SUPPLEMENTARY MATERIAL

Terpenoids from the roots of *Leontopodium longifolium* and their inhibitory activity on NO production in RAW264.7 cells

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ABSTRCT

Phytochemical investigation of the roots of *Leontopodium longifolium*, led to the isolation of a novel norsesquiterpene, named as longifolactone (**1**), along with three known diterpenes. The structures of these compounds were elucidated by analysis of HR-ESI-MS, UV, IR and 1D and 2D NMR spectroscopic data. The absolute configuration of the new compound was determined by electronic circular dichroism (ECD) using both experimental and calculated ECD spectra. Furthermore, their anti-inflammatory effects were evaluated in LPS-activated RAW264.7 cells to determine their effects on the release of NO. Longifolactone (**1**) showed weak cytotoxicity towards two human cancer cell lines.

Keywords: *Leontopodium longifolium*; Longifolatone; Sesquiterpene lactones; NO production inhibition

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EXPERIMENTAL

General Procedures

The melting point was determined on Kofler melting point apparatus which were uncorrected. Optical rotation was measured by a Perkin-Elmer 341 polarimeter IR spectra was recorded in KBr disks on a Bruker VERTEX 70 spectrophotometer. UV spectra was scanned from 200 to 1000 nm on a Shimadzu UV-2450 spectrotometer. HR-ESI-MS was conducted on a Bruker APEX II spectrometer. NMR were recorded on a Bruker Avance 500 spectrometer. Silica GF_{254} for thin layer chromatography (TLC) and Silica gel (200-300 mesh and 300-400 mesh) used for column chromatography (CC) were purchased from Qingdao Marine Chemical Factory in China.

Plant material

The roots of *L. longifolium* was collected in Huzhu, Qinghai Province, People's Republic of China in August 2014. The whole plant was identified by Prof. Yong Qiang Tian from College of Chemistry and Bioengineering, Lanzhou Jiaotong University, and the voucher specimen (NO. LP14005) is deposited in the Laboratory Natural Products Research, College of Chemistry and Bioengineering, Lanzhou Jiaotong University.

Extraction and isolation

Air-dried and minced roots (6.5 kg) of *L. longifolium* were extracted three times at room temperature with MeOH (50L each time) for 7 days. The MeOH extract was concentrated under reduced pressure to obtain the residue (280 g), and the residue was subjected to a silica gel column with mixtures of *n*-hexane- acetone (10:1 and 5:1, v/v) to give two fractions (Fr.1 and Fr. 2).

Fr.1 (*n*-hexane-acetone 10:1, 11 g) was subjected to silica gel CC eluted with PE-EtOAc (12:1 and 8:1, v/v) to get two subfractions (Fr.1A-Fr.1B), and then Fr.1B was repeatedly by silica gel CC eluting with *n*-hexane- CH_2Cl_2 (50:1) to get compound **4** (11 mg). Fr.2 (*n*-hexane-acetone 5:1, 10 g) was subjected to silica gel CC eluted with *n*-hexane-EtOAc (10:1, 5:1 and 3:1, v/v) to give three subfractions (Fr.2A-Fr.2C). Fr.2B (*n*-hexane-EtOAc 5:1, 0.4 g) was isolated by silica gel CC with CH_2Cl_2-acetone (30:1, v/v) and then purified by preparative thin-layer chromatography (TLC) to give compound **1** (4.2 mg). Fr.2C (*n*-hexane-EtOAc 3:1, 0.3 g) was further isolated by

silica gel CC with $CHCl_3$-MeOH (100;1, 50:1 and 10:1, v/v) as elution to get Fr.2C_1-Fr.2C_3. Compound **2** (23 mg) and **3** (8.8 mg) were finally purified by preparative TLC with $CHCl_3$- acetone (10:1) from Fr.2C_3.

Longifolactone (**1**): colorless solid, mp 72-74 ℃; $[\alpha]_D^{20}$ –40.0 (*c* 0.100, CH_3OH); IR (KBr) ν_{max}: 1726, 1691, 1647 cm^{-1}; UV (MeOH) λ_{max} (log ε): 240 (2.88) nm; HR-ESI-MS *m/z* 221.1539 $[M+H]^+$ (calcd. for $C_{14}H_{21}O_2^+$: 221.1536); ^1H and ^{13}C NMR data see Table 1.

Inhibitory activity on NO production in RAW264.7 cells

RAW264.7 cells at 5×10^3 per well were seeded in 96-well plates and incubated for 24 h, and the cells were treated with tested the test compounds at varying concentrations (2.5–40 μg/mL) and LPS (10 μg/mL) for 24 h. The supernatant was mixed with an equal volume of Griess reagent, and after incubating the mixed solution at room temperature for 5 min, the concentration of nitrite was measured by reading the absorption at 570 nm.

List of Supplementary Material

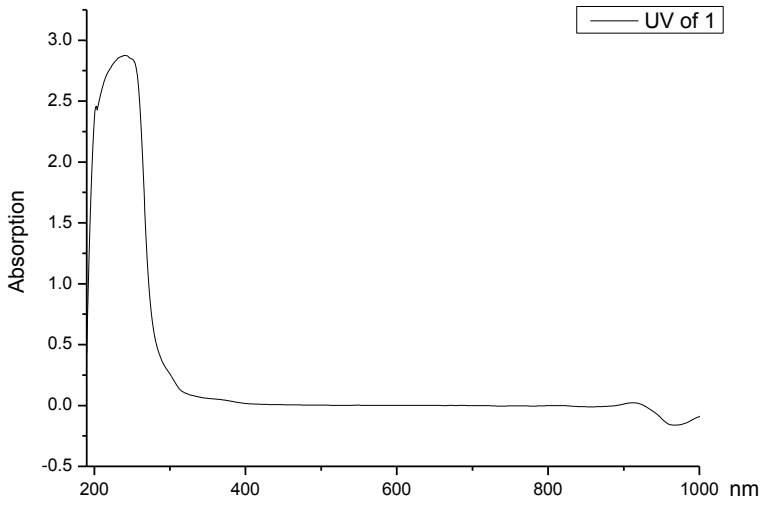


Figure S1. UV spectrum of compound **1**

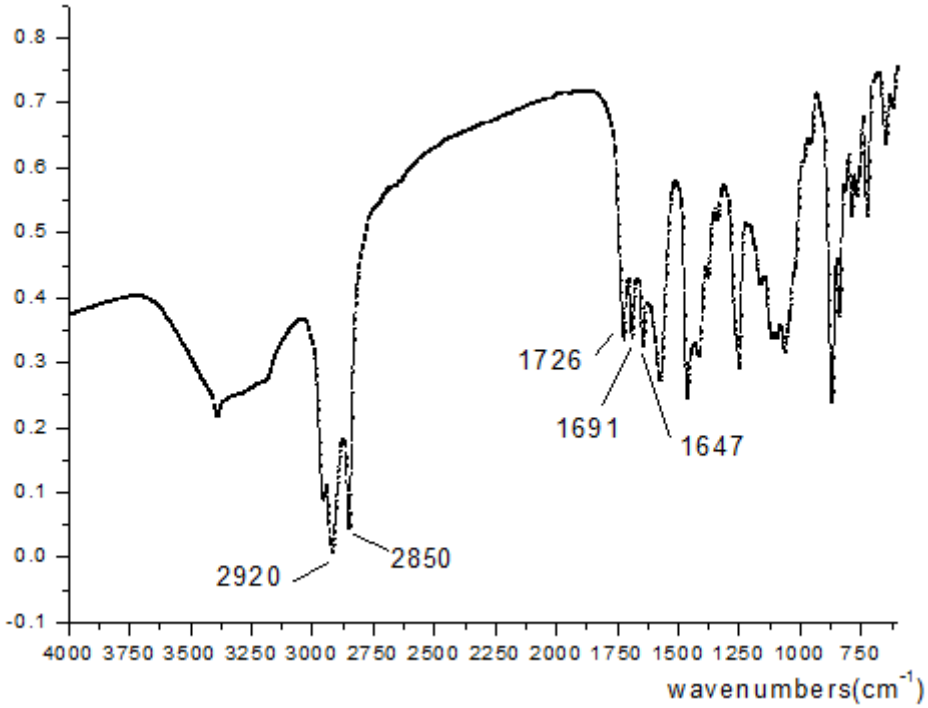


Figure S2. IR spectrum of compound **1**

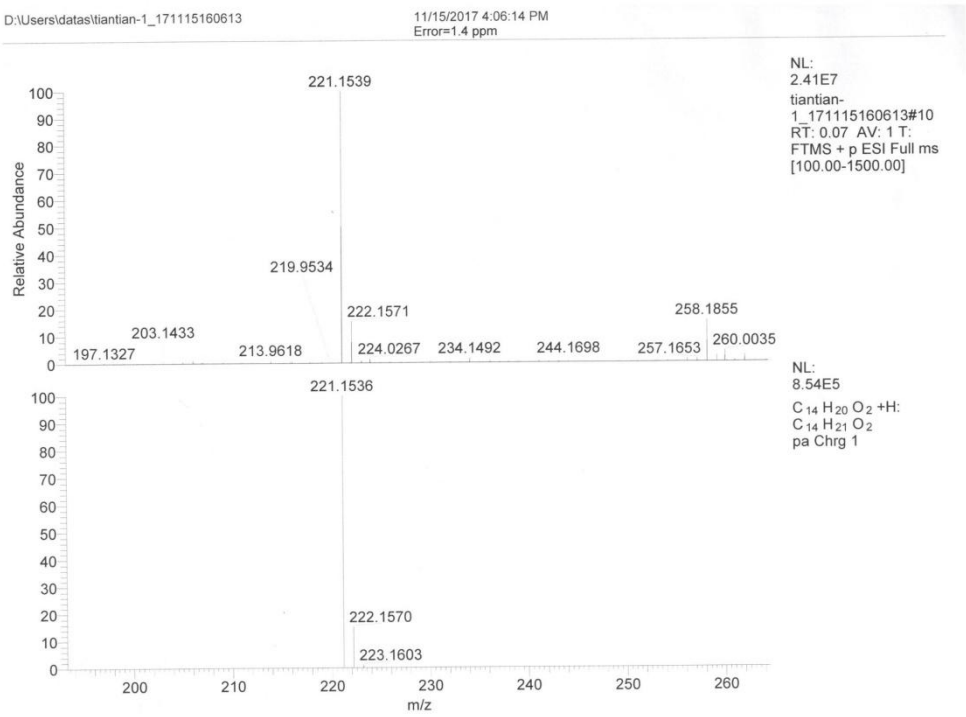


Figure S3. HR-EMI-MS spectrum of compound **1**

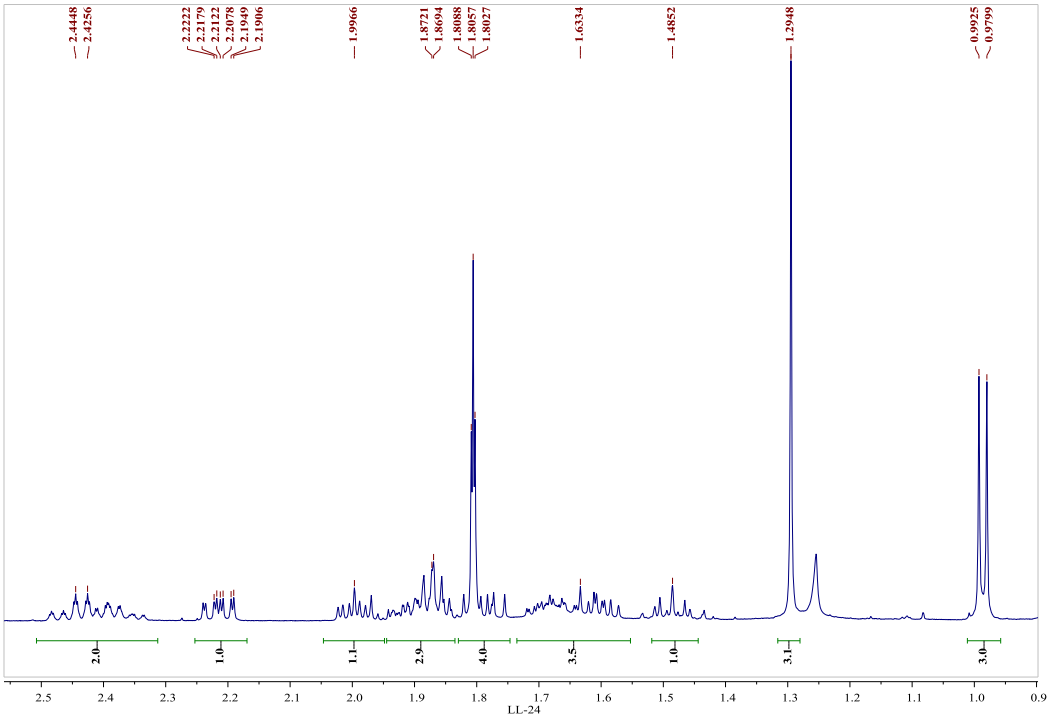


Figure S4. ^{1}H-NMR spectrum of compound **1**

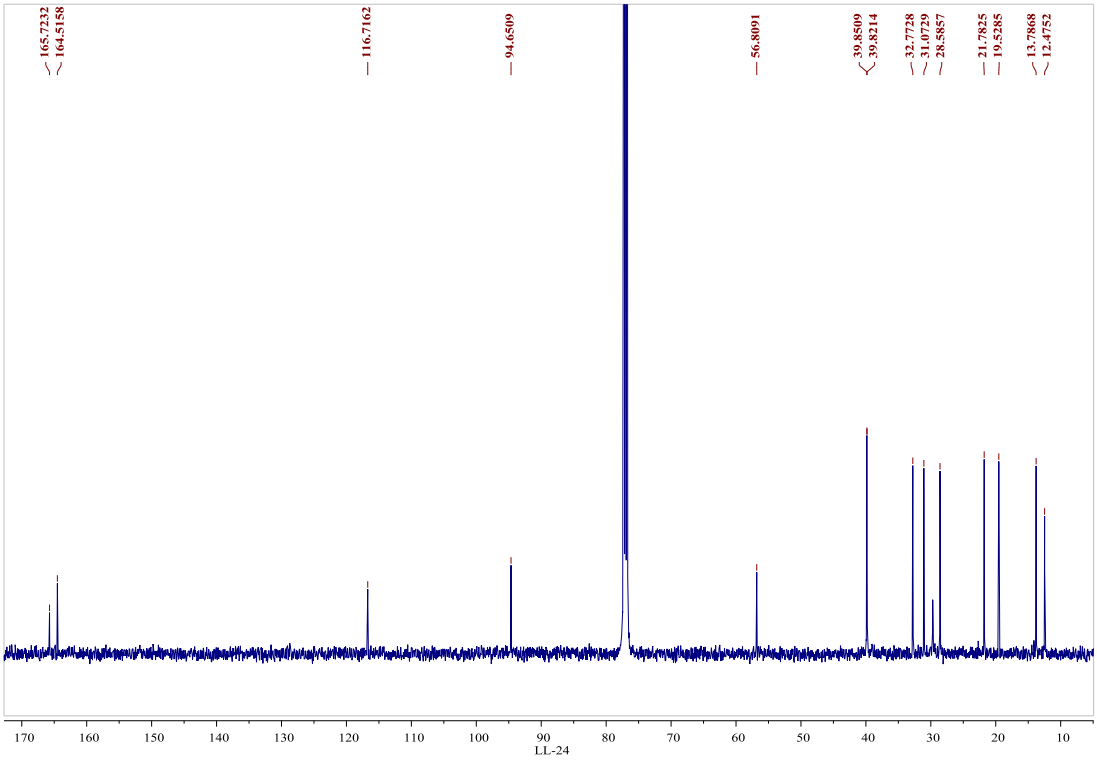


Figure S5. ^{13}C-NMR spectrum of compound **1**

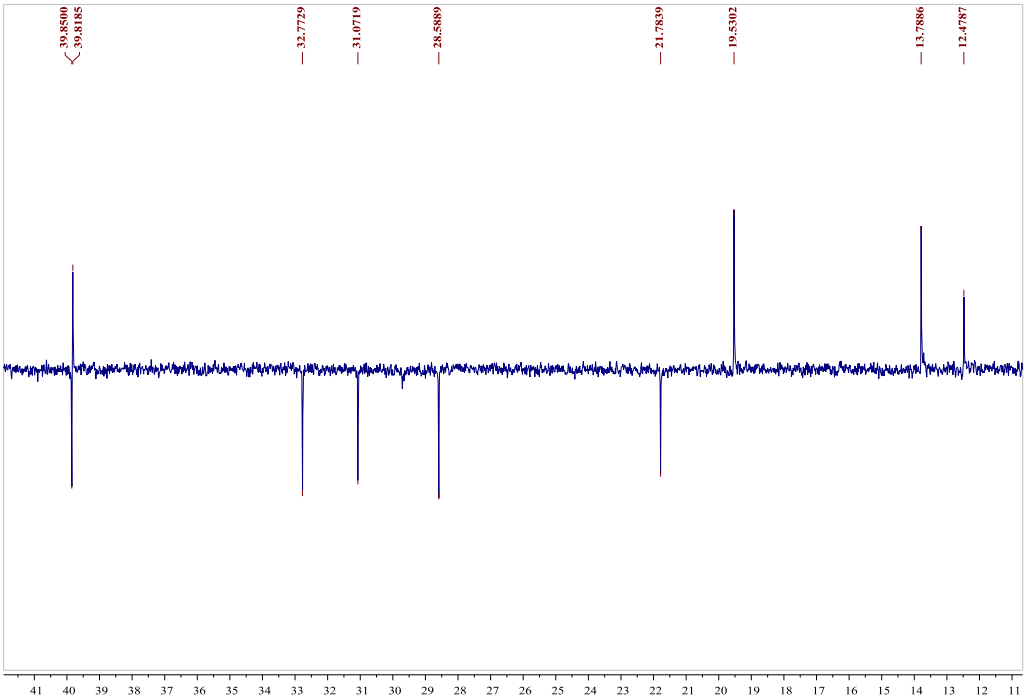


Figure S6. DEPT spectrum of compound **1**

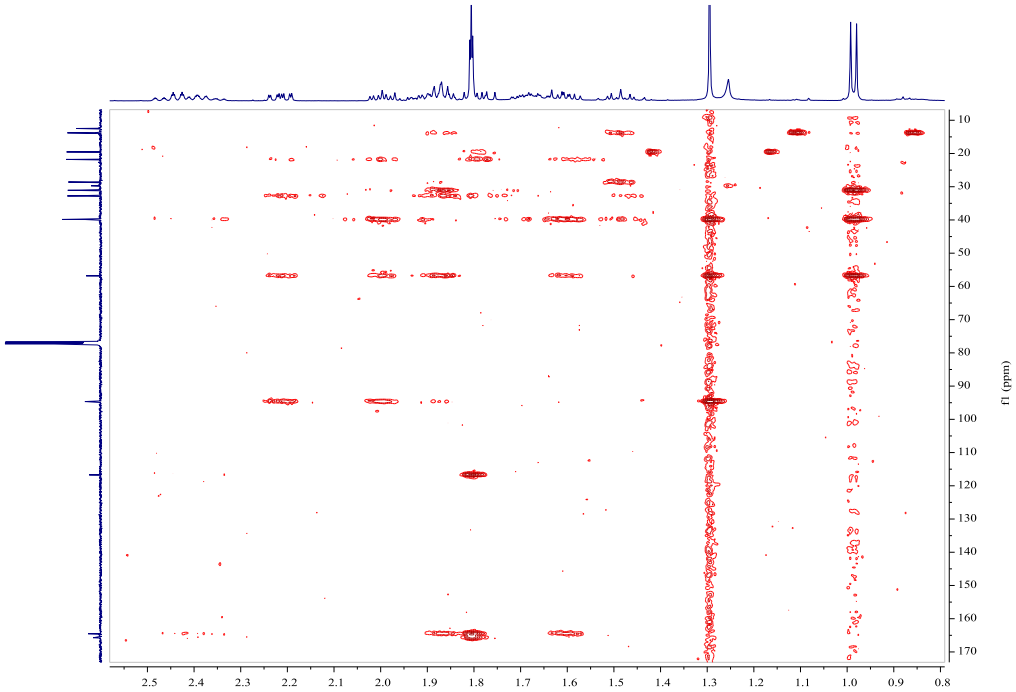


Figure S7. HMBC spectrum of compound **1**

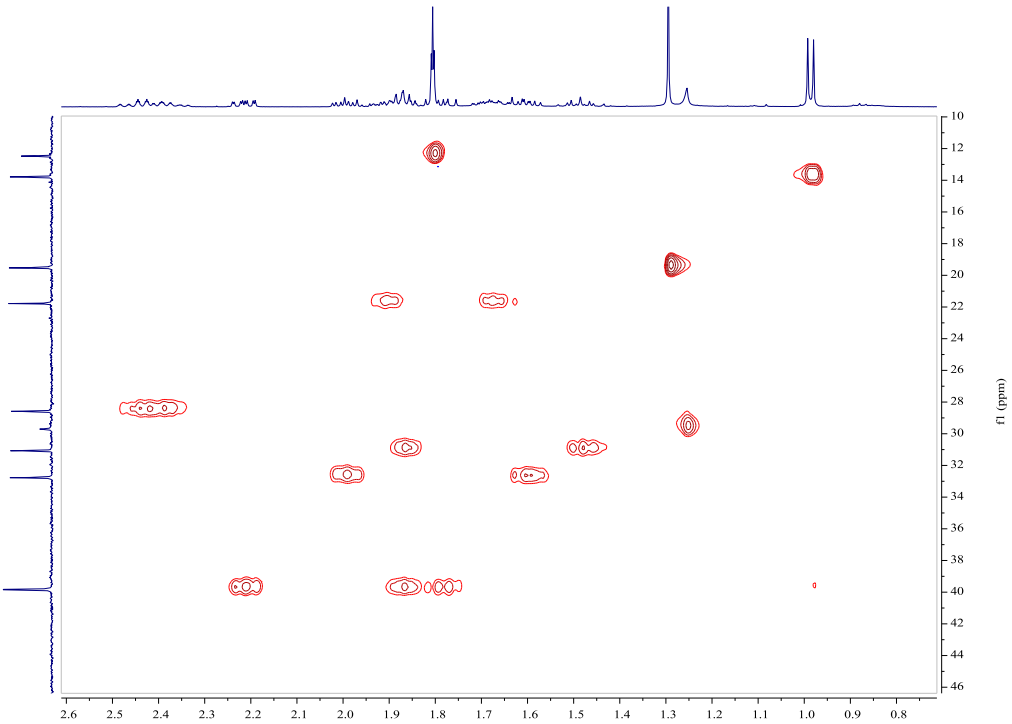


Figure S8. HMQC spectrum of compound **1**

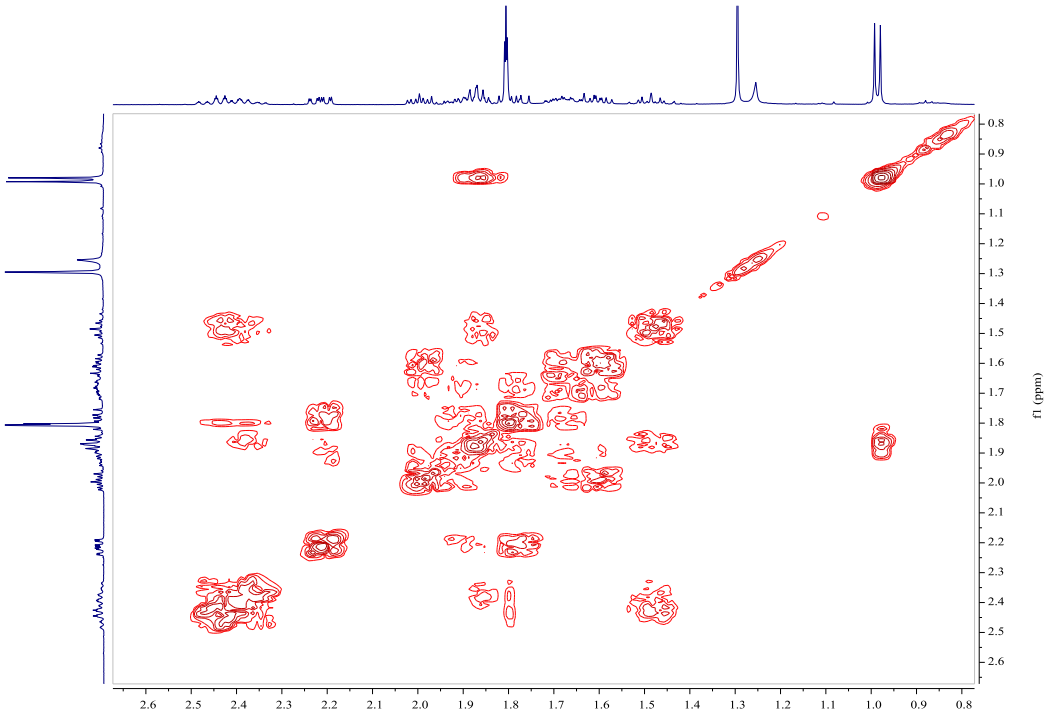


Figure S9. ^{1}H-^{1}H COSY spectrum of compound **1**

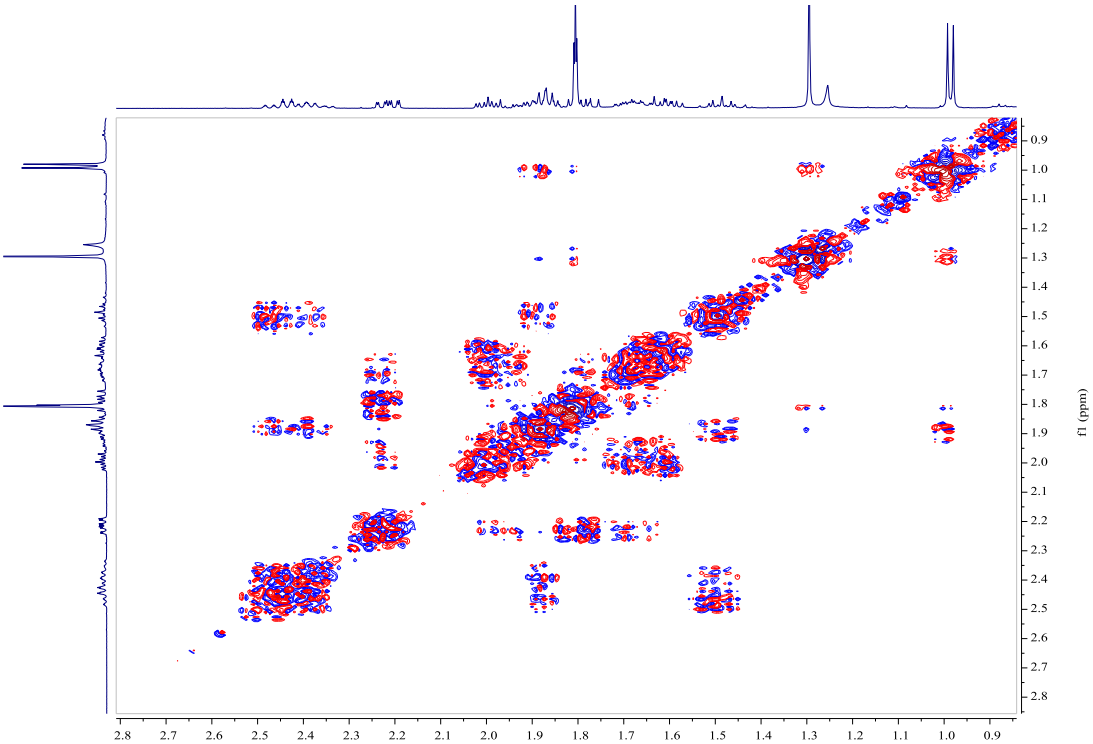


Figure S10. NOESY spectrum of compound **1**

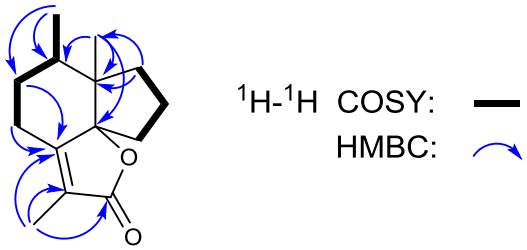
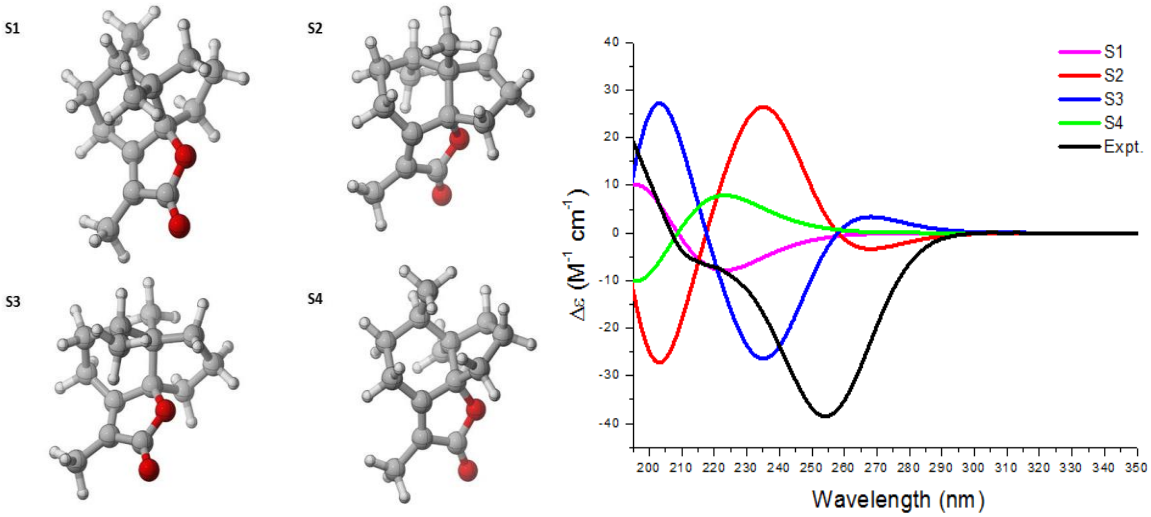


Figure S11. Key ^{1}H-^{1}H COSY and HMBC correlations of **1**.



a **b**

Figure S12. a is four potential isomers of compound **1**; **b** is experimental and calculated ECD spectra of **1**.

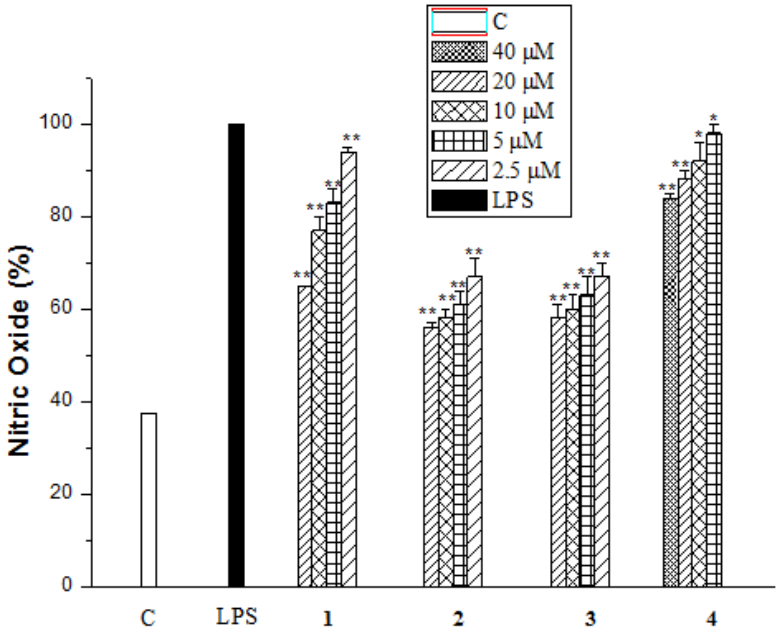


Figure S13. NO production in RAW246.7 cells treated with compounds **1**-**4**. C was used for blank control, LPS-induced NO production was used for positive control and represented at 100%. Data are expressed as mean ± SD from triplicate experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$ accepted as significant.

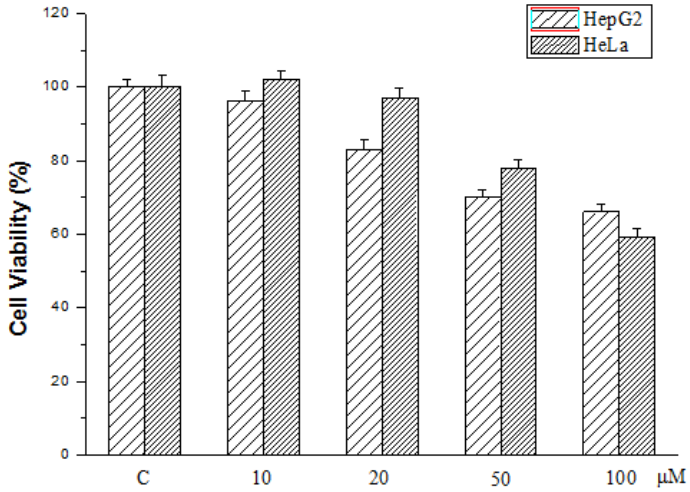


Figure S14. The cytotoxicity of longifolactone (**1**) against HepG2 and HeLa cells. Statistical analysis was conducted using one-way ANOVA followed by Tukey's t-test, and all data obtained from at least three replicate analyles.

Table S1. ^{1}H and ^{13}C NMR (DEPT) spectral data for **1**.*

position	δ_H (J in Hz)	δ_C
1	1.86 m	39.8 (d)
2	1.85 m, 1.48 m	31.0 (t)
3	2.44 m, 2.34 m	28.5 (t)
4	-	164.5 (s)
5	-	94.6 (s)
6	1.59 m, 1.99 ddd (10.3, 9.0, 3.8)	32.7 (t)
7	1.90 m, 1.68 m	21.7 (t)
8	2.21 ddd (13.8, 8.8, 1.9) 1.78 ddd (13.8, 10.3, 8.8)	39.8 (t)
9	-	56.8 (s)
10	-	116.7 (s)
11	-	165.7 (s)
12	0.98 d (6.3)	13.7 (q)
13	1.29 s	19.5 (q)
14	1.80 dd (1.6, 1.6)	12.4 (q)

* ^{1}H NMR was measured at 500 MHz in $CDCl_3$ and ^{13}C NMR (DEPT) was measured at 125 MHz in $CDCl_3$.

Table S2. The RAW264.7 cell viability (%) treated by compouds **1–4**.*

Concentration	10 μM	20 μM
1	97.14 ± 11.33	86.11 ± 4.56
2	92.31 ± 15.60	85.26 ± 6.13
3	103.87 ± 8.45	102.25 ± 12.63
4	85.43 ± 10.86	78.48 ± 7.20

*Results are expressed as the mean ± SD of at least triplicate experiments.