SUPPLEMENTARY MATERIAL

The new ether derivative of phenylpropanoid and bioactivity was investigated from the leaves of *Piper betle* L.

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ABSTRACT A new ether derivative of phenylpropanoid compound, γ-(γ'-isohydroxychavicol)-chavicol octanyl ether (**K1**) along with one known phenylpropanoid named *allyl*-pyrocatechol or hydroxychavicol (**2**) were isolated from *P. betle* var. *kali* collected from Tumluk district, West Bengal India. We first report the presence of compound **K1** in the genus *Piper*. Their structures were established on the basis of various spectroscopic analyses. Compounds **K1** and **2** showed excellent antioxidant DPPH free radical scavenging activity with IC_{50} values of 4.61 and 4.12 μg/mL compared to ascorbic acid as a standard antioxidant drug with IC_{50} value of 3.42 μg/mL, respectively. Evaluation of *in vitro* cytotoxic activities of compounds **K1** and **2** showed significant effects against human oral cancer cell lines (AW13516 and AW8507), human hepatoma cell lines (HEPG2 and PLC-PRF-5) and a human pancreatic cell line (MIA-PA-CA-2), compared to Doxorubicin® as a standard cytotoxic drug with GI_{50} values of <10 and 18.18 μg/mL.

Keywords: *Piper betle*; bioactive compounds; antioxidant; cytotoxic; SAR.

1. Experimental

1.1. Instruments

Melting point was determined by using an OPTIMELT (Automated melting point system, Stanford Research Systems, USA). The mass spectrum was recorded on WATERS-Q-T of Premier HAB213 using the (ESI-MS) electro spray Ionization technique. 1D NMR experiments were acquired in $CDCl_3$ as solvent at VNMRS-400 spectrometer, observing ^{1}H and ^{13}C at 400 MHz and 100 MHz, respectively. In the proton NMR spectra the coupling constants (*J*) are expressed in hertz (Hz). Chemical shifts (denoted as δ) of NMR are reported in parts per million (ppm) units relative to tetra methyl silane (TMS). 2D NMR spectra were acquired in $CDCl_3$, at VNMRS-400 MHz spectrophotometer, observing ^{1}H-^{1}H COSY and HMBC spectrum, respectively. HR-ESI-MS experiment was recorded on WATERS-SYNAPT G2 HDMS spectrometer and FT-IR spectrum was acquired on FT-IR-8400S-Shimadzu spectrophotometer with KBr pellets (Atiya et al., 2017a).

1.2. Chemical

All chemicals and solvents were of analytical grade (AR grade) and were purchased from S.D. fine chemicals Ltd., and Sigma Aldrich. Isolated compound was monitored by Thin-layer chromatography (TLC) was performed on TLC plates (Merck, Mumbai, India) precoated with silica gel 60 GF_{254} thickness 0.2 mm and activate at 110 °C for 1 hour. The spot was detected in either Iodine/UV light (254 and 365 nm). For column chromatography, silica gel 60 (# 230-240 mesh, Merck, Mumbai, India) was used.

1.3. Plant material

The fresh leaves of *P. betle* var. *kali* were collected from Tumluk district, Purba midinipur, West Bengal India, during the month of October 2011. The age of this plant was 1-2 years old. Plant leaves were collected from upper part of the plant specially branches. The voucher specimens were identified by a taxonomist, Dr. K. Karthigeyan (Scientist C) at the Central National Herbarium, Botanic Garden Howrah Kolkata, India. The voucher specimen (CNH/10B/2011/Tech.II/502) was deposited in the Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra, Ranchi, Jharkhand, India for future reference.

1.4. Extraction

Fresh leaves of *P. betle* var. *kali* (500g) were extracted in boiling water (4 L) with stirring for 5 hrs. The resulting extract was filtered through muslin cloth, centrifuged, and concentrated to one-

sixth of the original volume under reduced pressure at a temperature of 45 ± 5 °C. This concentrated extract was then extracted with chloroform in a separating funnel. The chloroform soluble fraction was concentrated under reduced pressure using a rotary evaporator to obtain a dark oily viscous brown mass (16.12 g) (Sharma et al. 2009).

1.5. Isolation of compounds

A small amount (7 g) of chloroform fraction was dissolved in chloroform (75 mL) and adsorbed on silica gel (# 60-120 mesh) for column chromatography. The slurry was air dried and chromatographed over a column (43 cm length x 3.5 cm width) packed with silica gel in *n*-hexane. The column was eluted successively with *n*-hexane, mixture of *n*-hexane and ethyl acetate (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9, v/v). The obtained fractions were collected separately and matched by TLC to check similarities. Similar fractions having same R_f values were put together. All the fractions were free from chlorophyll contamination and were characterized by spectral studies.

γ-(*γ′-isohydroxychavicol*)-*chavicol octanyl ether* **(K1)**

Dark orange yellow semi solid mass, soluble in chloroform; MF: $C_{26}H_{32}O_3$; MW: 392.23; Yield 20.02 mg; mp (°C) 40-45; ^{1}H NMR (400 MHz, $CDCl_3$, *δ*, ppm): 7.73 (1H, *d*, *J* = 2.5 Hz, H-2"), 7.55 (1H, *dd*, *J* = 2.5, 7.6 Hz, H-6"), 7.39 (1H, *d*, *J* = 7.6 Hz, Ar-H-5"), 7.22 (1H, *dd*, *J* = 2.1, 6.4 Hz, Ar-H-2), 7.20 (1H, *dd*, *J* = 2.1, 6.5 Hz, Ar-H-3), 6.82 (1H, *dd*, *J* = 2.3, 6.5 Hz, Ar-H-5), 6.78 (1H, *dd*, *J* = 2.3, 6.4 Hz, Ar-H-6), 5.41 (1H, m, -CH-*α*'), 4.38 (1H, m, -CH-*α*), 4.33 (1H, m, CH-*β*'), 4.27 (1H, m, CH-*γ*), 4.22 (1H, m, H-*γ*'), 4.09 (1H, m, -CH-*β*), 2.51 (2H, *t*, *J* = 6.3 Hz, CH_2-1'), 1.72 (12H, brs, CH_2-2'-7'), 0.92 (3H, brs, CH_3-8'); ^{13}C NMR (100 MHz, $CDCl_3$, *δ*, ppm): 156.66 (C-4), 147.05 (C-3"), 146.66 (C-4"), 127.00 (C-1), 127.08 (C-2), 130.16 (C-6), 129.92 (C-*α*), 129.92 (C- *γ*), 129.92 (C-1"), 128.89 (C-*γ*'), 124.95 (C-*α*'), 123.94 (C- *β*), 122.30 (C-*β*'), 121.02 (C-6"), 117.71 (C-5"), 116.11 (C-3), 116.11 (C-2"), 115.95 (C-5), 65.51 (C-1'), 31.91 (C-2'), 30.17 (C-3'), 29.68 (C-4'), 29.64 (C-5'), 29.34 (C-6'), 22.67 (C-7'), 14.09 (C-8').

Allylpyrocatechol or Hydroxychavicol: (**2**)

Brownish red semi solid mass, soluble in chloroform; MF: $C_9H_{10}O_2$; MW: 150.06; Yield 60.34 mg; mp (°C) 30-32; ^{1}H NMR (400 MHz, $CDCl_3$, *δ*, ppm): 6.81 (1H, *d*, *J* = 8.1 Hz, Ar-H-5), 6.70 (1H, *d*, *J* = 2.2 Hz, Ar-H-2), 6.65 (1H, *dd*, *J* = 2.2,8.1 Hz, Ar-H-6), 5.94 (1H, m, CH-*β*), 5.09 (2H, m, =CH_2-*γ*), 3.27 (2H, *d*, *J* = 9.2 Hz, CH_2-*α*); ^{13}C NMR (100 MHz, $CDCl_3$, *δ*, ppm):143.46

(C-3), 141.67 (C-4), 137.60 (C-β), 133.20 (C-1), 120.96 (C-6), 115.67 (C-5), 115.54 (C-2), 115.31 (C-γ), 39.48 (C-α).

1.6. Total phenolic content

Total amount of phenolic contents were measured with Folin–Ciocalteu reagent, with slight modification (Atiya et al., 2017a; Baba & Malik 2015). Total phenolic content was expressed in milligram equivalents of gallic acid (GAE) per gram of fresh weight of plant material (mg GAE/g fresh weight).

1.7. Evaluation of antioxidant activity by DPPH radical scavenging method

Free radical scavenging activity was measured by 1, 1- diphenyl-2-picryl hydrazyl (DPPH) (Shekhar & Anju 2014; Atiya et al., 2017a).

1.8. In vitro cytotoxic activity

As reported previously (Houghton et al. 2007). Cell culture for the anticancer activities of each test samples were conducted at Advanced Center for Treatment, Research and Education in Cancer (ACTREC), Mumbai where the human cell lines were maintained in ideal laboratory conditions. Cells lines were harvested in suitable media supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and 1% Penicillin-Streptomycin in an adherent tissue culture microtiter plate at 37 °C with 5.0% CO_2. 96-wells microtiter plate were seeded with 5 × 103 cells per well and incubated again at 37 °C with 5% CO_2, 95% air and 100 % relative humidity for 24 hours prior to addition of experimental test samples. When the seeded plates achieved confluency, the cells were treated with different percentage of test samples in different concentrations. Evaluation of anti-cancer activity by sulforhodamine-B assay (SRB), the test samples were solubilized in appropriate solvent at 400-fold and stored frozen prior to use. At the time of drug addition, an aliquot of frozen test concentrate was defrost and diluted to 10 times the desired final maximum test concentration with complete medium containing test article at a concentration of 100, 200, 400 and 800 μg/mL. Aliquots of 10 μL of these different dilutions were added to the appropriate microtiter wells already containing 90 μL of cell suspension, resulting in the required final test sample concentrations of 10-80 μg/mL. For each of the experiments a known anticancer drug was used as a positive control. For the endpoint measurement, after samples addition, plates were incubated at standard conditions for 48 hours. Thereafter, 100 μL of 10% cold trichloro-acetic acid (TCA) was gently added to each well of the plates and incubated for 50 minutes at 4 °C to fix the cells attached to the bottom of the wells.

After fixation, the supernatant was discarded; the plates were washed five times with distilled water in order to remove excessive fixative and dead cells and kept overnight at 4 °C. Once the plate was dried, 100 μL of SRB dye (0.4%, w/v in 1% acetic acid) was added to each of the wells, and plates were incubated for 25 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing four times with 1% acetic acid. Viable cells take up SRB dye and stain pink. The plates were allowed to air dried. Bound stain was subsequently eluted with 100 μL of 10 mM Trizma base [tris (hydroxymethyl) amino methane (pH 10.5)]. Plates were kept over mechanical shaker for 5-10 minutes for complete mixing of bound dye with tris base. Blank wells contained medium but no cells and the control wells contained cells but no test samples. The absorbance was read on an Elisa Plate Reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test wells to the average absorbance of the control walls × 100. Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of test sample at the four concentration levels (Ti)]; the percentage growth was calculated at each of the sample concentration levels. Percentage growth inhibition (GI_{50}) was calculated as: [(Ti-Tz)/(C-Tz)] × 100. TGI = Drug concentration resulting in total growth inhibition (TGI) will calculated from Ti=Tz and LC_{50} = Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning indicating a net loss of 50% cells following treatment is calculated from [(Ti-Tz)/Tz × 100 = -50.

Statistical analysis

All the experiments were performed in triplicates and the data were reported as the mean ± SD. The results obtained were analyzed using one-way ANOVA method using Graph Pad Prism version 5.0. A difference was considered statistically significant if $p<0.05$.

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Figures:

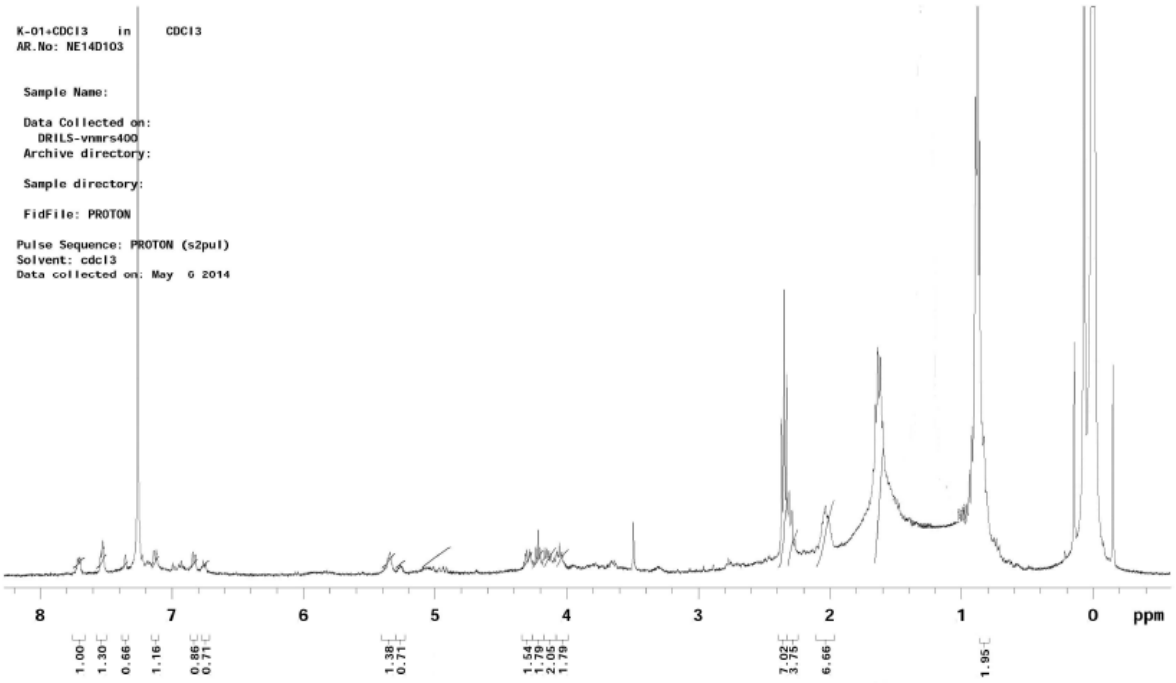


Figure S1. 400 MHz-^{1}H-NMR Spectrum of Compound **K1** in $CDCl_3$.

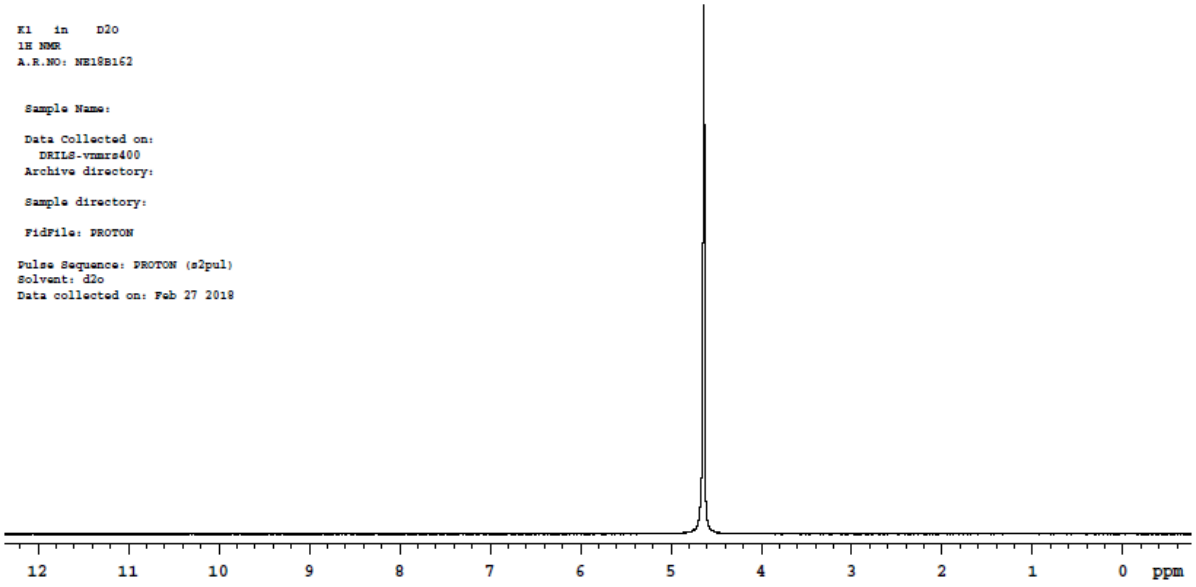


Figure S2. MHz-^{1}H-NMR Spectrum of Compound **K1** in D2O.

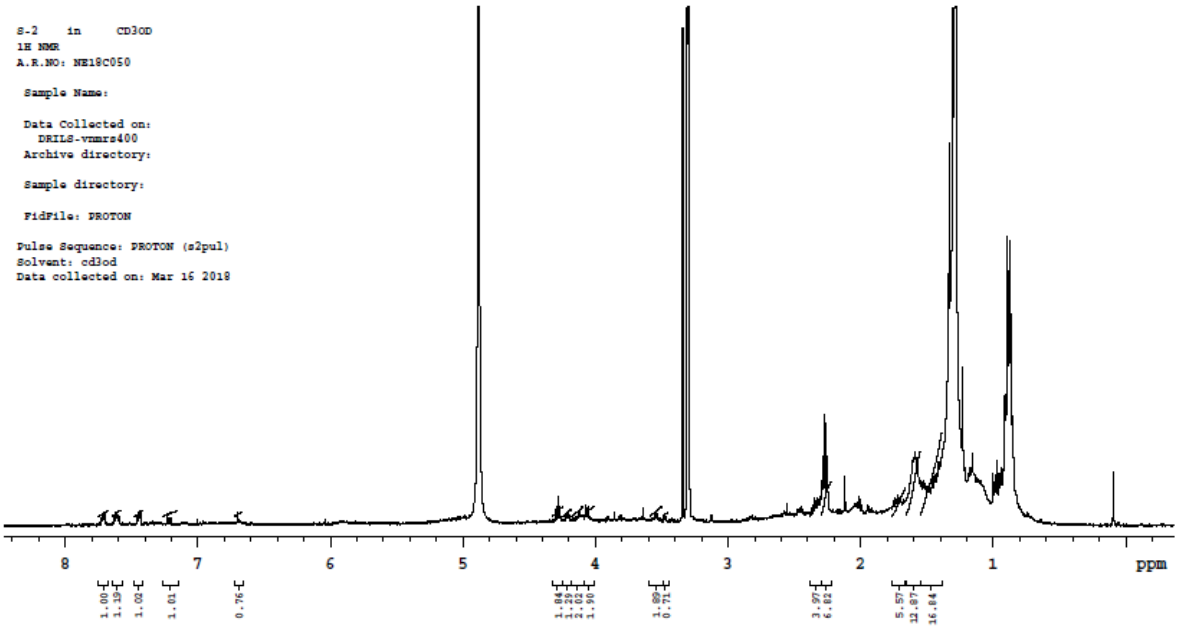


Figure S3. 400 MHz-^{1}H-NMR Spectrum of Compound **K1** in CD3OD.

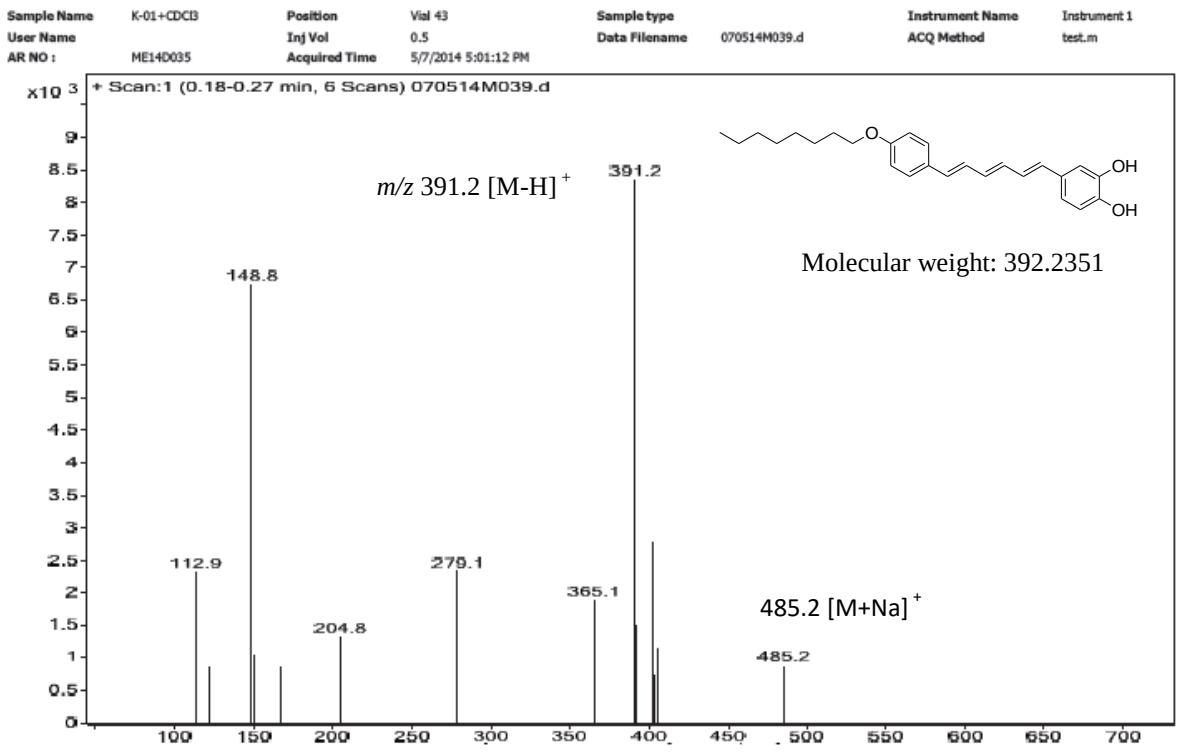
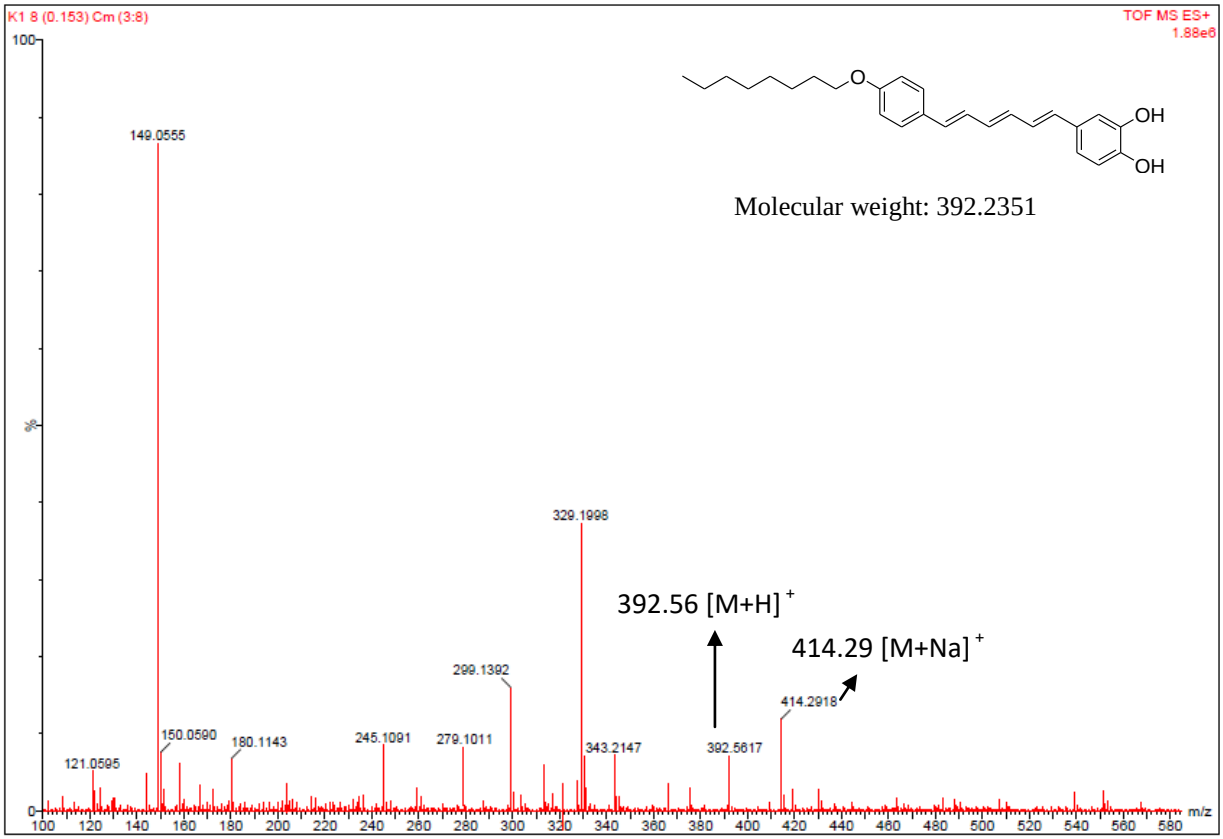


Figure S4. ESI-MS Spectrum of Compound **K1**.



Molecular weight: 392.2351

Figure S5. HR-ESI-MS Spectrum of Compound K**1**.

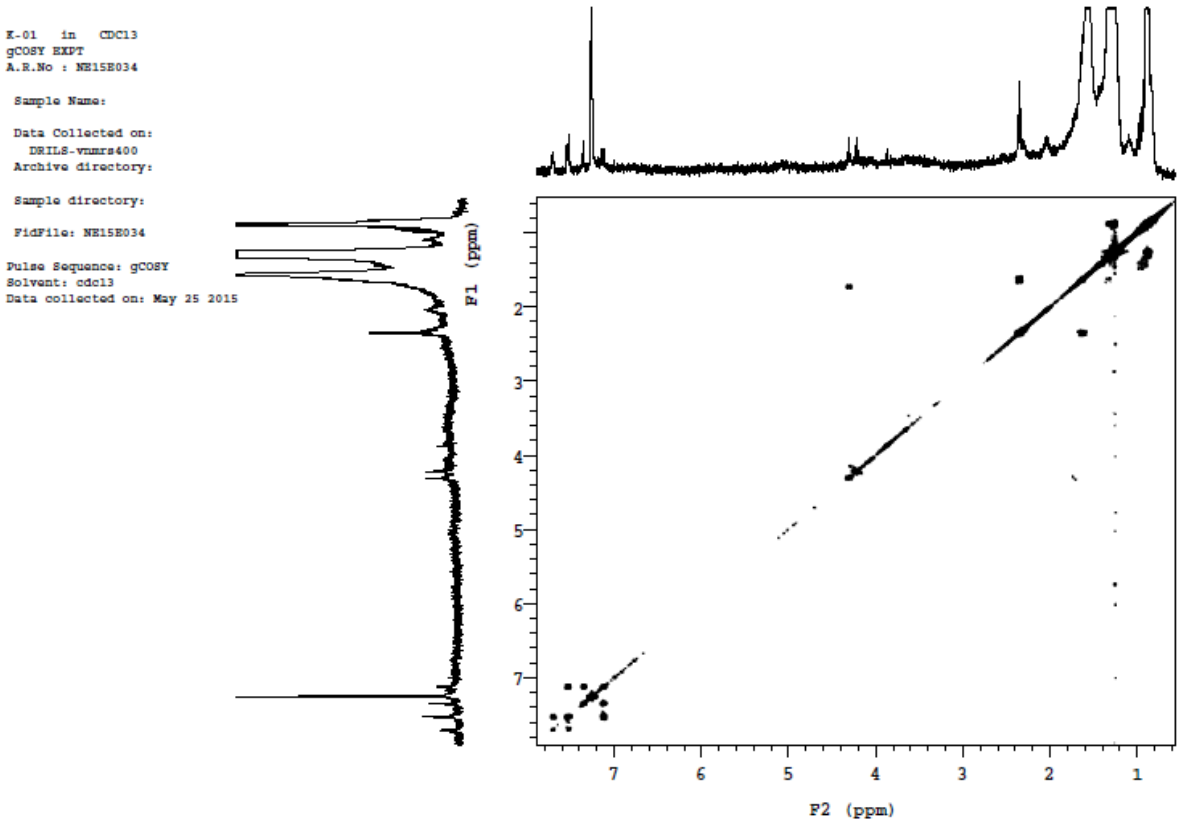


Figure S6. ^{1}H-^{1}H COSY Spectrum of compound K**1**.

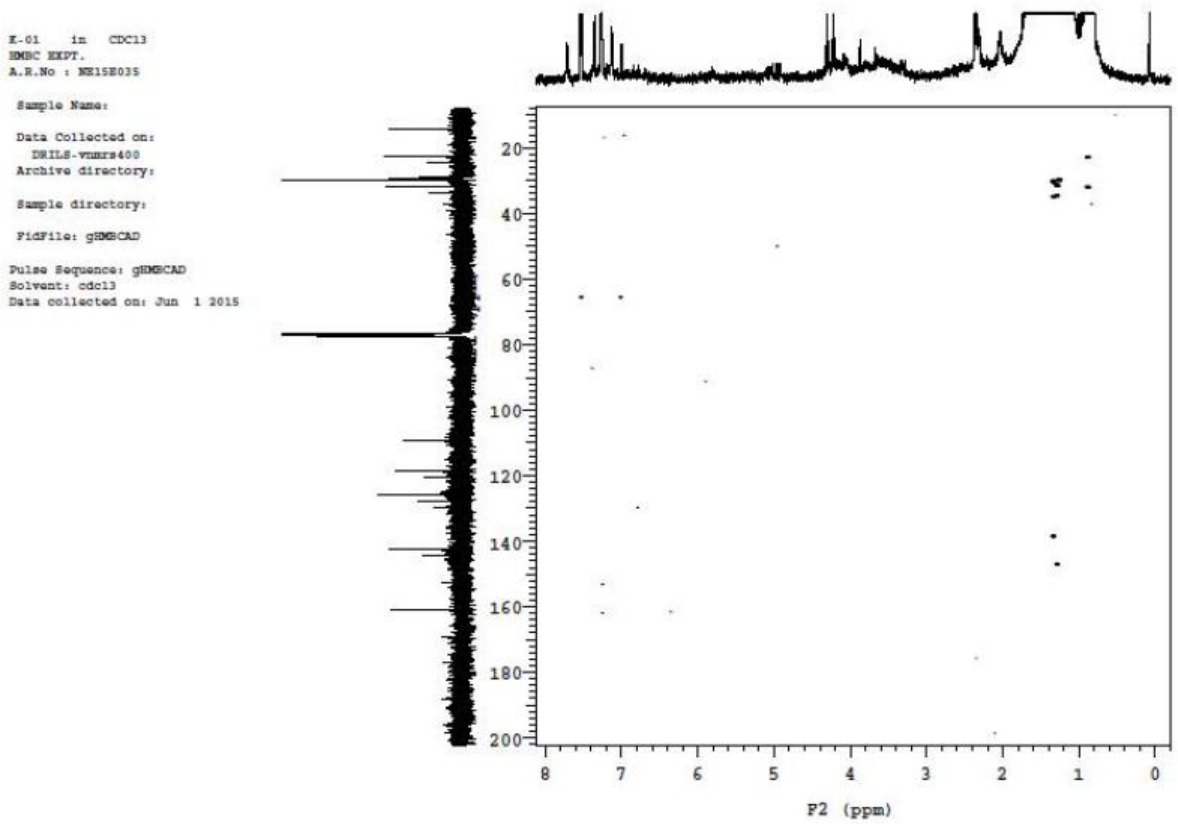


Figure S7. HMBC Spectrum of compound K**1**.

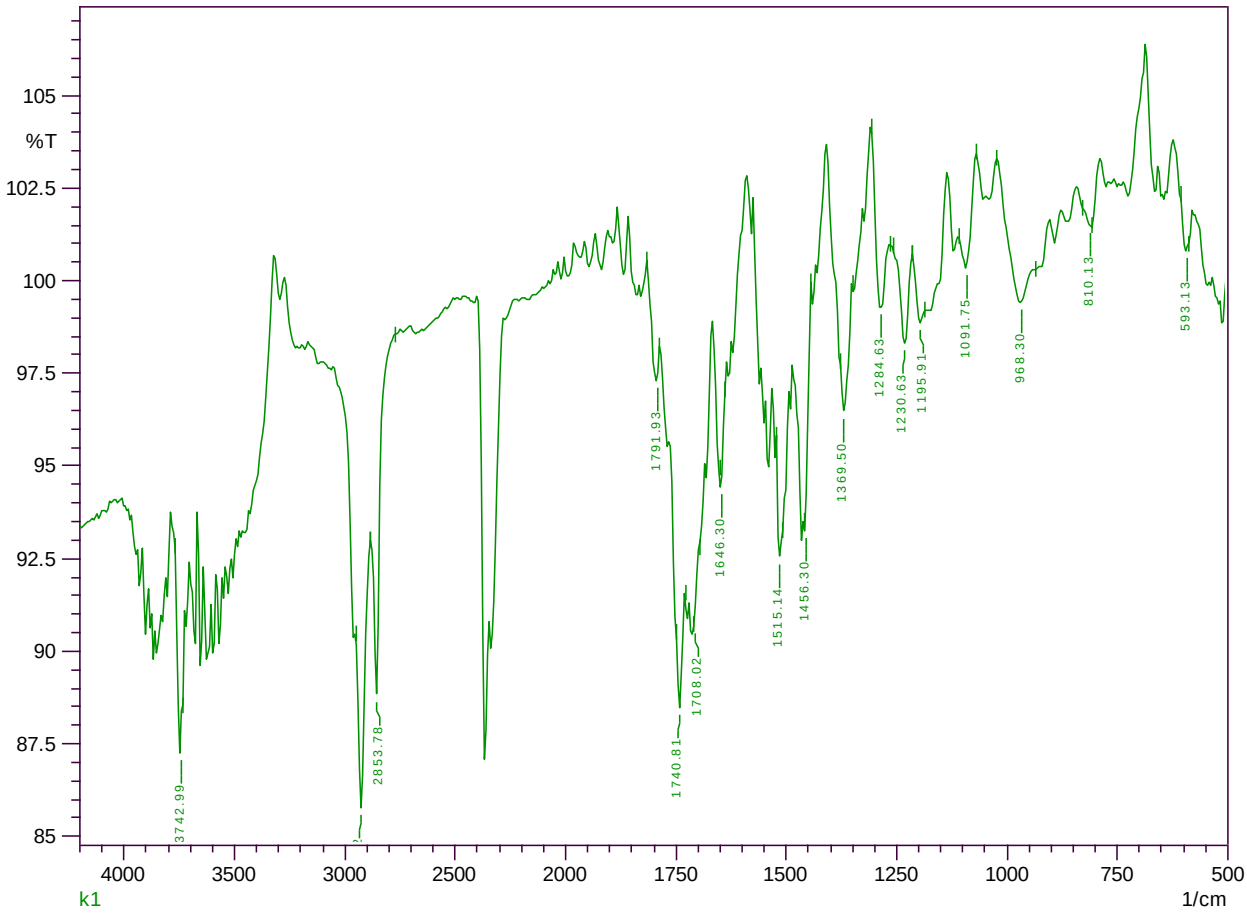
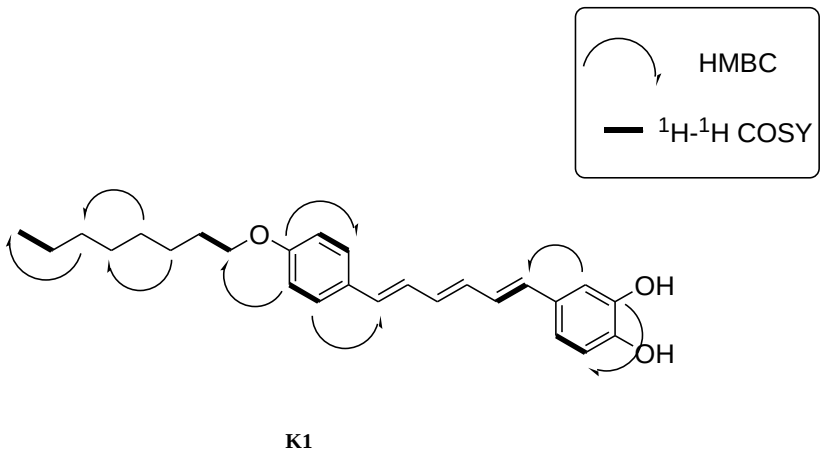


Figure S8. FT-IR Spectrum of Compound **K1.**



K1

Figure S9. Key ^{1}H-^{1}H COSY and HMBC correlation of isolated compound **K1**

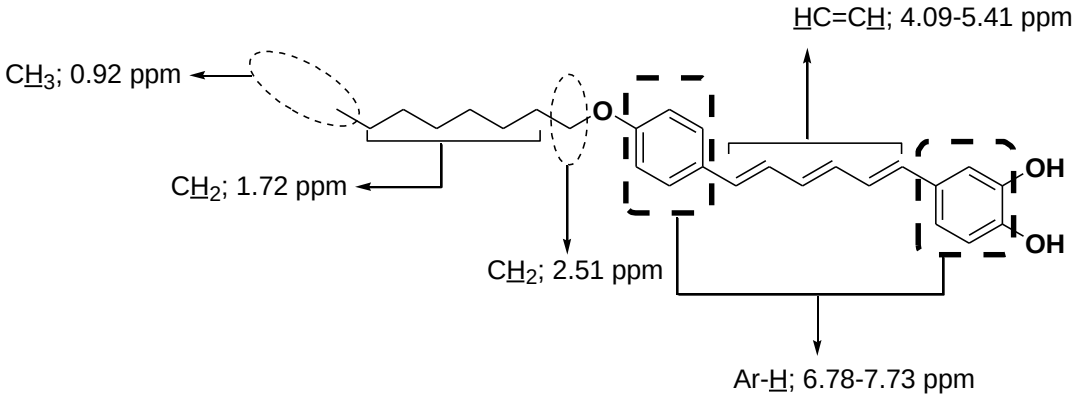


Figure S10. Basic proton peaks which were noticed in ^{1}H NMR spectrum of compounds **K1**.

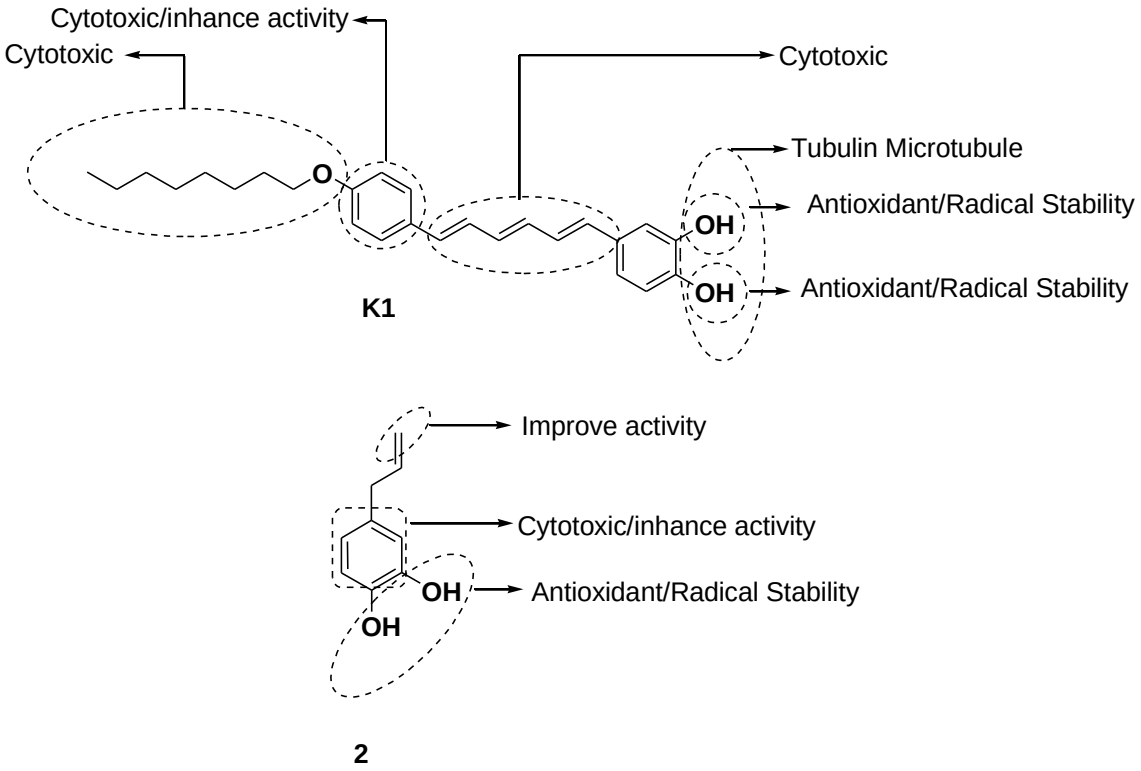


Figure S11. The structure activity relationships (SAR) of **K1** and **2** (Wu et al. 2014).

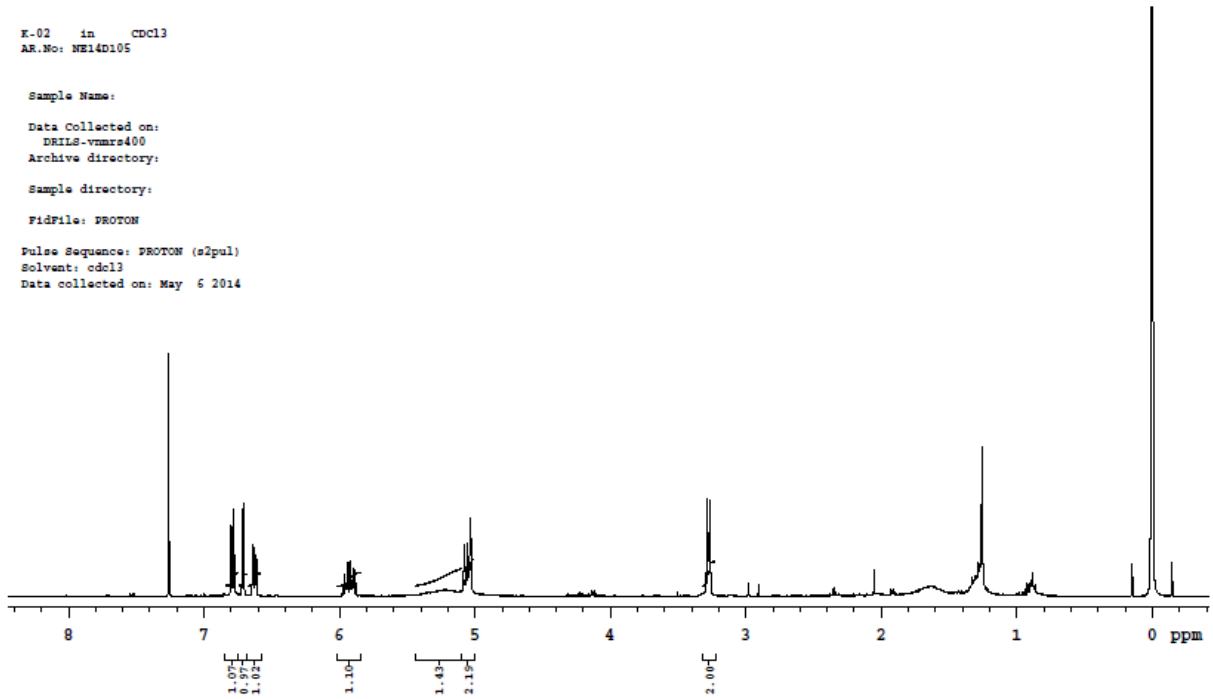


Figure S12. 400 MHz-^{1}H-NMR Spectrum of Compound **2** in $CDCl_3$.

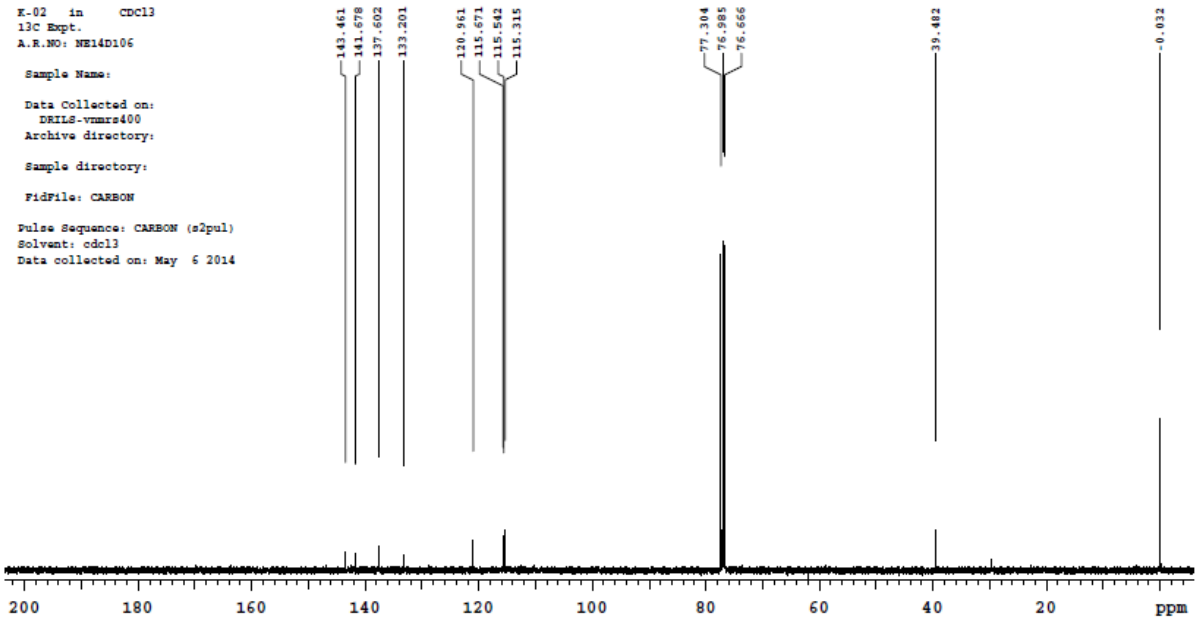


Figure S13. 100 MHz-^{13}C-NMR Spectrum of Compound **2** in $CDCl_3$.

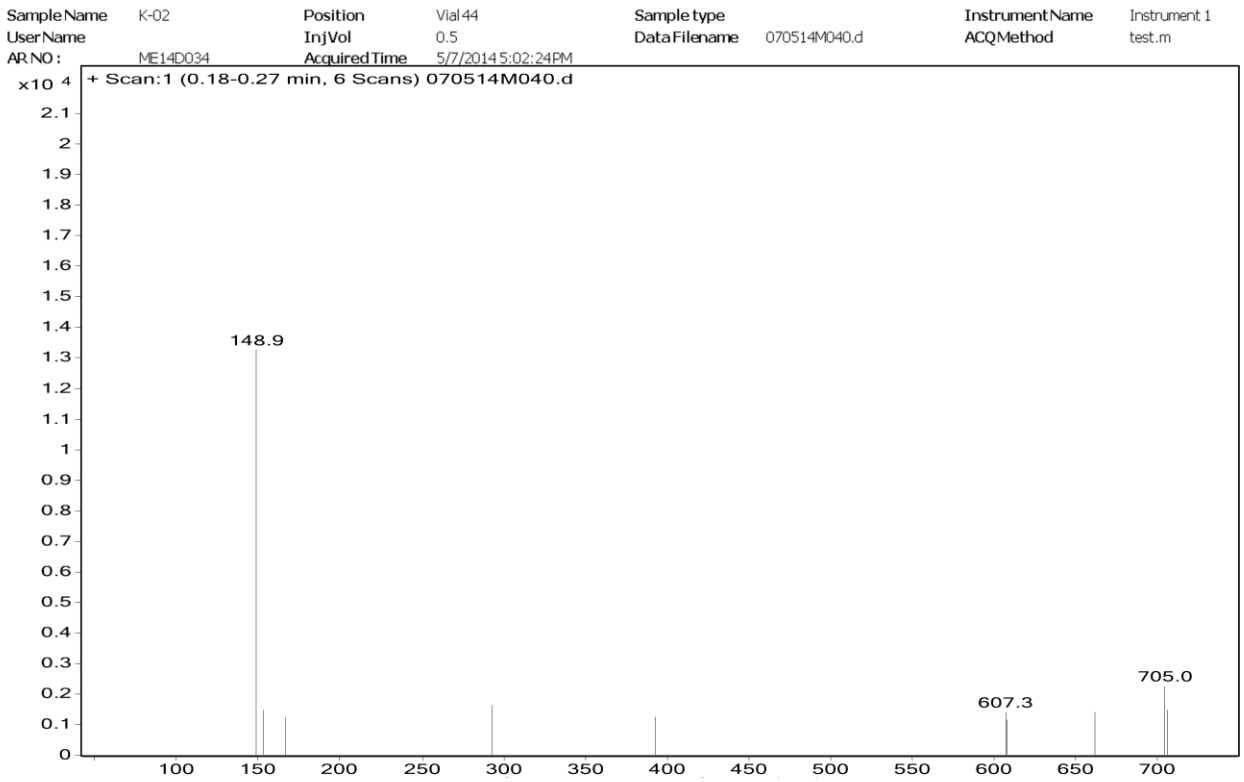


Figure S14. ESI-MS Spectrum of Compound **2**.

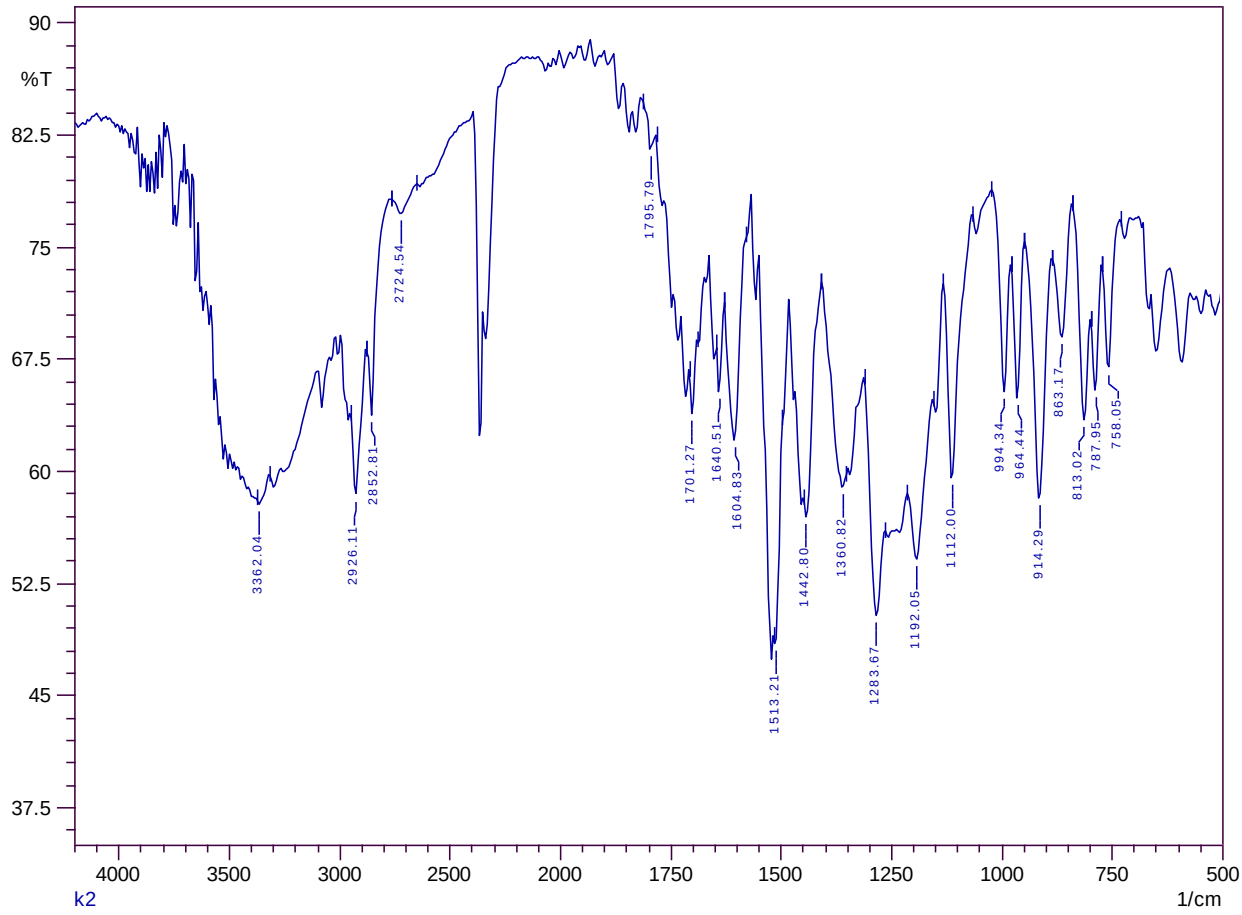


Figure S15. FT-IR Spectrum of Compound **2**.

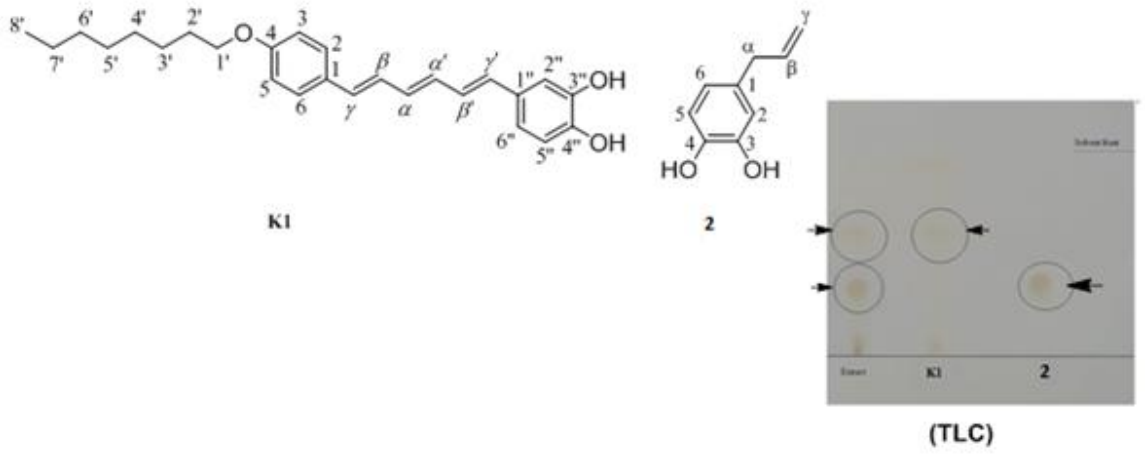


Figure S16. TLC profile of the compounds **K1** and **2** (*n*-hexane-ethyl acetate, 8:2) were visualized with the help of iodine vapours.