SUPPLEMENTARY MATERIAL

Anti-diabetic activities of phenolic compounds of Alternaria sp. an endophyte isolated from the leaves of desert plants growing in Egypt

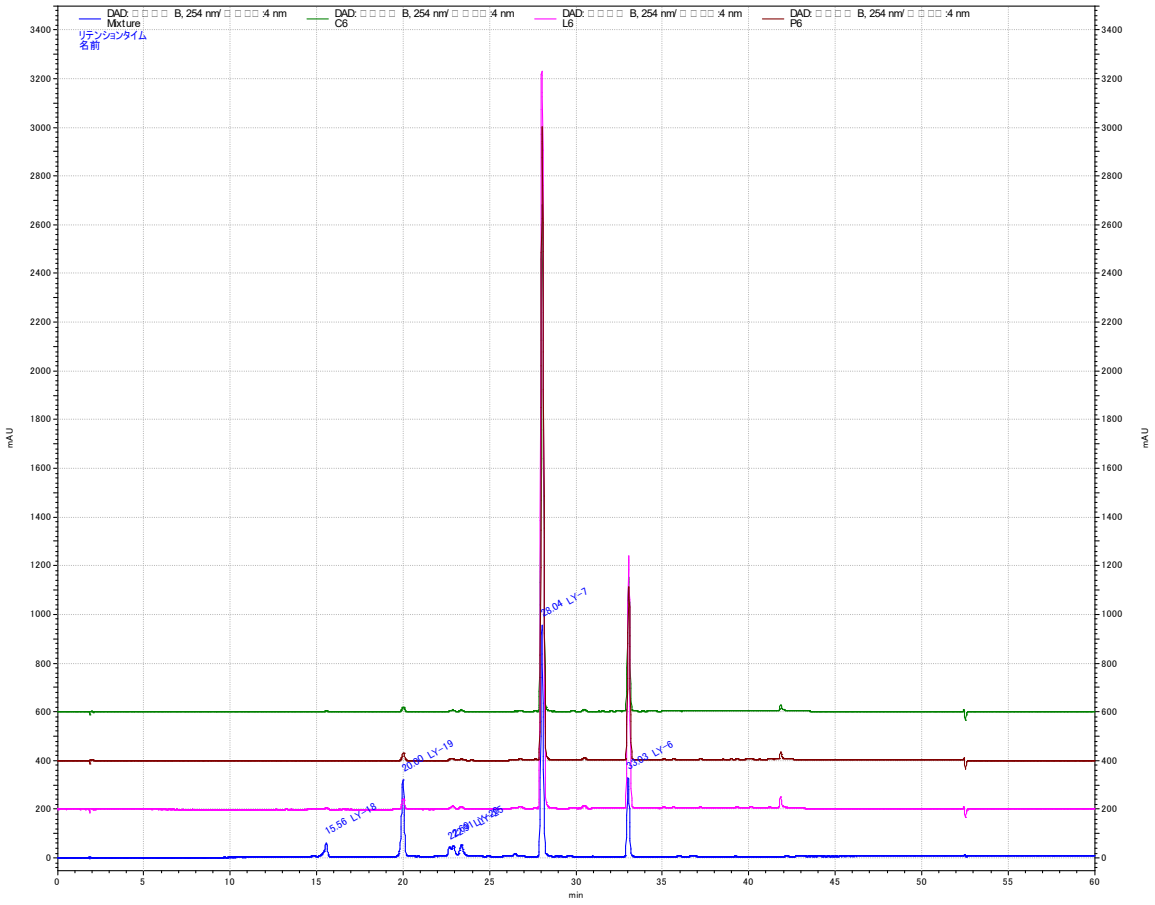
Ahmed Elbermawi [1,*], Ahmed R. Ali [2,*], Yhiya Amen [1], Ahmed Ashour [1], Kadria F. Ahmad [1], El-Sayed S. Mansour [1], Ahmed F. Halim [1]

[1] Department of Pharmacognosy Faculty of Pharmacy, Mansoura University, Mansoura, 35516 Egypt.
[2] Department of Medicinal Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.
* Ahmed Elbermawi: asbeder@mans.edu.eg; Tel.: +20 10-0481-1533, Ahmed R. Ali: ahmed_reda5588@mans.edu.eg; Tel: +20 10-9838-4072.

Abstract: Six phenolic compounds; talaroflavone (**1**), alternarienoic Acid (**2**), altenuene (**3**), altenusin (**4**), alternariol (**5**), alternariol-5-*O*-methyl ether (**6**), were isolated from the solid rice culture media of *Alternaria* sp. an endophyte isolated from the fresh leaves of three desert plants; *Lycium schweinfurthii* Dammer (Solanaceae), *Pancratium maritimum* L. (Amaryllidaceae) and *Cynanchum acutum* L. (Apocynaceae). Compounds **2**, **3**, and **4** exhibited potent α-glucosidase and lipase inhibitory activities suggesting that they might act as naturally occurring anti-diabetic candidates. The same compounds showed potent binding in the active site for both enzymes with desirable pharmacokinetic properties. The isolated bioactive compounds were not exclusive to a certain host plant which reveals the dominant ecological standpoints for consequent optimization. This could lead to a cost-effective and reproducible yield compliant to commercial scale-up.

Keywords: Alternaria sp., anti-diabetic, α-glucosidase; lipase.

Figure S1: HPLC chromatogram of mix of compounds (blue) *vs* the MeOH extract of the three plants isolated Alternaria sp. metabolites; pink: *Lycium*, brown: *Pancracium* and green: *Cynanchum*.



First peak (compound 1, R_t=15.57), second peak (compound 2, R_t=20.01), third peak (compound 3, R_t=22.69), fourh peak (compound 4, R_t=22.84), fifth peak (compound 5, R_t=28.01) and the last peak (compound 6, R_t=33.05).

Experimental Section

General

^{1}H and ^{13}C-NMR spectra were obtained on a Bruker DRX 600 NMR spectrometer (Bruker Daltonics INC., MA, USA) using TMS as internal standard for chemical shifts. Chemical shifts (δ) were expressed in ppm with reference to the TMS resonance. HR-FAB-MS were measured with a JEOL JMS 700 spectrometer (JEOL, Japan) or HR–ESI–MS was determined using LC–MS–IT–TOF (Shimadzu, Tokyo, Japan). The MS instrument was operated using an ESI source in both positive and negative ionization modes with survey scans acquired from *m/z* 100–2000 for MS and *m/z* 50–1500 for MS/MS. The ionization parameters were as follows: probe voltage, ± 4.5 kV; nebulizer gas flow, 1.5 L/min; CDL temperature, 200°C; heat block temperature, 200°C. MPLC Column parameters: GL Sciences Inc (20X250 mm, 5 µm), UV detector at 254 nm, Flow rate: 10 ml/min. HPLC analysis was carried out using Inertsil ODS-3 column (5 µm, 4.6 x 150 nm, GL sciences, Tokyo, Japan) attached to Agilent 1220 Infinity LC system, equipped with a binary solvent deliver system, an auto sampler and a photodiode array detector (Agilent Technologies, California, USA).Dimethylsulfoxide (DMSO) and other organic solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sephadex LH-20 was purchased from GE Healthcare (Uppsala, Sweden). Diaion HP 20 was purchased from Mitsubishi Chemical Corporation, Japan.Silica gel (75–120 mesh) and RP-C_{18} silica gel (38–63 µm) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Thin layer chromatography (TLC) silica gel 60 F_{254} was purchased from Merck (Darmstadt, Germany). The developed chromatograms were visualized under 254 nm. UV light and the spots were made visible by spraying with vanillin/H_2SO_4 reagent before warming in an oven pre-heated to 110°C for 5 min.

Fungal material

Suaeda vera (Amaranthaceae), *Heliotropium curassavicum* (Boraginaceae), *Limoniastrum monopetalum* (Plumbaginaceae), *Cynanchum acutum* (Apocynaceae), *Lycium schweinfurthii* (Solanaceae), *Pancracium maritimum* (Amaryllidaceae), *Ficus carica* (Moraceae), *Asphodelus microcarpus* Salzm. & Viv. (Asphodelaceae), *Psidium guajava* (Myrtaceae) and *Thymelaea hirsute* (Thymelaeaceae) leaves were collected from International coastal road, 40 km away from Gamasa towards Alexandria, Egypt in December 2016. The plant identity was confirmed by Dr. Ibrahim Mashaly, Professor of Ecology, Faculty of Sciences, Mansoura University. Fresh leaves

were cut into very small pieces, washed with sterilized water, and then the surface is thoroughly treated with 70% ethanol for 1-2 minutes and dried under the laminar flow to avoid surface contaminating microbes. The leaf fragments were inoculated in petri dishes containing malt agar (MA) medium (Kjer *et al.*, 2010), and incubated for five days. When fungal hyphae almost cover the surface of the malt agar plate, cultures were then re-inoculated onto fresh malt agar media until obtaining a pure colony.

The isolated strain was identified according to a molecular biological protocol by DNA amplification and sequencing of the ITS region as described by (Kjer *et al.*, 2010). The GenBank accession number of the fungus is (LC430328).

Production and isolation of fungal metabolites:

The isolated fungus from *L. shweinfurthii* was grown on solid rice culture media prepared by autoclaving 100 g of rice and 100 ml of water in a 1 L flasks, then fermentation was performed in five flasks for 30 days at room temperature under stationary conditions. To each 1L flask about 400 ml EtOAc were added and left overnight to allow complete extraction and left for 3 days. After filtration, re-extraction was done with fresh EtOAc three times till exhaustion. The combined EtOAc extracts were washed with distilled water and then evaporated under reduced pressure till dryness. The dry residues were dissolved in 90% MeOH and defatted by shaking with n-hexane to yield 3.2 g MeOH extract from solid rice culture media.

Isolation of compounds

The extract was fractionated on Diaion HP 20 by elution with H_2O/MeOH (100:0, 50:50, 25:75 and 0:100).

Fractions eluted with 50 % MeOH (93.60 mg) were subjected to fine purification using reversed phase open column chromatography, isocratically eluted with H_2O/MeOH (40:60) to afford compounds **1** at R_f value 0.62 & **2** at R_f value 0.74, quenched as dark blue spots under UV light at 254 nm on precoated RP-C_{18} F_{254} plates using the solvent system H_2O–MeOH (40:60). Fractions eluted with 75 % MeOH (74.42 mg) were subjected to chromatographic purification using MPLC gradiently eluted with H_2O-MeOH (50% -100%, 50 min.), compound **3** was purified from the fraction eluted at t_R 18.1 min. using preparative reversed phase TLC developed with H_2O/MeOH

(30:70) with R_f value 0.6, while compound **4** was isolated in pure form from the fraction eluted at t_R 22.2 min.

Fractions eluted with 100 % MeOH (400.70 mg) were subjected to chromatographic purification using Sephadex LH-20 (100 g) eluted with MeOH to afford 67 sub-fractions 2 ml each. The combined sub-fraction (33-67, 130 mg) was subjected to fine purification using MPLC, the same condition as mentioned before, to isolate compound **5** at t_R 20.4 min. & **6** at t_R 25.3 min.

Enzymatic assays

1.1. Inhibition of α-glucosidase activity

The *α*-glucosidase inhibitory activity was assayed according to a previously described method (Fatmawati et al. 2011). Several concentrations (with a three-fold dilution) as followings: 300, 100, 33.3, 11.1, 3.7, 1.23 and 0.41, were used in the assay. A 0.1 mL portion of the sample solution or positive control solution dissolved in DMSO and 0.1 mL of *α*-glucosidase enzyme (5 units/mL) in 0.15 M HEPES buffer were added to 0.1 M sucrose solution in 0.15 M HEPES buffer and then incubated at 37 °C for 30 min. After incubation, the reaction was ended by heating at 100 °C for 10 min. The formation of glucose was determined by the glucose oxidase method, using a BF-5S Biosensor (Oji Scientific Instrument, Hyogo, Japan). IC_{50} values were determined for pure compounds isolated from *Alternaria sp*. an endophyte isolated from *L. schweinfurthii* fresh leaves.

Table S1: **IC_{50} (µM) values of the different compounds from rice culture MeOH fractions of *Alternaria sp.* The results are expressed as average ± SD (n=3).**

Compound	IC_{50} (µM)
Acarbose	283±14.65
Compound **1**	>300
Compound **2**	7.95±1.2
Compound **3**	40.38±3.7
Compound **4**	46.14±0.84
Compound **5**	179.88±4.65
Compound **6**	236.25±6.22

1.2. Inhibition of pancreatic lipase

The pancreatic lipase inhibition activity was measured by an *in vitro* enzyme reaction, which used 4-methylumbelliferyl oleate (4-MUO) as a substrate (MIZUTANI *et al.*, 2010), (Nakai *et al.*, 2005). Several concentrations (with a three-fold dilution) as followings: 300, 100, 33.3, 11.1, 3.7, 1.23 and 0.41, were used in the assay. The samples dissolved in DMSO were first diluted with a buffer solution consisting of 13 mM Tris–HCl, 150 mM NaCl and 1.3 mM $CaCl_2$ (pH = 8). Then 25 µL of the sample solution was mixed with 50 µL of a 0.25 mM 4-MUO solution (dissolved in the buffer) in the wells of a 96-well plate, followed by the addition of 25 µL of the lipase (Wako, Osaka, Japan) solution (50 U/mL). After incubation at 25 °C for 30 min, 100 µL of 0.1 M sodium citrate (pH 4.2) was added to stop the enzyme reaction. The amount of 4-MUO released by the action of the lipase was measured with a fluorometrical microplate reader (FlexStation 3 Microplate Reader, Molecular Devices, Orleans Drive Sunnyvale, CA, USA) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Orlistat (final concentration: 0.5 µg/mL) was used as a positive control for the inhibition of lipase activity.

Table S2: **IC_{50} (µM) values of the different compounds from rice culture MeOH fractions of *Alternaria sp.* against lipase enzyme. The results are expressed as average ± SD (n=3).**

Compound	IC_{50} (µM)
Orlistat	1.35 ± 0.1
Compound **1**	73.82±0.82
Compound **2**	20.82±0.95
Compound **3**	3.18±0.79
Compound **4**	21.46±0.97
Compound **5**	56.85±0.77
Compound **6**	70.60±0.86

Figure S2: HR-ESI-MS of compound 1

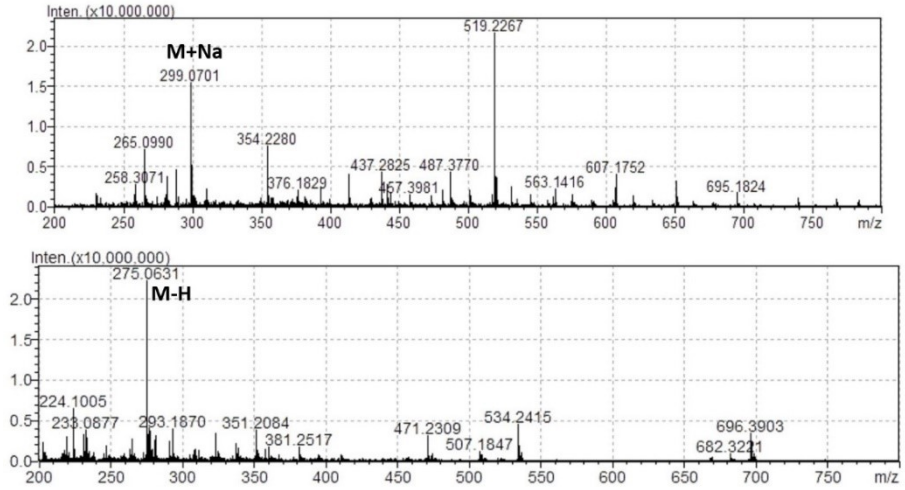


Figure S3: ^{1}H NMR of compound 1

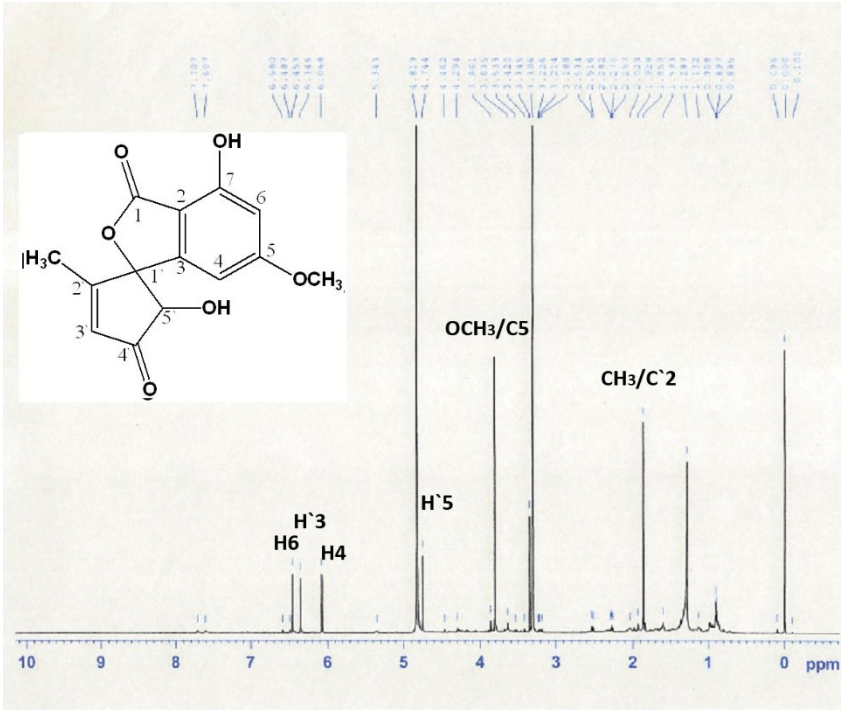


Figure S4: ^{1}H NMR of compound 1 (expansion from 5.3-8.0 ppm)

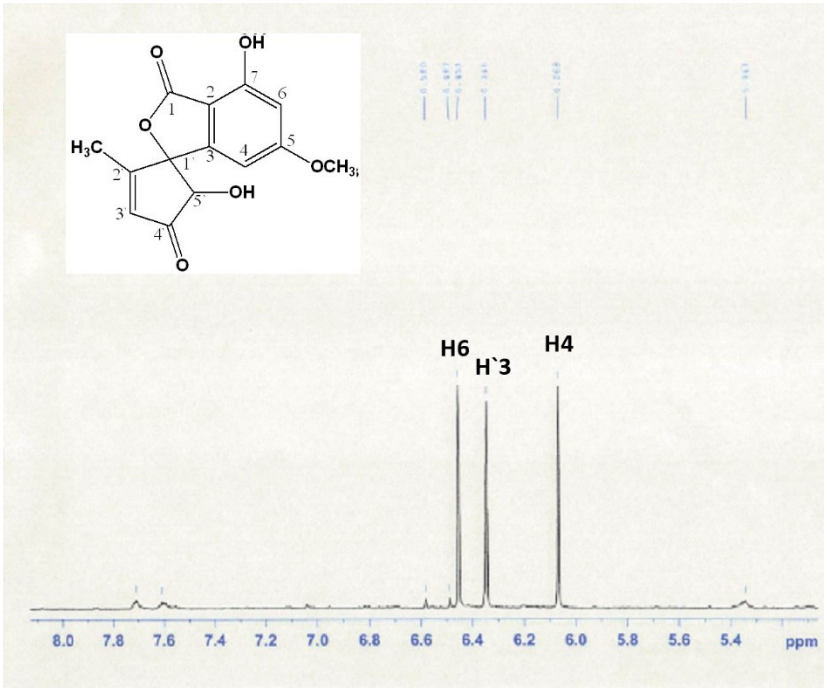


Figure S5: ^{1}H NMR of compound 1 (expansion from 3.2-4.8 ppm)

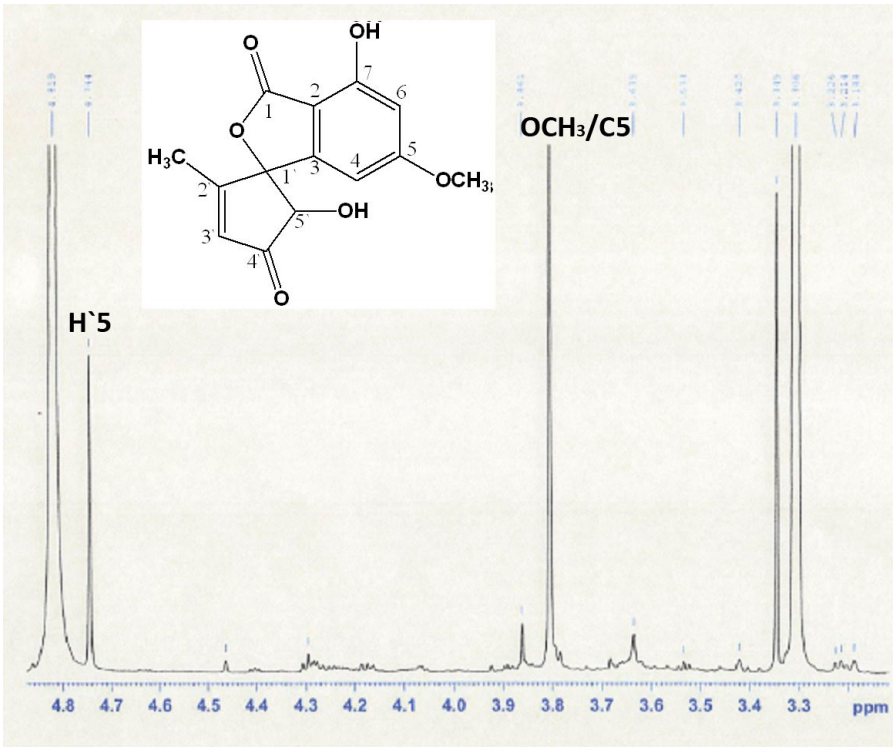


Figure S6: ^{1}H NMR of compound 1 (expansion from 0.9-2.5 ppm)

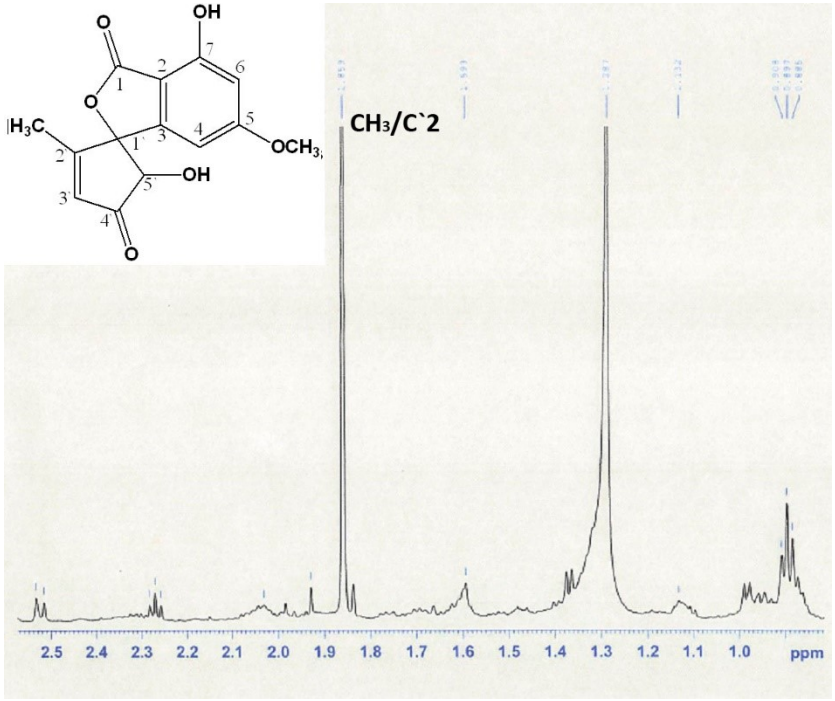


Figure S7: ^{13}C NMR of compound 1

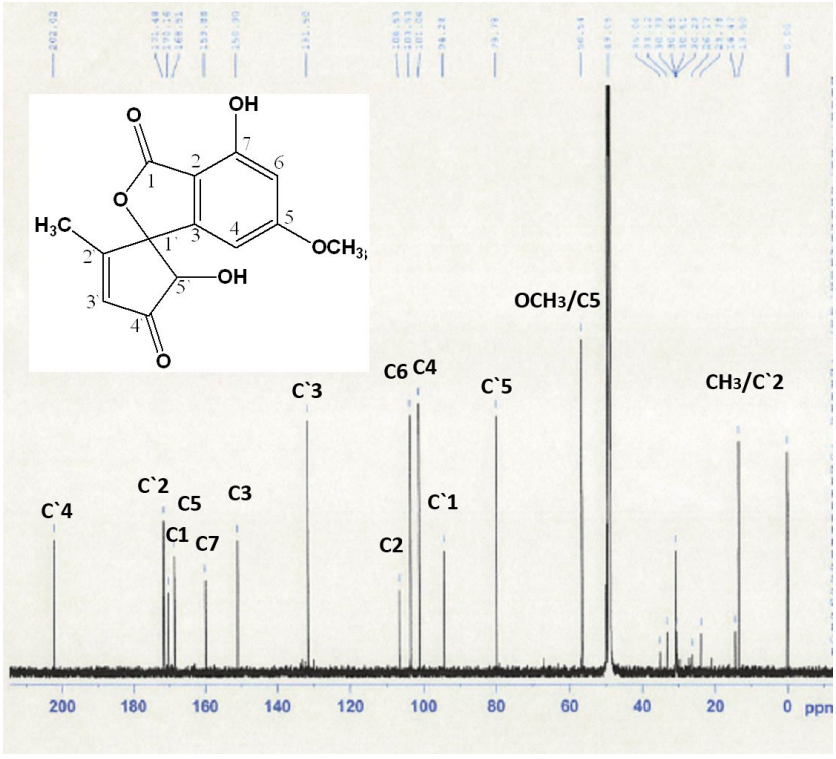


Figure S8: HSQC of compound 1

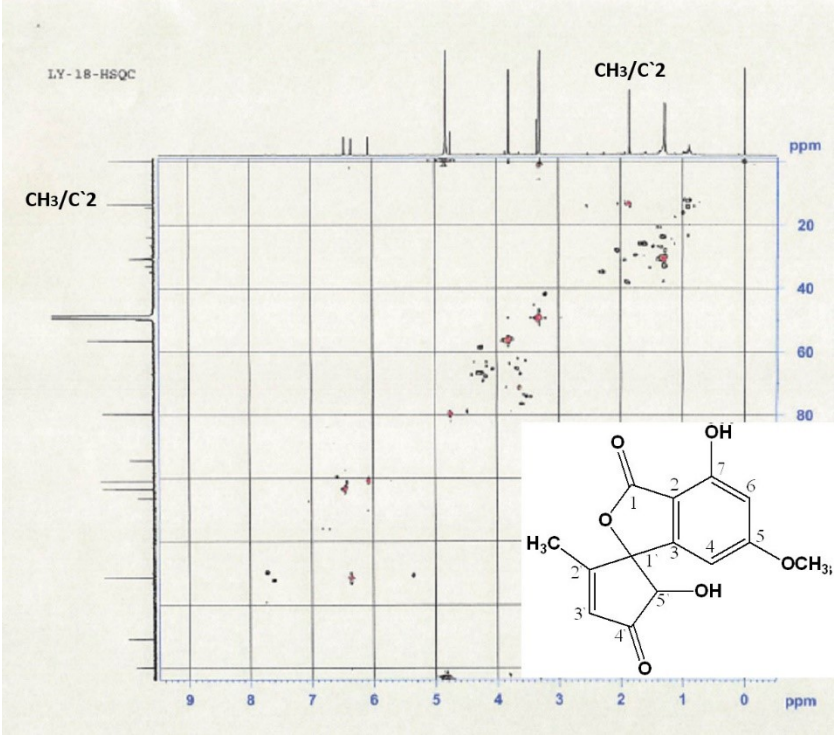


Figure S9: HSQC of compound 1 (expansion from 5.4-7.8 ppm)

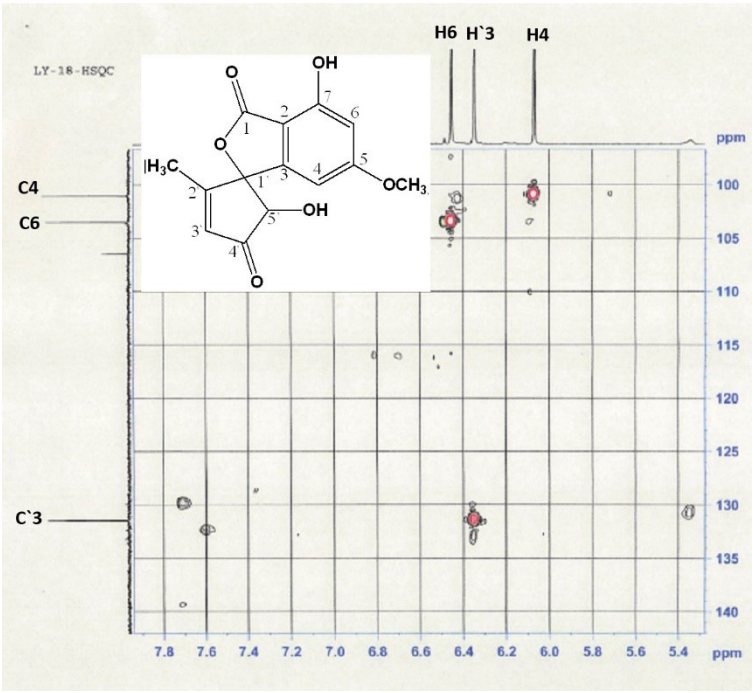


Figure S10: HSQC of compound 1 (expansion from 3.0-4.8 ppm)

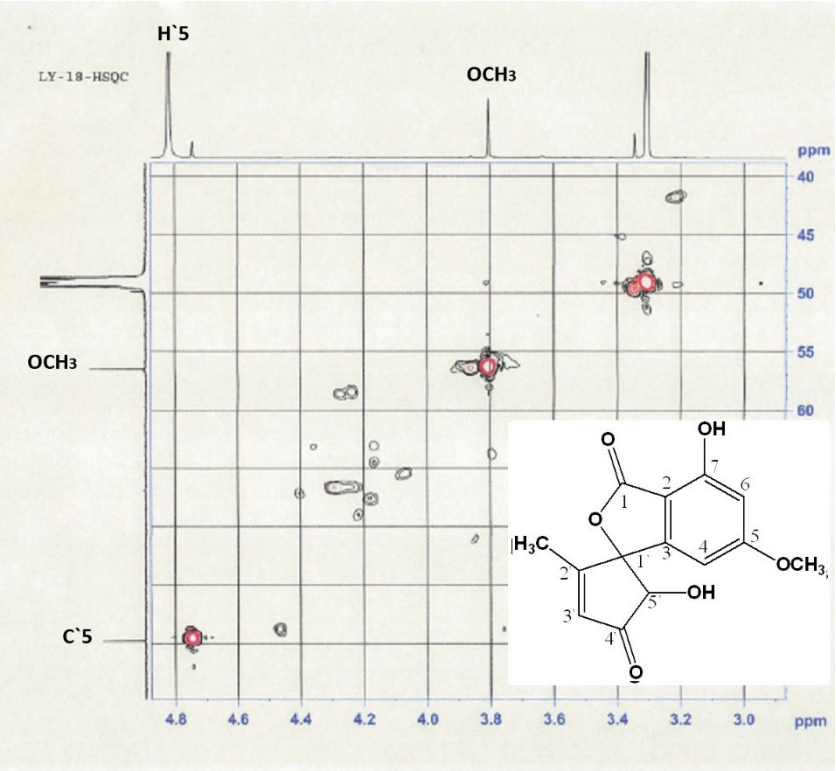


Figure S11: HMBC of compound 1 expansion from 3.7-6.5 ppm

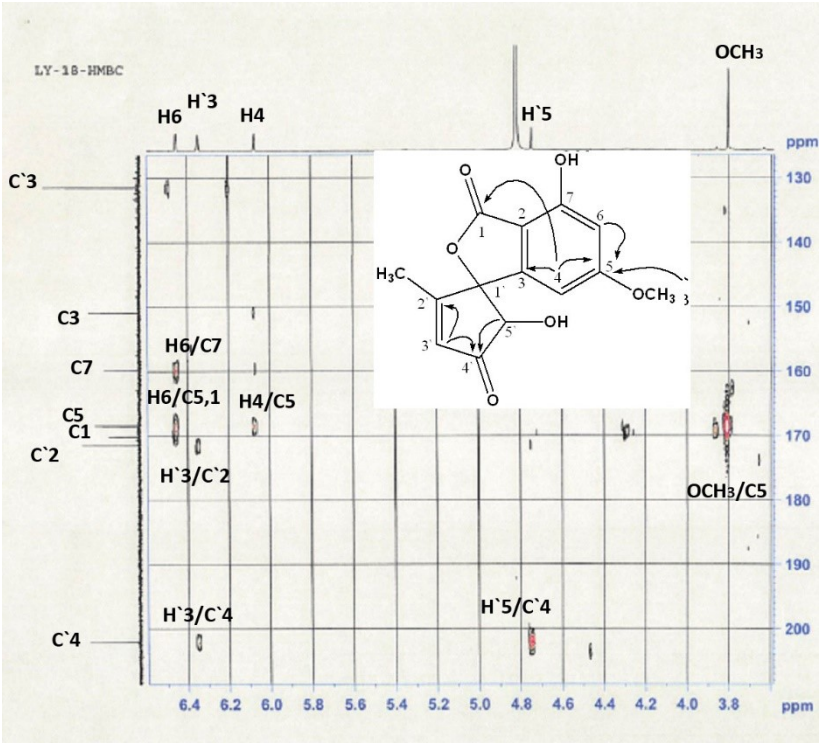


Figure S12: HMBC of compound 1 expansion from 3.6-6.6 ppm

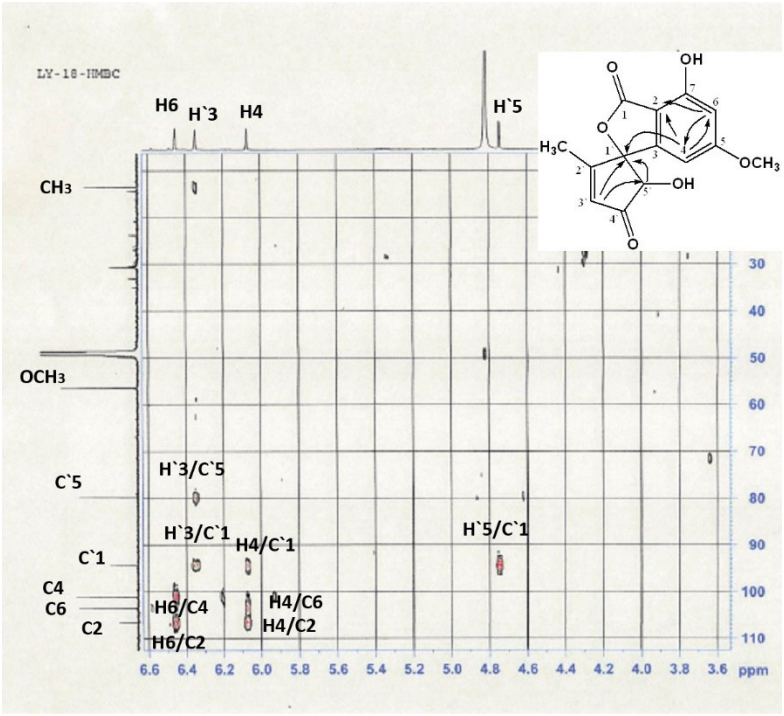


Figure S13: HMBC of compound 1 expansion from 1.3-2.6 ppm

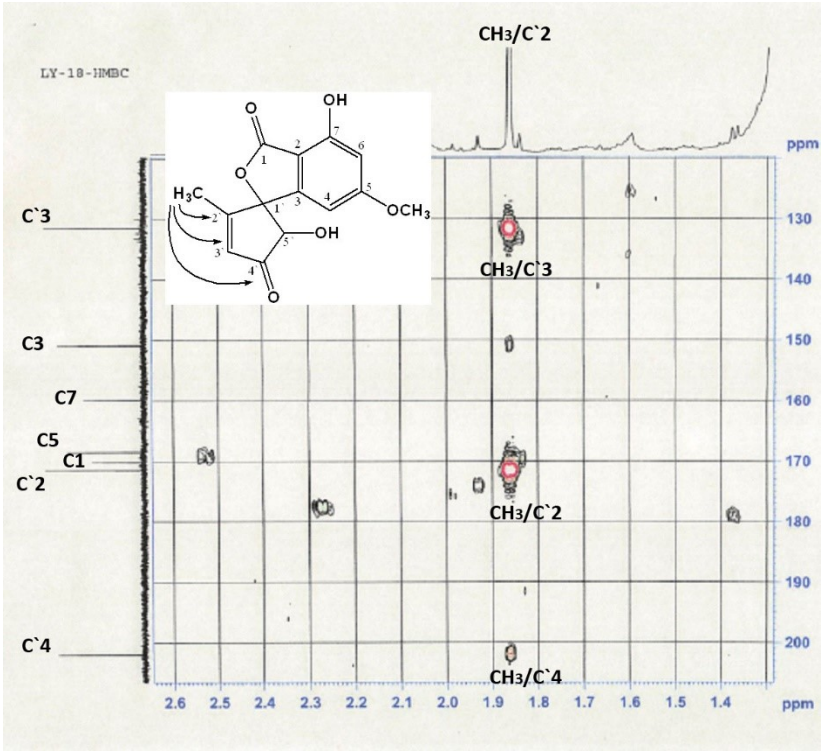


Figure S14: HR-FAB-MS of compound 2

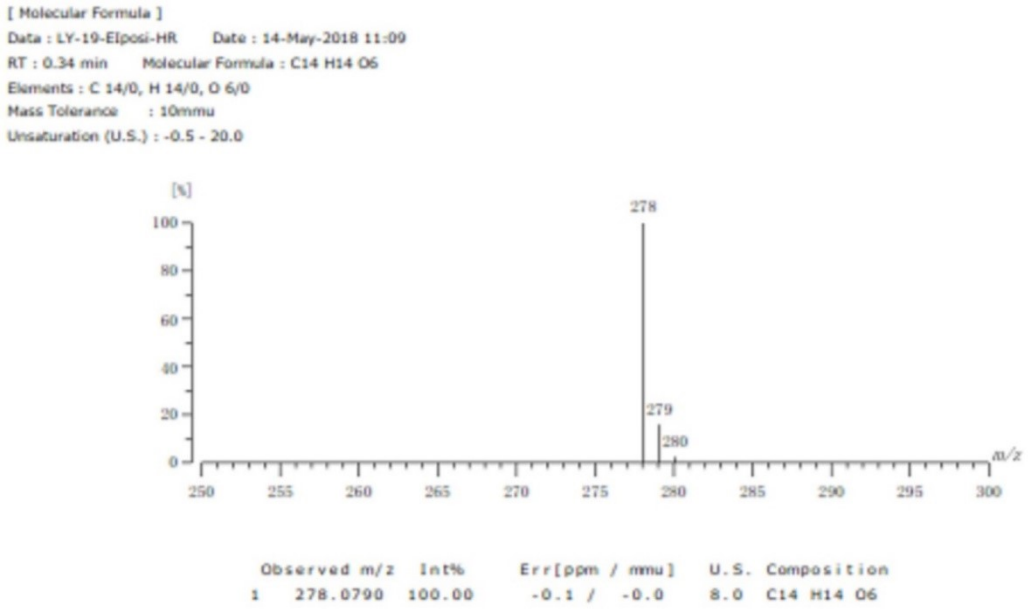


Figure S15: ^{1}H NMR of compound 2

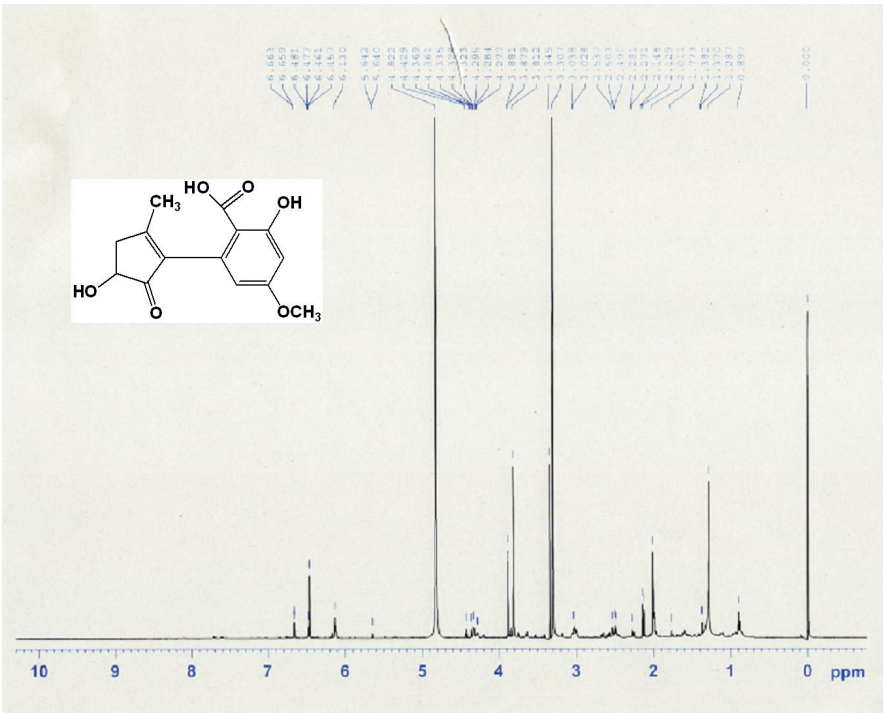


Figure S16: ^{1}H NMR of compound 2 (expansion from 5.6-6.7 ppm)

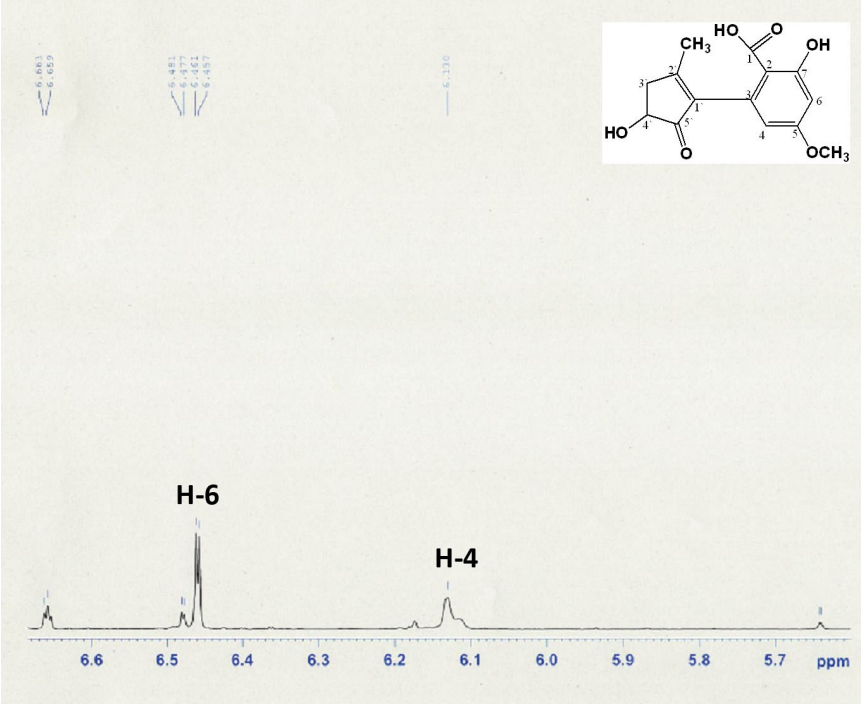


Figure S17: ^{1}H NMR of compound 2 (expansion from 3.75-4.45 ppm)

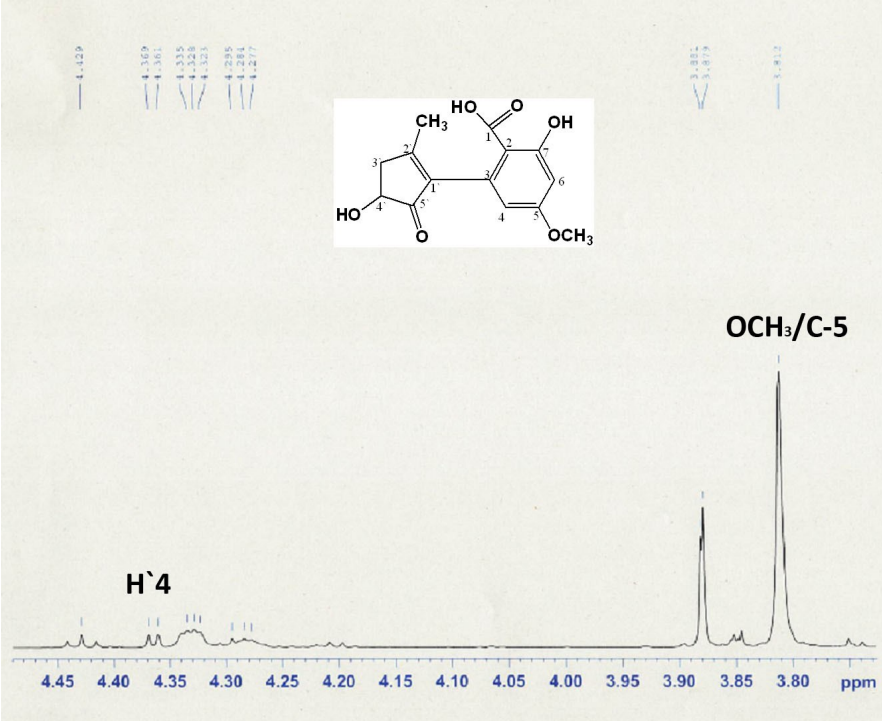


Figure S18: ^{1}H NMR of compound 2 (expansion from 0.8-3.1 ppm)

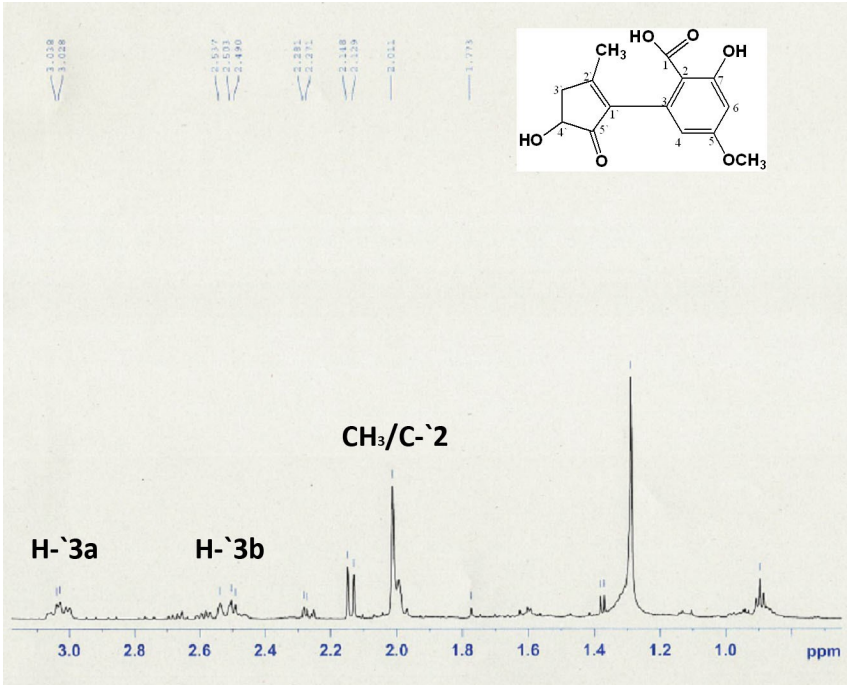


Figure S19: ^{13}C NMR of compound 2

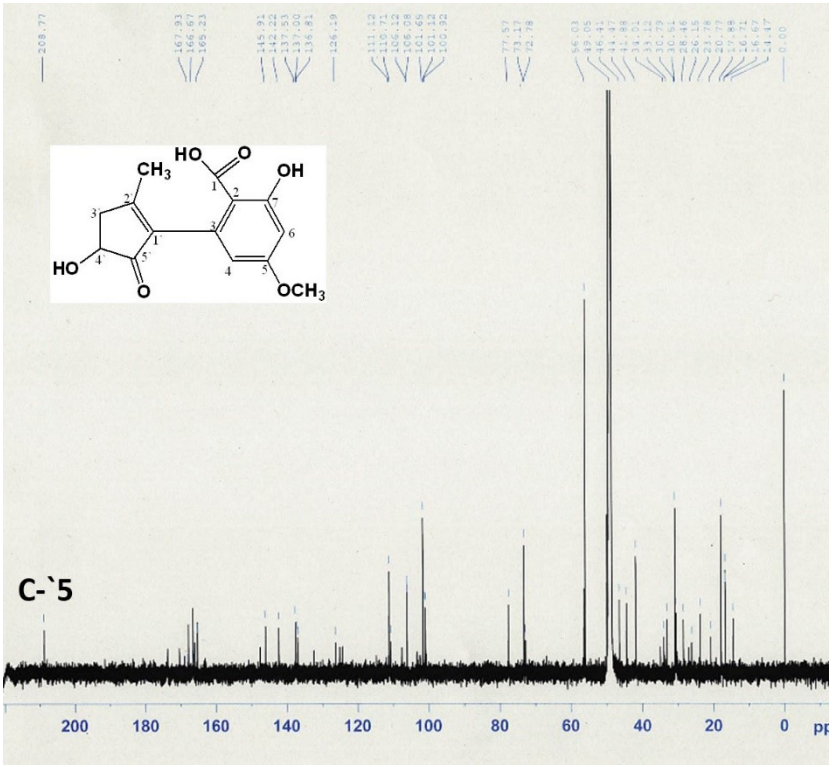


Figure S20: ^{13}C NMR of compound 2 (expansion from 100-170 ppm)

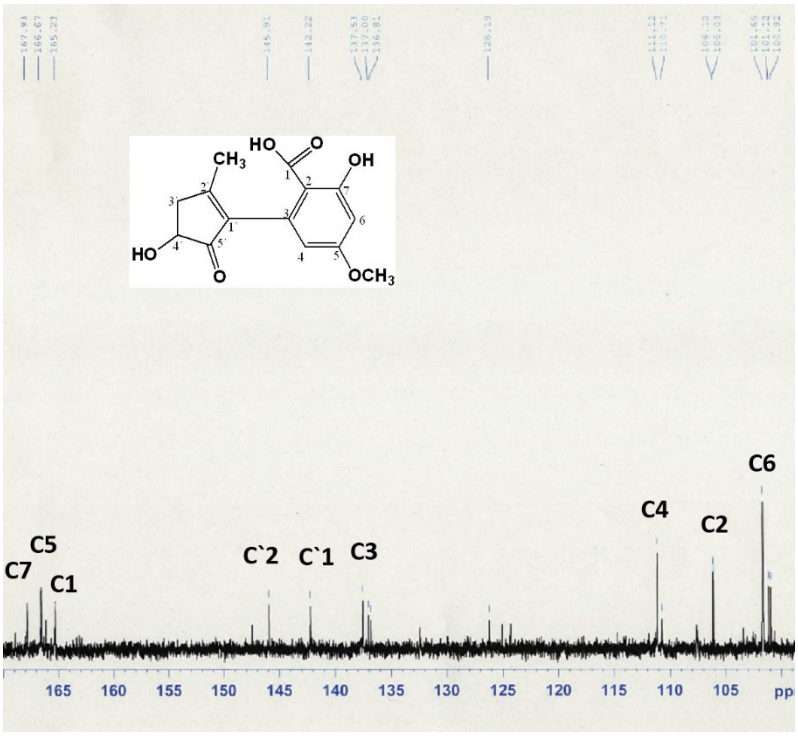


Figure S21: ^{13}C NMR of compound 2 (expansion from 15-80 ppm)

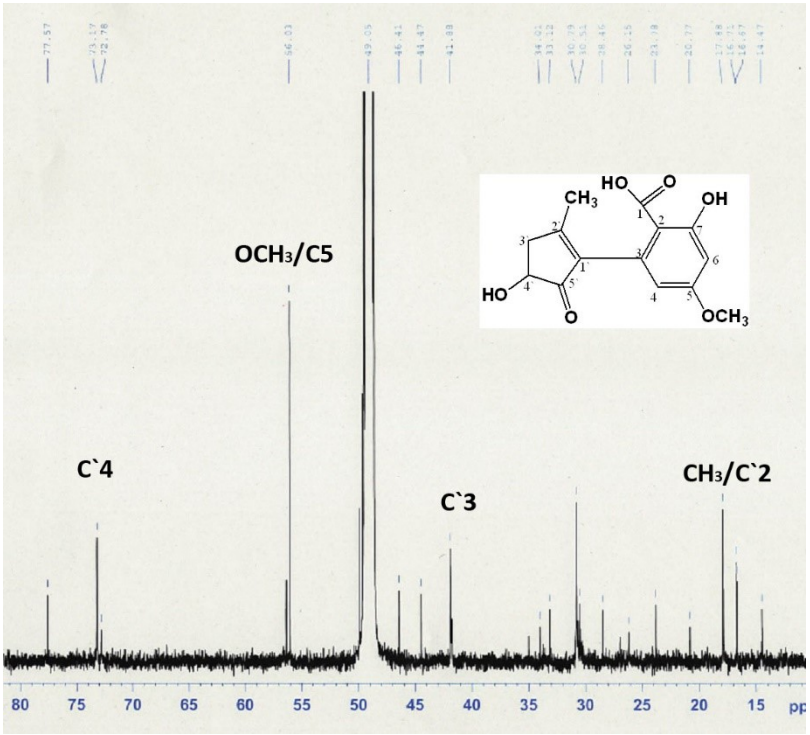


Figure S22: HSQC of compound 2 expansion from 5.4-7.8 ppm

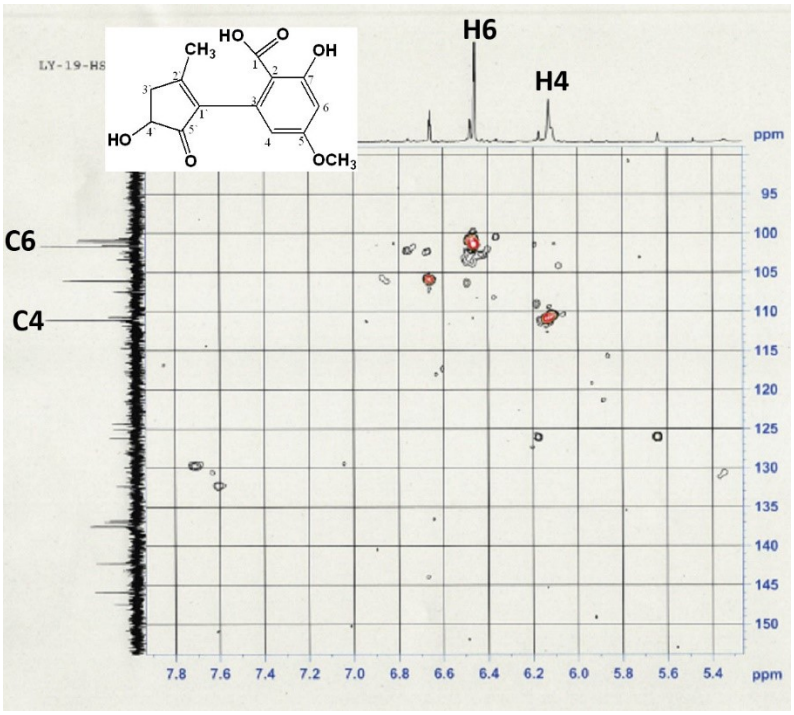


Figure S23: HSQC of compound 2 expansion from 3.1-4.9 ppm

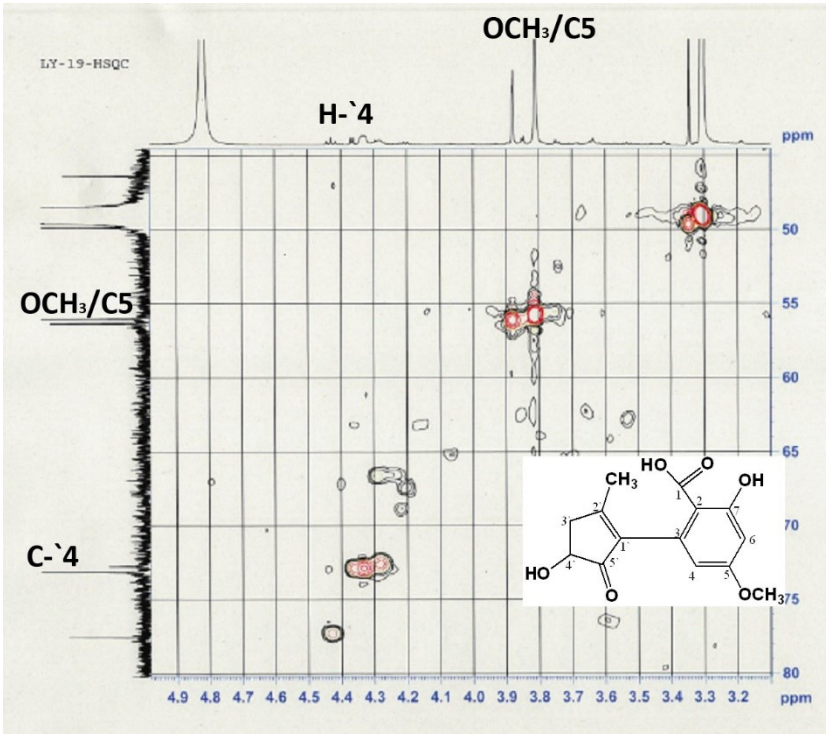


Figure S24: HSQC of compound 2 expansion from 0.8-3.4 ppm

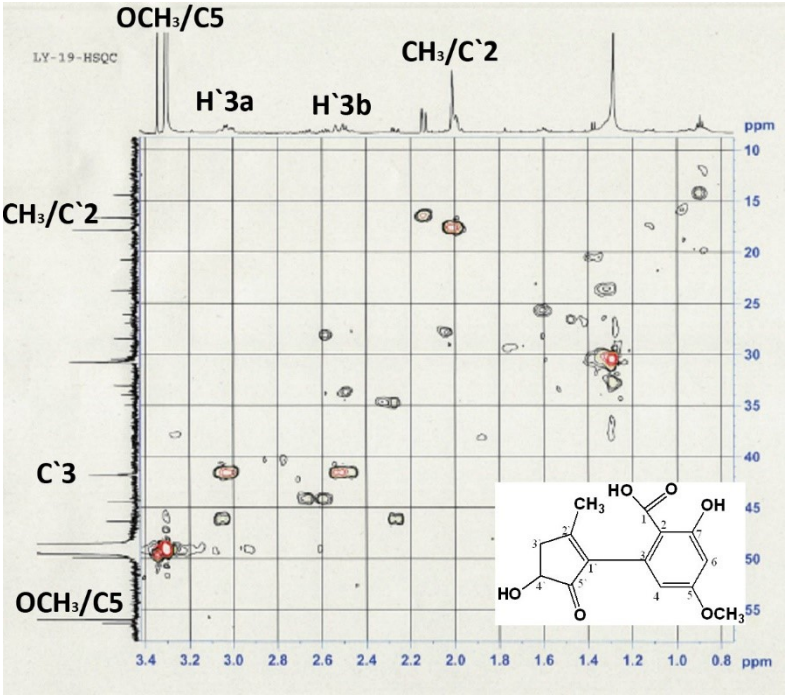


Figure S25: HMBC of compound 2 expansion from 3.7-7.0 ppm

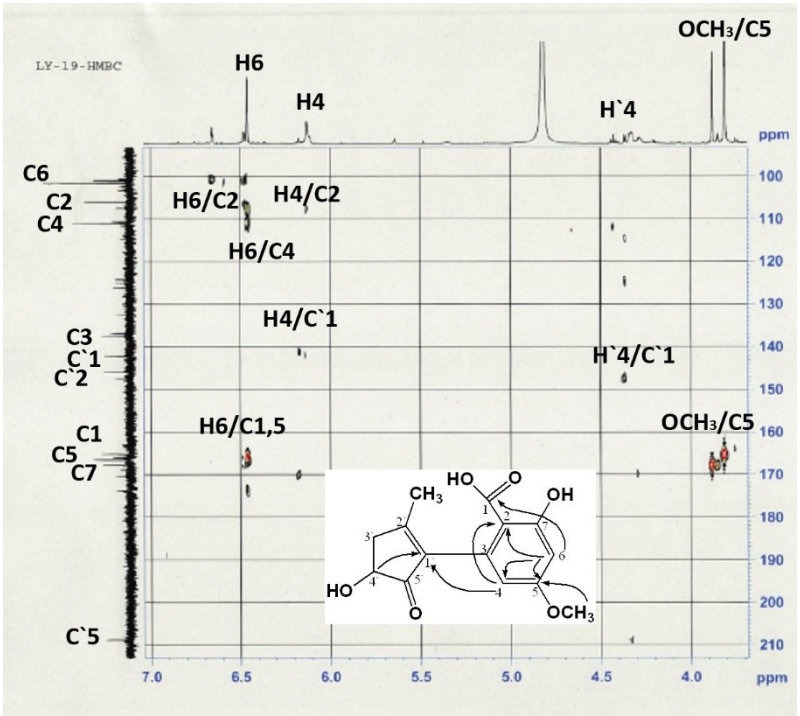


Figure S26: HMBC of compound 2 expansion from 1.3-3.2 ppm

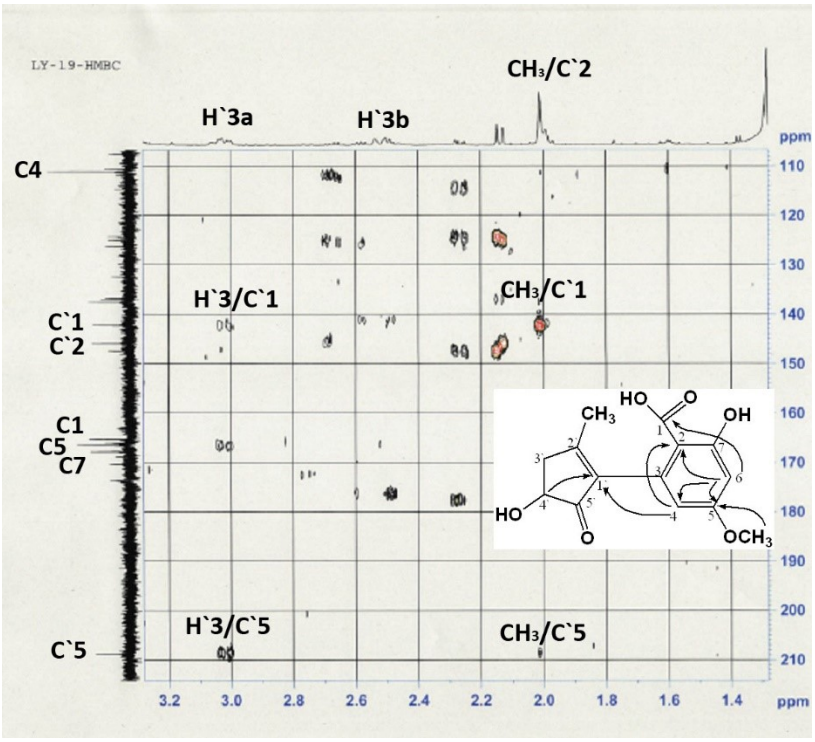


Figure S27: HMBC of compound 2 expansion from 0.8-2.8 ppm

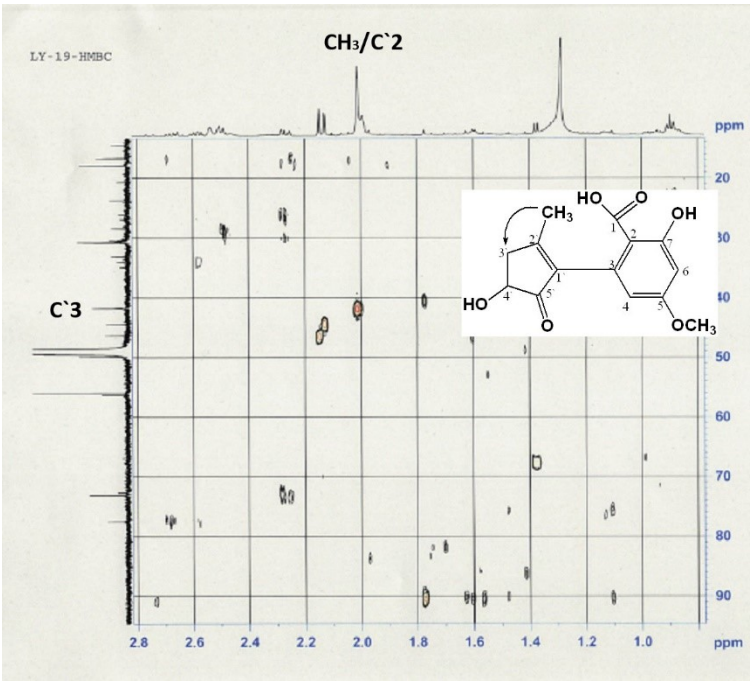


Figure S28: HR-ESI-MS of compound 3

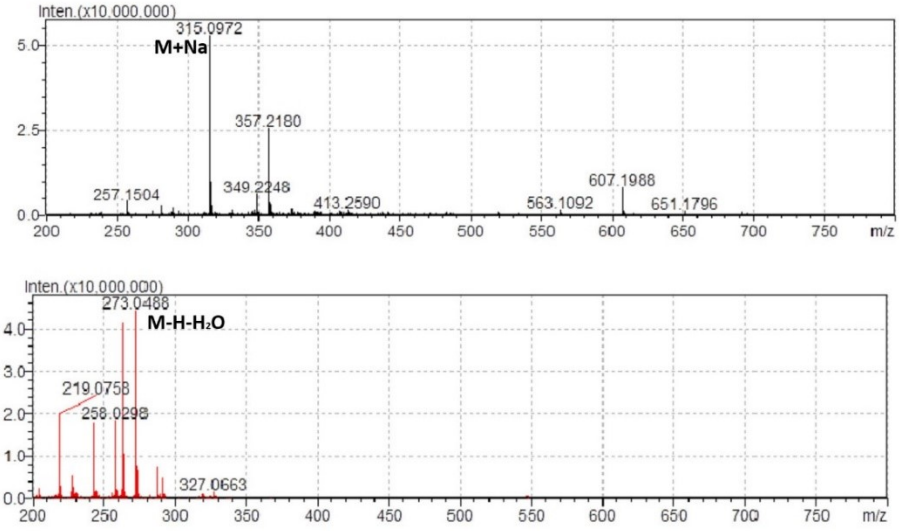


Figure S29: ^{1}H NMR of compound 3

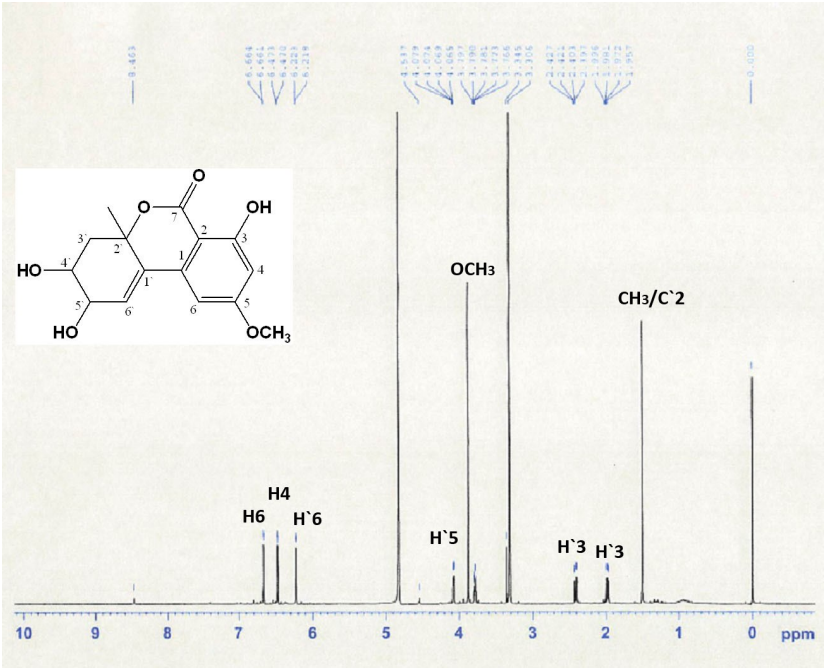


Figure S30: ^{1}H NMR of compound 3 (expansion from 6.15-6.85 ppm)

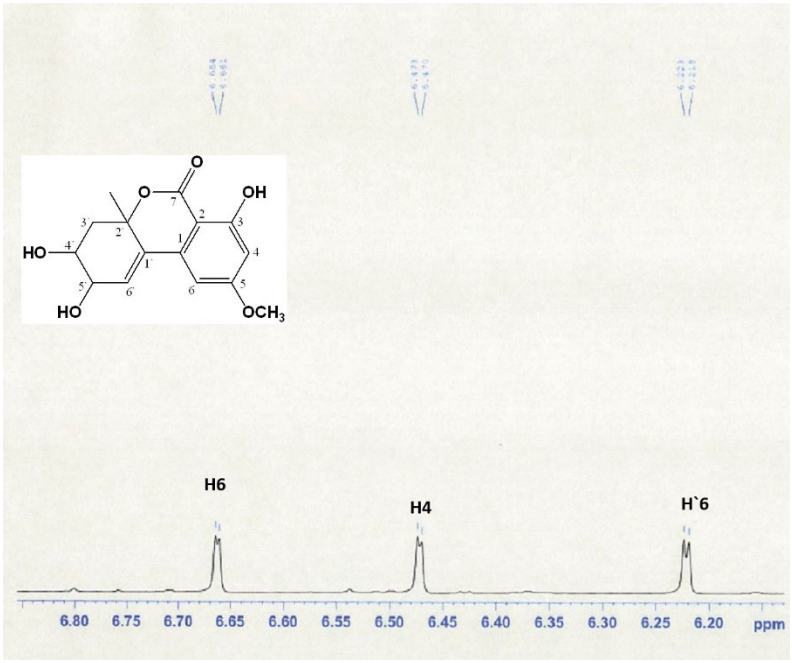


Figure S31: ^{1}H NMR of compound 3 (expansion from 3.3-4.2 ppm)

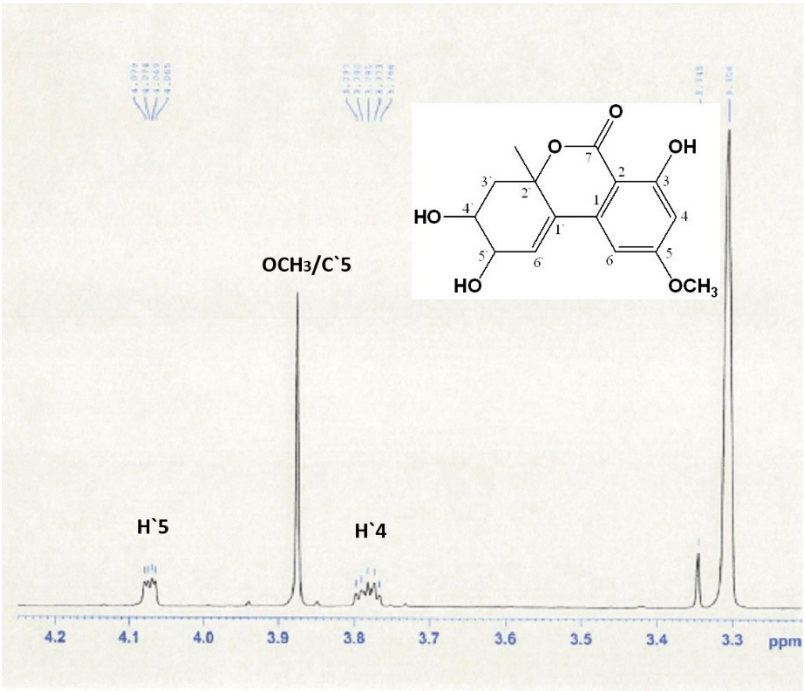


Figure S32: ^{1}H NMR of compound 3 (expansion from 1.5-2.5 ppm)

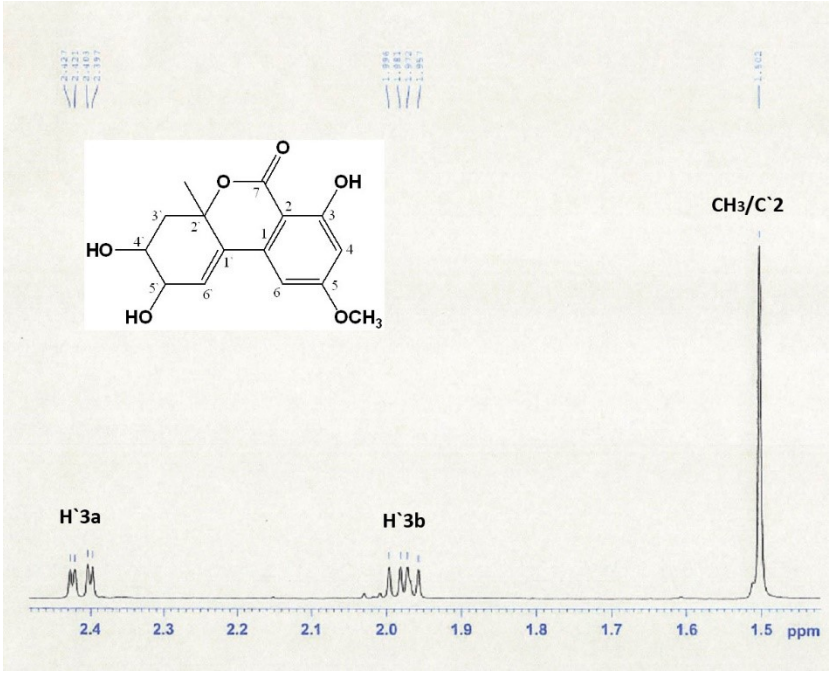


Figure S33: ^{13}C NMR of compound 3

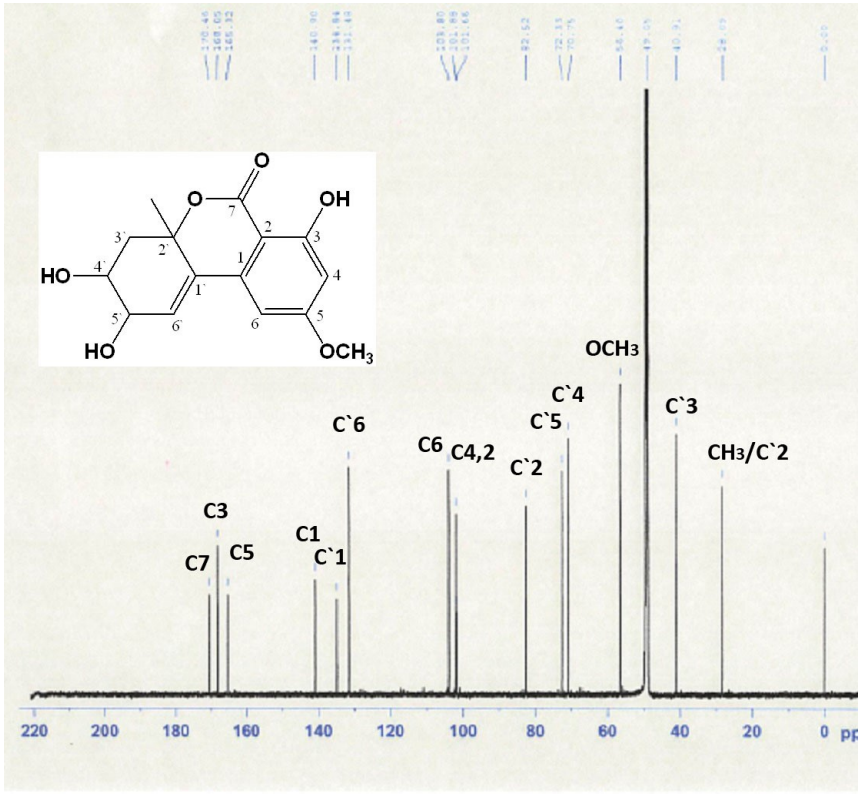


Figure S34: HSQC of compound 3 expansion from 5.7-7.5 ppm

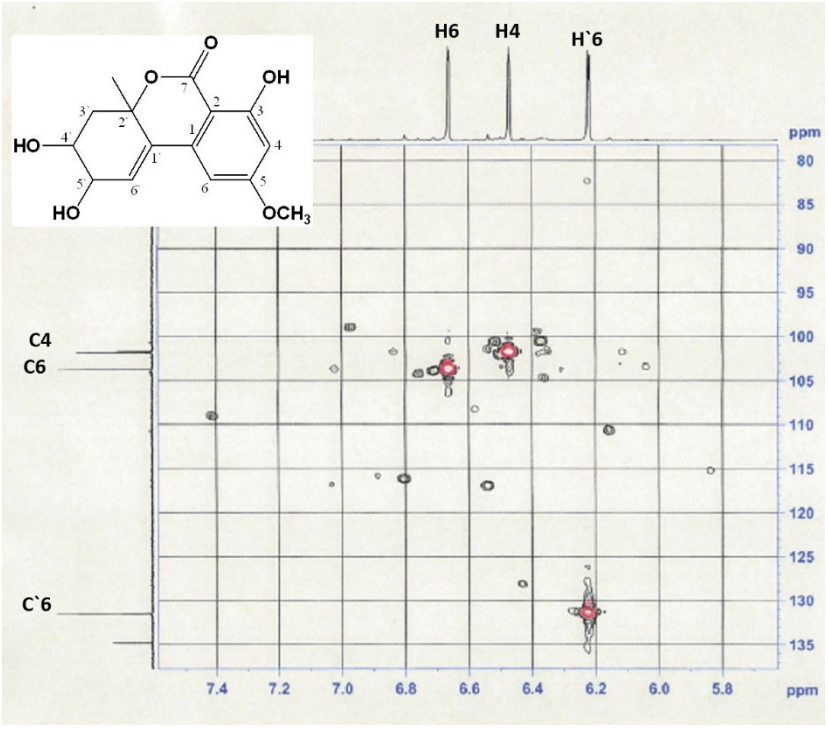


Figure S35: HSQC of compound 3 expansion from 3.6-4.4 ppm

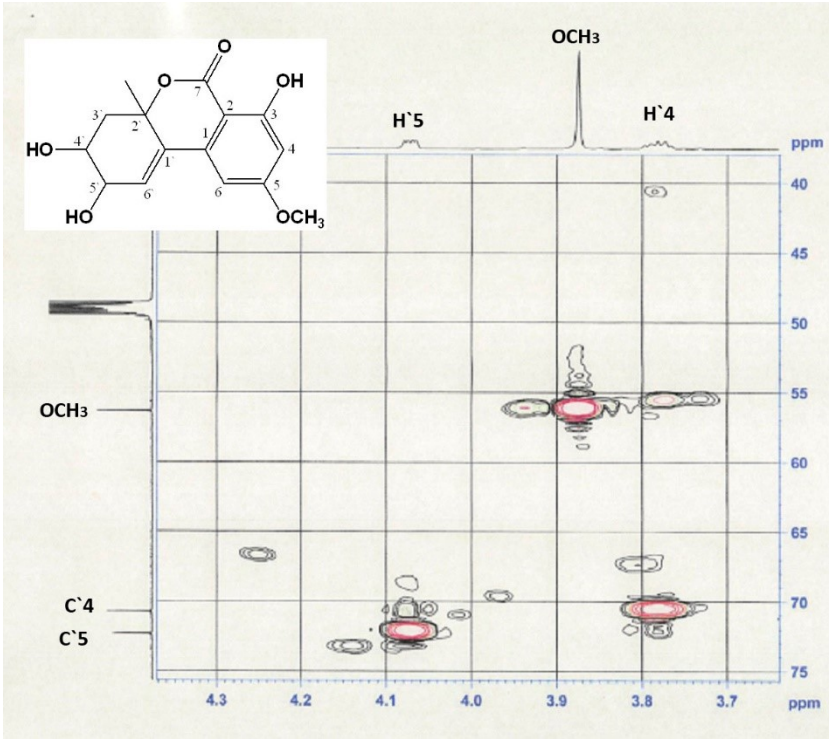


Figure S36: HSQC of compound 3 expansion from 1.2-2.5 ppm

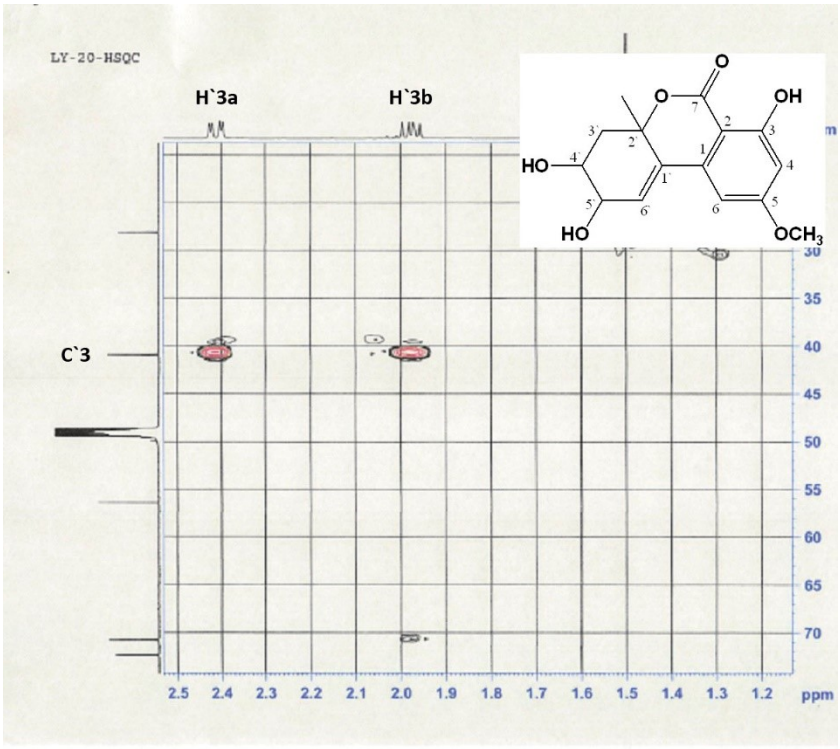


Figure S37: HMBC of compound 3 expansion from 5.9-7.1 ppm

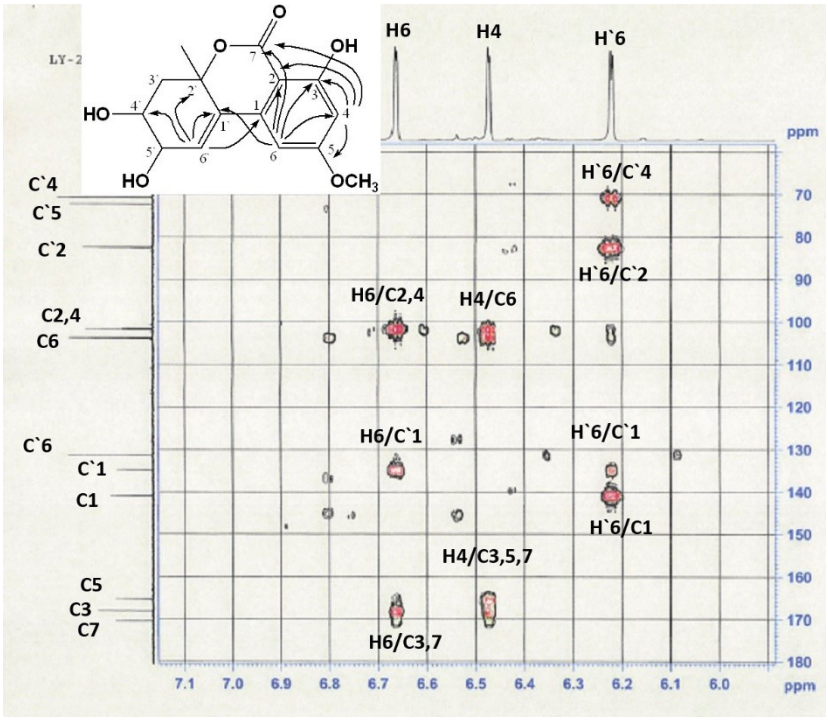


Figure S38: HMBC of compound 3 expansion from 1.6-4.2 ppm

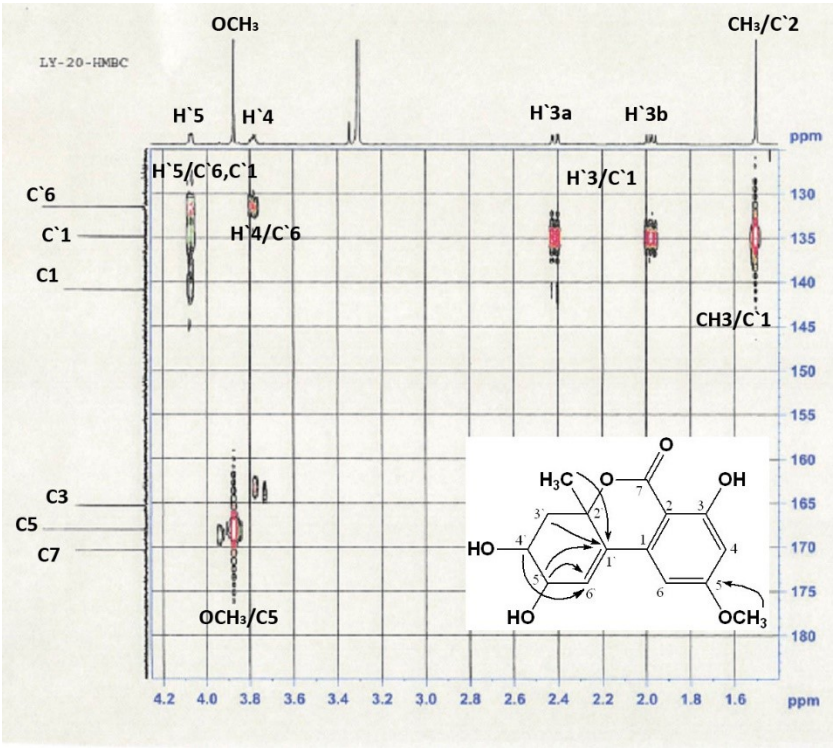


Figure S39: HMBC of compound 3 expansion from 3.7-4.9 ppm

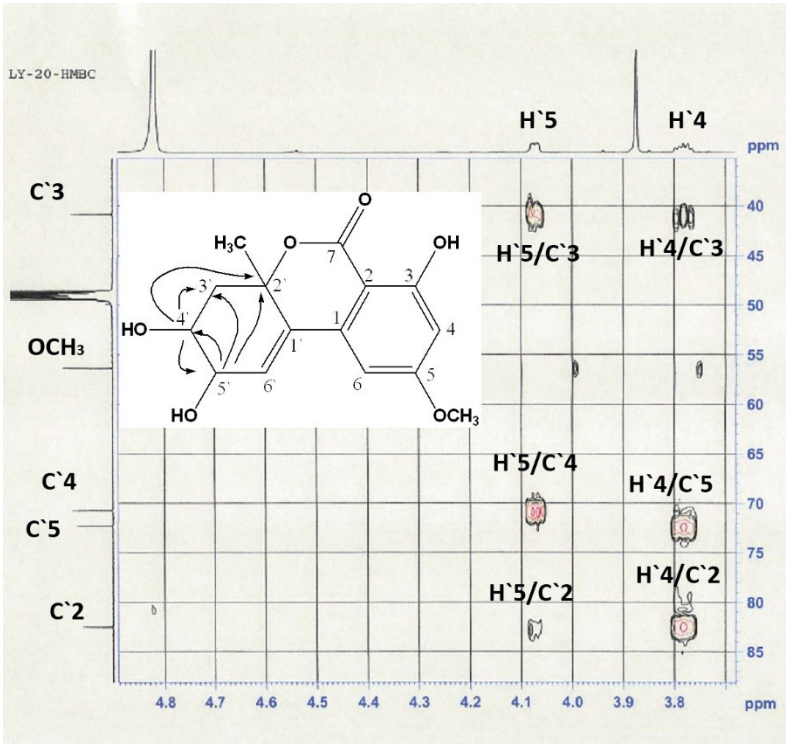


Figure S40: HR-FAB^{+}-MS of compound 4

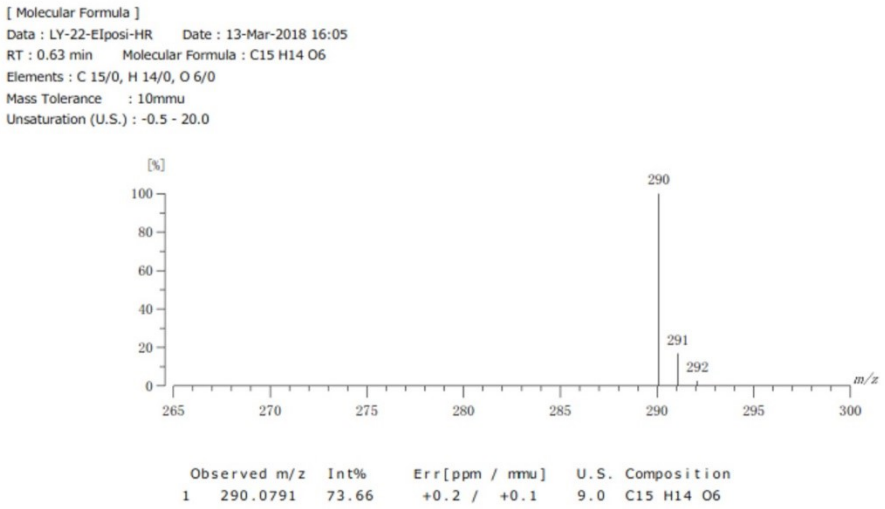


Figure S41: ^{1}H NMR of compound 4

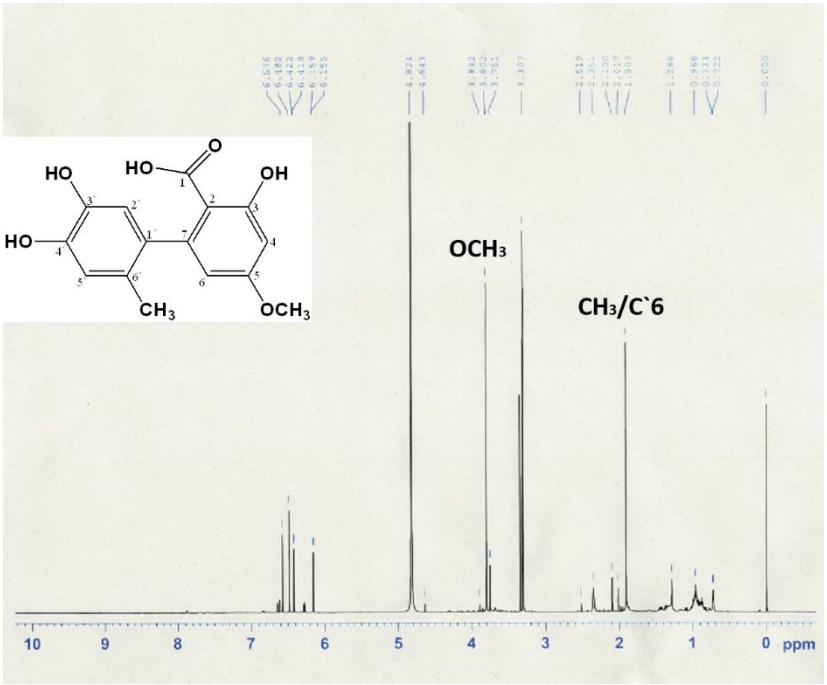


Figure S42: ^{1}H NMR of compound 4 (expansion from 6.15-6.60 ppm)

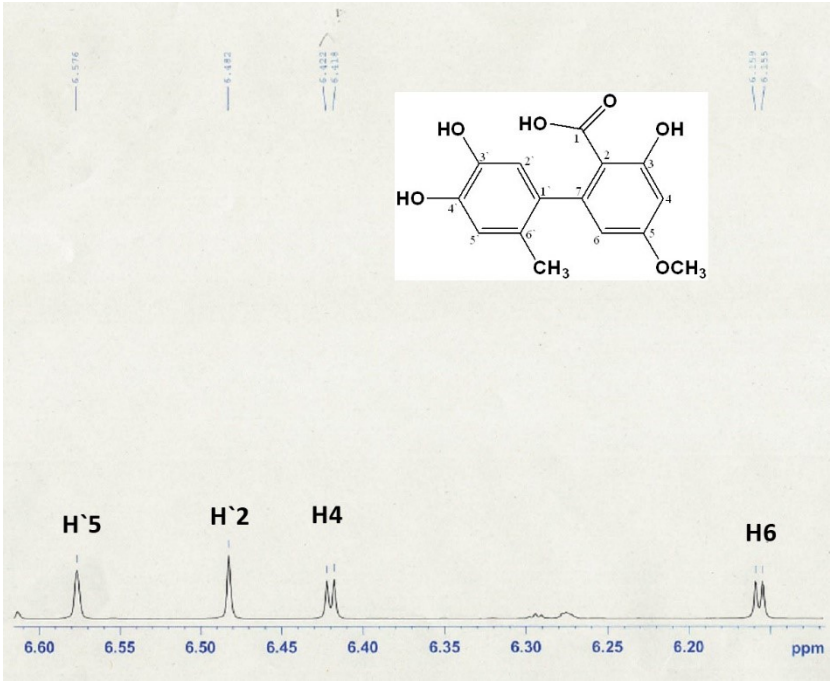


Figure S43: ^{13}C NMR of compound 4

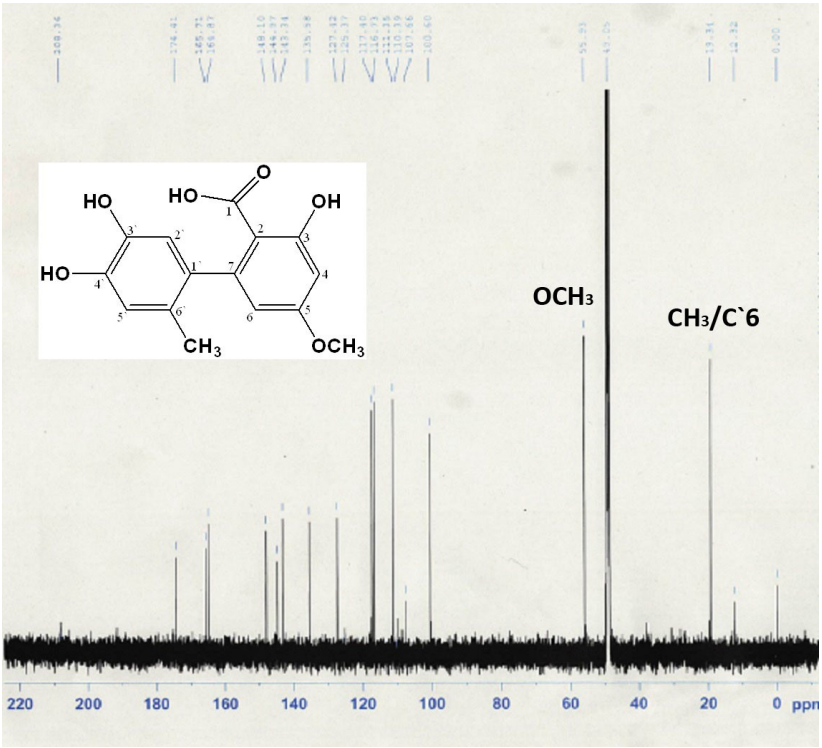


Figure S44: ^{13}C NMR of compound 4 (expansion from 100-175 ppm)

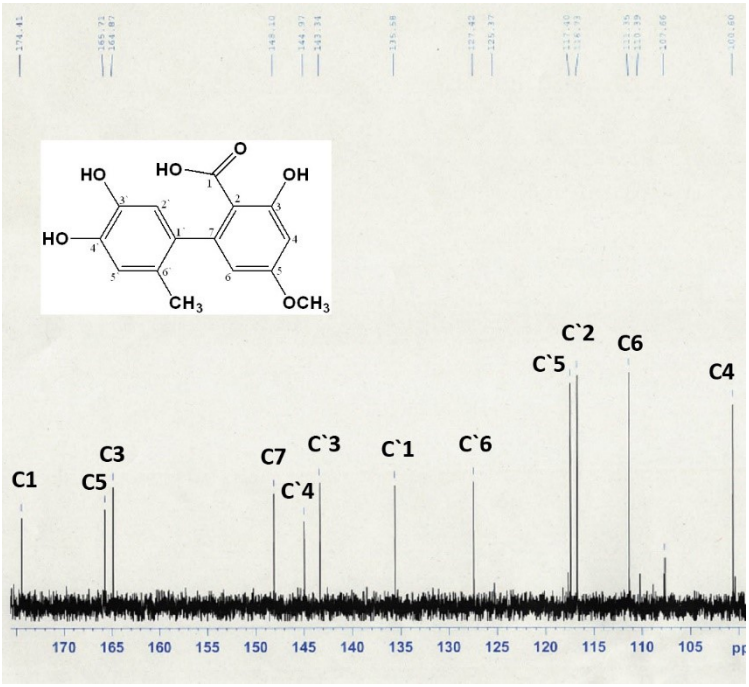


Figure S45: HSQC of compound 4

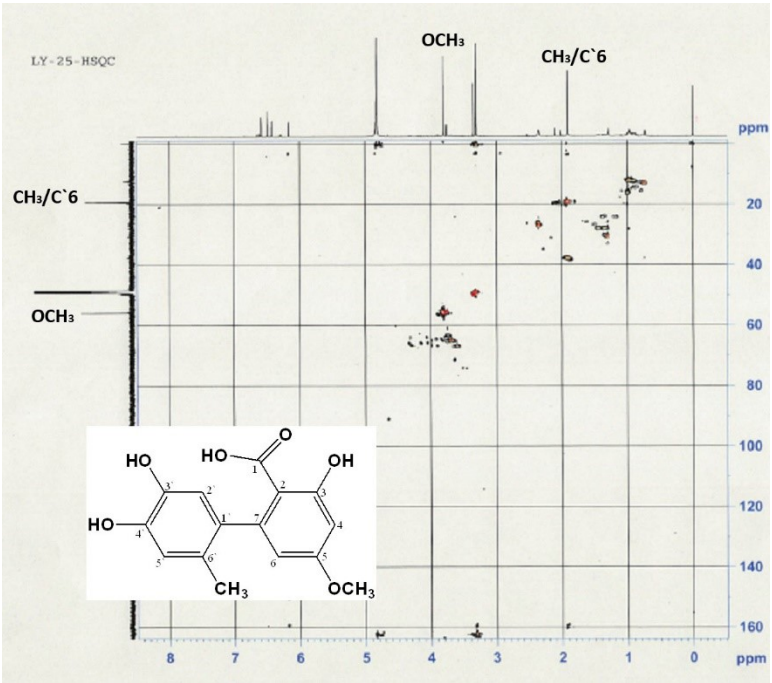


Figure S46: HSQC of compound 4 (expansion from 6.0-8.0 ppm)

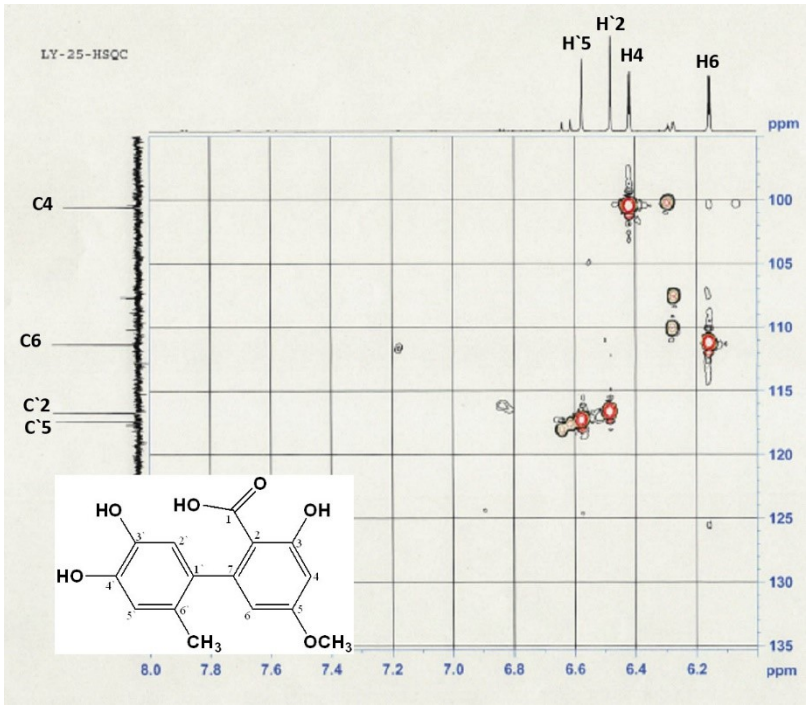


Figure S47: HSQC of compound 4 (expansion from 0.7-2.6 ppm)

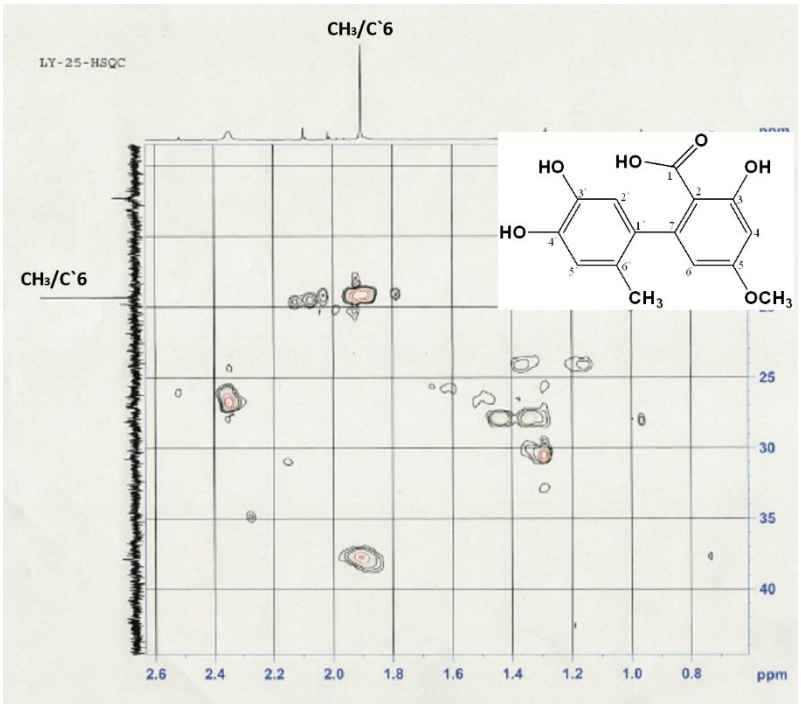


Figure S48: HMBC of compound 4 expansion from 5.9-6.8 ppm

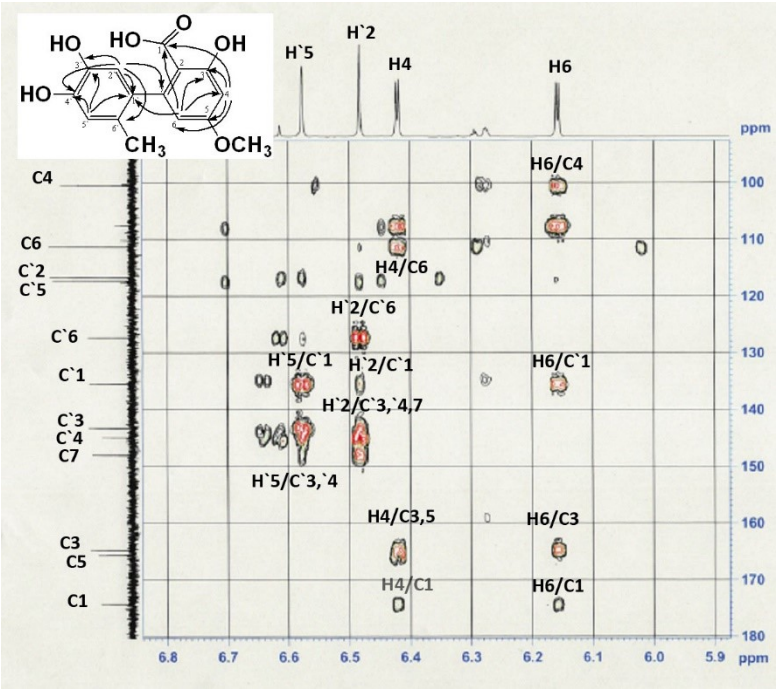


Figure S49: HMBC of compound 4 (expansion from 3.6-5.0 ppm)

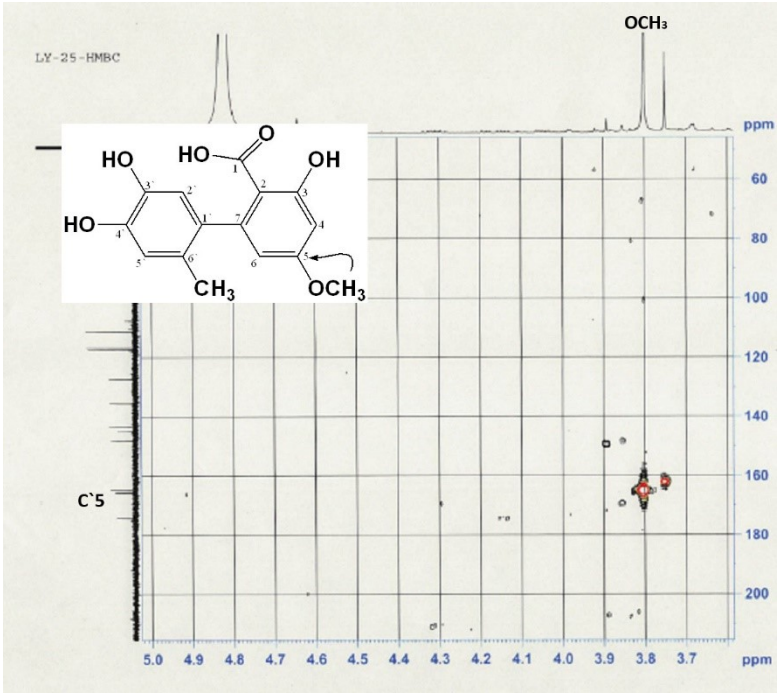


Figure S50: HMBC of compound 4 (expansion from 1.0-2.7 ppm)

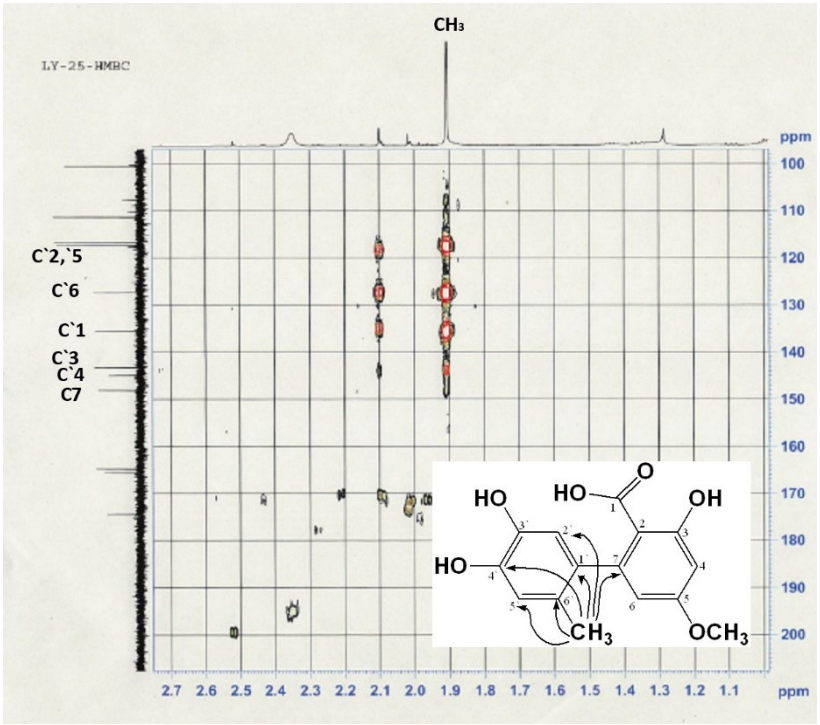


Figure S51 : HR-FAB-MS of compound 5

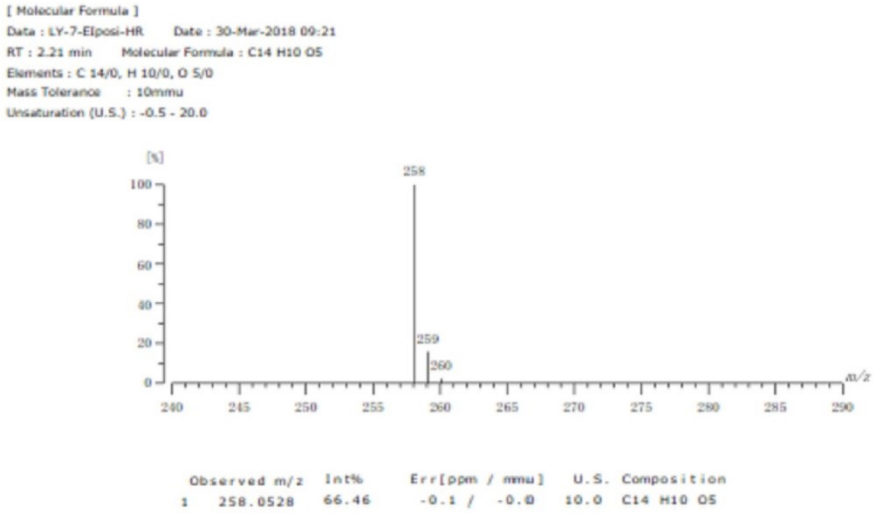


Figure S52 : ^{1}H NMR of compound 5

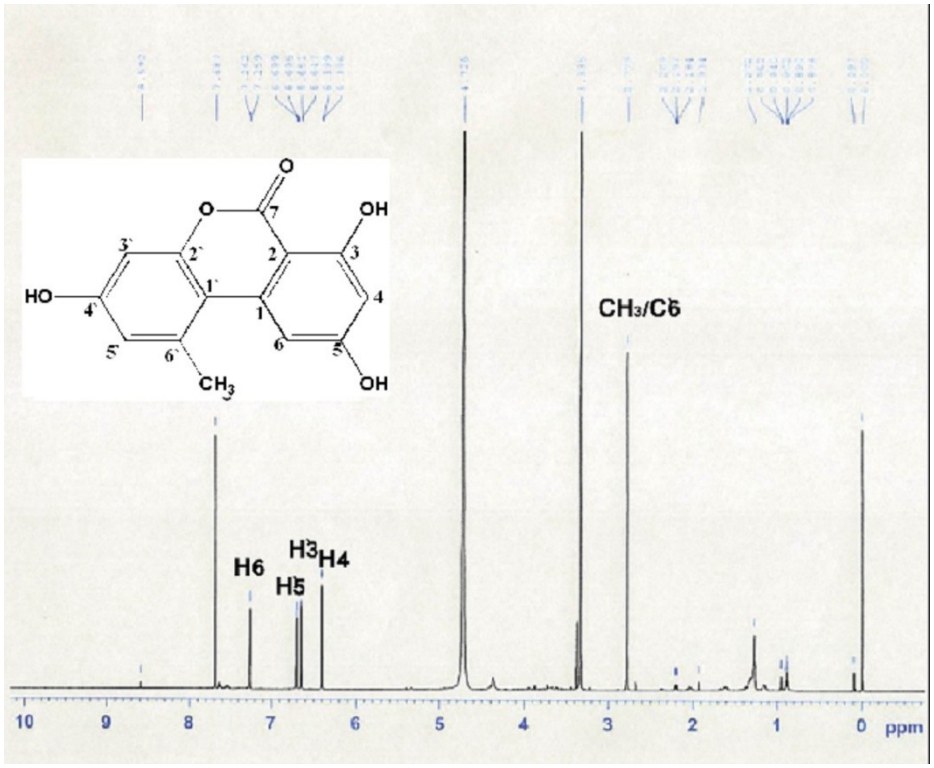


Figure S53: ^{13}C NMR of compound 5

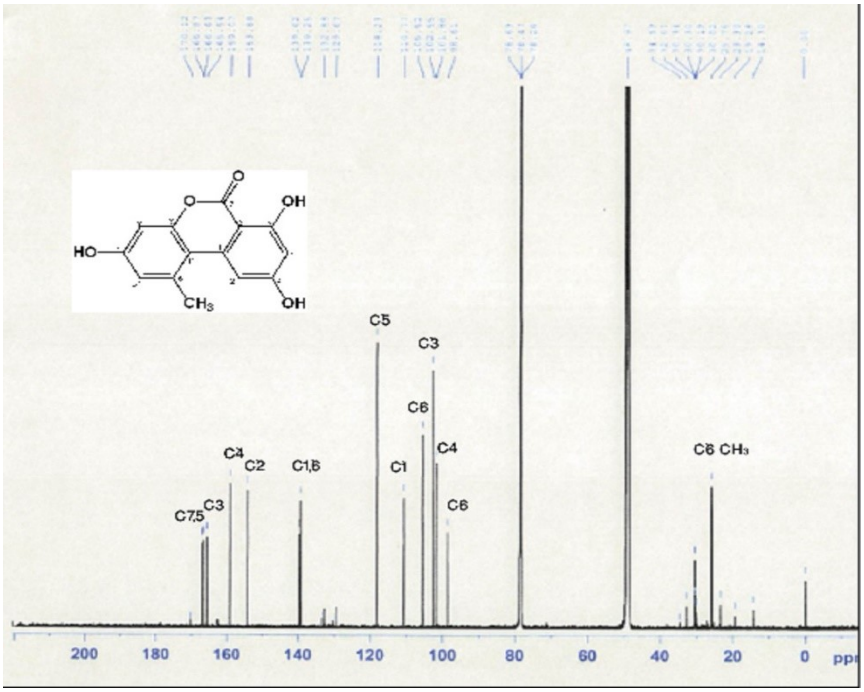


Figure S54: HSQC of compound 5 (expansion from 6-8 ppm)

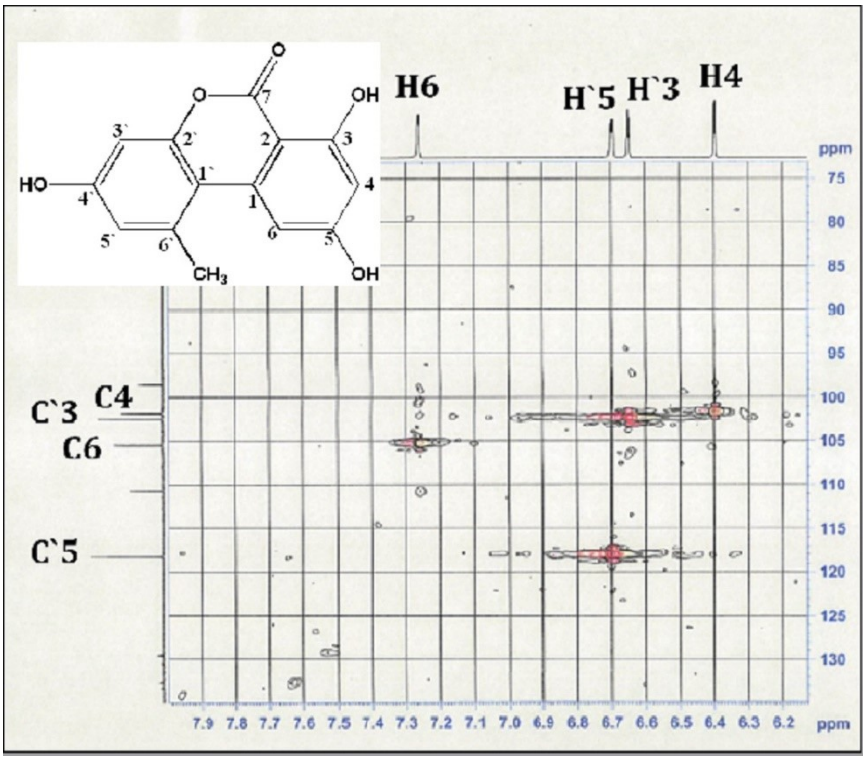


Figure S55: HSQC of compound 5 (expansion from 1-4 ppm)

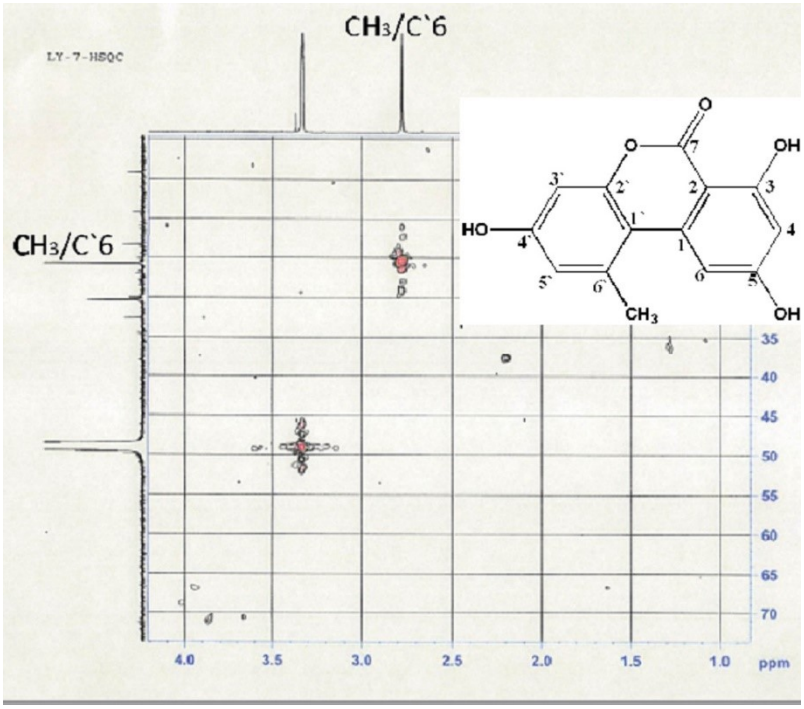


Figure S56: HMBC of compound 5 (expansion from 6.2-7.9 ppm)

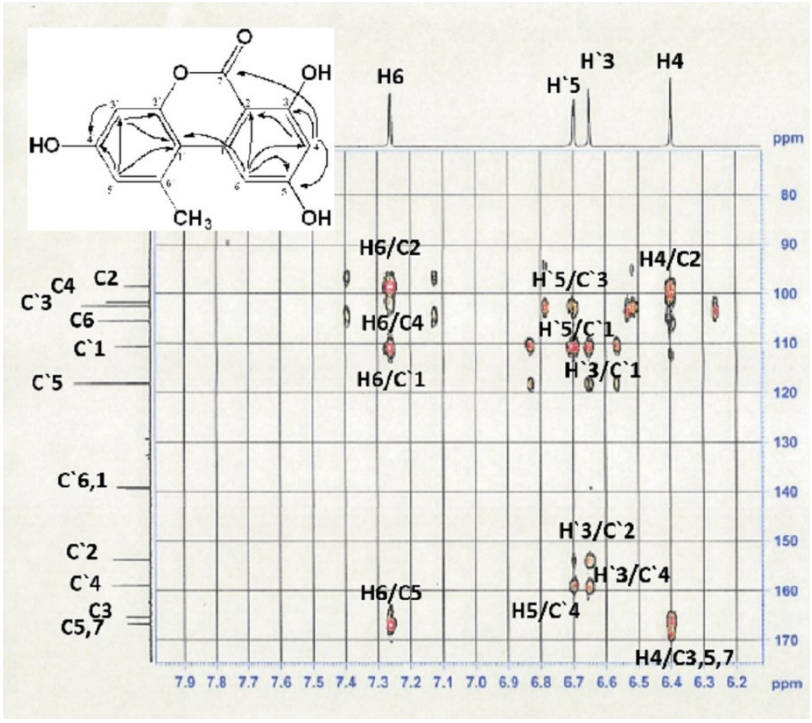


Figure S57: HMBC of compound 5 (expansion from 1.4-4 ppm)

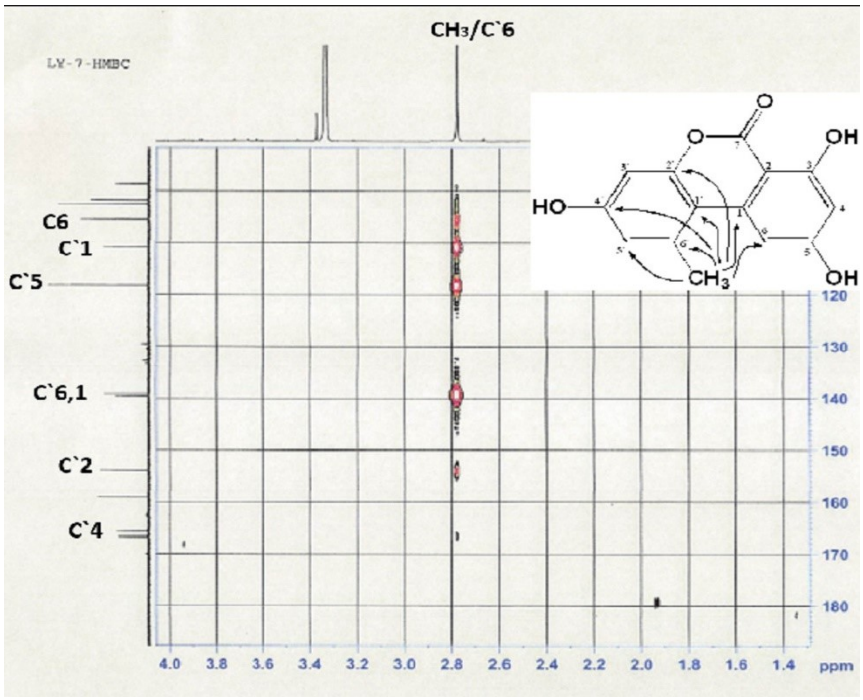


Figure S58: HR-FAB^{+}-MS of compound 6

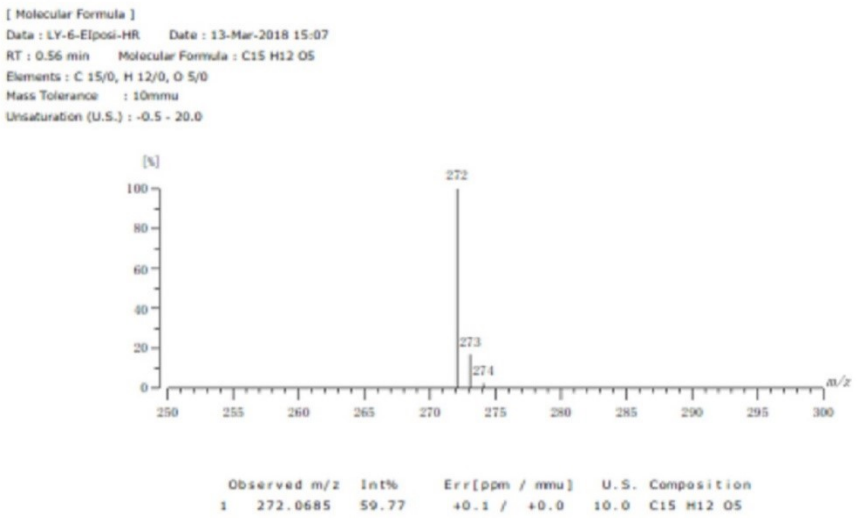


Figure S59: ^{1}H NMR of compound 6

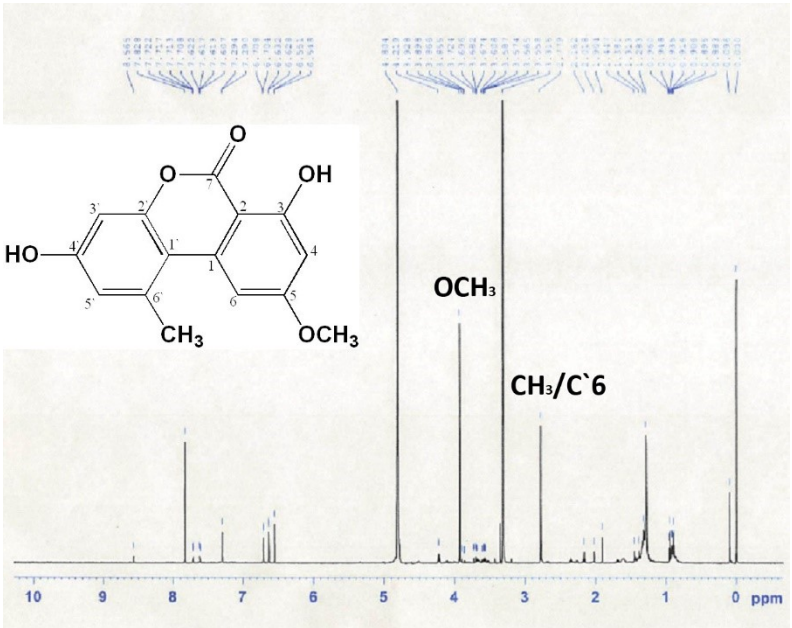


Figure S60: ^{1}H NMR of compound 6 (expansion from 6.5-7.9 ppm)

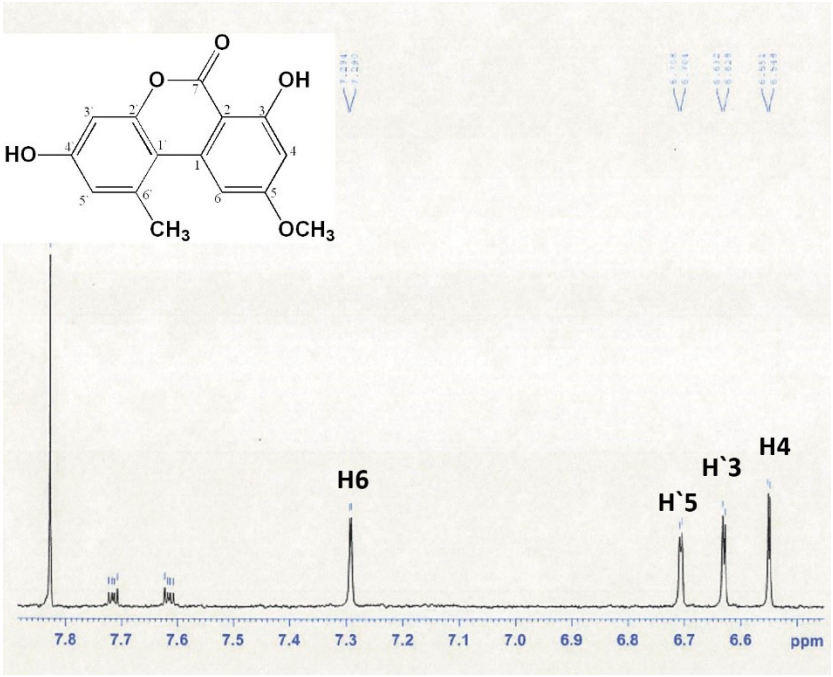


Figure S61: ^{13}C NMR of compound 6

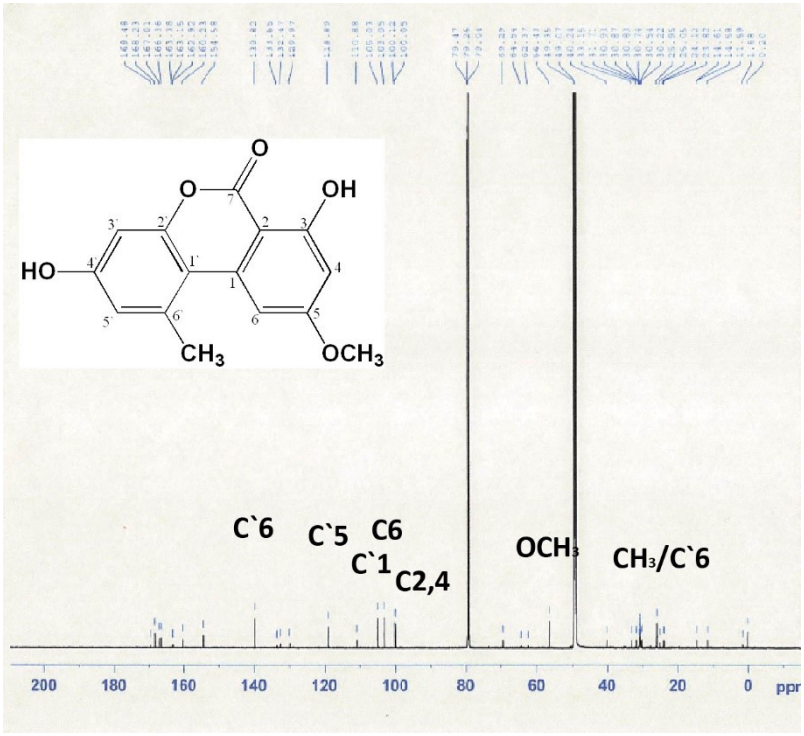


Figure S62: ^{13}C NMR of compound 6 expansion from 154-170 ppm

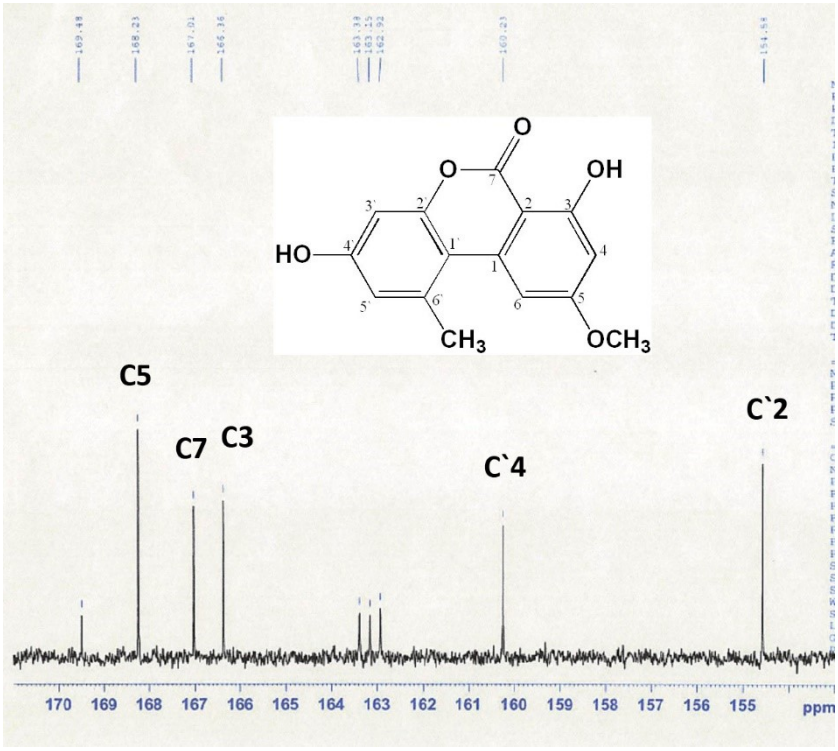


Figure S63: HSQC of compound 6 expansion from 6.4-7.9 ppm

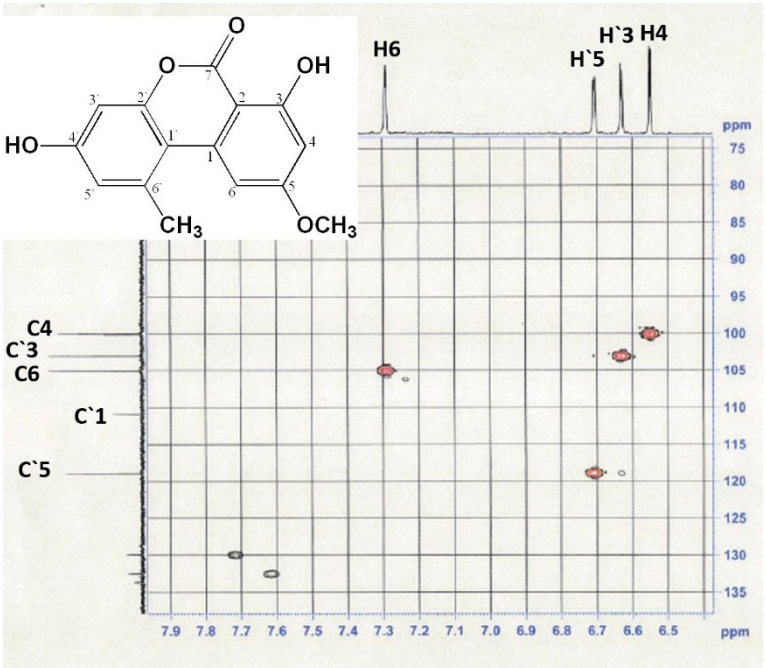


Figure S64: HSQC of compound 6 expansion from 3.5-4.4 ppm

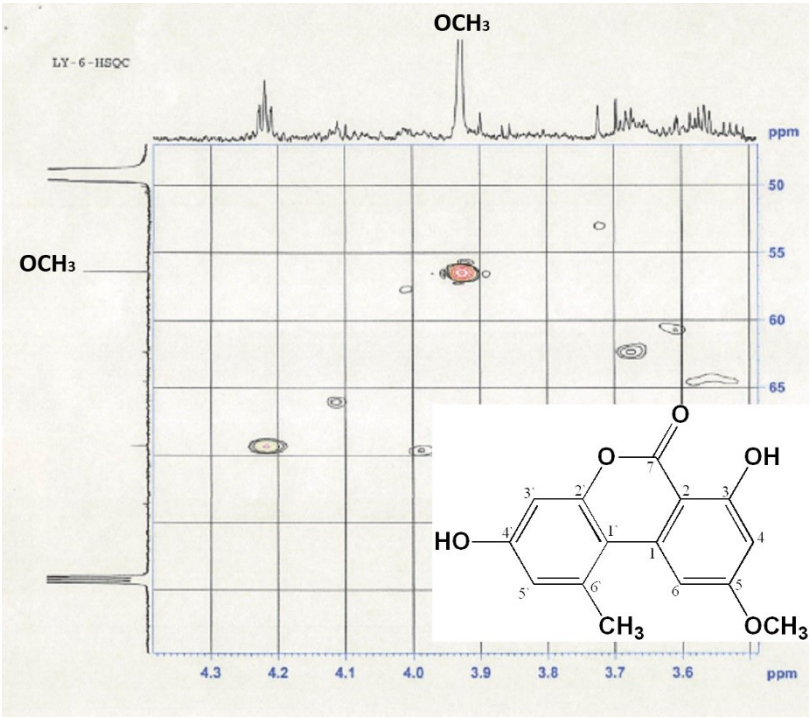


Figure S65: HSQC of compound 6 expansion from 0.8-2.8 ppm

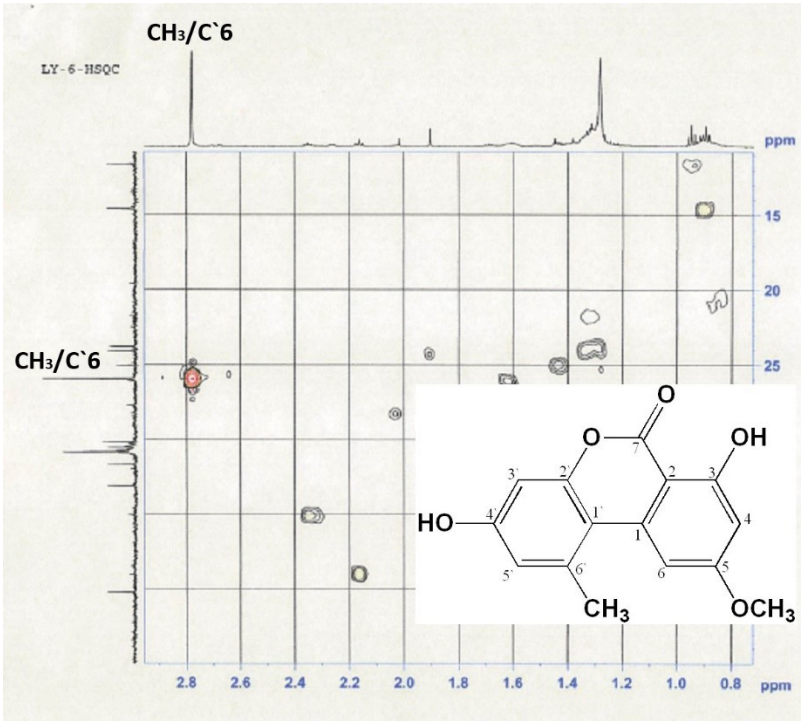


Figure S66: HMBC of compound 6 expansion from 6.3-7.4 ppm

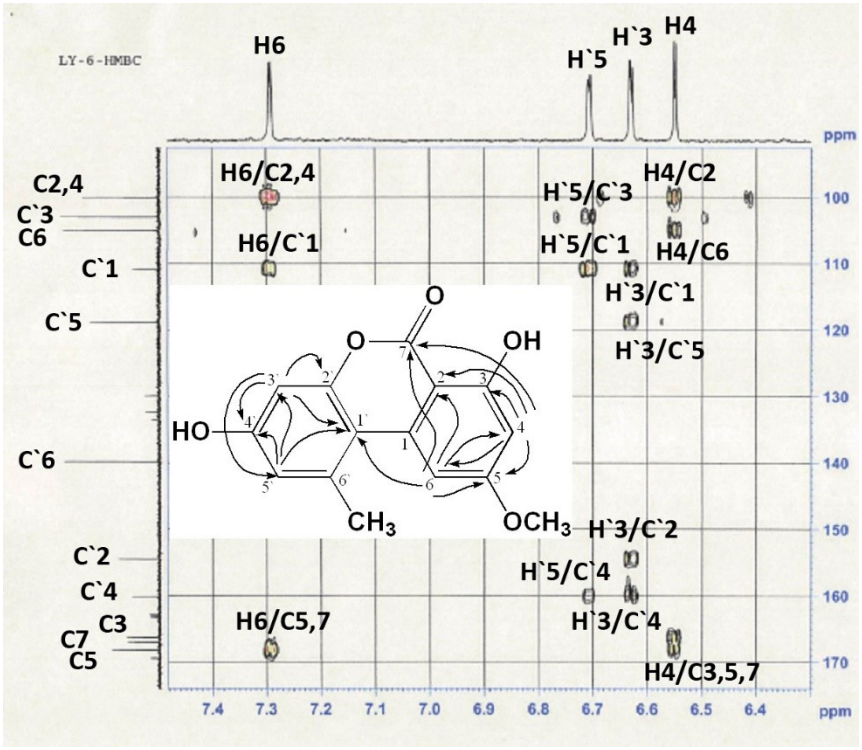


Figure S67: HMBC of compound 6 expansion from 1.4-4.0 ppm