^{13}C NMR (100 MHz, Acetone- d_6): δ 171.53, 167.14, 166.40, 159.99, 152.91, 149.55, 147.10, 115.05, 114.60, 112.49, 108.31, 106.56,

100.79, 56.87, 40.41, 39.38, 27.01, 25.97, 15.49, 14.99 ppm. HRESIMS (m/z): 343.1554 [$M - H$]⁻ (calcd for $C_{20}H_{23}O_5$, 343.1545).

2.4.9. Decarboxystenosporic acid (**9**): White amorphous powder; 1H NMR (400 MHz, Acetone- d_6): δ 11.24 (1H, s), 6.67 – 6.65 (1H, m), 6.63 – 6.61 (1H, m), 6.60 – 6.58 (1H, m), 6.46 (1H, d, $J = 2.56$ Hz), 6.40 (1H, d, $J = 2.69$ Hz), 3.87 (3H, s), 2.99 – 2.95 (2H, m), 2.59 (2H, t, $J = 7.58$, 15.40 Hz), 1.75 – 1.68 (2H, m), 1.66 – 1.59 (2H, m), 1.37 – 1.32 (4H, m), 0.96 (6H, t, $J = 7.33$, 14.67 Hz); ^{13}C NMR (100 MHz, Acetone- d_6): δ 171.51, 167.11, 166.38, 159.99, 152.91, 149.54, 147.34, 114.98, 114.54, 112.48, 108.25, 106.57, 100.78, 56.87, 40.40, 37.28, 33.13, 32.60, 27.01, 24.12, 15.49, 15.27 ppm. HRESIMS (m/z): 371.1862 [$M - H$]⁻ (calcd for $C_{22}H_{27}O_5$, 371.1858).

2.4.10. Methyldivaricatinic acid (**10**): White amorphous powder; 1H NMR (400 MHz, Acetone- d_6): δ 11.53 (1H, s), 6.37 (1H, d, $J = 2.69$ Hz), 6.32 (1H, d, $J = 2.69$ Hz), 3.95 (3H, s), 3.82 (3H, s), 2.87 – 2.83 (2H, m), 1.61 – 1.51 (2H, m), 0.95 (3H, t, $J = 7.45$, 14.79 Hz); ^{13}C : (100 MHz, Acetone- d_6): δ 173.57, 167.04, 165.95, 149.39, 112.13, 106.71, 100.69, 56.74, 53.39, 40.26, 26.72, 15.45 ppm. HRESIMS (m/z): 223.0964 [$M - H$]⁻ (calcd for $C_{12}H_{15}O_4$, 223.0970).

2.4.11. Divaricatinic acid (**11**): White amorphous powder; 1H NMR (400 MHz, Acetone- d_6): δ 6.35 (1H, d, $J = 2.56$ Hz), 6.32 (1H, d, $J = 2.56$ Hz), 3.82 (3H, s), 2.95 – 2.91 (2H, m), 1.65 – 1.56 (2H, m), 0.93 (3H, t, $J = 7.33$, 14.67 Hz); ^{13}C NMR (100 MHz, Acetone- d_6): δ 174.89, 167.90, 165.99, 150.07, 111.97, 106.13, 100.54, 56.70, 40.13, 26.81, 15.44 ppm. HRESIMS (m/z): 209.0805 [$M - H$]⁻ (calcd for $C_{11}H_{13}O_4$, 209.0814).

2.4.12. Olivetolic acid (**12**): White amorphous powder; 1H NMR (400 MHz, Acetone- d_6): δ 6.28 (1H, d, $J = 2.4$ Hz), 6.21 (1H, d, $J = 2.4$ Hz), 2.93 – 2.89 (2H, m), 1.61 – 1.54 (2H, m), 1.34 – 1.32 (4H, m), 0.88 (3H, t, $J = 6.8$, 14.0 Hz); ^{13}C NMR (100 MHz, Acetone- d_6): δ 175.00, 167.88, 164.28, 150.79, 112.51, 105.36, 102.56, 38.12, 33.70, 33.45, 24.11, 15.30 ppm. HRESIMS (m/z): 223.0961 [$M - H$]⁻ (calcd for $C_{12}H_{15}O_4$, 223.0970).

2.4.13. Divarinolmonomethylether (**13**): White amorphous powder; 1H NMR (400 MHz, Acetone- d_6): δ 6.29 (1H, d, $J = 2.5$ Hz), 6.26 (1H, d, $J = 2.45$ Hz), 6.23 (1H, d, $J = 2.4$ Hz), 3.72 (3H, s), 2.48 – 2.44 (2H, m), 1.62 – 1.56 (2H, m), 0.93 (3H, t, $J = 6.7$, 14.5 Hz); ^{13}C NMR (100 MHz, Acetone- d_6): δ 162.81, 160.24, 146.60, 109.73, 107.18, 100.49, 56.24, 39.76, 26.07, 15.04 ppm. HRESIMS (m/z): 165.0916 [$M - H$]⁻ (calcd for $C_{10}H_{13}O_2$, 165.0916).

2.5.14. Atranol (**14**): White amorphous powder; 1H NMR (400 MHz, Acetone- d_6): δ ppm 10.28 (s, 1H), 6.24 (s, 2H), 2.03 (s, 3H); ^{13}C NMR (100 MHz, Acetone- d_6): 195.18, 164.03 (2C), 152.56, 110.27, 109.42, 109.42, 23.38 ppm. HRESIMS (m/z): 151.0399 [$M - H$]⁻ (calcd for $C_8H_7O_3$, 151.0395).



Figure S1: The authentic *Ramalina conduplicans*.

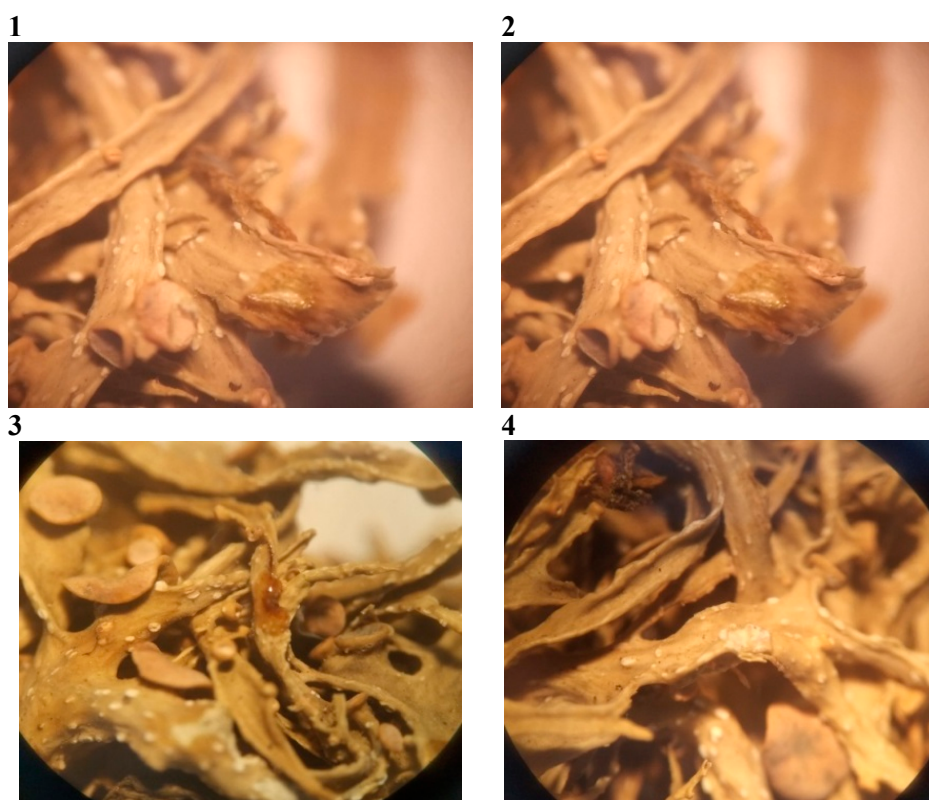


Figure S2: The spot tests for identification of *Ramalina conduplicans*. 1 = after K test (pale yellow), 2 = after C test (negative), 3 = after KC test (negative), 4 = after P test (negative).

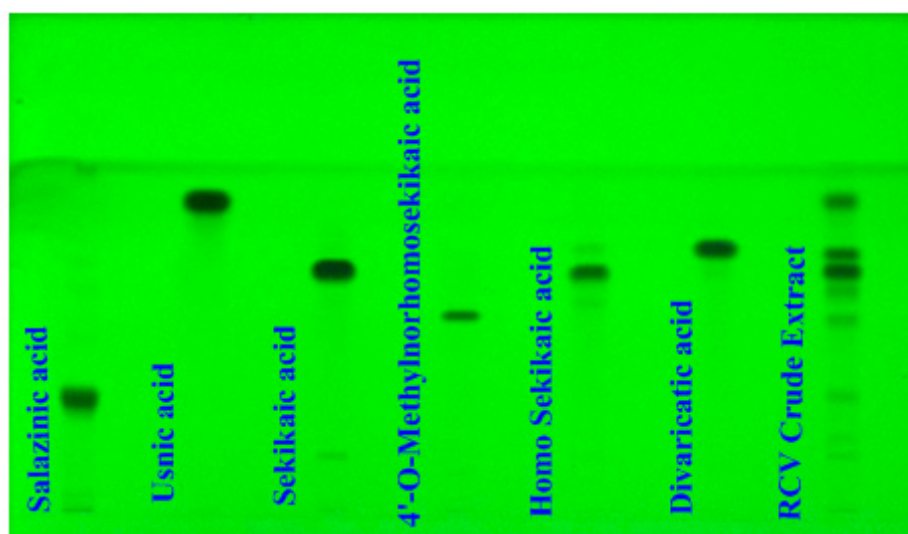


Figure S3: Depicts the HPTLC profiles for *R. conduplicans* extract along the standard compounds at 254 nm, TLC system: toluene/dioxane/acetic acid (72.50:24.20:3.30).

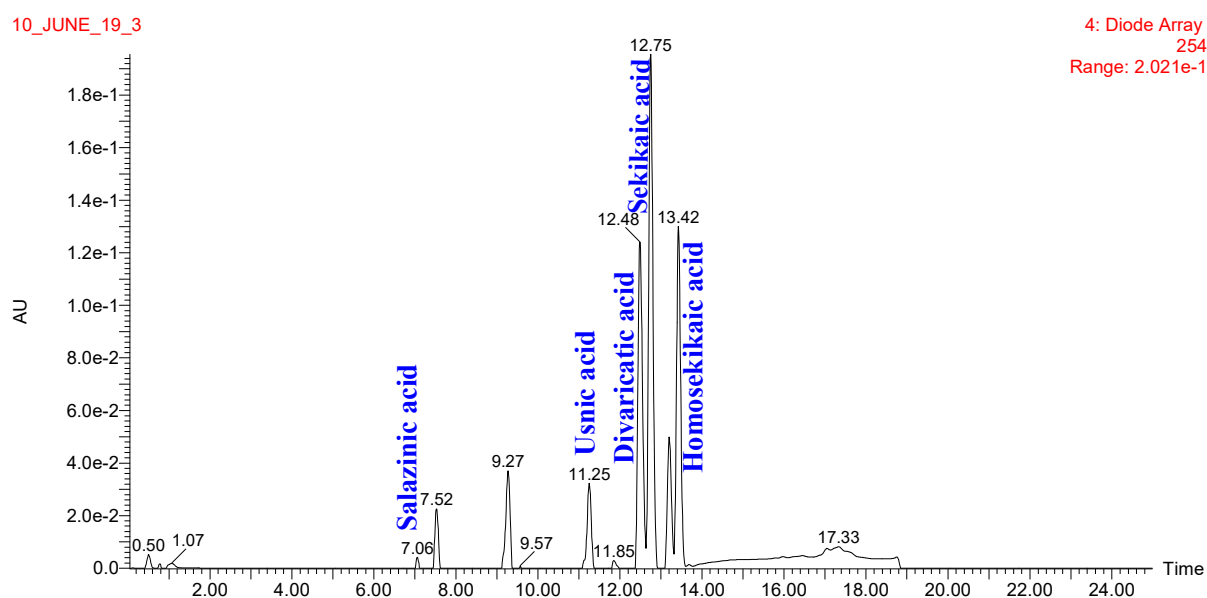
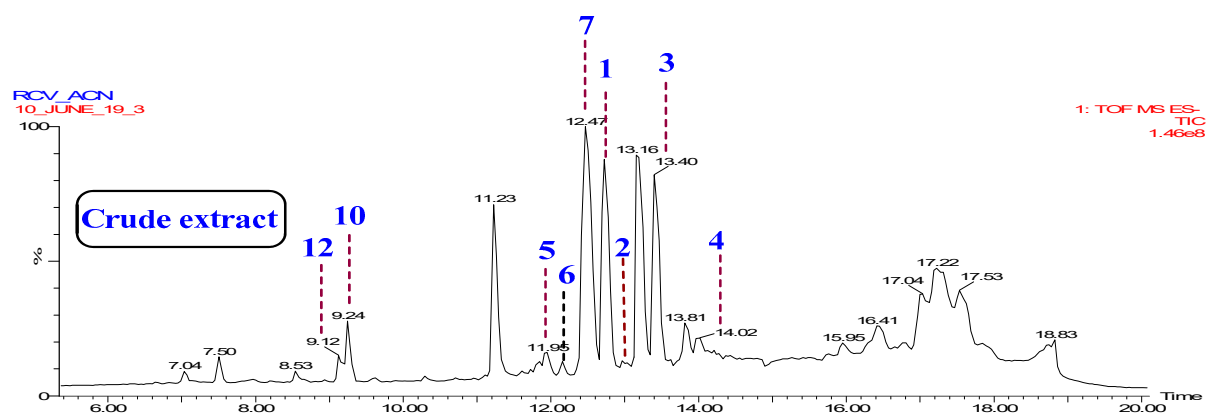
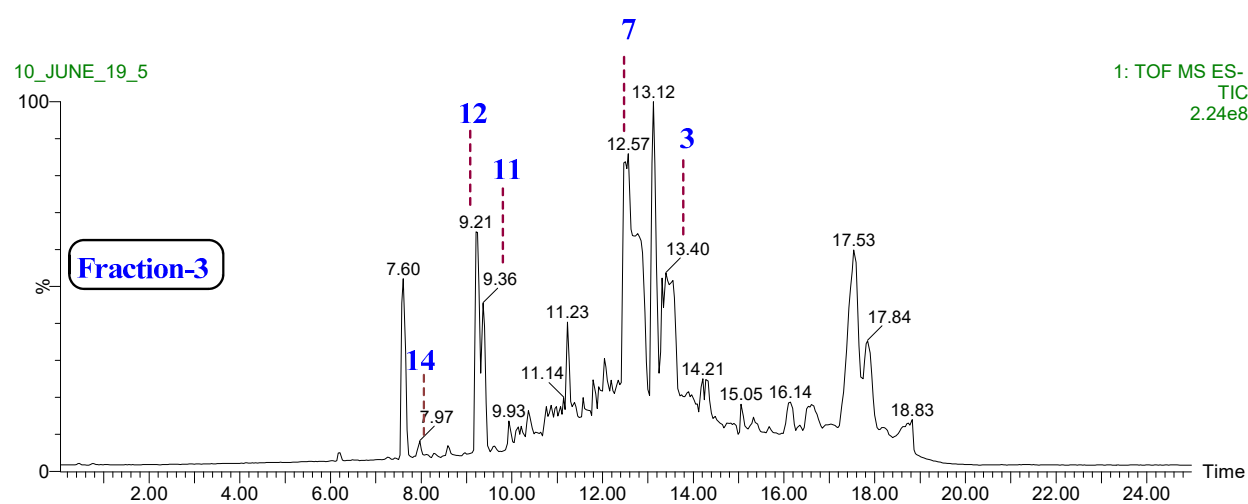


Figure S4: UPLC-PDA chromatogram of acetone extract of *R. conduplicans*.

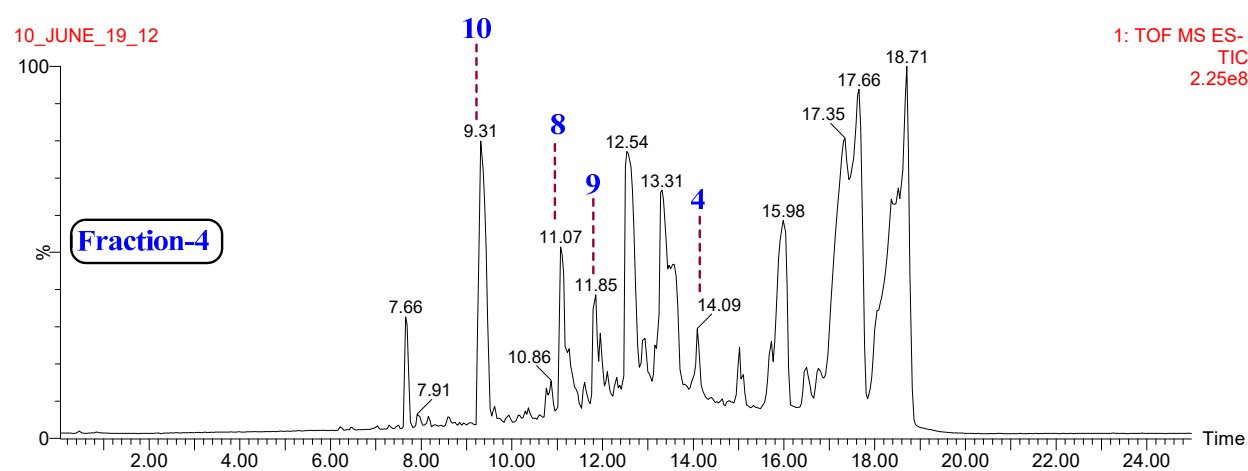
A)



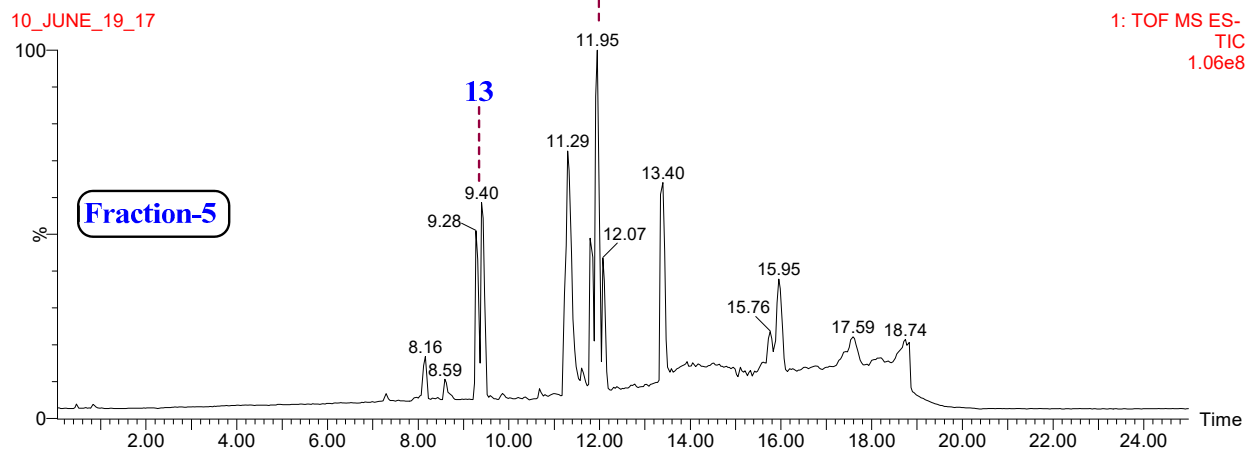
B)



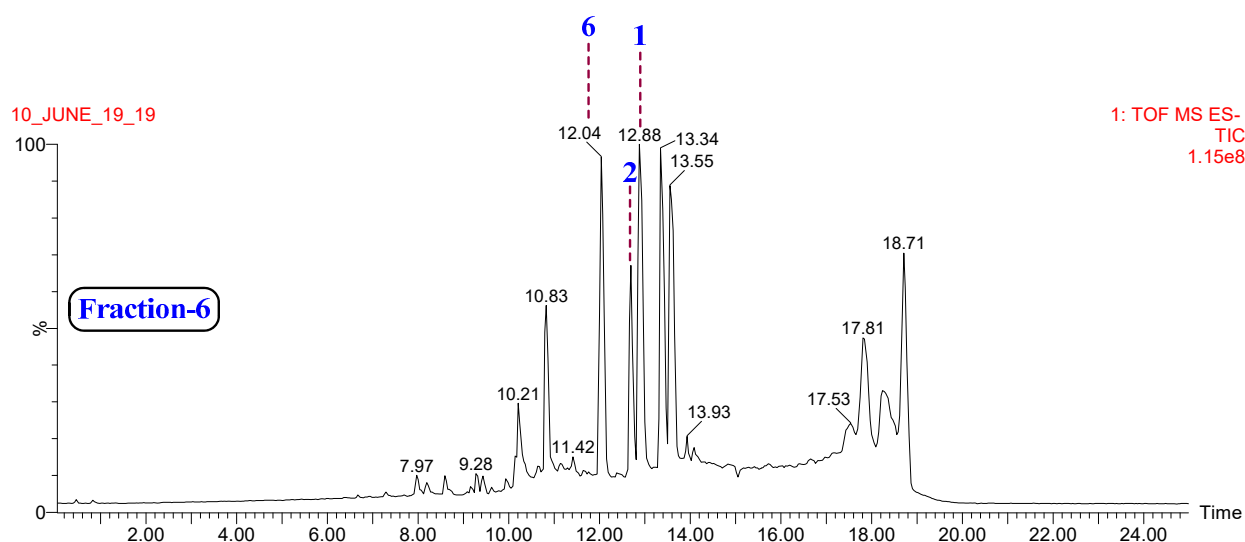
C)



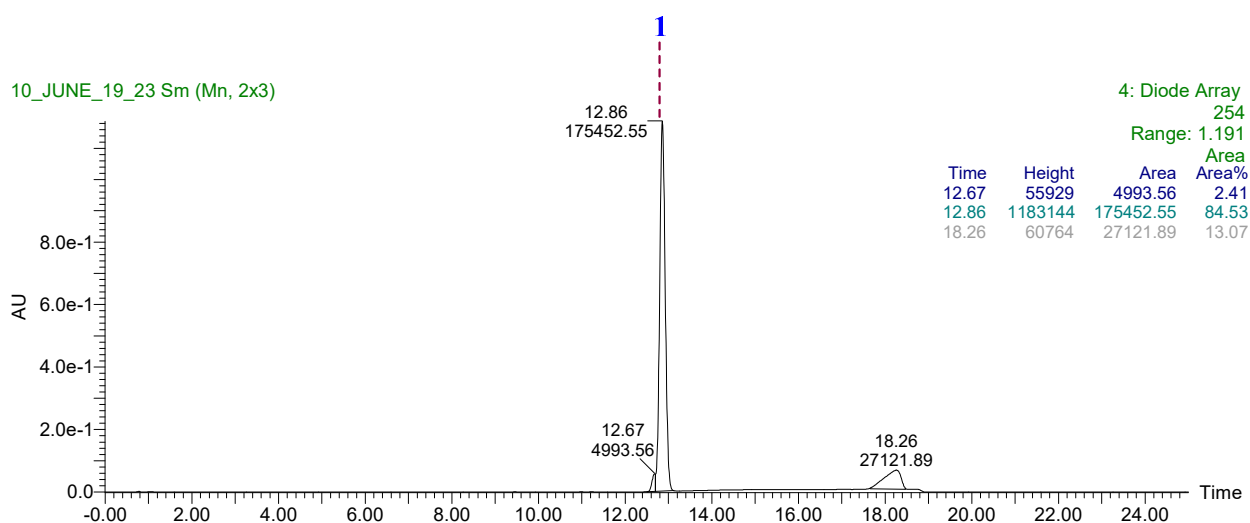
D)



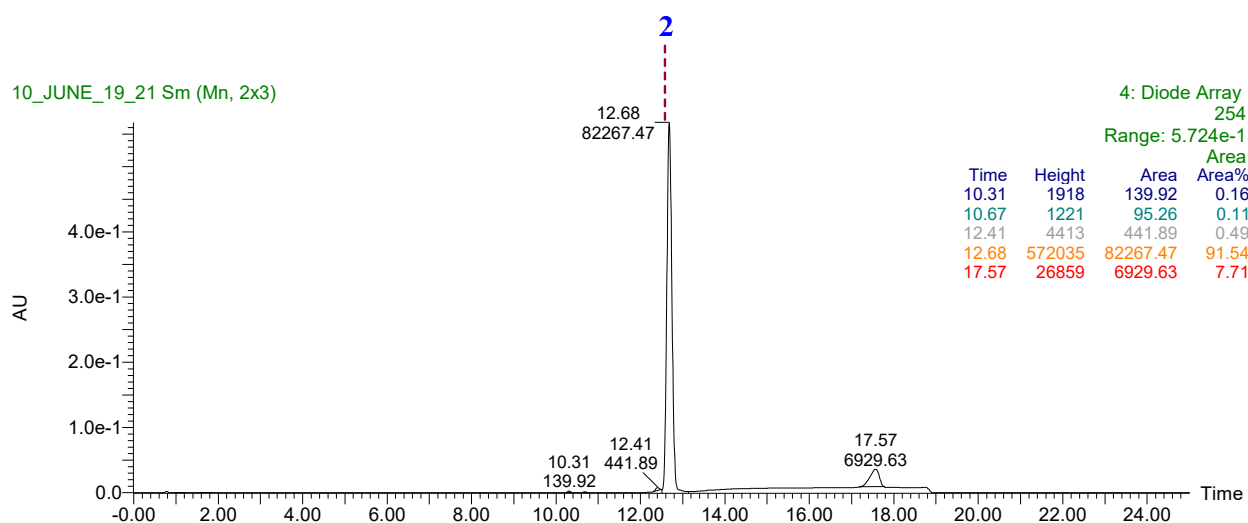
E)



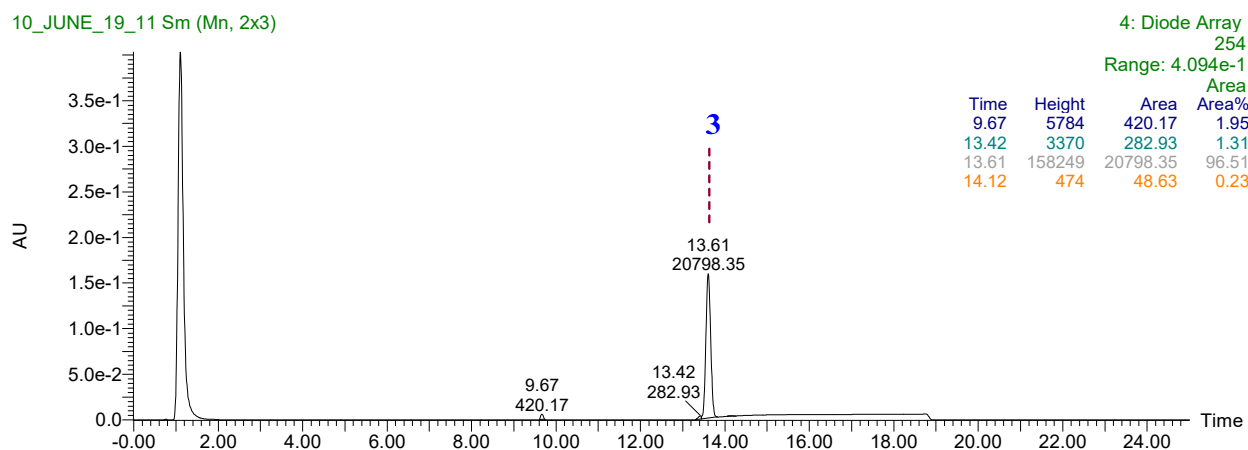
F)



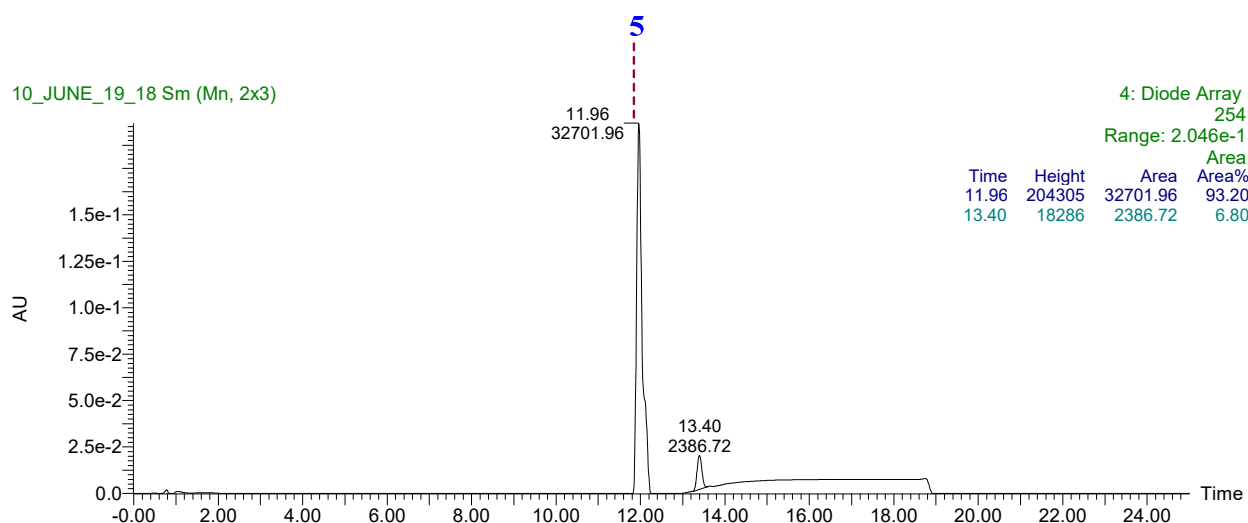
G)



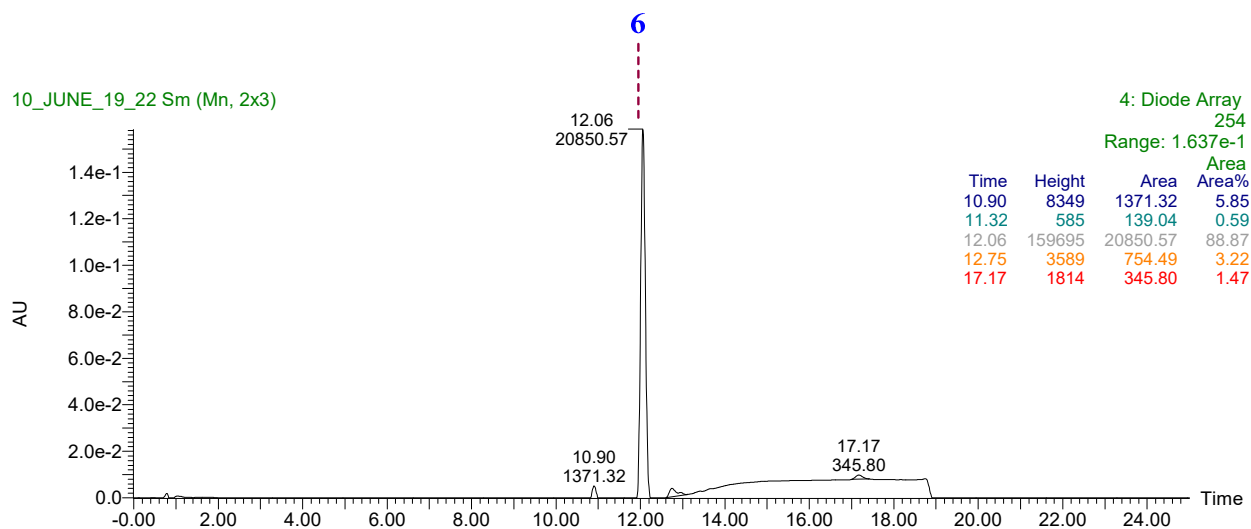
H)



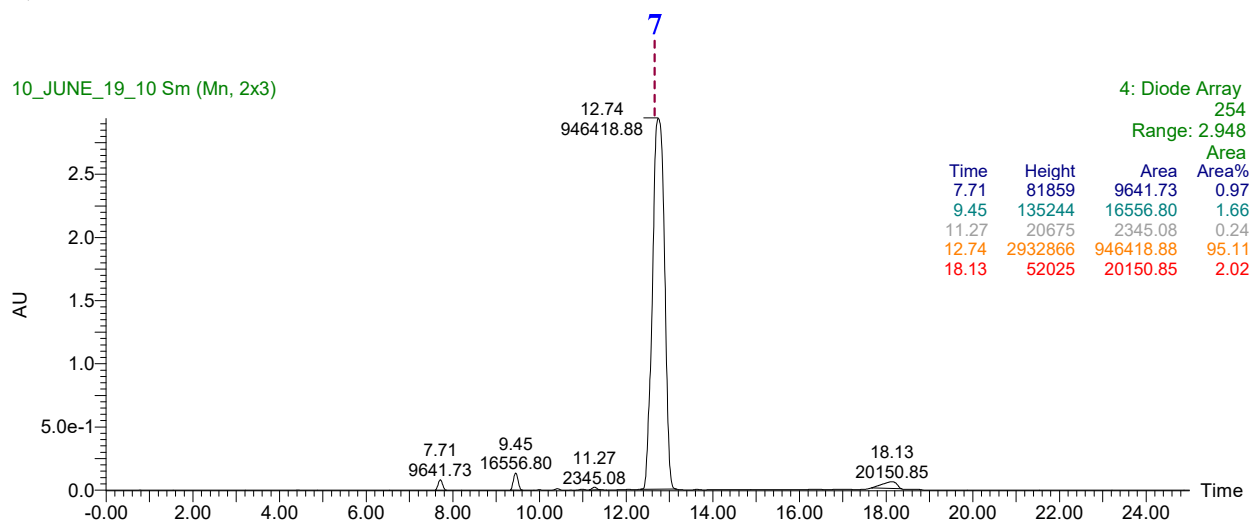
I)



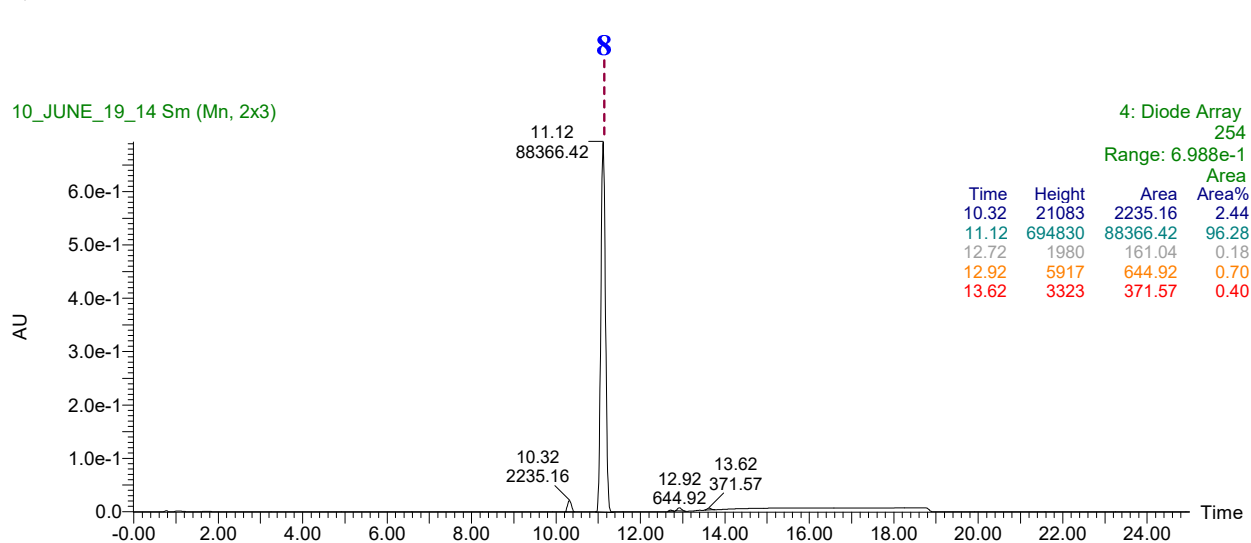
J)



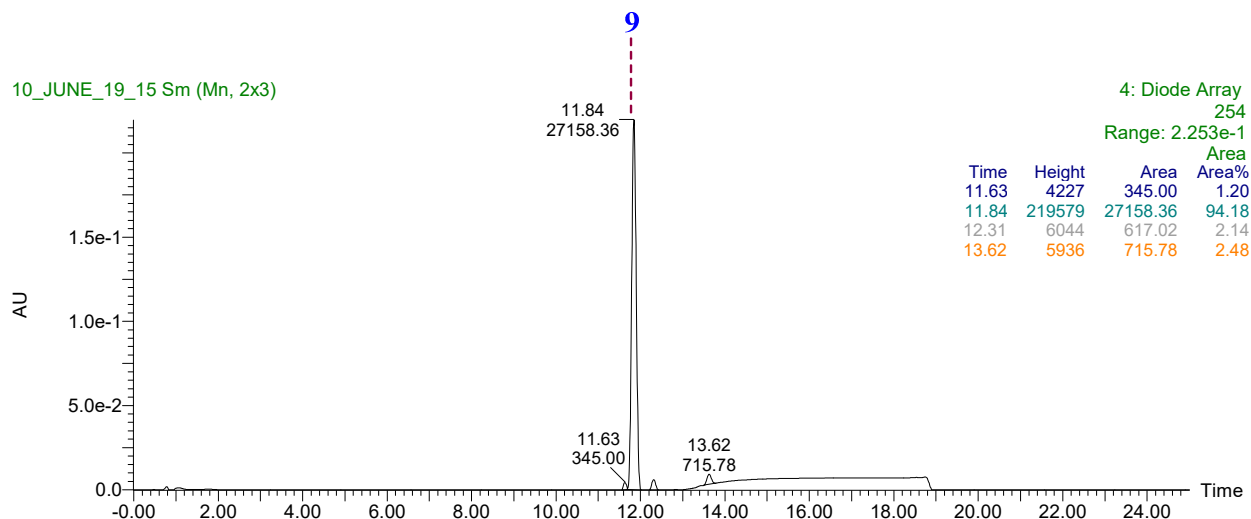
K)



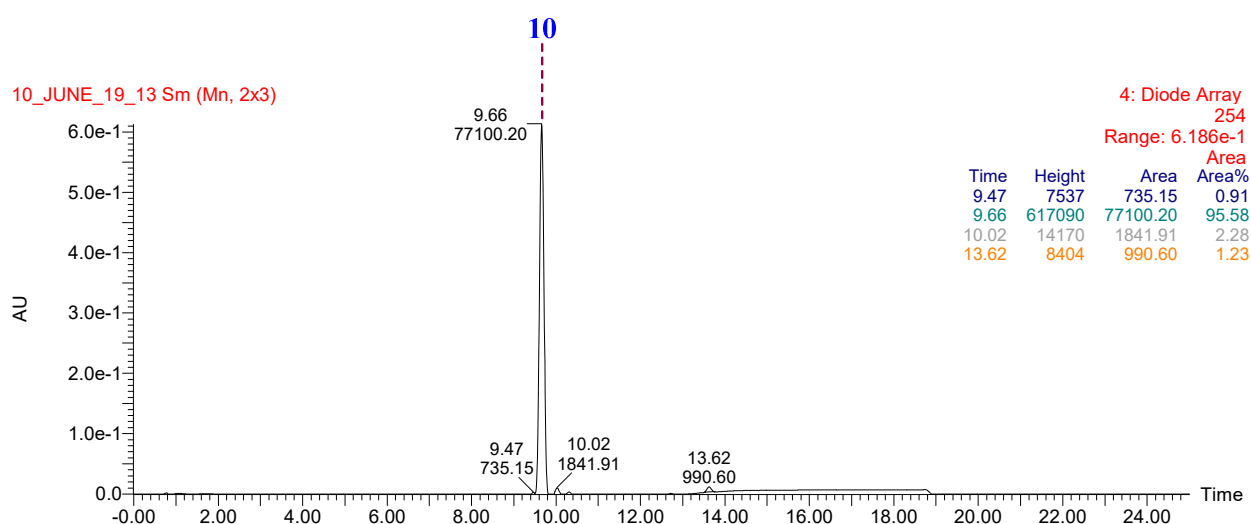
L)



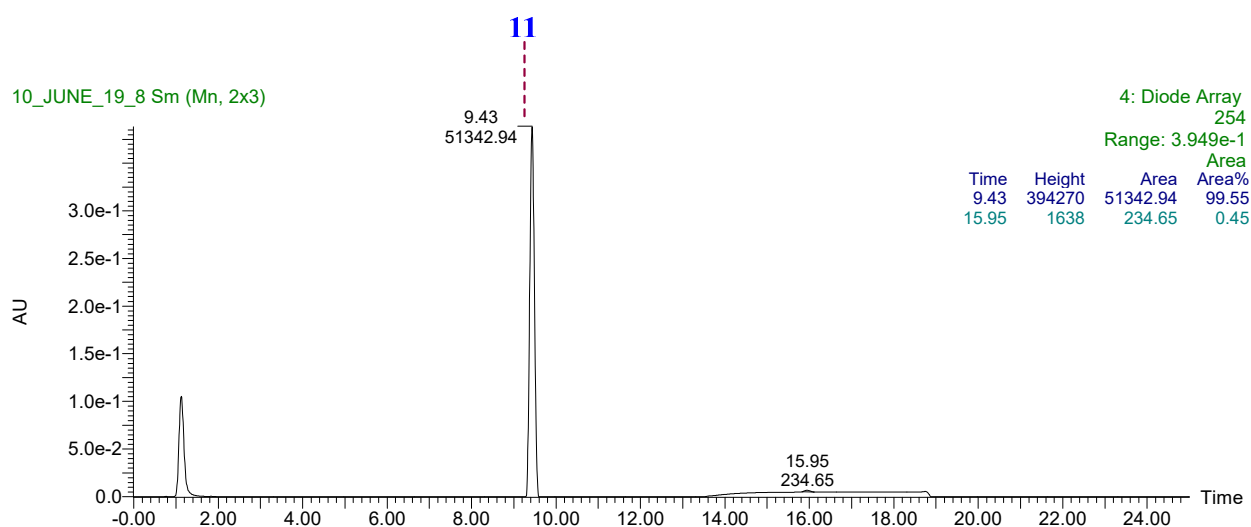
M)



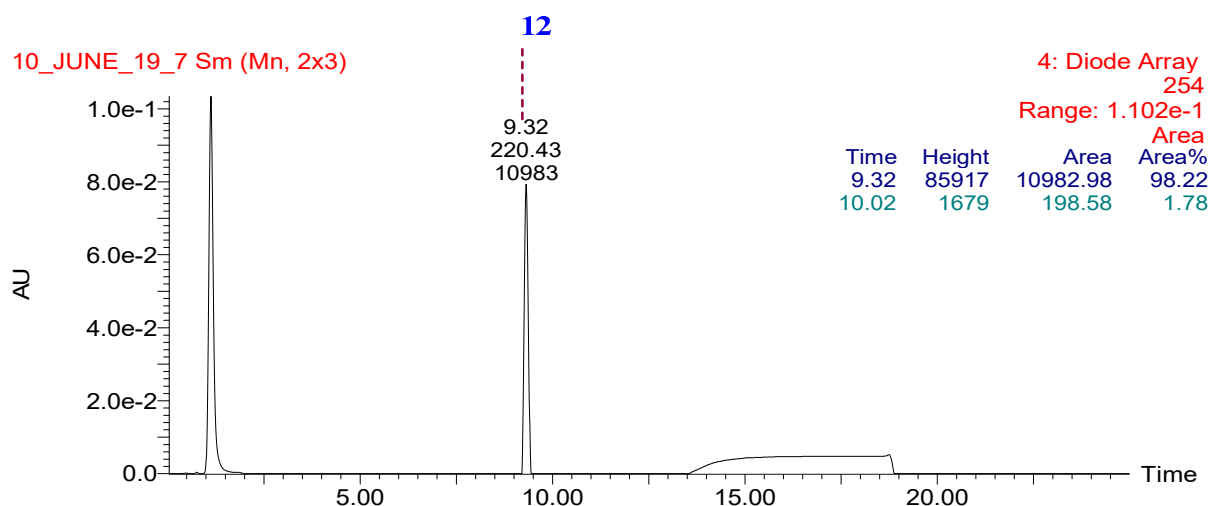
N)



O)



P)



Q)

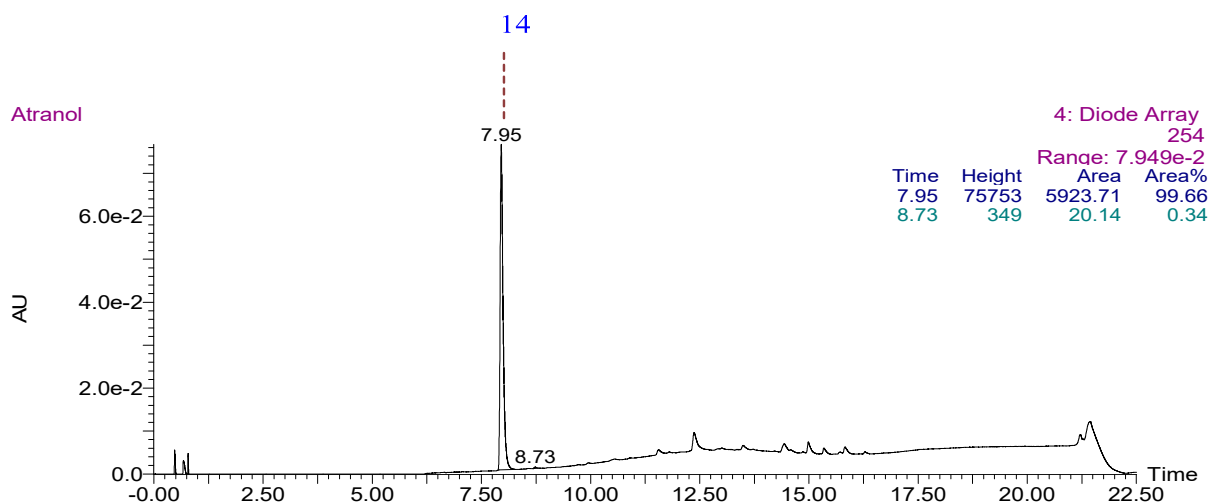


Figure S5: Shows the Total Ion Chromatogram (TIC) of fractions and pure compounds. A = Extract; B = Fraction 3; C = Fraction 4; D = Fraction 5; E = Fraction 6; F = Compound 1; G = Compound 2; H = Compound 3; I = Compound 5; J = Compound 6; K = Compound 7; L = Compound 8; M = Compound 9; N = Compound 10; O = Compound 11; P = Compound 12; Q = Compound 14.

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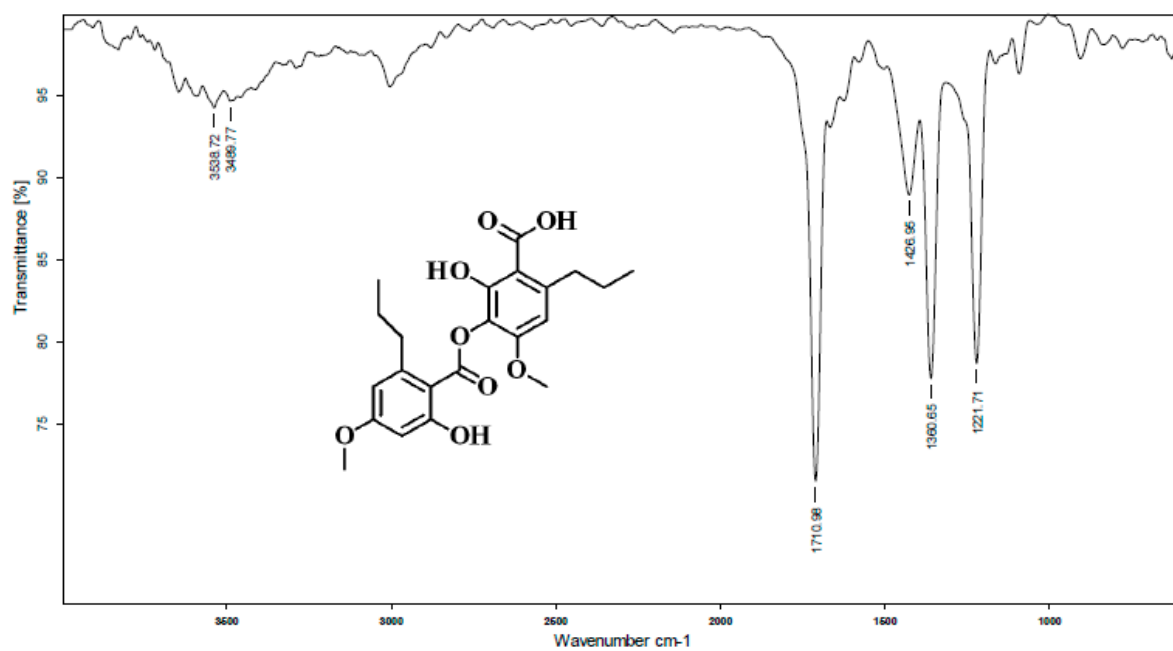


Figure S6: FTIR spectrum of Sekikaic acid (1).

Minimum:				-1.5				
Maximum:	5.0	5.0	50.0					
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf (%)	Formula
417.1553	417.1549	0.4	1.0	10.5	484.1	n/a	n/a	C22 H25 O8



10_JUNE_19_23 330 (12.829) AM2 (Ar,22000.0,0.00,0.00); ABS; Cm (329:333)

2: TOF MS ES-
7.75e7

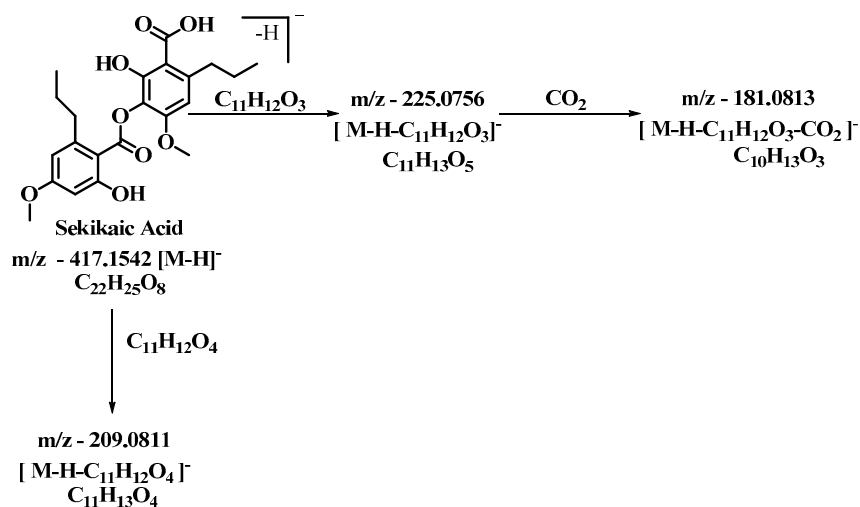
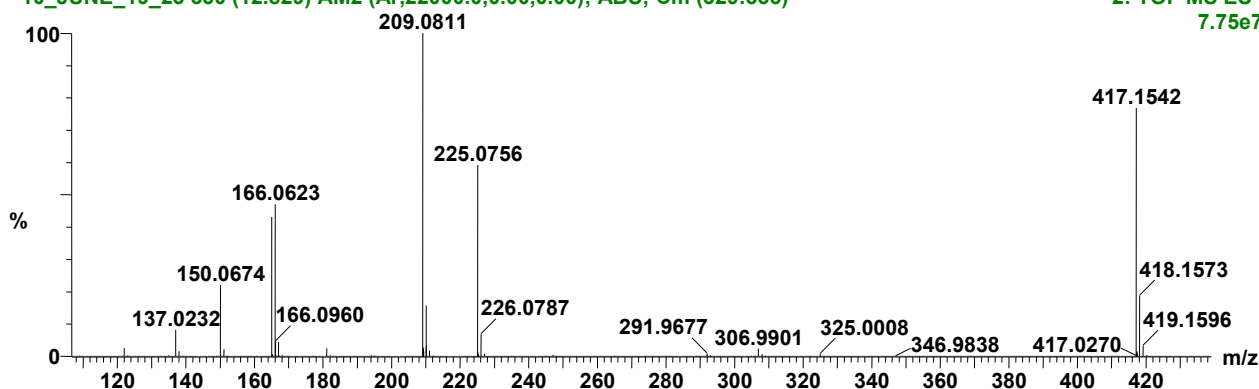


Figure S7: HRESIMS, MS/MS spectra and fragmentation pattern of Sekikaic acid (1).

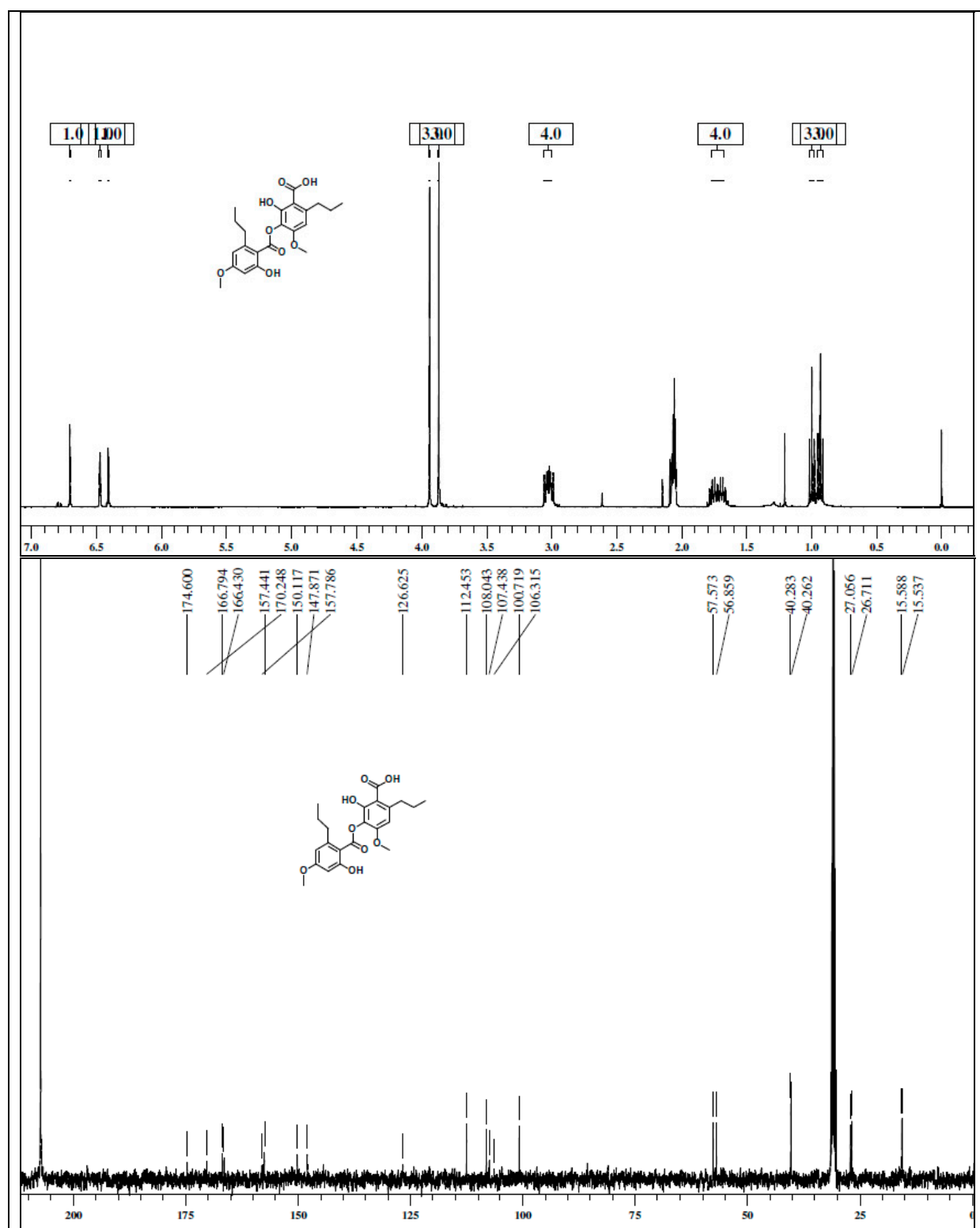


Figure S8: ¹H & ¹³C NMR spectrum of Sekikaic acid (**1**) (400 & 100 MHz, CD₃COCD₃).

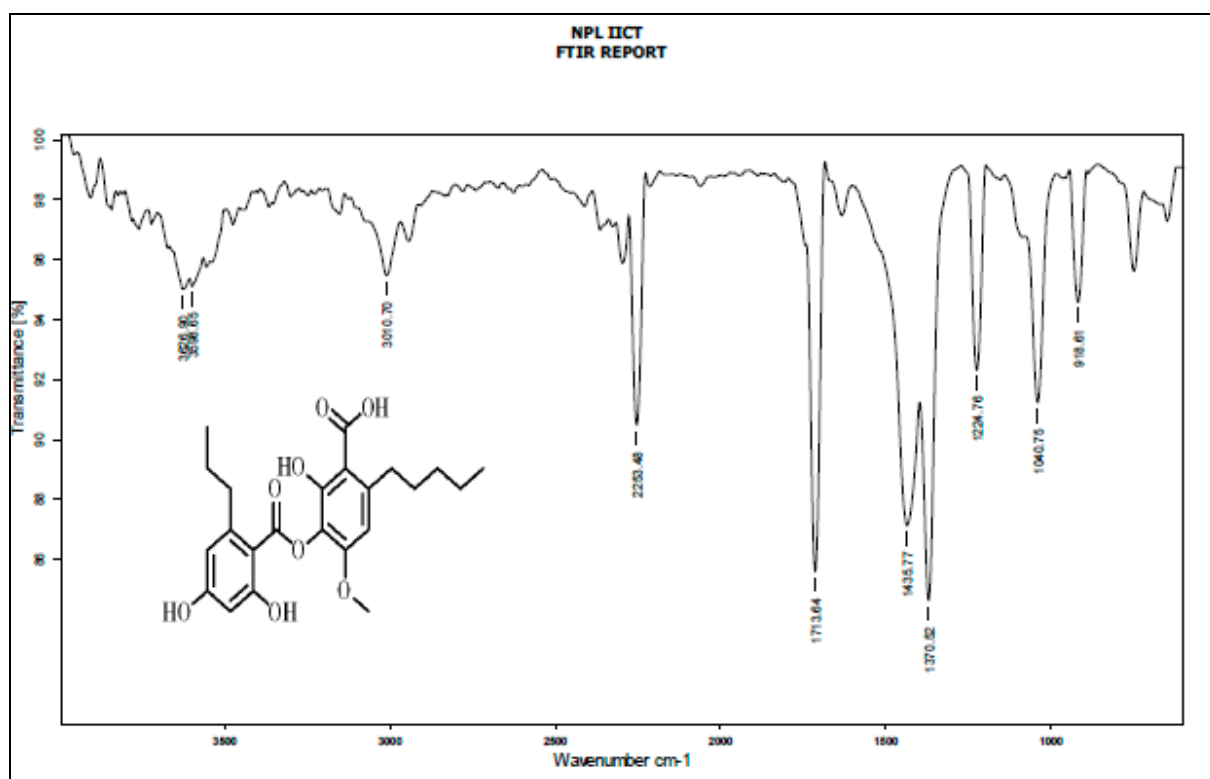
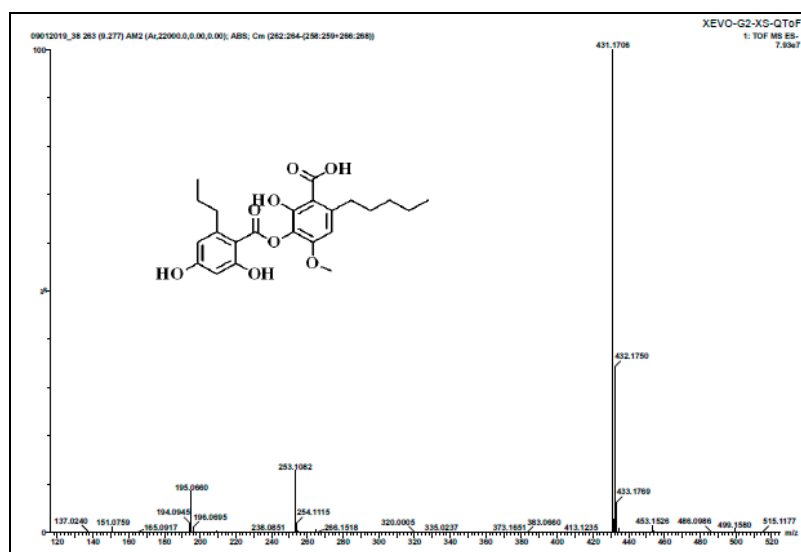


Figure S9: FTIR spectrum of 4'-O-Methylnorhomosekikaic acid (**2**).

Minimum: -1.5
Maximum: 5.0 5.0 50.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf (%)	Formula
431.1706	431.1706	0.0	0.0	10.5	693.9	n/a	n/a	C ₂₃ H ₂₇ O ₈



10_JUNE_19_21 325 (12.639) AM2 (Ar,22000.0,0.00,0.00); ABS; Cm (325:328-(315:320+331:348))

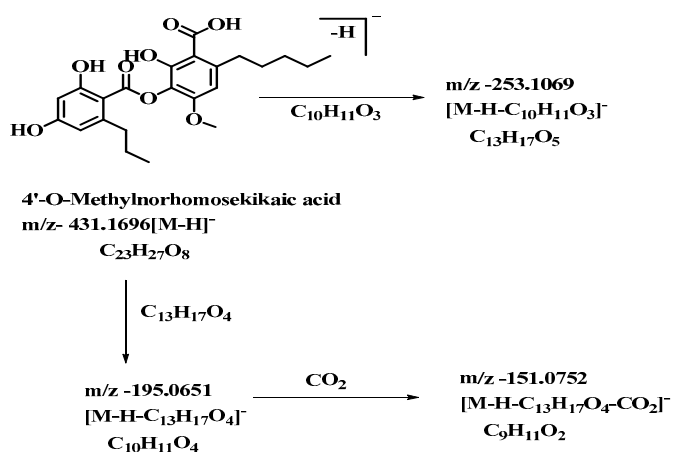
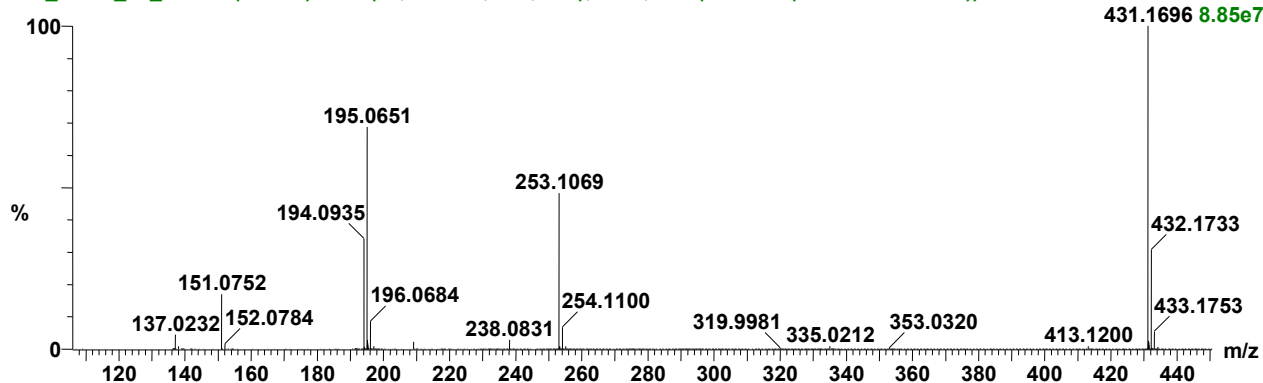


Figure S10: HRESIMS, MS/MS spectra and fragmentation pattern of 4'-O-Methylnorhomosekikaic acid (2).

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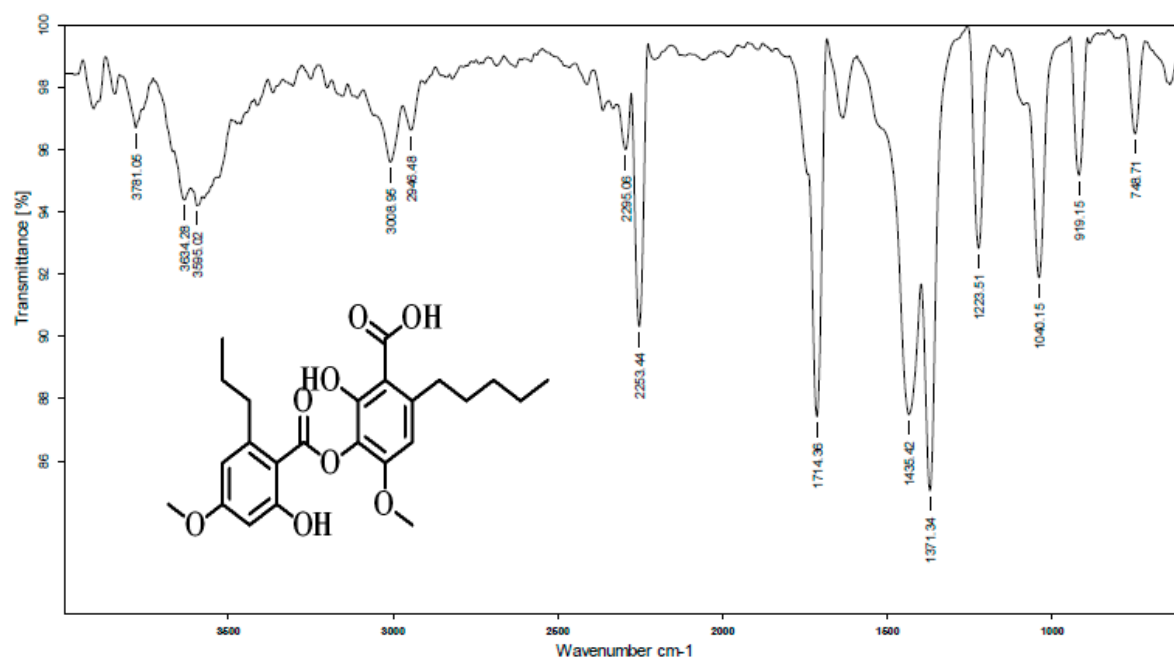


Figure S12: FTIR spectrum of Homosekikaic acid (3).

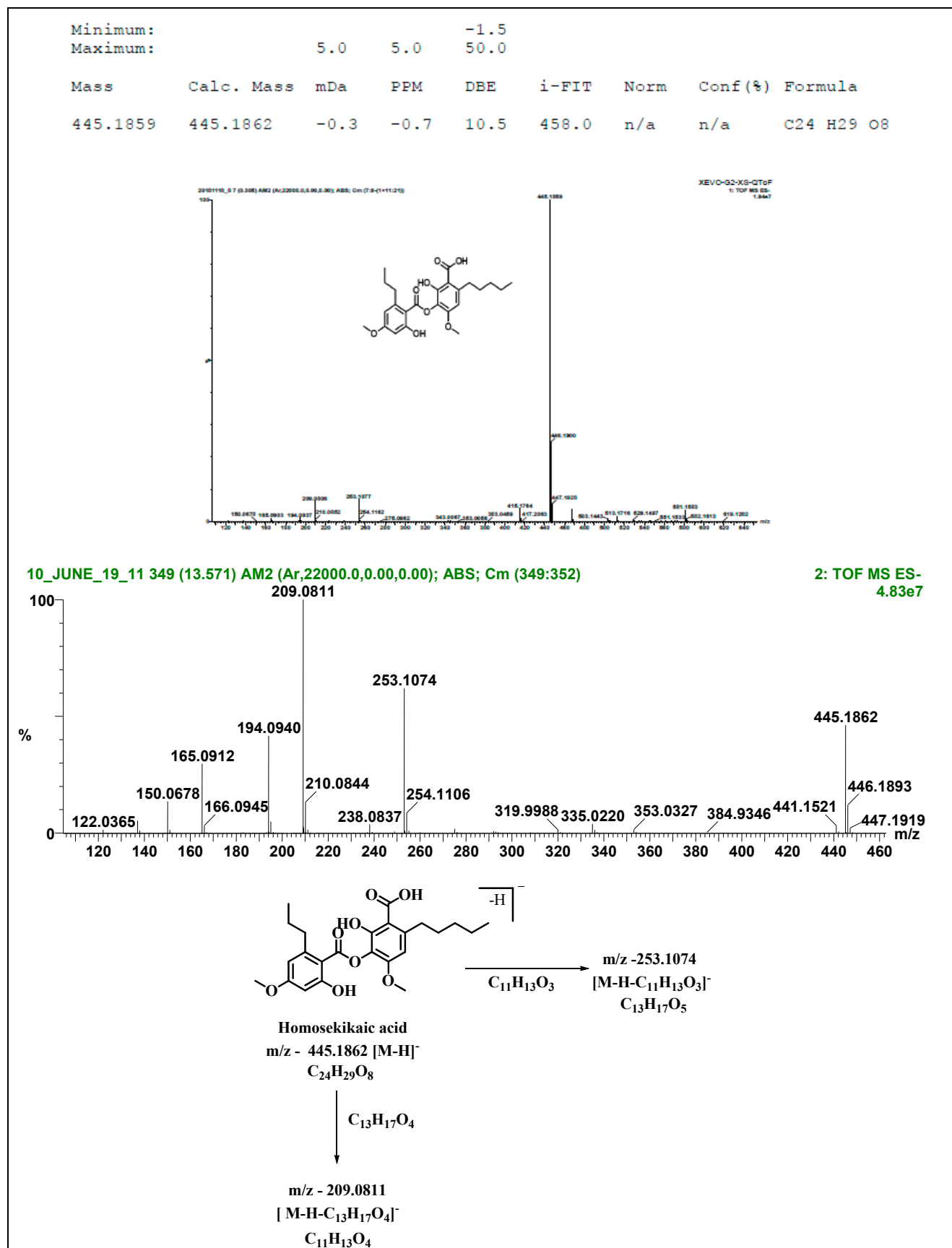


Figure S13: HRESIMS, MS/MS spectra and fragmentation pattern of Homosekikaic acid (3).

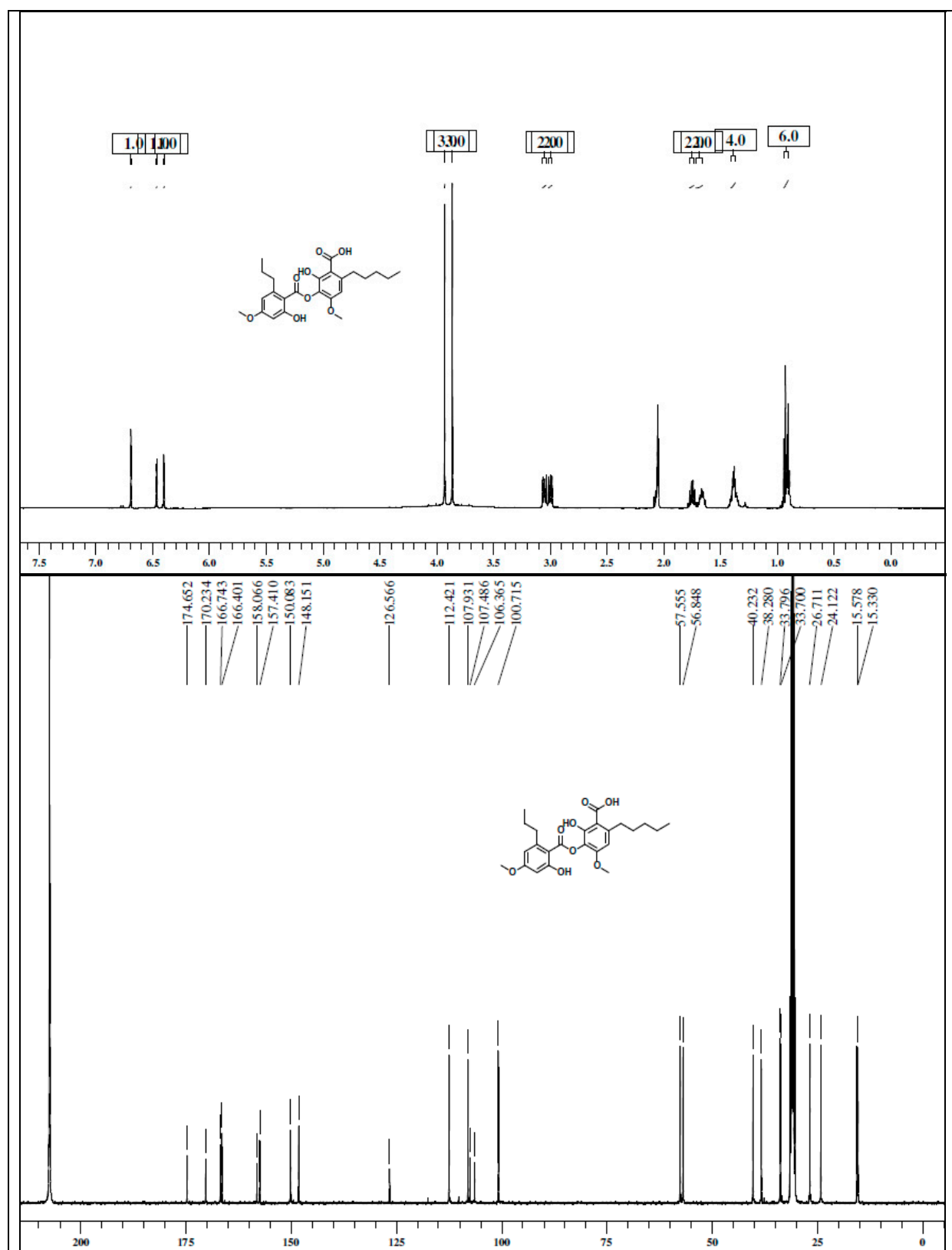


Figure S14: ¹H & ¹³C NMR Spectrum of Homosekikaic acid (**3**) (400 & 100 MHz, CD₃COCD₃).

Minimum:				-1.5				
Maximum:	5.0	10.0	50.0					
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
473.2176	473.2175	0.1	0.2	10.5	595.0	n/a	n/a	C26 H33 O8

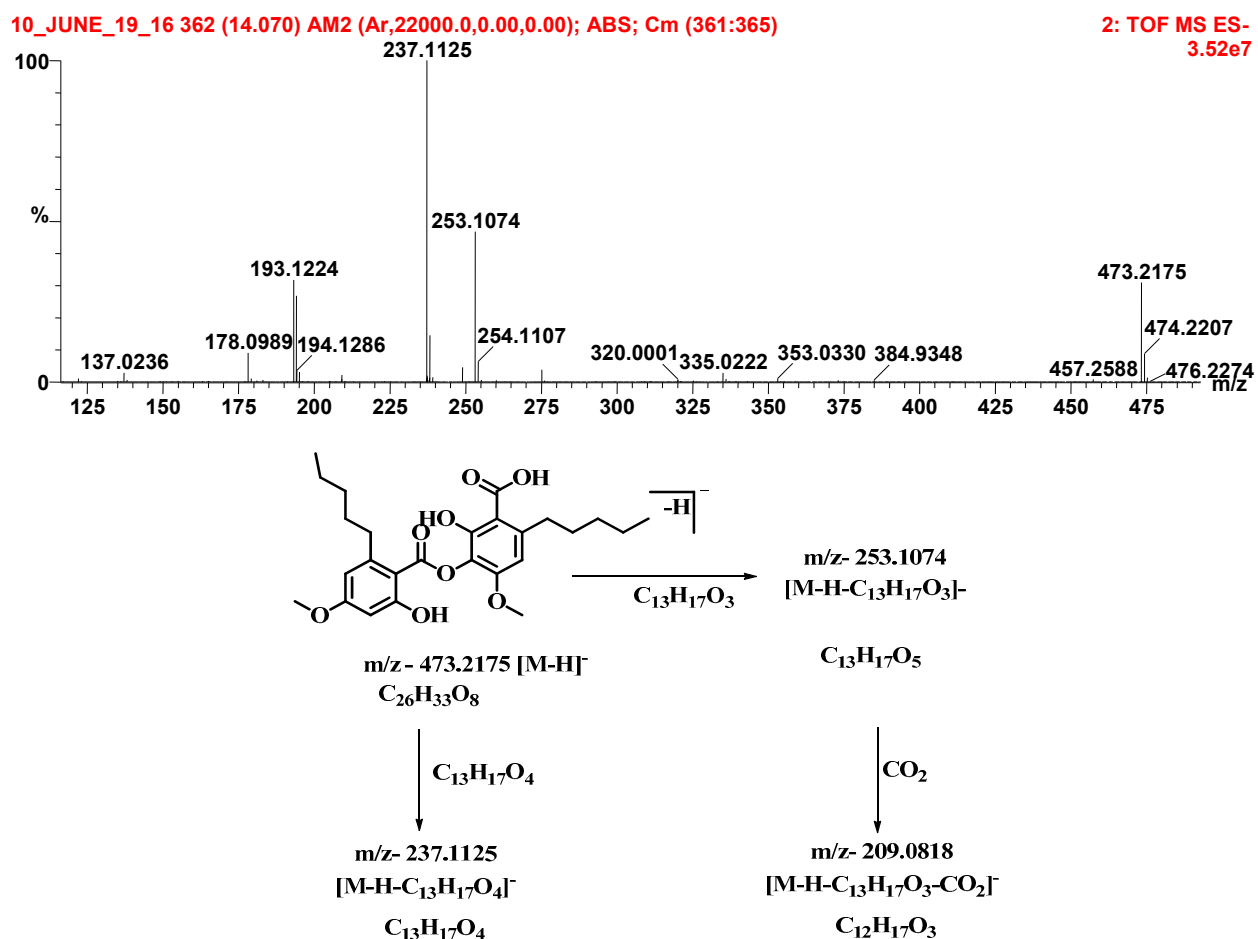
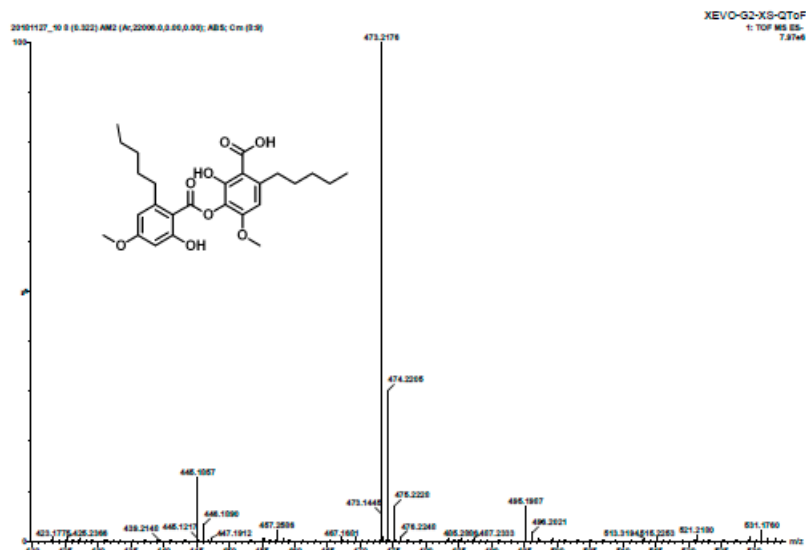


Figure S15: HRESIMS, MS/MS spectra and fragmentation pattern of Hyperhomosekikaic acid (**4**).

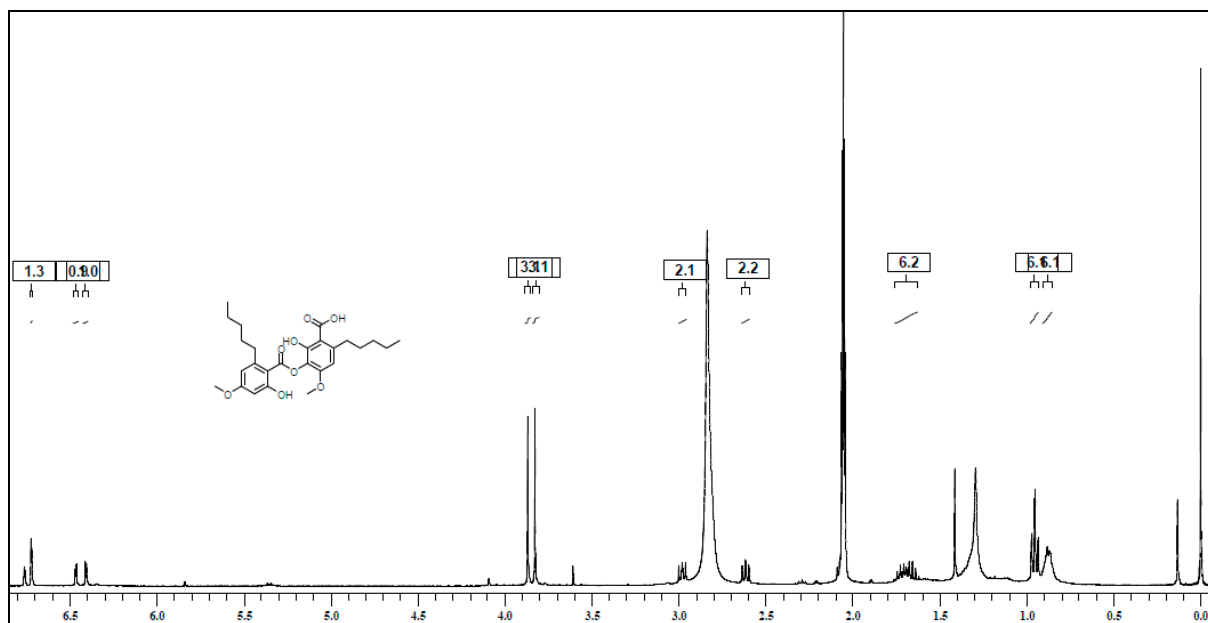


Figure S16: ^1H NMR Spectrum of Hyperhomosekikaic acid (**4**) (400 MHz, CD_3COCD_3).

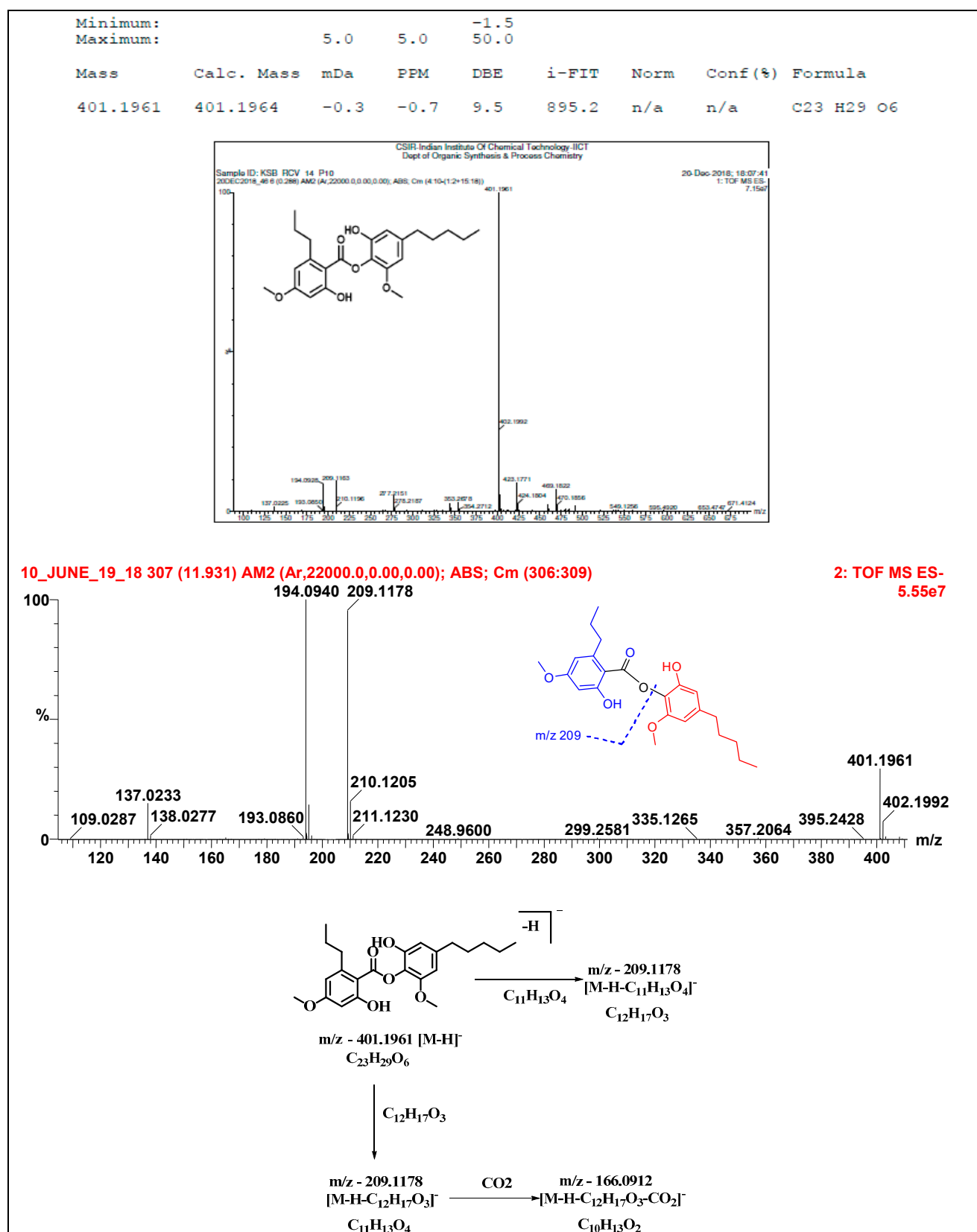


Figure S17: HRESIMS, MS/MS spectra and fragmentation pattern of compound (5).

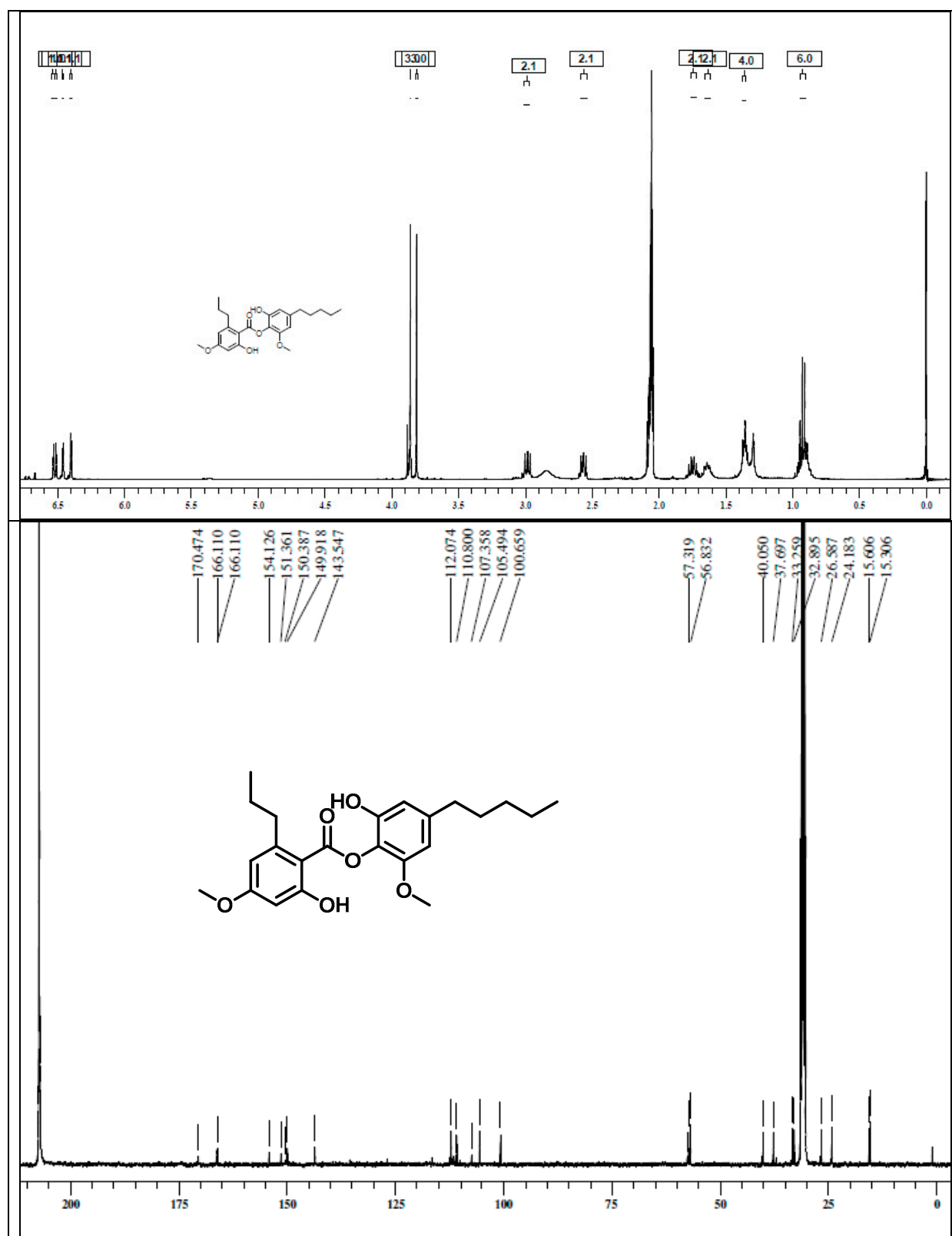


Figure S18: ¹H & ¹³C NMR Spectrum of Compound (5) (400 & 100 MHz, CD₃COCD₃).

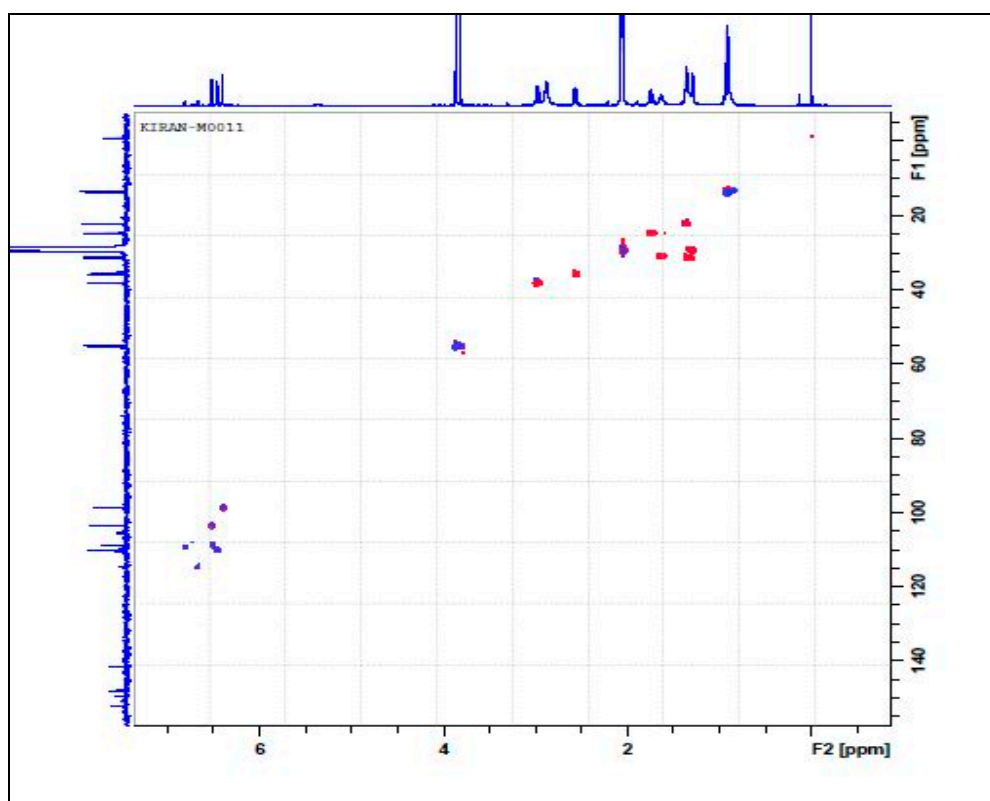


Figure S20: HSQC Spectrum of Compound 5.

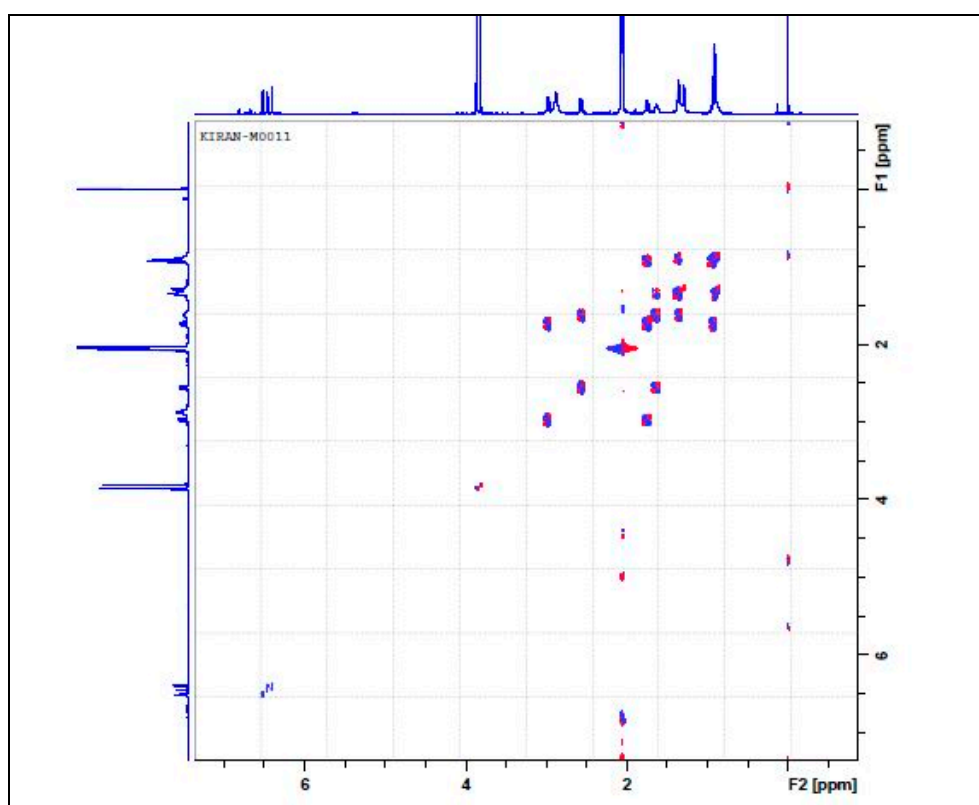


Figure S21: DQF-COSY Spectrum of Compound 5.

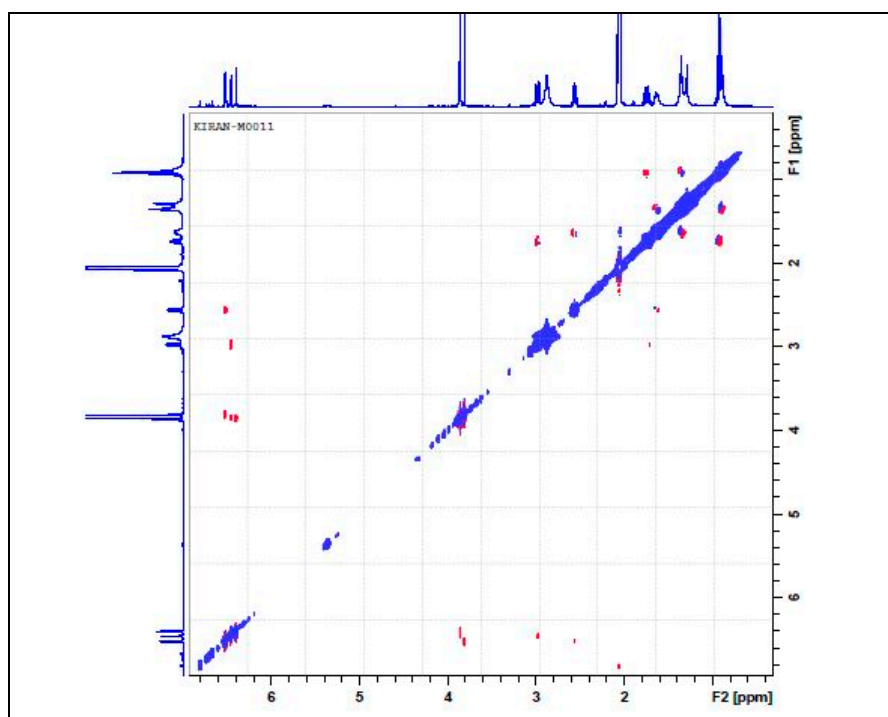


Figure S22: NOESY Spectrum of Compound **5**.

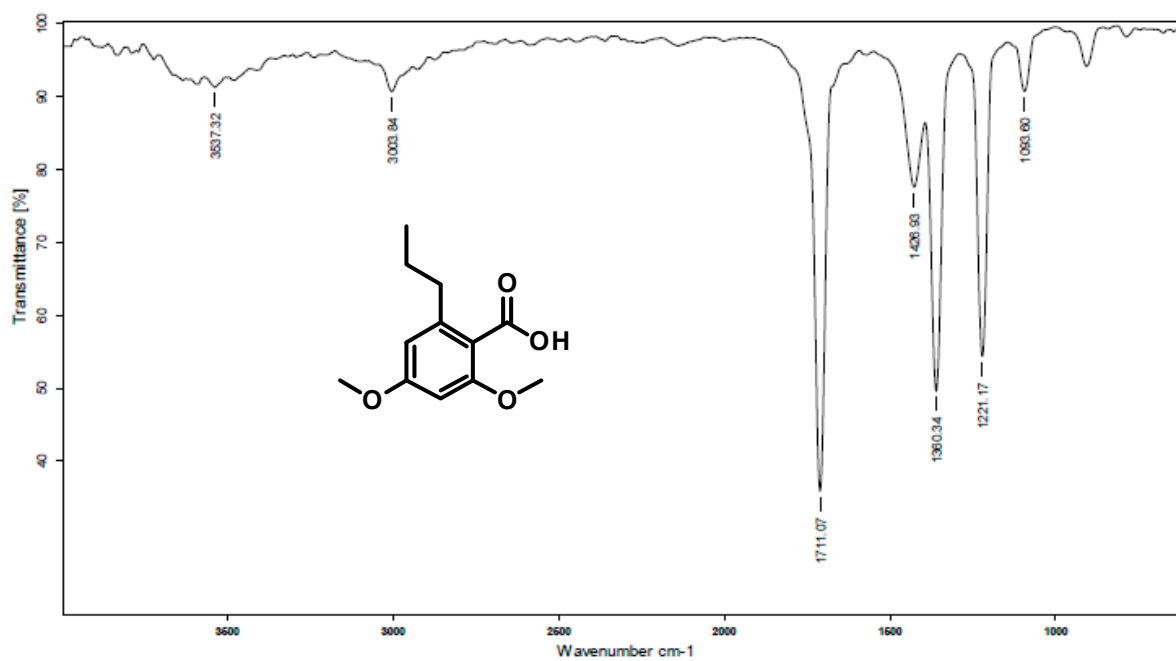


Figure S23: FTIR spectrum of Compound **6**.

Minimum:				-1.5				
Maximum:	5.0	5.0		50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
223.0975	223.0970	0.5	2.2	5.5	607.7	n/a	n/a	C ₁₂ H ₁₅ O ₄

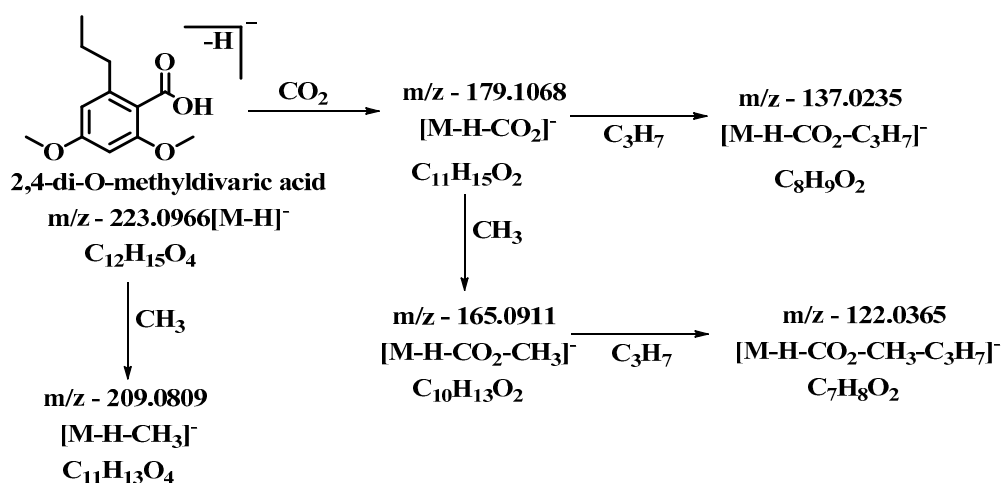
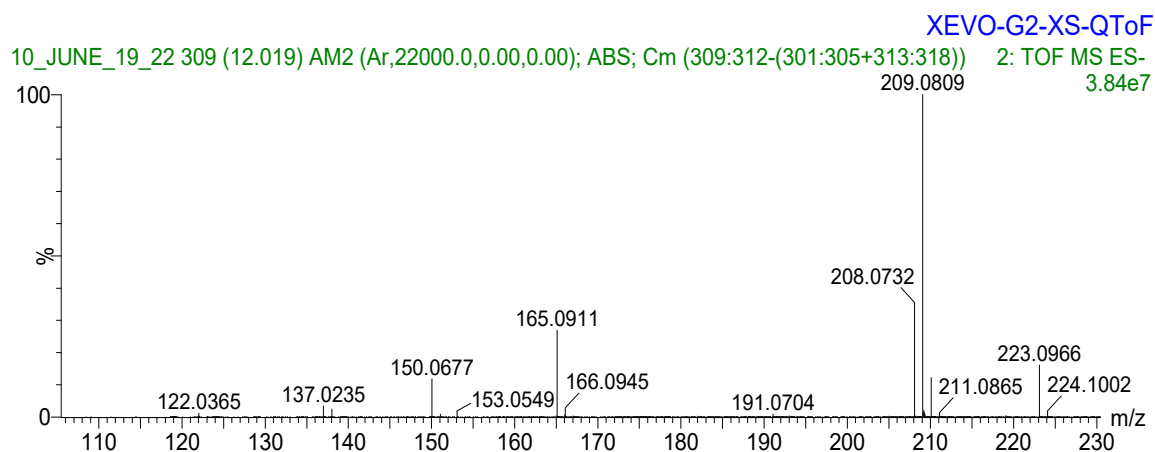
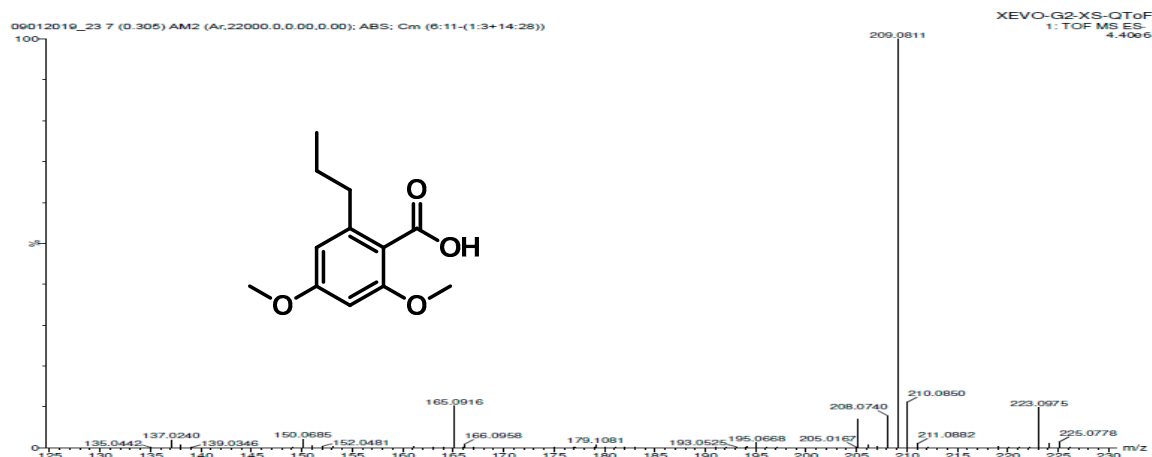


Figure S24: HRESIMS, MS/MS spectra and fragmentation pattern of 2, 4-di-O-Methyldivarc acid (6).

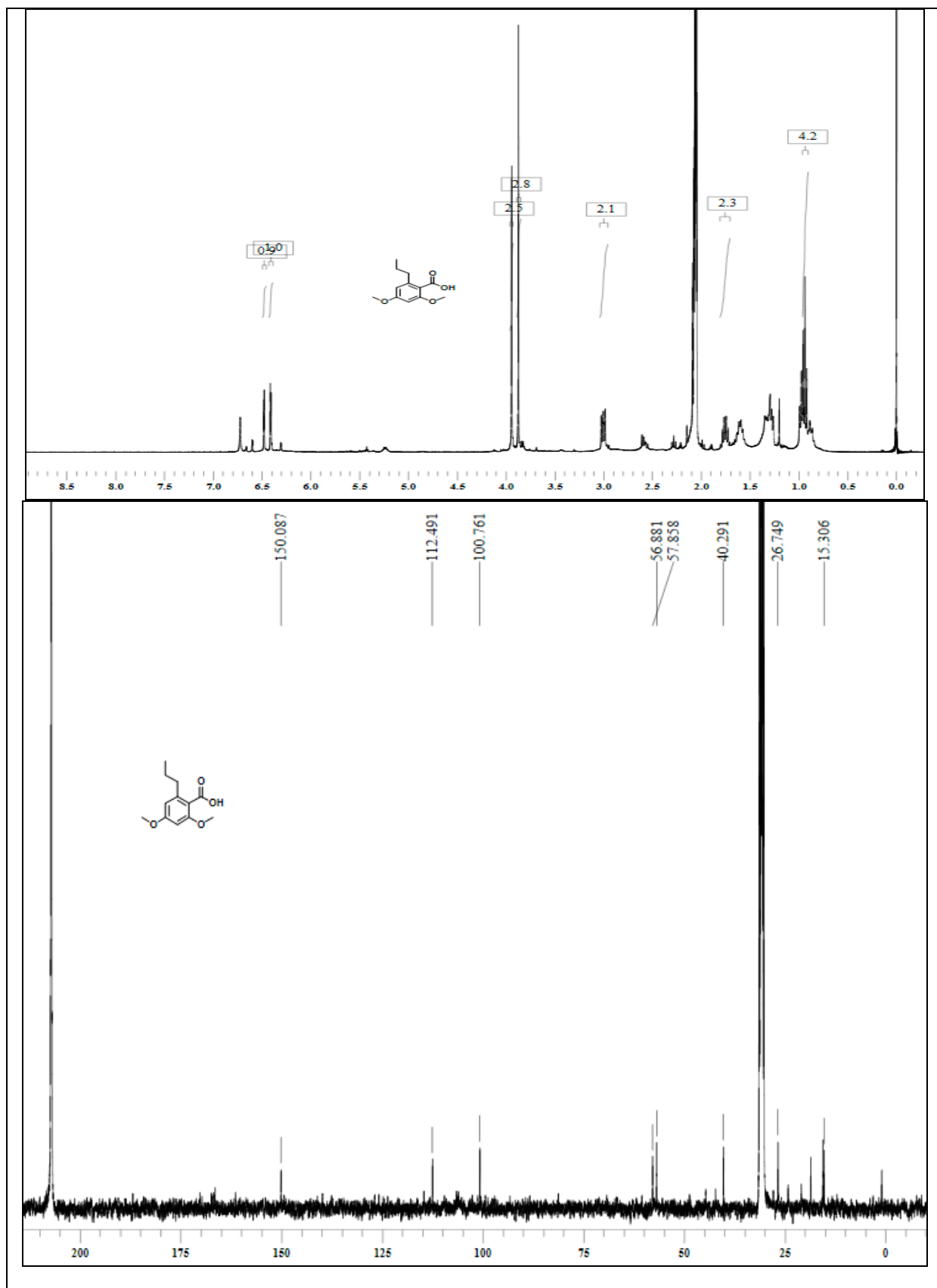


Figure S25: ^1H & ^{13}C NMR Spectrum of 2,4-di-O-Methyldivaic acid (**6**) (400 & 100 MHz, CD_3COCD_3).

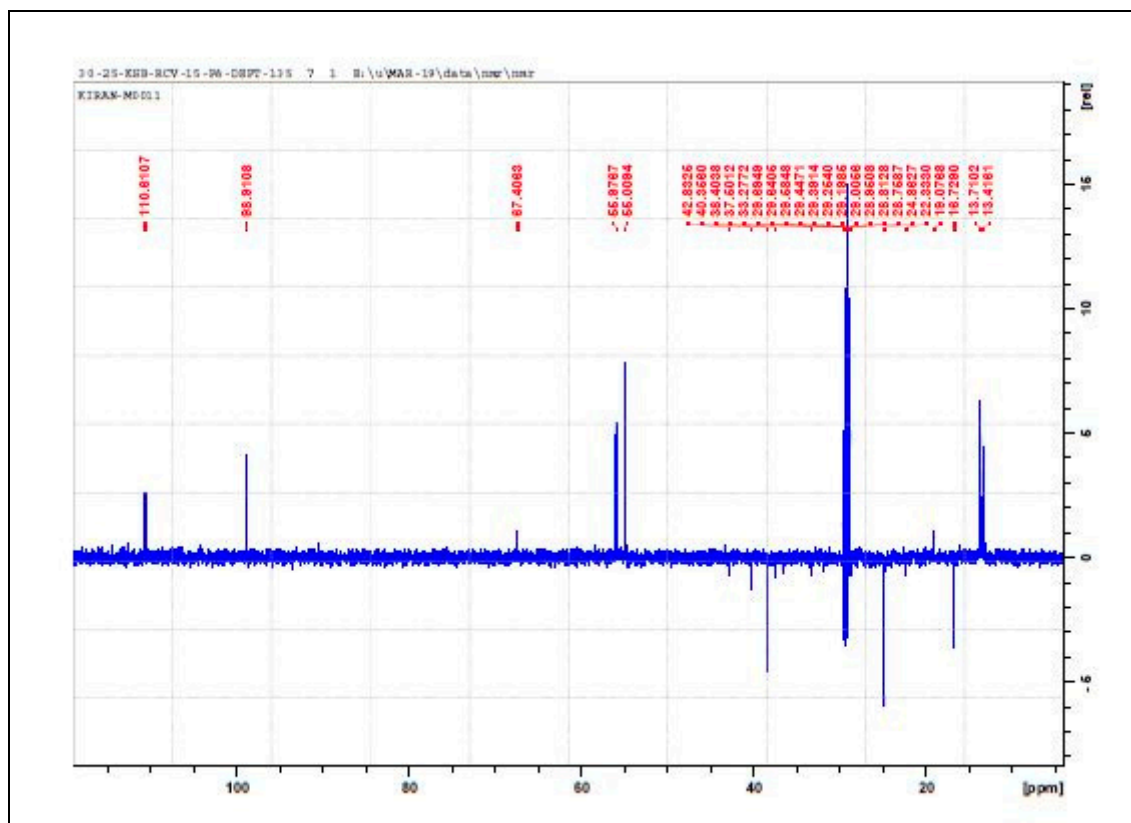


Figure S26: DEPT 135° NMR spectra of 2,4-di-O-Methyldivaic acid (**6**).

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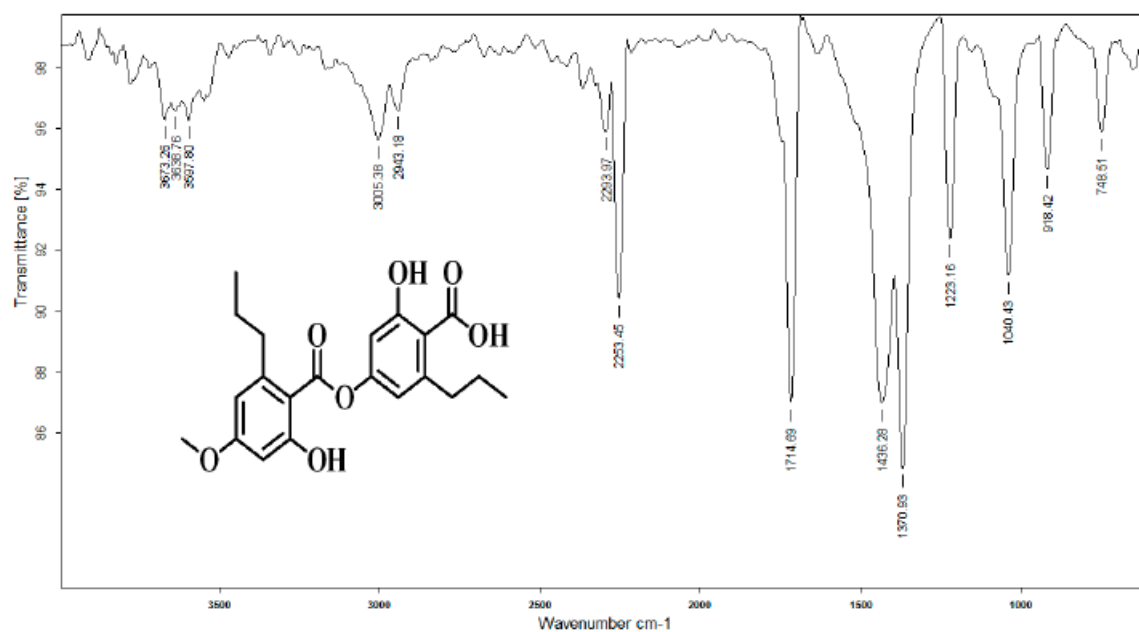


Figure S27: FTIR spectrum of Divaricatic acid (7).

20101110_6.D (0.322) AM2 (Kc22066.0,0.00,0.90): A55; Cn (0-10)

100

367.1461

350.1490

338.1517

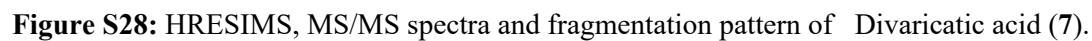
455.1323

623.1155

133.0649 150.1754 177.0544 196.0682 209.0754 240.0605 256.1820 325.0947 339.2119 356.0995 417.1710 446.1045 466.1472 517.1157 524.1370 556.0773 584.0919

0

Chemical structure: CCOC1=CC=C(C(=C1)O)C(=O)OC2=CC=C(C(=C2)O)C(=O)OCC



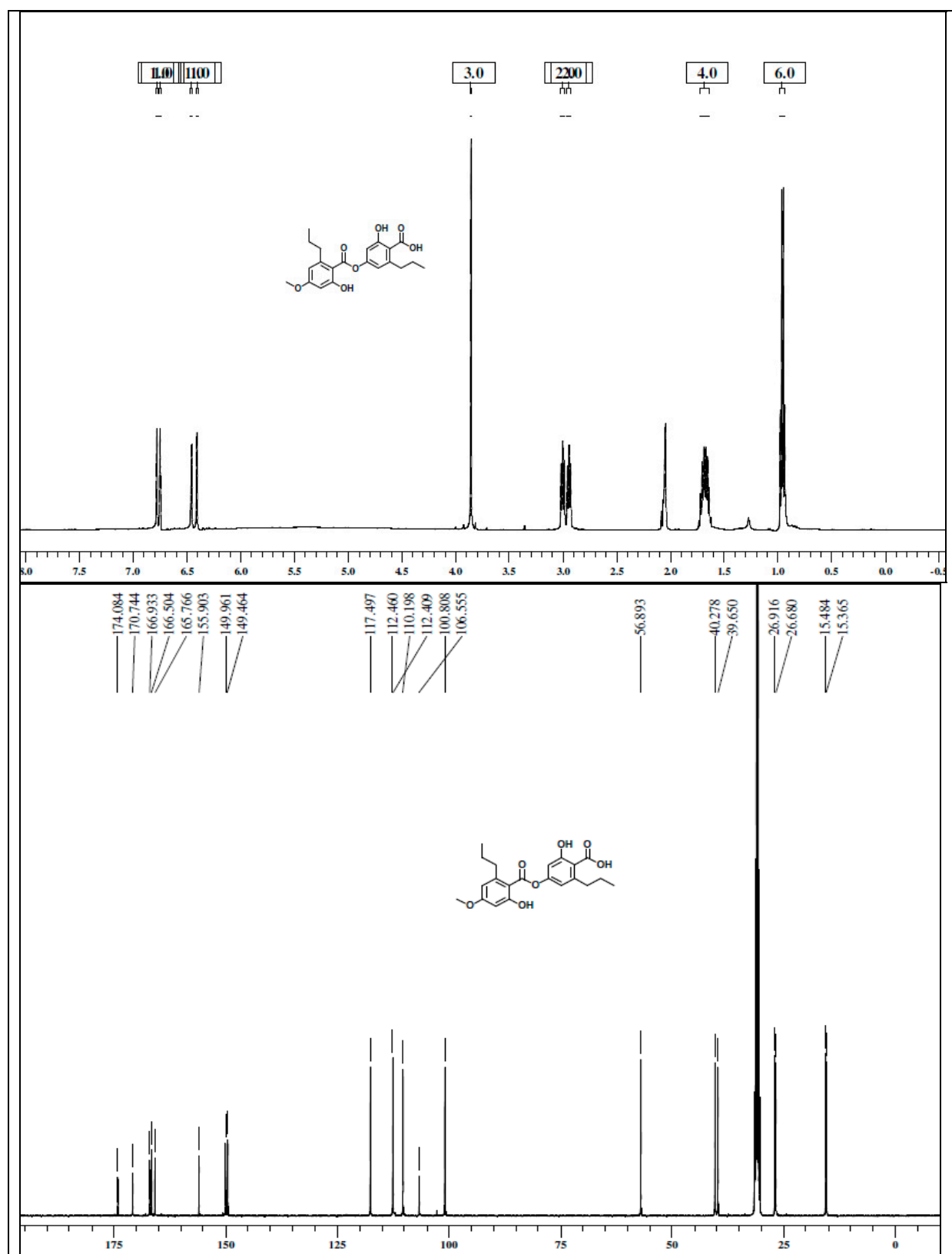


Figure S29: ¹H & ¹³C NMR Spectrum of Divaricatic acid (**7**) (400 & 100 MHz, CD₃COCD₃).

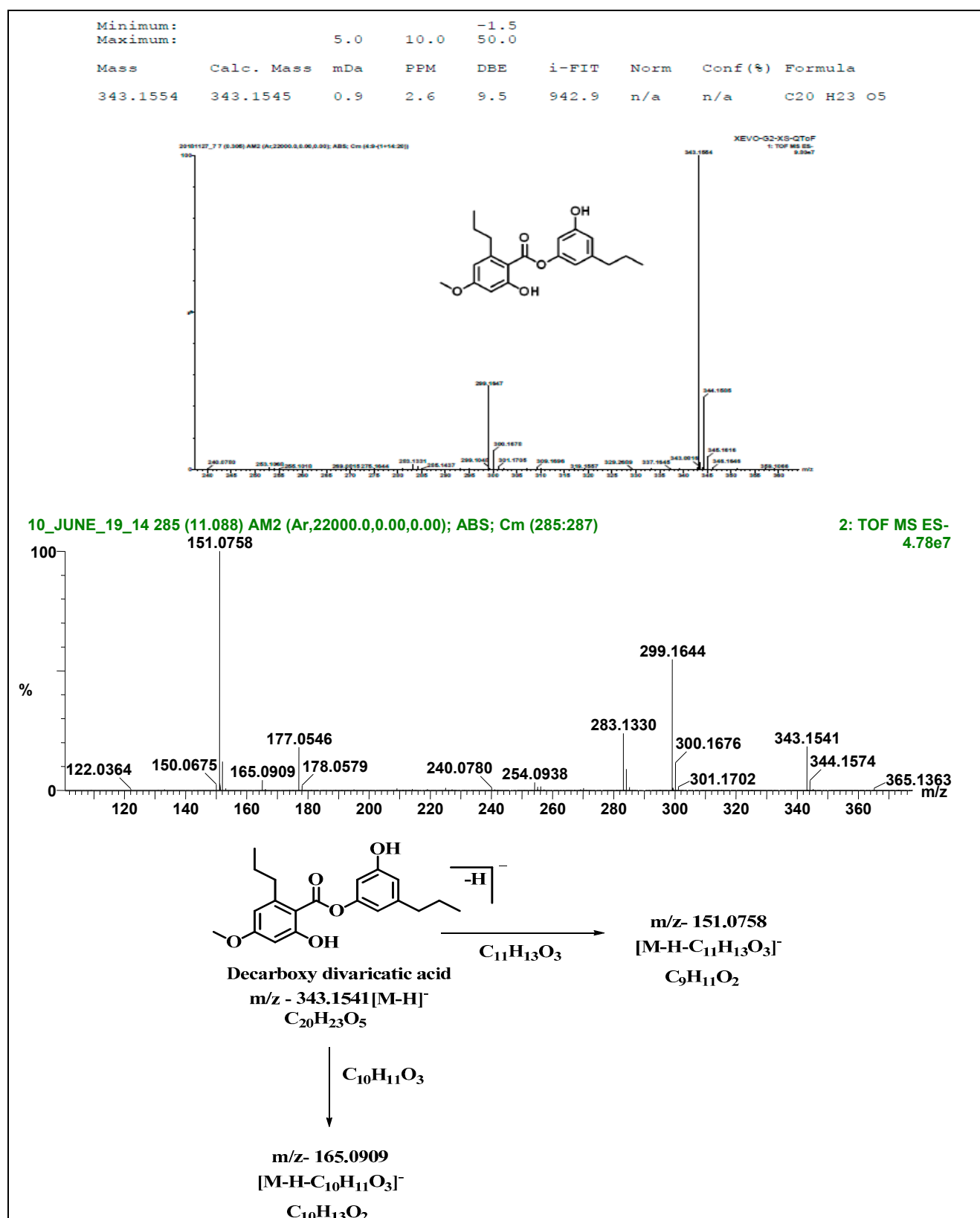


Figure S30: HRESIMS, MS/MS spectra and fragmentation pattern of Decarboxydivaric acid (8).

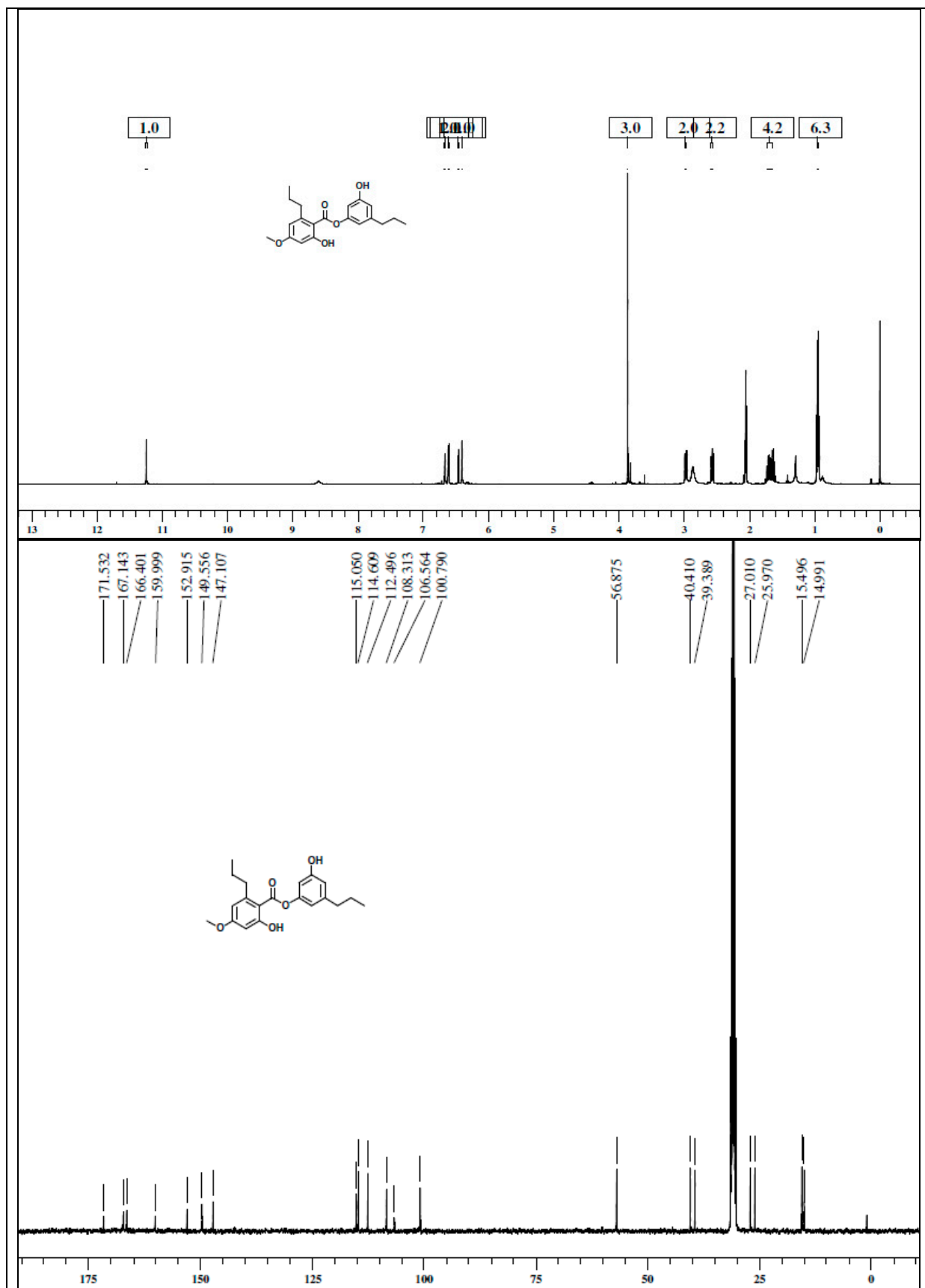
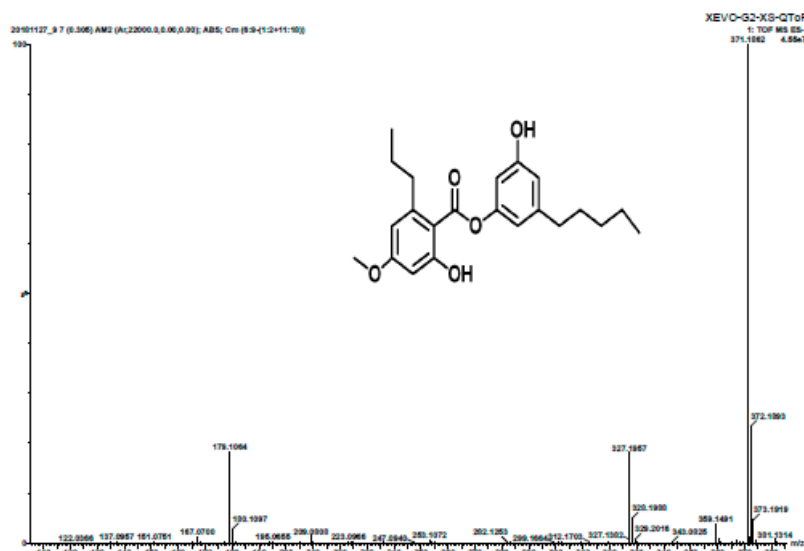


Figure S31: ¹H & ¹³C NMR Spectrum of Decarboxydivaric acid (**8**) (400 & 100 MHz, CD₃COCD₃).

Minimum:				-1.5				
Maximum:		5.0	5.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf (%)	Formula
371.1862	371.1858	0.4	1.1	9.5	764.4	n/a	n/a	C ₂₂ H ₂₇ O ₅



10_JUNE_19_15 304 (11.810) AM2 (Ar,22000.0,0.00,0.00); ABS; Cm (303:306)

2: TOF MS ES-
5.69e7

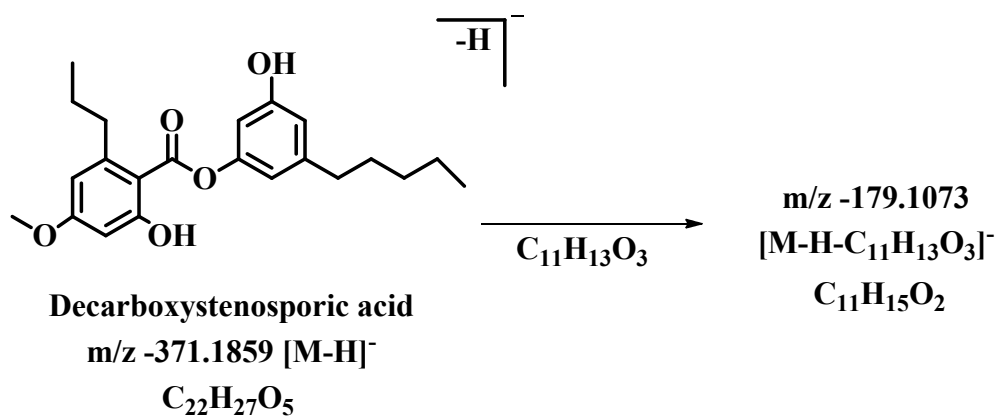
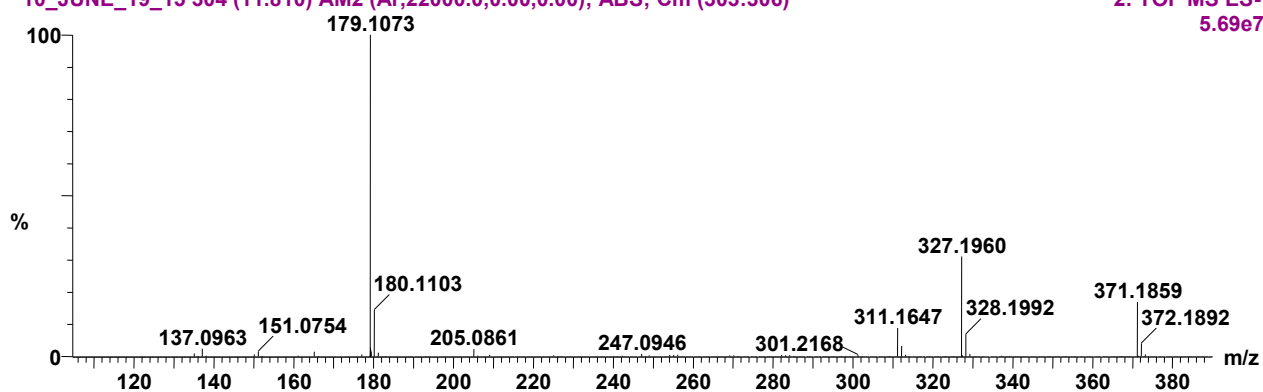


Figure S32: HRESIMS, MS/MS spectra and fragmentation pattern of Decarboxystenosporic acid (9).

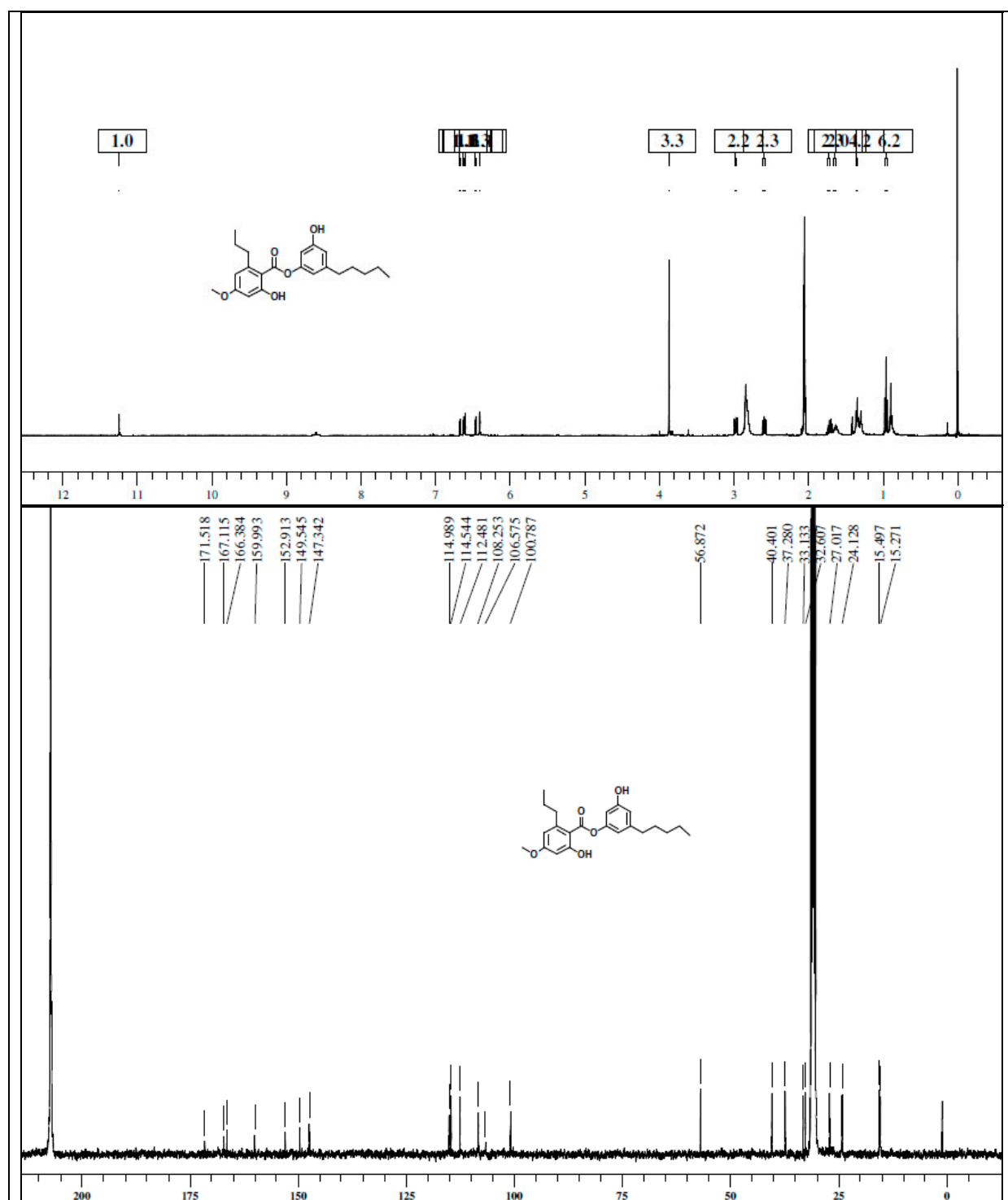
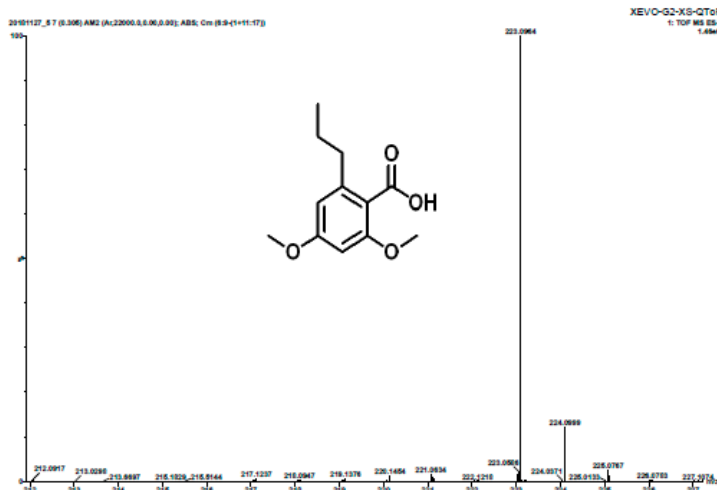


Figure S33: ¹H & ¹³C NMR Spectrum of Decarboxystenosporic acid (**9**) (400 & 100 MHz, CD₃COCD₃).

Minimum:				-1.5				
Maximum:	5.0	10.0		50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
223.0964	223.0970	-0.6	-2.7	5.5	533.5	n/a	n/a	C12 H15 O4



10_JUNE_19_20 239 (9.293) AM2 (Ar,22000.0,0.00,0.00); ABS; Cm (238:240)

2: TOF MS ES-
9.24e6

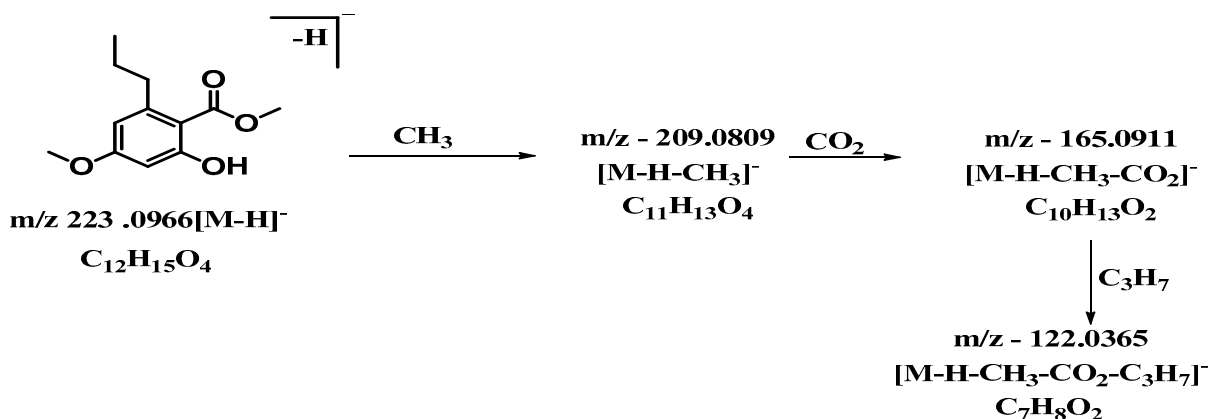
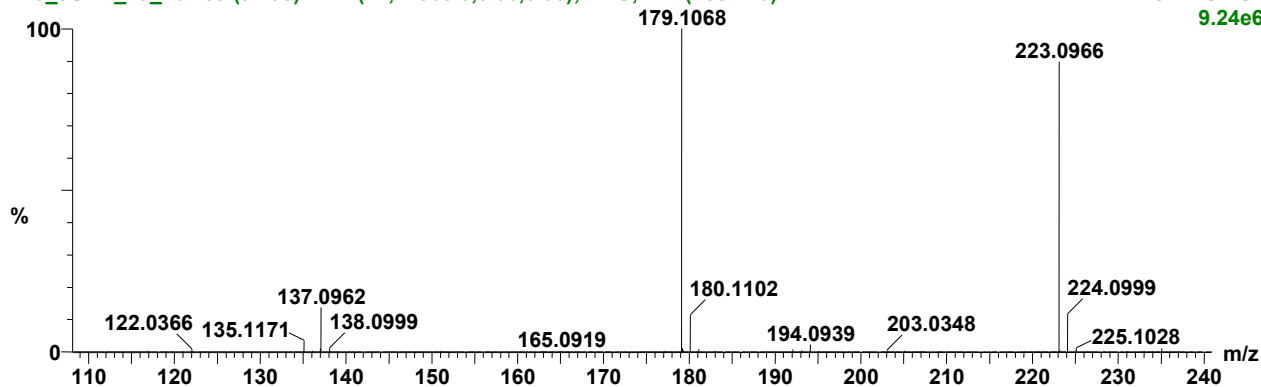


Figure S34: HRESIMS, MS/MS spectra and fragmentation pattern of Methyldivaricatin (10).

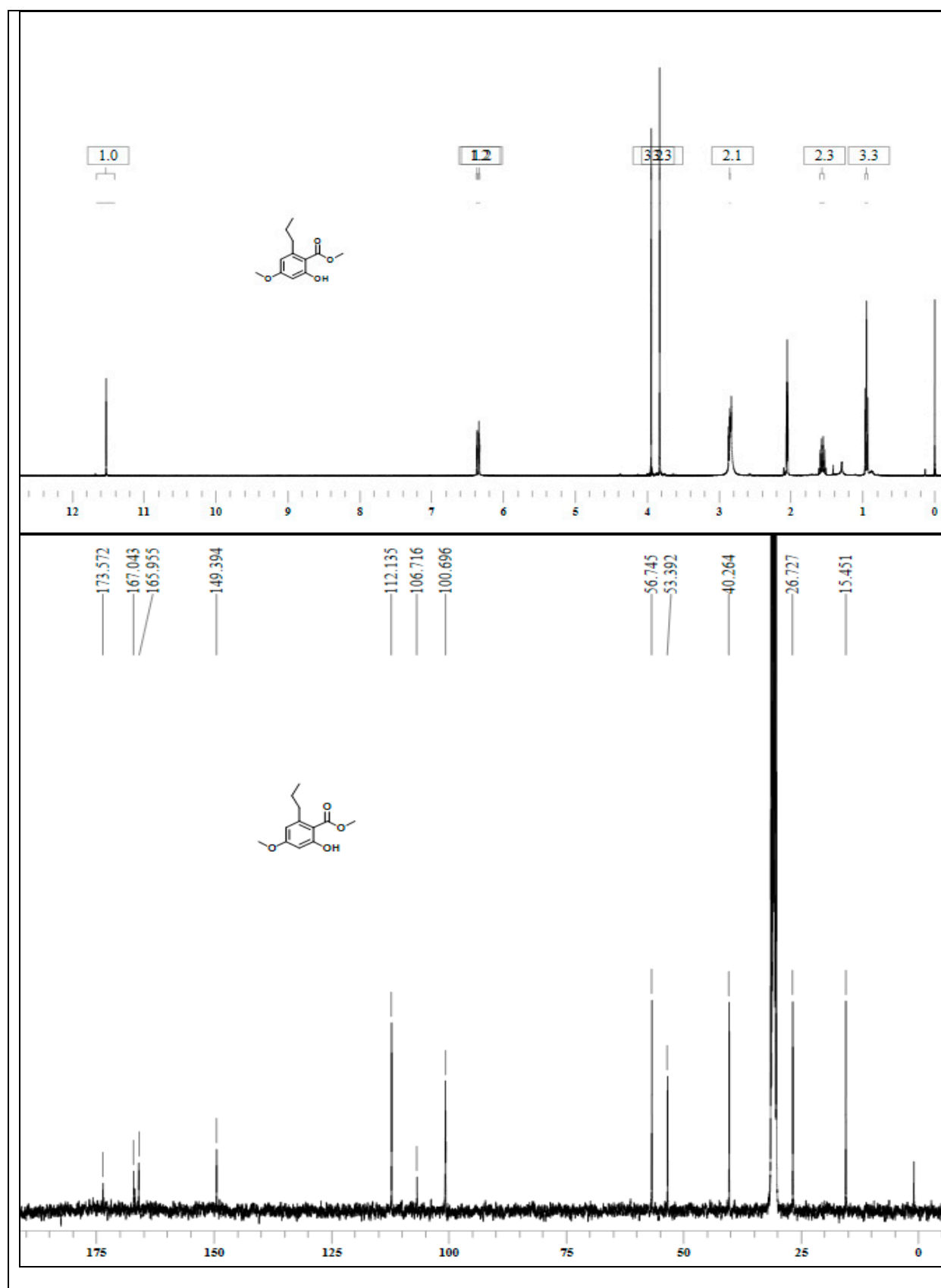
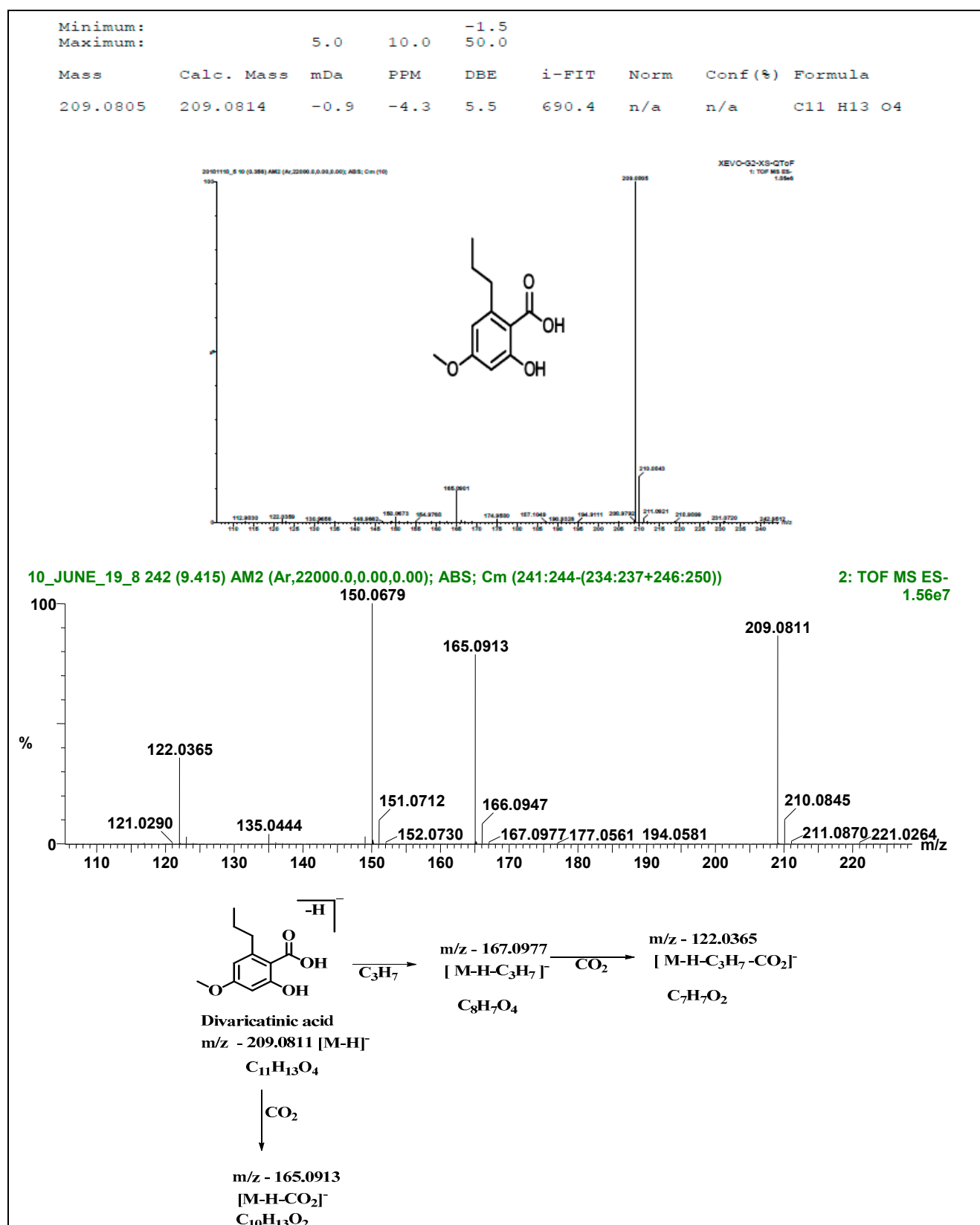


Figure S35: ^1H & ^{13}C NMR Spectrum Methyldivaricinate (10) (400 & 100 MHz, CD_3COCD_3).



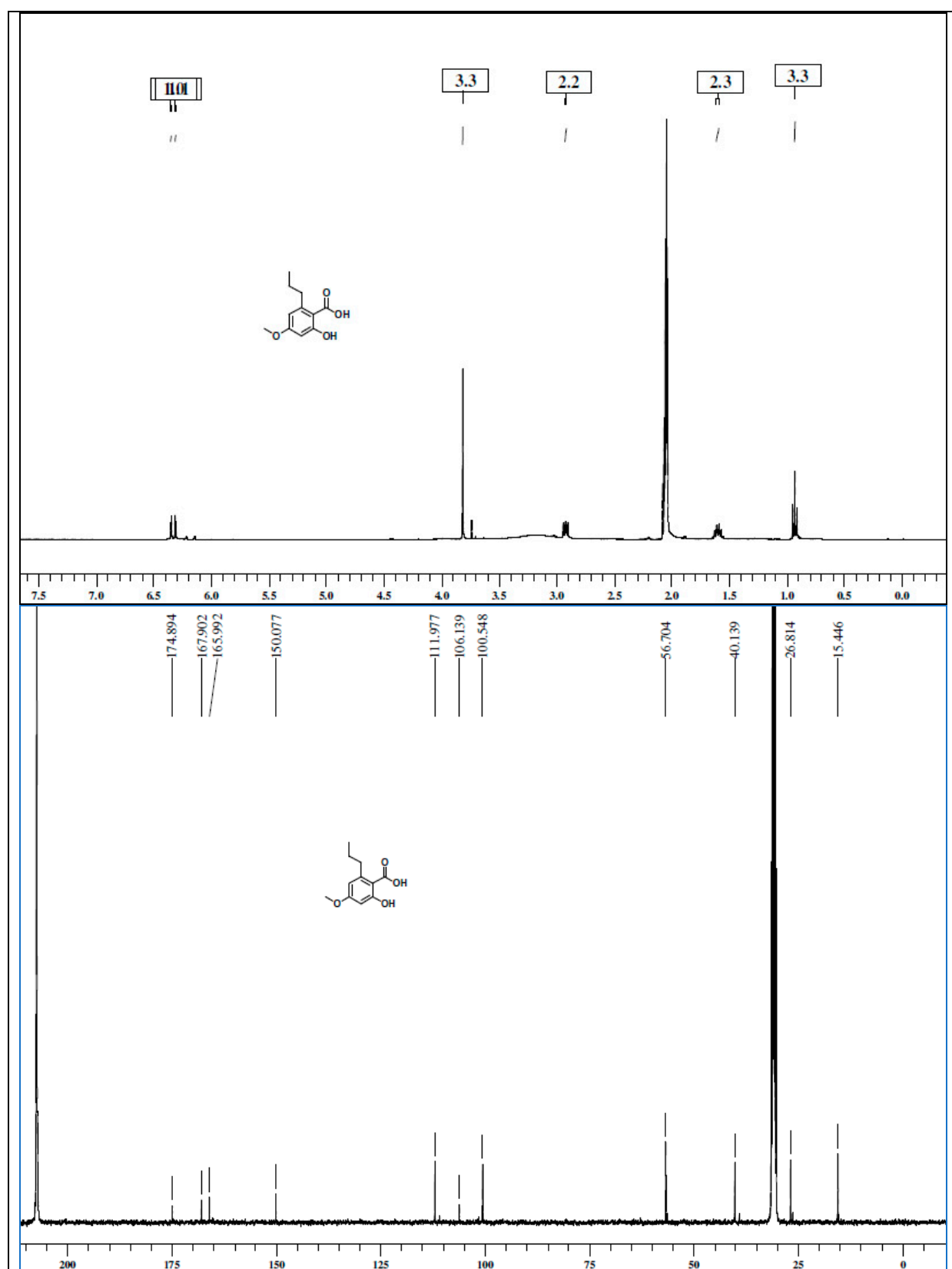


Figure S37: ^1H & ^{13}C NMR Spectrum Divaricatinic acid (11) (400 & 100 MHz, CD_3COCD_3).

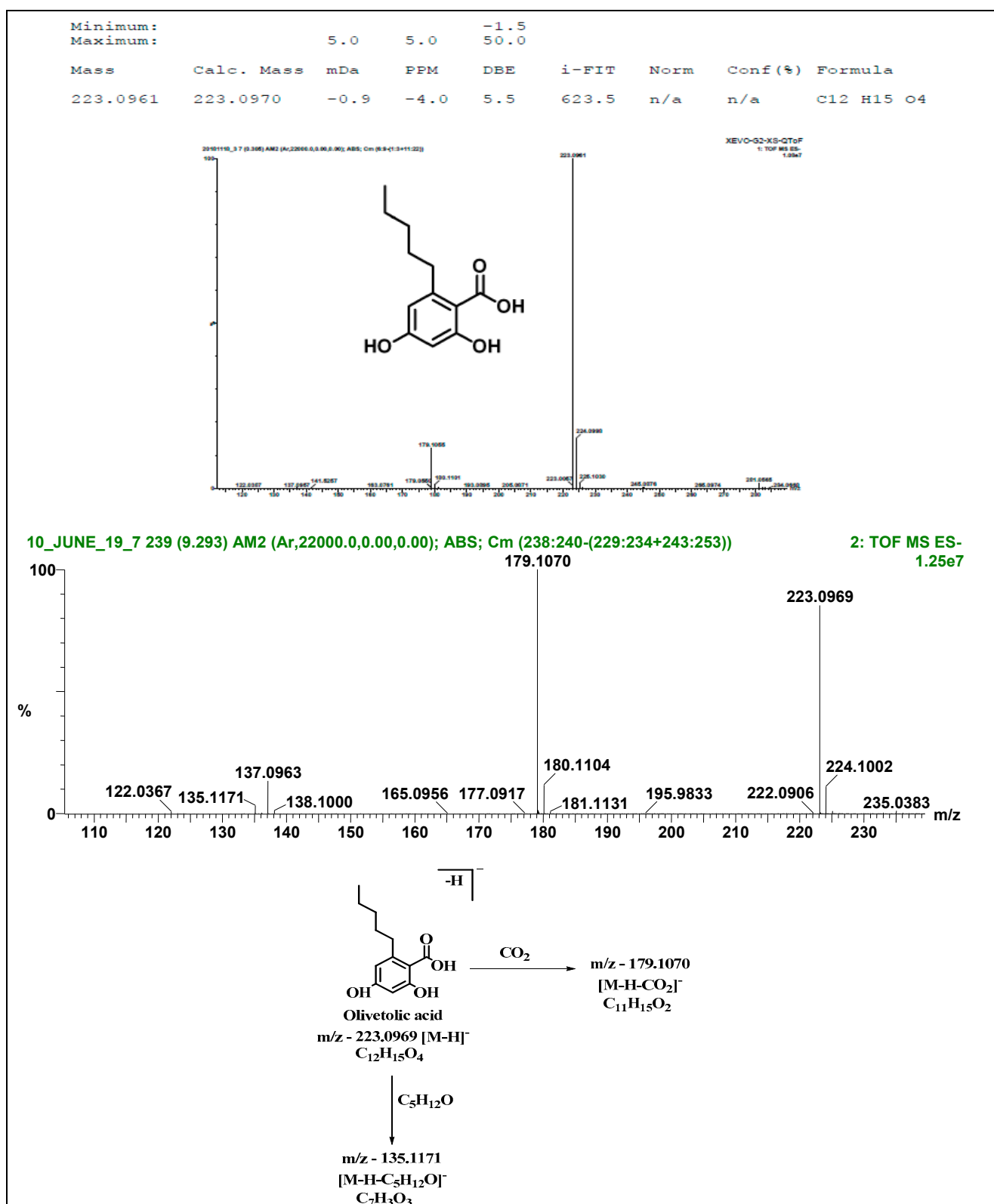


Figure S38: HRESIMS, MS/MS spectra and fragmentation pattern of Olivetolic acid (**12**).

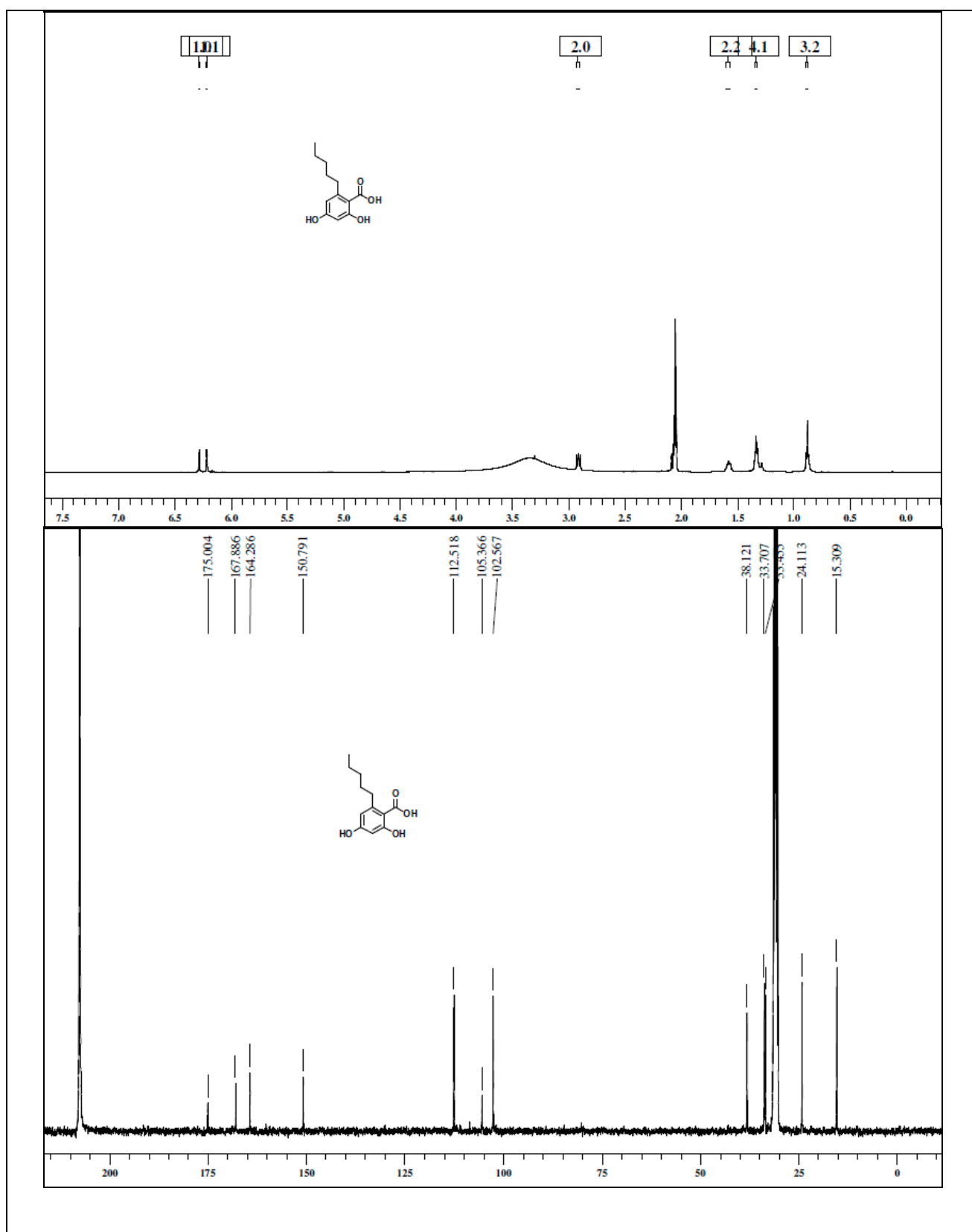
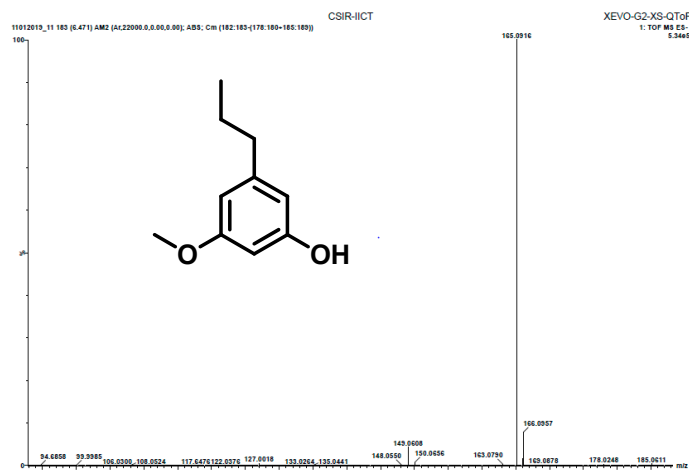


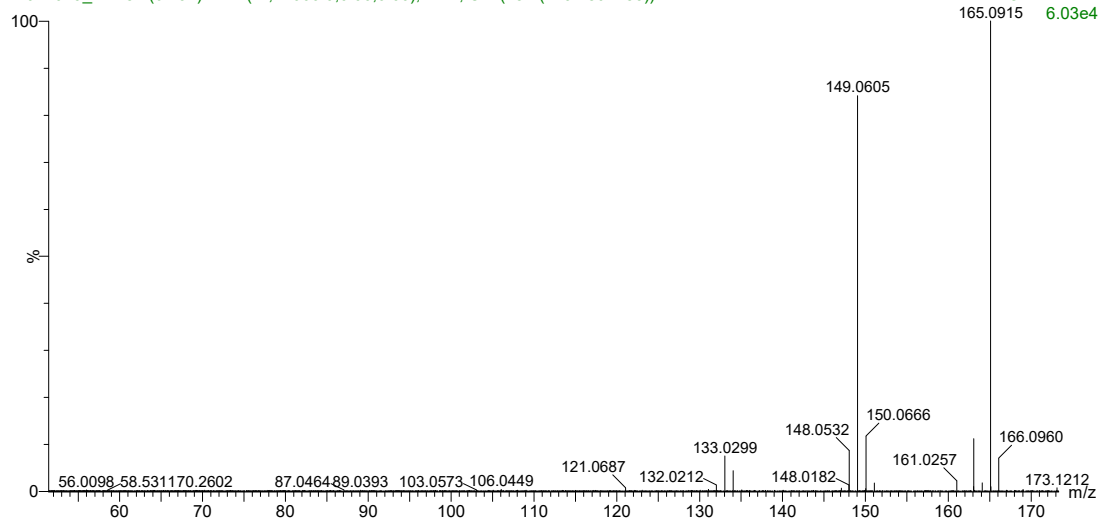
Figure S39: ^1H & ^{13}C NMR Spectrum of Olivetolic acid (**12**) (400 & 100 MHz, CD_3COCD_3).

Minimum:				-1.5				
Maximum:		5.0	5.0	50.0				

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
165.0916	165.0916	0.0	0.0	4.5	512.3	n/a	n/a	C ₁₀ H ₁₃ O ₂



11012019_11 182 (6.434) AM2 (Ar,22000.0,0.00,0.00); ABS; Cm (182-(179:180+183))



Divarinolmonomethylether
m/z - 165.0916 [M-H]⁻
C₁₀H₁₃O₂

Figure S40: HRESIMS, MS/MS Spectra and fragmentation pattern of Divarinolmonomethylether (13).

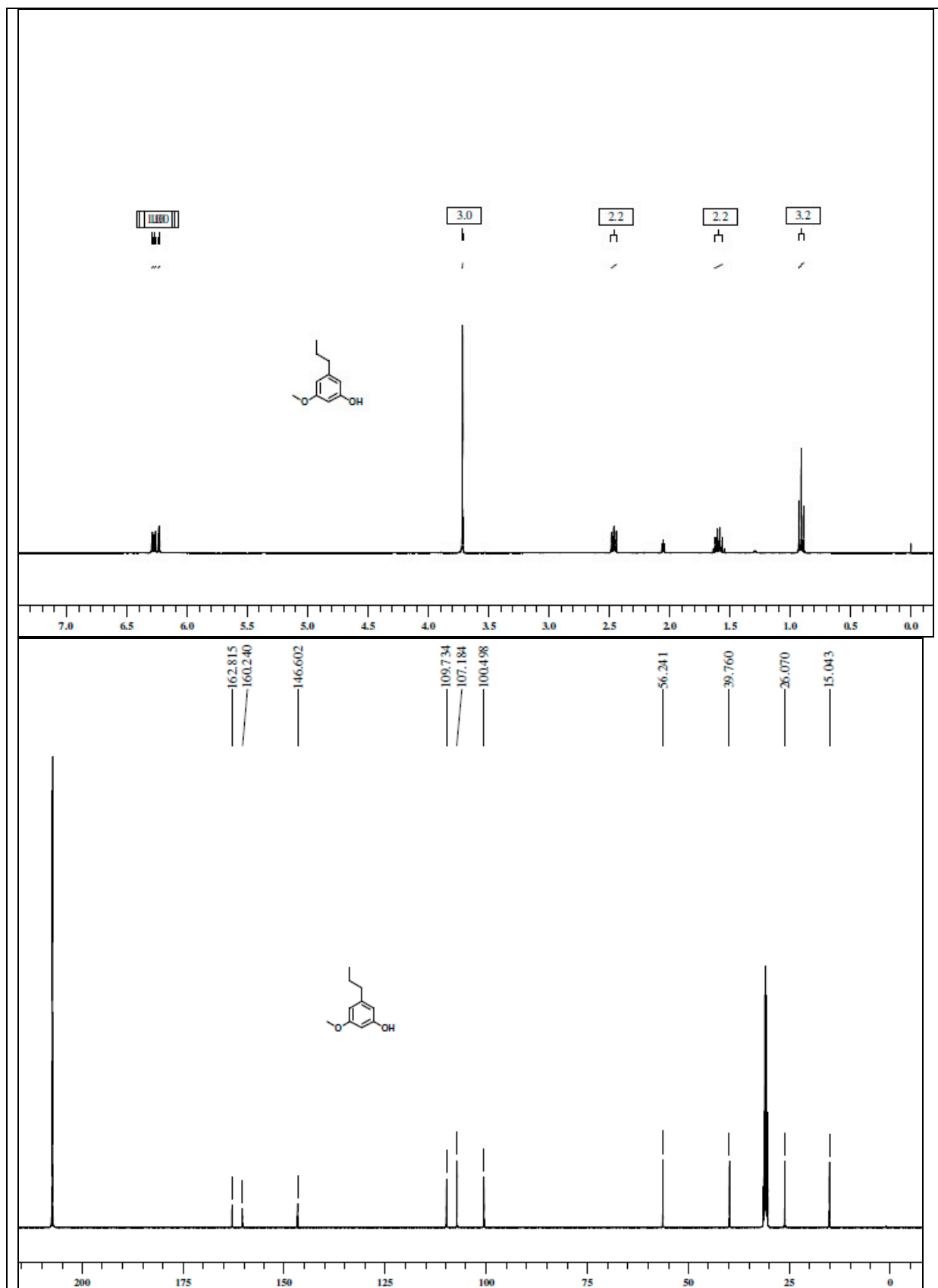
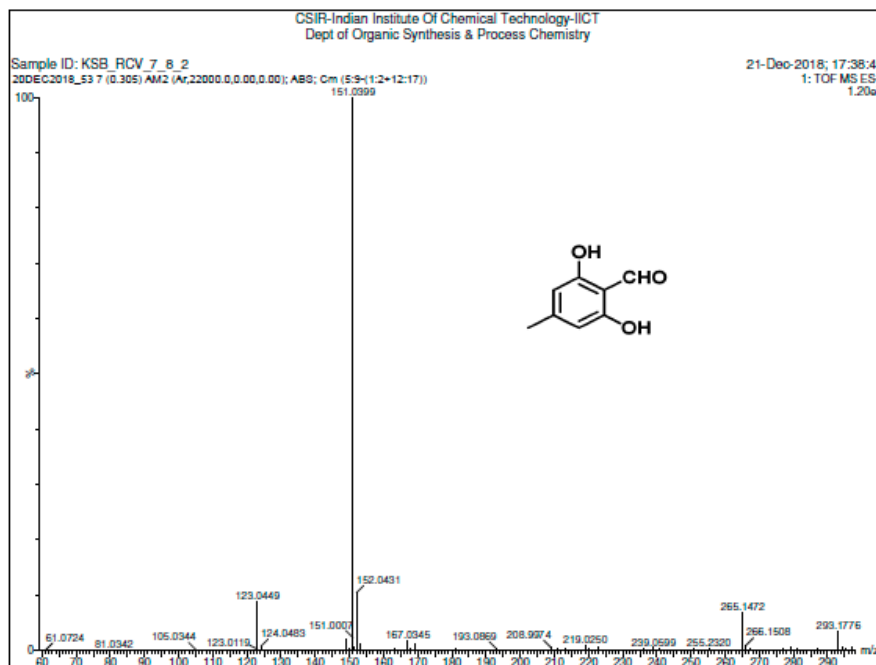


Figure S41: ^1H & ^{13}C NMR Spectrum of Divarinolmonomethylether (**13**) (400 & 100 MHz, CD_3COCD_3).

Minimum:				-1.5					
Maximum:		5.0	5.0	50.0					
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf (%)	Formula	
151.0399	151.0395	0.4	2.6	5.5	924.3	n/a	n/a	C8 H7 O3	



XEVO-G2-XS-QToF

Atranol 234 (8.002) AM2 (Ar,7000.0,554.26,0.00,LS 10); ABS; Cm (233:237-(224:230+239:247))

2: TOF MS ES-
2.18e6

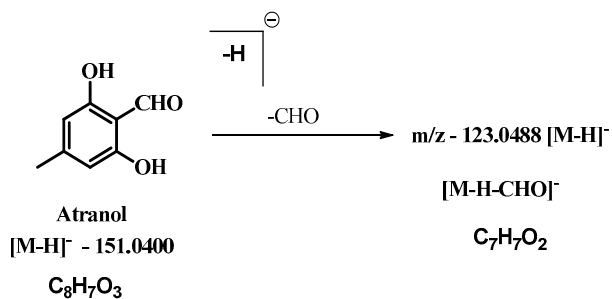
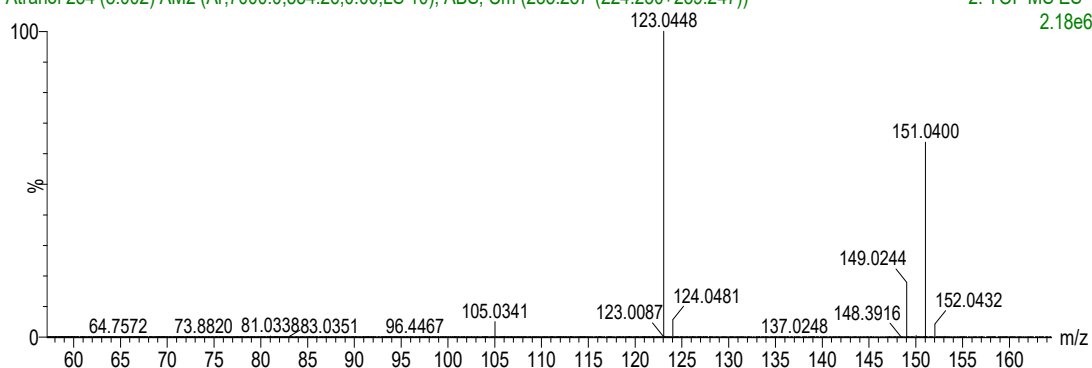


Figure S42: HRESIMS, MS/MS Spectra and fragmentation pattern of Atranol (14).

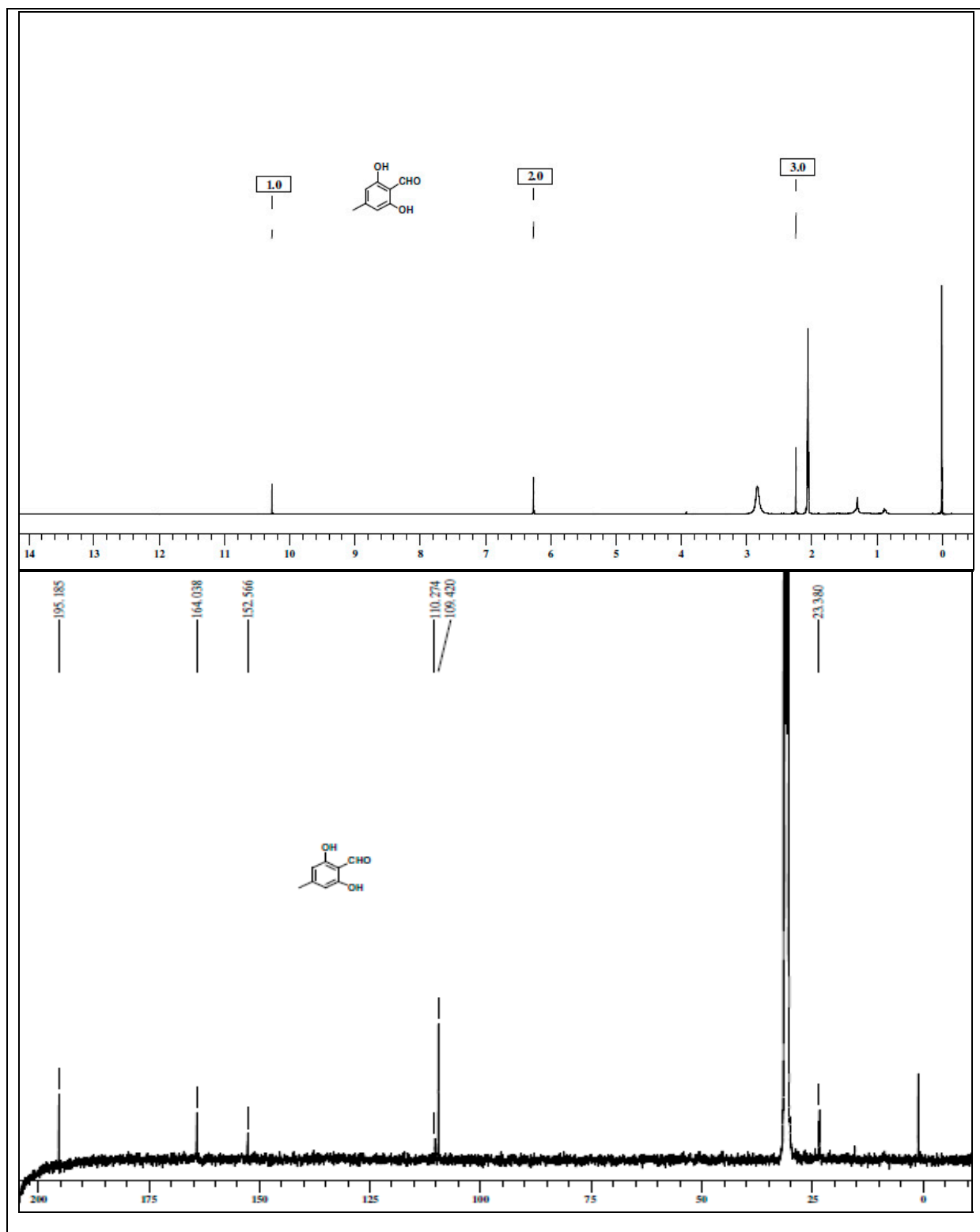


Figure S43: ^1H & ^{13}C NMR Spectrum of Atranol (**14**) (400 & 100 MHz, CD_3COCD_3).

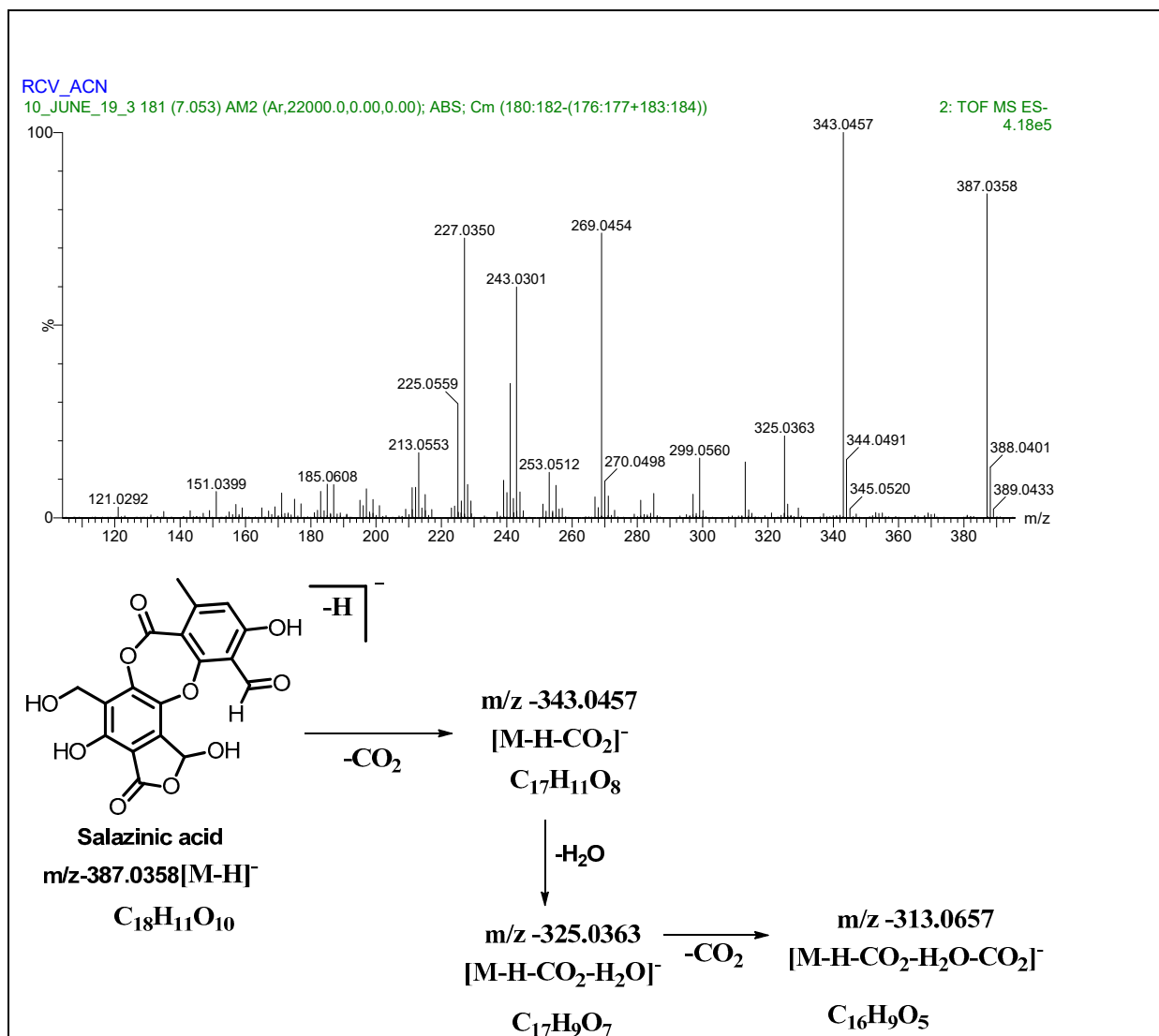


Figure S44: MS/MS Spectra and fragmentation pattern of Salazinic acid.

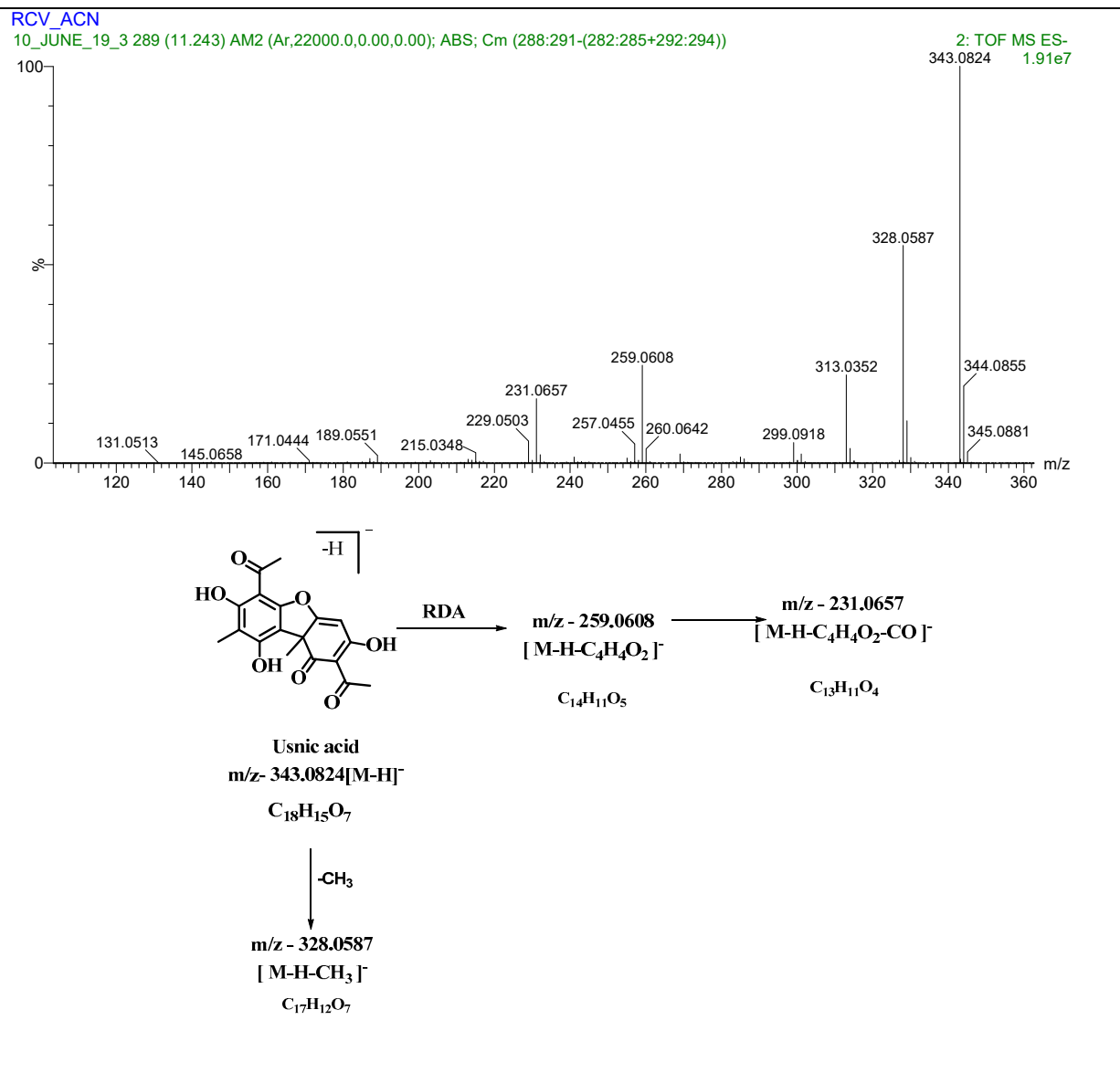


Figure S45: MS/MS Spectra and fragmentation pattern of Usnic acid.

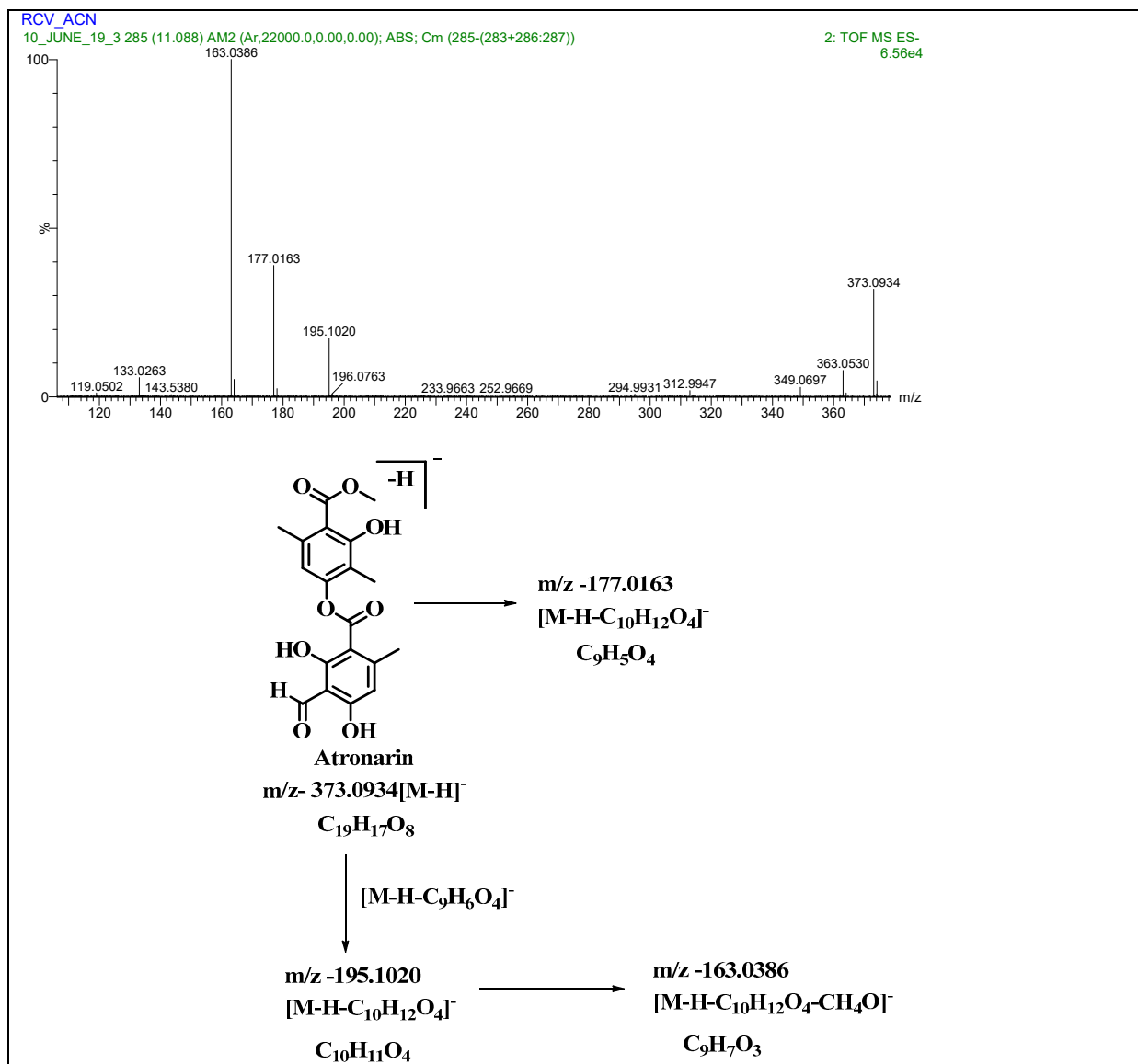


Figure S46: MS/MS Spectra and fragmentation pattern of Atronarin.

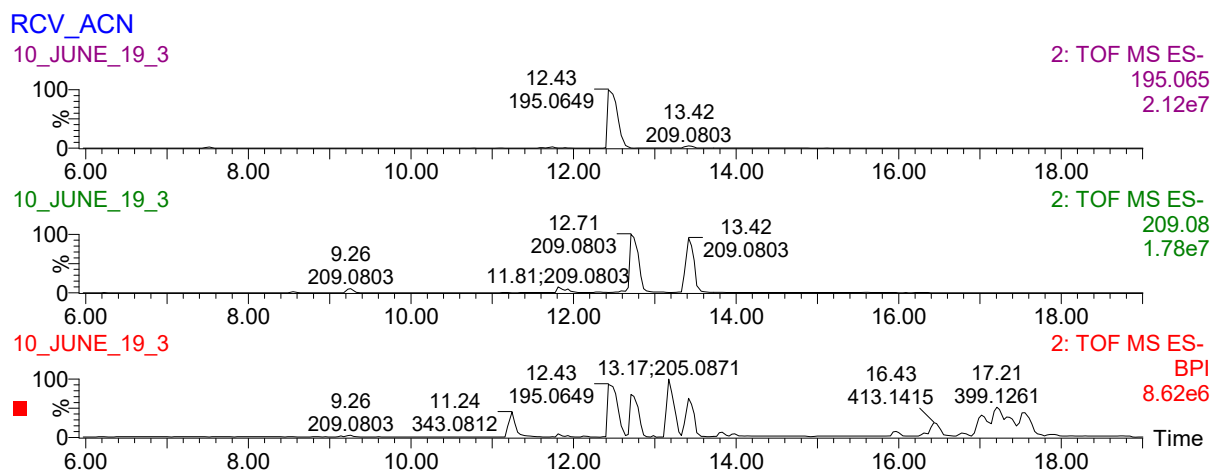


Figure S47: TIC of *R.conduplicans*: a) 209 ion extracted chromatogram: b) 195 ion extracted chromatogram.

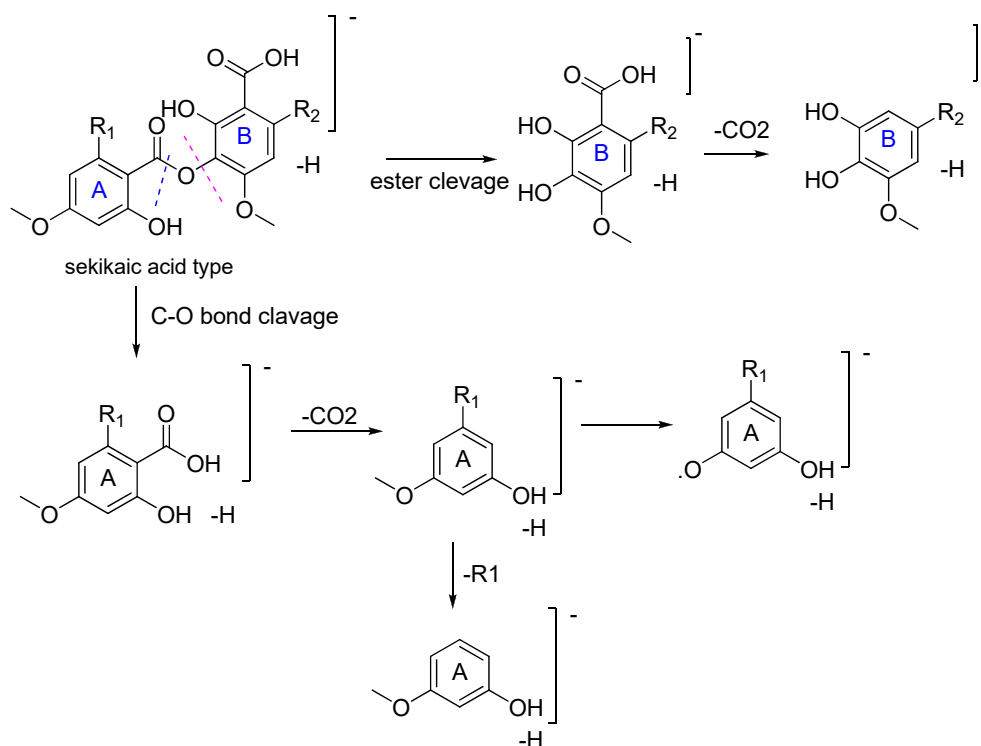


Figure S48: Common fragmentation of depsides, based on ESI (-) MS/MS of Sekikaic acid (1).

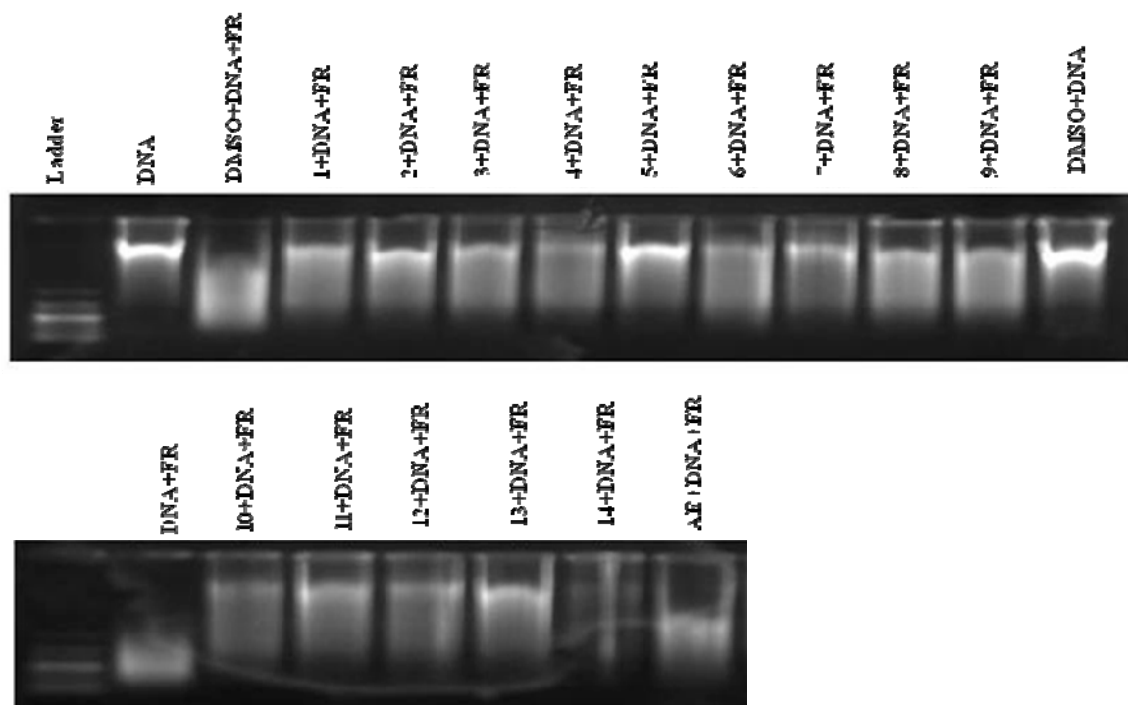


Figure S49. *In vitro* DNA damage assay. Compounds (1-14) and *R. conduplicans*. AE were incubated with DNA and Fenton Reagent and DNA damage was recorded with Agarose Gel electrophoresis.