SUPPLEMENTARY MATERIAL

Cytotoxic and immunomodulatory phenol derivatives from a marine sponge-derived fungus *Ascomycota* sp. VK12

Tran Hong Quang[a], Nguyen Viet Phong[a], Tran Thi Hong Hanh[a], Nguyen Xuan Cuong[a], Nguyen Thi Thanh Ngan[b], Hyuncheol Oh[c], Nguyen Hoai Nam[a,*] and Chau Van Minh[a]

[a]*Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam;* [b]*Institute of Genome Research, Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam;* [c]*College of Pharmacy, Wonkwang University, Iksan 54538, Republic of Korea*

*To whom correspondence should be addressed:
Email: namnguyenhoai@imbc.vast.vn (Nguyen Hoai Nam)

Abstract

Chemical investigation of the marine-derived endophytic fungus *Ascomycota* sp. VK12 resulted in isolation and identification of a new compound, (3*R*)-(3′,5′-dihydroxyphenyl)butan-2-one (**1**) and five known ones: AGI-7 (**2**), sescandelin (**3**), sescandelin-B (**4**), 4-hydroxybenzaldehyde (B), and hydroxysydonic acid (**6**). The absolute configuration of **1** was determined by time-dependent density functional theory (TDDFT) electronic circular dichroism (ECD), specific optical rotation, and NMR calculations. Compounds **1** and **2** showed cytotoxicity toward HepG2, MCF-7, and SK-Mel2 carcinoma cells, with IC_{50} values ranging from 48.6 to 96.5 μM. Compounds **1**, **2**, **4-6** displayed NO inhibitory effects in LPS-stimulated BV2 cells, with IC_{50} values in a range from 24.2 to 76.5 μM. Compound **2** further inhibited PGE_2 overproduction, with an IC_{50} value of 25.3 μM. The inhibitory effects of **2** toward NO and PGE_2 overproduction were found to have a close relationship with its suppression of iNOS and COX-2 protein expression, respectively.

Keywords: marine-derived fungus, *Ascomycota*, phenylbutan-2-one, cytotoxic, anti-inflammatory

Experimental

1. General experimental procedures

Optical rotations were determined on a JASCO P-2000 polarimeter (Tokyo, Japan). HR-IDA-TOF-MS were recorded on a X500 QTOF mass spectrometer (Sciex, USA). Circular dichroism (CD) spectra were acquired on a Chirascan spectrometer (Applied Photophysics, UK). The ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on an AVANCE III HD 500 FT-NMR spectrometer (Bruker, Germany) with tetramethylsilane (TMS) as an internal standard. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck, Darmstadt, Germany) and YMC*GEL (ODS-A, 12 nm, S-150 μm, YMC Co., Ltd., Japan) resins. TLC used pre-coated silica gel 60 F_{254} (Merck) and RP-18 F_{254S} plates (Merck), and compounds were visualised by spraying with aqueous 10% H2SO4 and heating for 3–5 min. Preparative high performance liquid chromatography (HPLC) was performed on an Agilent 1200 system (Agilent Technologies, USA) equipped with a G1361A preparative pump, a G1315D diode array detector, a G2260A preparative autosampler, and a COSMOSIL 5C_{18}-MS-II Packed column (250 × 10 mm, 5 μm).

2. Fungal material

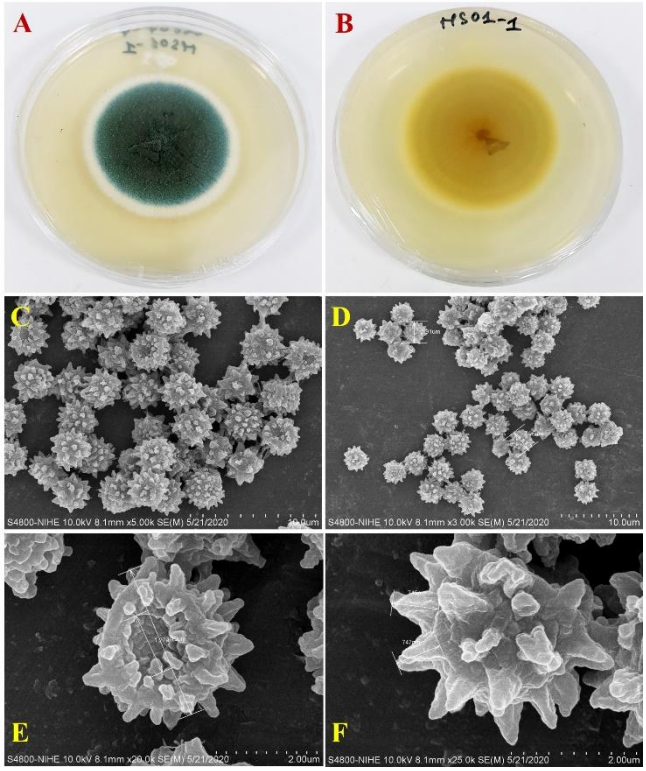


Figure S1. Morphological characteristics of *Ascomycota* sp. VK12 grown for 10 days on potato dextrose agar (PDA) at 25 ^{o}C. Obverse side (A) and reverse side (B) of the grown colony; C-F: images taken using a Hitachi S-4800 scanning electron microscope.

3. Fungal identification

The fungal sample was disrupted by bead-beating method and gDNA was extracted using a commercial kit (Qiagen, Germany) according to the manufacturer's protocol. The fungal ITS gene was amplified using the primer pair ITS1 and ITS4. The total reaction volume (20 μL) contained template DNA, 1.0 pmol of each primer, deoxynucleotide triphosphates (dNTPs, 200 μM each), 0.4 U Taq DNA polymerase, and supplied buffer. PCR was then performed as the following protocol: one cycle (98 ^{o}C for 2 min) for initial denaturation; 25 cycles (98 ^{o}C for 5 s; 60 ^{o}C for 30 s; 72 ^{o}C for 30 s) for annealing and extension, and one cycle (72 ^{o}C for 10 min) for final extension of the amplified DNA. After electrophoresis at 100 V for 30 min (Figure S1), the PCR product (approximate size 583 bp) was purified by PerfectPrep gel cleanup kit according to the manufacturer's protocol and then submitted to sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were analyzed using the Bioedit v7.2. BLAST search of the FASTA sequence was performed on NCBI. The result showed that the sequence of the fungal strain VK12 has the closest match (98.56 %) with that of the reported fungal strain *Ascomycota* sp. L276 (NCBI accession No. KU535800.1). By analyses using Clustal X2, BioEdit and Mega X programs, the phylogenetic tree was constructed (Figure S2). As indicated by the phylogenetic tree, the bootstrap values are 100 % based on 1000 replications shown at branching points and the Bar is 0.02 substitutions per nucleotide position. On the basis of the data obtained, the fungal strain was identified as *Ascomycota* sp. VK12. The sequence data was deposited at NCBI, with the accession No. MK641454.1.

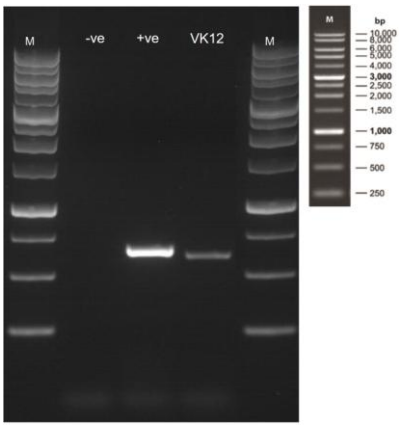


Figure S2. Gel electrophoresis of PCR product of the fungal strain VK12. M: DNA ladder; -ve: PCR no-template control; +ve: Positive control (DNA extracted from *Rigidoporus* sp.) used as template

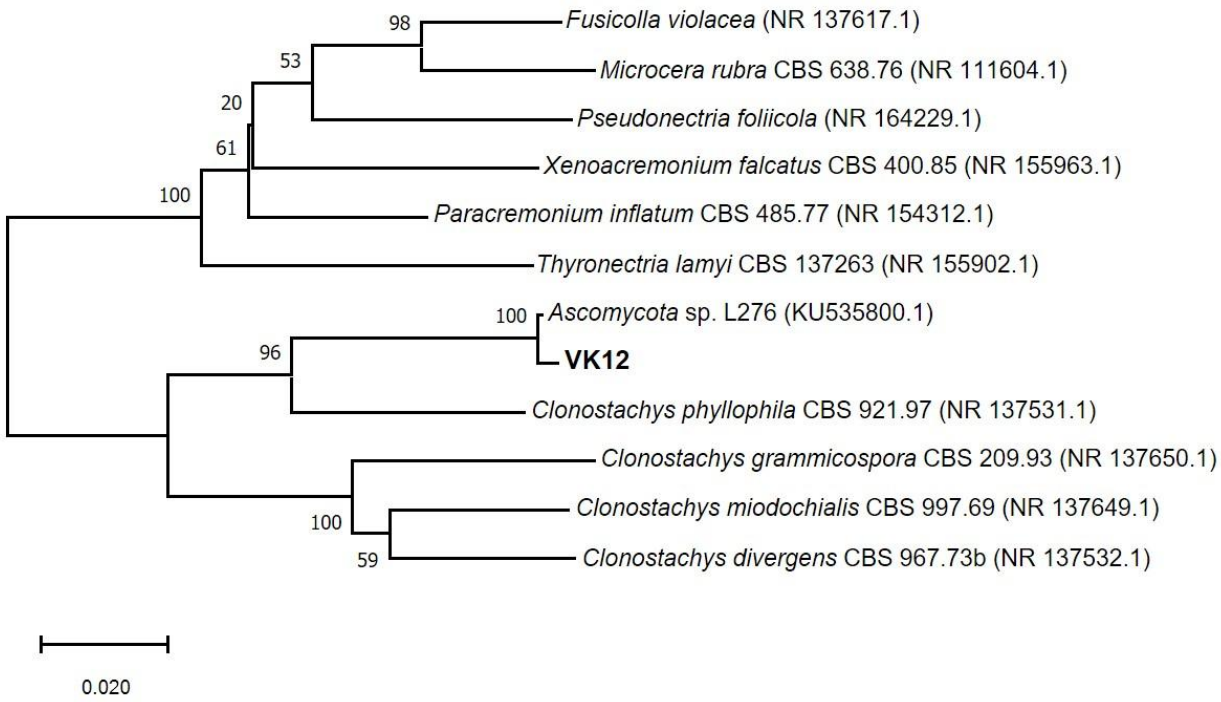
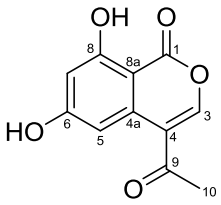


Figure S3. Phylogenetic tree of the fungal strain VK12. Maximum-likelihood phylogenetic tree based on ITS region, showing the taxonomic position of strain VK12 in the genus *Ascomycota* sp. L276. Bootstrap value is 100 % based on 1000 replications are shown at branching points. Bar, 0.02 substitutions per nucleotide position

- ITS sequence of the fungal strain VK12:

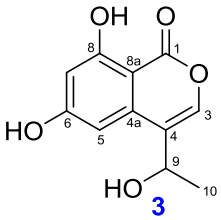
GTGGGCAAGCGGGAGGACTTACCGAGTTTACACTCCCAAACCCATGTGAATCTTACCTT
TTCGTTGCTTCGGCGGGATCGCCCCGGGCGCCATCGTGTGCCCCGGATCCAGGCGCCCG
CCGGGGGACTTAAACTCTTGTATTACTTTGTTTTCTCTGAGTGGATTACACAAATAATCA
AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
AAAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC
CCGCCAGTATTCTGGCGGGCATGCCTGTCTGAGCGTCATTTCAACCCTCATGCCCTAGC
GGCGTGGTGTTGGGGCTCGGCCTGAACTTGCTTCGGGCCGTCCCCTAAATCTAGTGGCG
GACCCGCTGTGGCCTCCTCTGCGTAGTAGTAATATTTCGCATGTGGAGAGCAGCA.

4. ^{1}H and ^{13}C NMR data of compounds 2-6



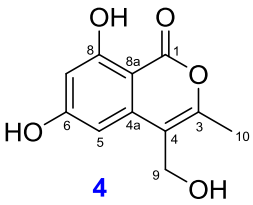
2

AGI-7 (**2**): ^{1}H NMR (500 MHz, DMSO-d$_6$): δ_H 8.52 (1H, s, H-3), 7.55 (1H, d, *J* = 2.0 Hz, H-5), 6.40 (1H, d, *J* = 2.0 Hz, H-7), 2.48 (3H, s, H$_3$-10), 10.97 (1H, s, 8-OH). ^{13}C NMR (125 MHz, DMSO-d$_6$): δ_C 163.6 (C-1), 154.9 (C-3), 116.7 (C-4), 134.9 (C-4a), 104.0 (C-5), 166.2 (C-6), 102.6 (C-7), 163.1 (C-8), 98.3 (C-8a), 196.2 (C-9), 28.3 (C-10).



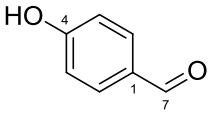
3

Sescandelin (**3**): ^{1}H NMR (500 MHz, CD$_3$OD): δ_H 7.38 (1H, d, *J* = 0.5 Hz, H-3), 6.64 (1H, d, *J* = 2.0 Hz, H-5), 6.39 (1H, d, *J* = 2.0 Hz, H-7), 4.92 (1H, d, *J* = 6.5 Hz, H-9), 1.54 (3H, d, *J* = 6.5 Hz, H$_3$-10). ^{13}C NMR (125 MHz, CD$_3$OD): δ_C 167.5 (C-1), 142.7 (C-3), 123.4 (C-4), 139.0 (C-4a), 102.4 (C-5), 167.2 (C-6), 103.1 (C-7), 165.5 (C-8), 100.4 (C-8a), 65.2 (C-9), 23.2 (C-10).



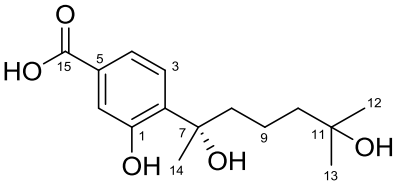
4

Sescandelin-B (**4**): ^{1}H NMR (500 MHz, DMSO-d$_6$): δ_H 6.64 (1H, d, *J* = 2.0 Hz, H-5), 6.33 (1H, d, *J* = 2.0 Hz, H-7), 4.42 (2H, s, H$_2$-9), 2.30 (3H, s, H$_3$-10), 11.16 (1H, s, 8-OH). ^{13}C NMR (125 MHz, DMSO-d$_6$): δ_C 165.0 (C-1), 152.4 (C-3), 113.2 (C-4), 139.7 (C-4a), 101.4 (C-5), 165.7 (C-6), 101.1 (C-7), 162.9 (C-8), 98.2 (C-8a), 55.9 (C-9), 16.6 (C-10).



5

4-Hydroxybenzaldehyde (**5**): ^{1}H NMR (500 MHz, CD$_3$OD): δ_H 7.80 (2H, dd, *J* = 2.0, 9.0 Hz, H-2 and H-6), 6.94 (2H, dd, *J* = 2.0, 9.0 Hz, H-3 and H-5), 9.79 (1H, s, H-7). ^{13}C NMR (125 MHz, CD$_3$OD): δ_C 130.3 (C-1), 133.4 (C-2 and C-6), 116.9 (C-3 and C-5), 165.2 (C-4), 192.8 (C-7).



6

Hydroxysydonic acid (**6**): ^{1}H NMR (500 MHz, CD$_3$OD): δ_H 7.30 (1H, d, *J* = 8.0 Hz, H-3), 7.48 (1H, dd, *J* = 2.0, 8.0 Hz, H-4), 7.40 (1H, d, *J* = 2.0 Hz, H-6), 1.13 (3H, s, H$_3$-12), 1.12 (3H, s, H$_3$-13), 1.63 (3H, s, H$_3$-14). ^{13}C NMR (125 MHz, CD$_3$OD): δ_C 156.9 (C-1), 137.9 (C-2), 127.8 (C-3), 121.6 (C-4), 131.6 (C-4a), 118.7 (C-5), 156.9 (C-6), 78.0 (C-7), 44.0 (C-8), 20.0 (C-9), 45.0 (C-10), 71.5 (C-11), 29.2 (C-12), 29.1 (C-13), 28.9 (C-14),

169.9 (C-15).

5. Cytotoxic assay

Cancer cells were cultivated in a humidified atmosphere of 5% CO_2 at 37°C for 48 h. Cell viability was examined by SRB assay for cell density determination, based on the measurement of cellular protein content (Monks et al. 1991). Viable cells were seeded in the growth medium (180 μL) into 96 well microwell plates (4×10^4 cells per well) and allowed to attach overnight. Test samples were added carefully into well of 96-well plates and the cultivation was continued under the same conditions for another 72 h. Thereafter, the medium was removed and the remaining cell monolayers are fixed with the cold 20% (w/v) TCA for 1 h at 4^oC and stained by 1X SRB staining solution at room temperature for 30 min, after which the unbound dye is removed by washing repeatedly with 1% (v/v) acetic acid. The protein-bound dye is dissolved in 10 mM Tris base solution for OD determination at 515 nm on an ELISA Plate Reader (Bio-Rad). DMSO 10% was used as blank sample while ellipticine was used as positive control. The cytotoxicity was indicated as half inhibition concentration (IC_{50}), which was calculated by program TableCurve Version 4.0 from concentration range 100 μg/ml; 20 μg/ml; 4 μg/ml; 0.8 μg/ml. The experiment was prepared in triplicate. The inhibition rate (IR) of cells was calculated by the following formula IR% = [100% – [(absorbance$_t$ – absorbance$_0$)/(absorbance$_c$ – absorbance$_0$)] × 100], in which: IR: Inhibition rate of cell growth, absorbance$_t$: average optical density value at day 3; absorbance$_0$: average optical density value at time-zero; absorbance$_c$: average optical density value of the blank DMSO control sample.

6. Anti-inflammatory assay

Cell culture

BV2 microglia cells were maintained at a density of 5×10^5 cells/mL in DMEM medium supplemented with 10 % heat-inactivated FBS, penicillin G (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM), and incubated at 37 ^oC in a humidified atmosphere containing 5 % CO_2. The effects of various experimental treatments on cell viability were evaluated by determining mitochondrial reductase function with an assay based on the reduction of the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into formazan crystals. The formation of formazan is proportional to the number of functional mitochondria in the living cells (Mosmann 1983).

Viability assay

50 µL of MTT (2.5 mg/mL) was added to cell suspension (1×10^5 cells/mL in each well of the 96-well plates) at a final concentration of 0.5 mg/mL, and the mixture was further incubated for 3 h at 37 ^{o}C. The formed formazan was dissolved in acidic 2-propanol, and absorbance was measured at 540 nm. Absorbance of the formazan formed in control (untreated) cells was considered as 100% viability (Mosmann 1983).

NO inhibitory assay

BV2 microglia cells were pretreated for 3 h with different concentrations of tested compounds, and treated 24 h with LPS (1 µg/mL). The nitrite concentration in the medium, an indicator of NO production, was measured with the Griess reaction. Each supernatant (100 µL) was mixed with an equal volume of the Griess reagent (Solution A: 222488; Solution B: S438081, Sigma), and the absorbance of the mixture at 525 nm was determined using an ELISA plate reader (Quang et al. 2018).

PGE_2 determination

The level of PGE_2 present in each sample was determined by using a commercially available kit from R&D Systems (Minneapolis, MN) in accordance with the manufacturer's instructions. The assay was repeated independently three times. Briefly, BV2 microglial cells were cultured in 24-well plates, pre-incubated for 3 h with different concentrations of compounds, and then stimulated for 24 h with LPS. The cell culture supernatants were collected immediately after treatment and spun at 13,000 × *g* for 2 min to remove particulate matter. The medium was added to a 96-well plate pre-coated with affinity-purified polyclonal antibodies specific for PGE_2. An enzyme-linked polyclonal antibody specific for PGE_2 was added to the wells, left to react for 24 h, and washed to remove any unbound antibody-enzyme reagent. After the addition of a substrate solution, the intensity of color produced was measured at 450 nm, which was proportional to the amount of PGE_2 present (Quang et al. 2018).

Western blot analysis

BV2 microglial cells were harvested and pelleted by centrifugation at 16,000 rpm for 15 min. The pelleted cells were washed with phosphate-buffered saline (PBS) and lysed with 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM PMSF, 5 mg/mL aprotinin, 5

mg/mL pepstatin A, and 1 mg/mL chymostatin). The protein concentration was determined by using a Lowry protein assay kit (P5626; Sigma-Aldrich), and an equal amount of protein from each sample was resolved by using 7.5% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto a Hybond™ enhanced chemiluminescence nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% (*w/v*) skim milk and sequentially incubated with the primary antibody (Santa Cruz Biotechnology, CA, USA) and the horseradish peroxidase-conjugated secondary antibody followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The intensities of band signals were quantified densitometrically by using ImageJ software (National Institutes of Health, Bethesda, MD). Molecular weight markers and the internal standards, β-actin and PCNA, were also run on the gel. The analysis was repeated independently three times (Quang et al. 2018).

Statistical analysis

The data are expressed as the mean ± SD of at least three independent experiments. To compare three or more groups, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests was carried out. Data were analysed by using GraphPad Prism software, version 5.01 (GraphPad Software Inc., San Diego, CA, USA).

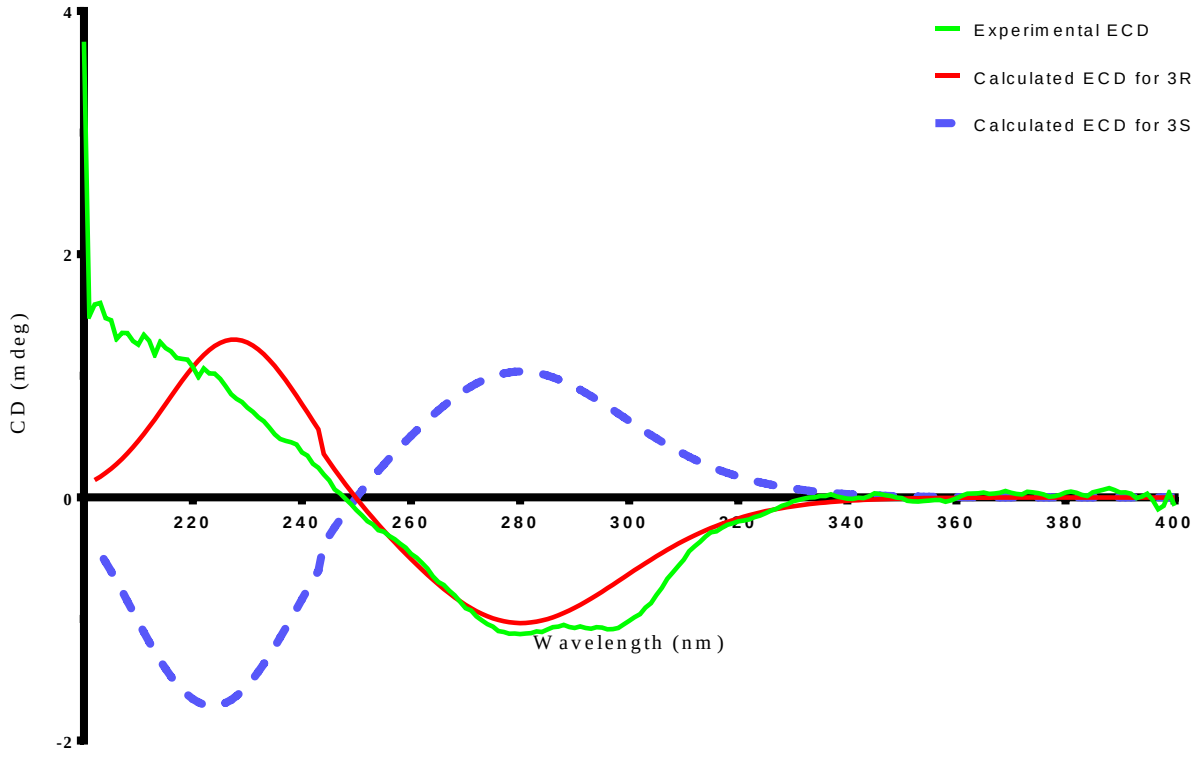


Figure S4. The experimental and calculated ECD spectra for compound **1**

Table S1. Experimental ^{1}H and ^{13}C NMR data of **1** and calculated data for 3*R* and 3*S* stereoisomers

C	**1**				3*R* isomer[e]		3*S* isomer[e]	
	$\delta_{C, exp}$[a,b]	$\delta_{H, exp}$[a,c]	$\delta_{C, exp}$[d,b]	$\delta_{H,exp}$[d,c]	$\delta_{C, cal}$	$\delta_{H, cal}$	$\delta_{C, cal}$	$\delta_{H, cal}$
1	28.2	2.06, s	28.3	2.08, s	27.0	1.85, s	27.0	1.85, s
2	211.5		209.4		206.7		206.7	
3	54.6	3.68, q (7.0)	53.5	3.63, q (7.0)	53.2	3.34, q (6.6)	53.2	3.34, q (6.5)
4	17.3	1.30, d (7.0)	16.9	1.35, d (7.0)	18.1	1.28, d (6.6)	18.1	1.27, d (6.5)
1′	144.3		143.2		143.6		143.7	
2′	107.3	6.18, br s	107.4	6.27, br s	105.0	5.90, s	105.0	5.91, s
3′	160.1		157.4		157.1		157.5	
4′	102.4	6.18, br s	101.8	6.27, br s	99.1	5.90, s	99.1	5.91, s
5′	160.1		157.4		157.4		157.5	
6′	107.3	6.18, br s	107.4	6.27, br s	105.0	5.72, s	105.0	5.72, s

[a]Recorded in CD_3OD, [b]500 MHz, [c]125 MHz, [d]in $CDCl_3$, [e]Quantum-chemical calculations of ^{1}H and ^{13}C NMR chemical shifts for 3S and 3R stereoisomers using Spartan'18 software (Wavefunction Inc., Irvine, CA, USA) (Hehre et al. 2019)

Table S2. Cytotoxicity of compounds **1-6** toward HepG2, MCF7, and SK-Mel2 cancer cells

Compounds	**IC_{50} values (μM)[a]**		
	HepG2	**MCF7**	**SK-Mel2**
1	65.9 ± 7.8	57.7 ± 6.7	96.5 ± 2.4
2	75.3 ± 2.0	48.6 ± 3.0	89.7 ± 2.7
3	> 100	> 100	> 100
4	> 100	> 100	> 100
5	> 100	> 100	> 100
6	> 100	> 100	> 100
Ellipticine[b]	1.8 ± 0.2	1.4 ± 0.1	2.1 ± 0.2

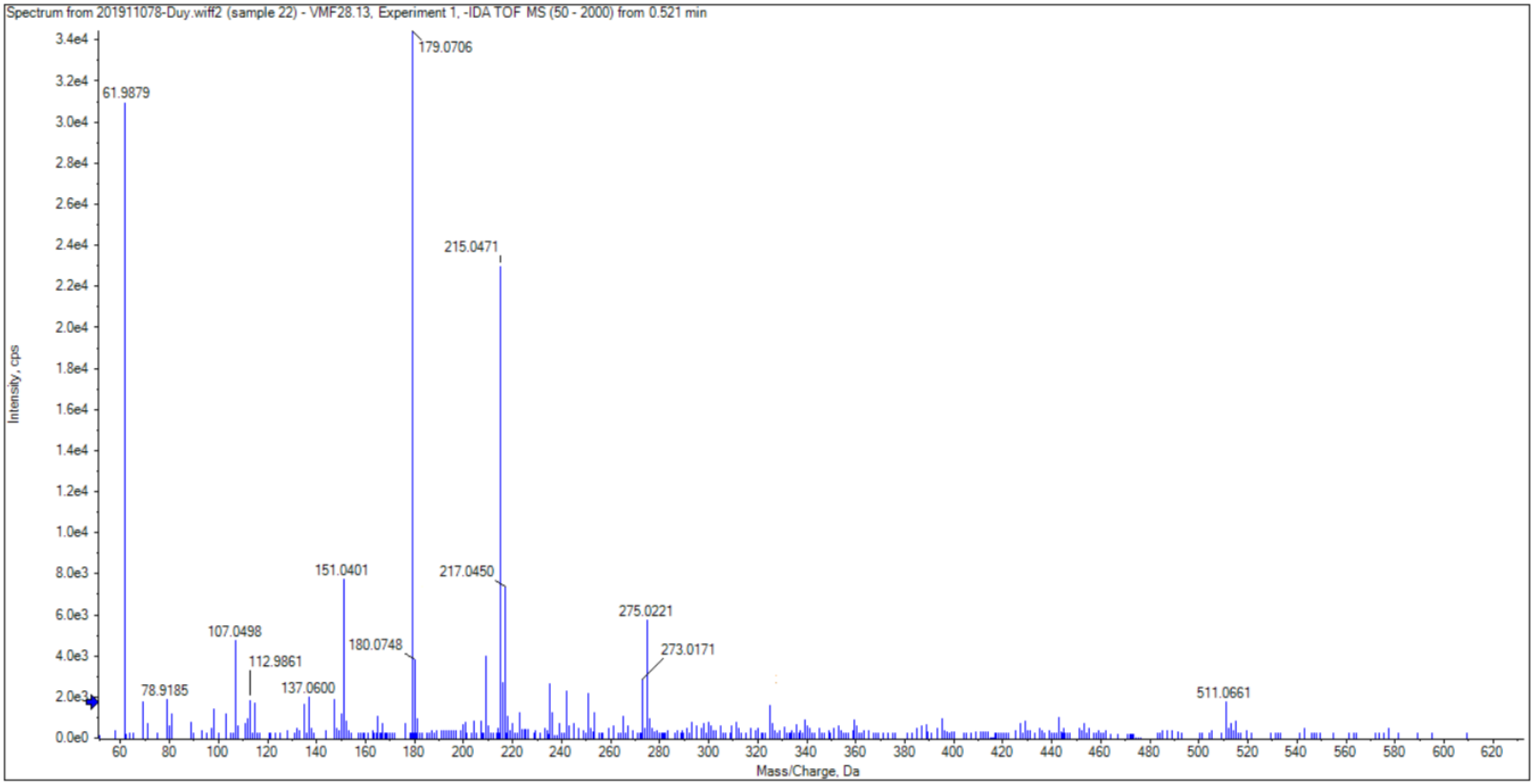
[a] Mean ± SD (n = 3), [b] positive control

Table S3. Nitric oxide inhibitory effects of compounds **1-6**

Compounds	**IC_{50} values (μM)[a]**
1	76.5 ± 3.1
2	24.2 ± 1.2
3	>80 μM
4	37.2 ± 1.9
5	40.6 ± 2.0
6	41.5 ± 2.1
Butein[b]	4.5 ± 0.2

[a] Mean ± SD (n = 3), [b] positive control

7. HRTOFMS and NMR spectra for compound 1



Hit	Formula	m/z	RDB	ppm	MS Rank	MSMS ppm	MSMS Rank	Found
1	C10H12O3	179.07137	10.0	-3.8	1			NA/NA

Figure S5. HR-IDA-TOF mass spectrum of compound **1**

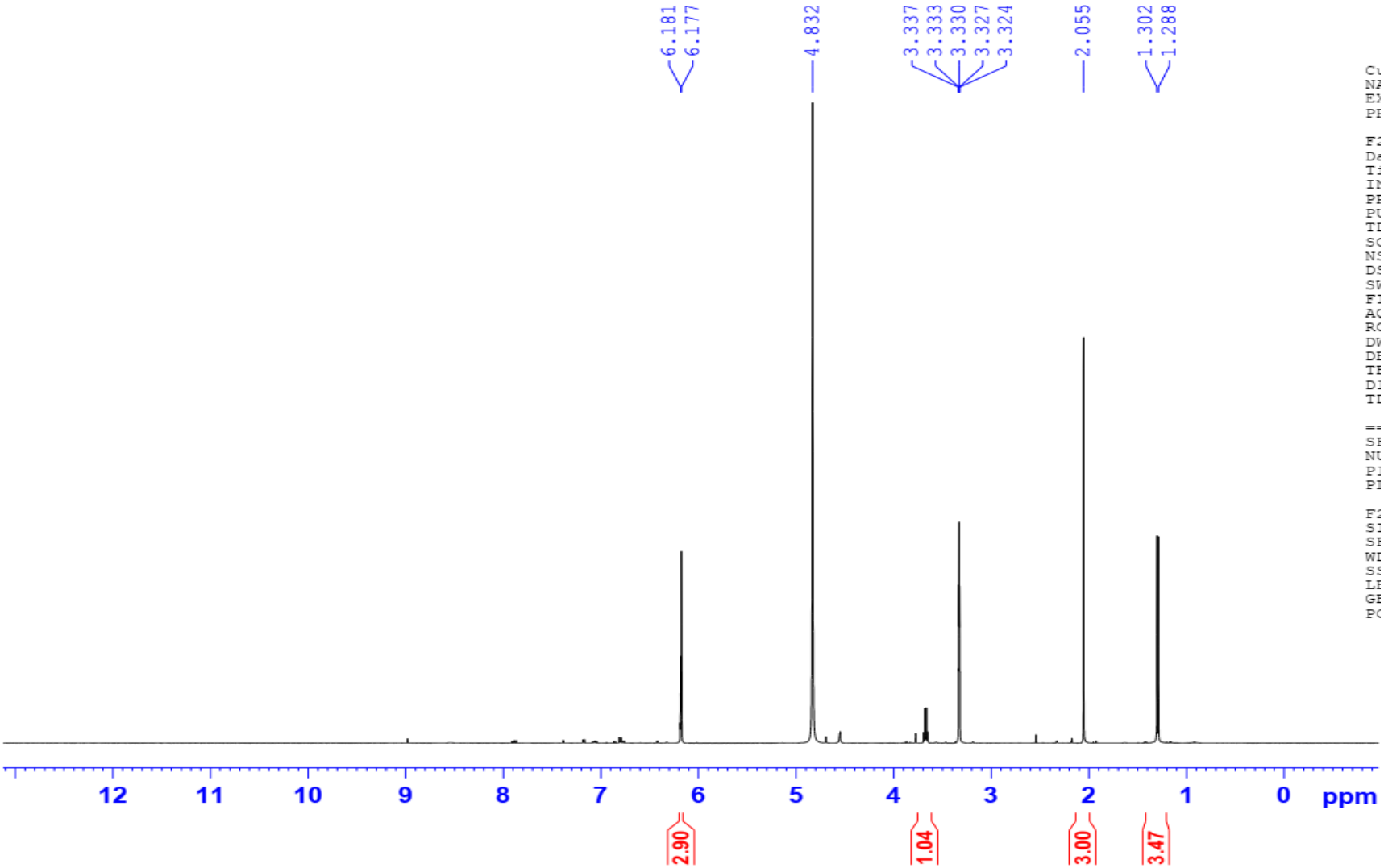


Figure S6. ^{1}H NMR (CD_3OD, 500 MHz) spectrum of compound **1**

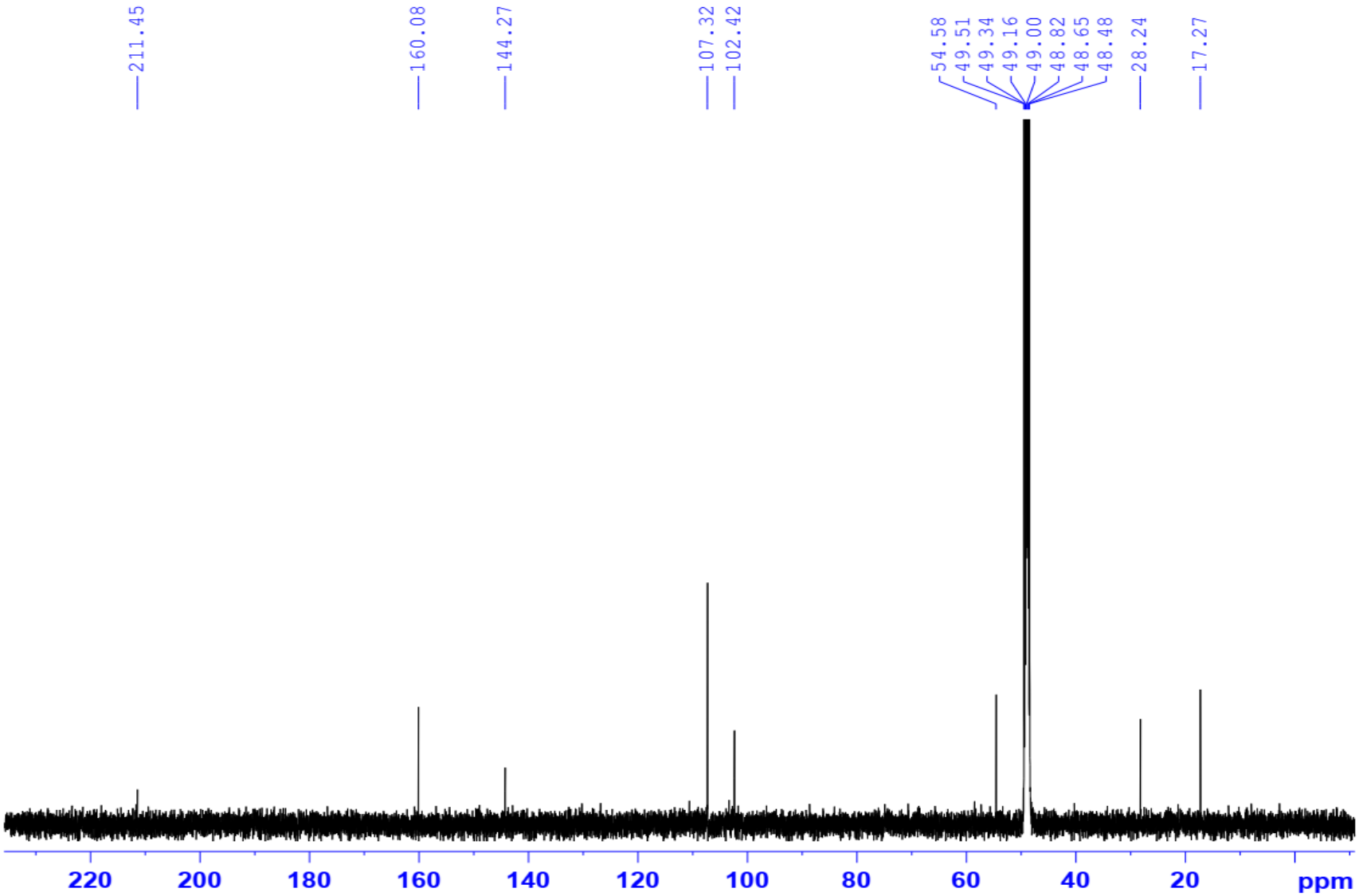


Figure S7. ^{13}C NMR (CD_3OD, 125 MHz) spectrum of compound **1**

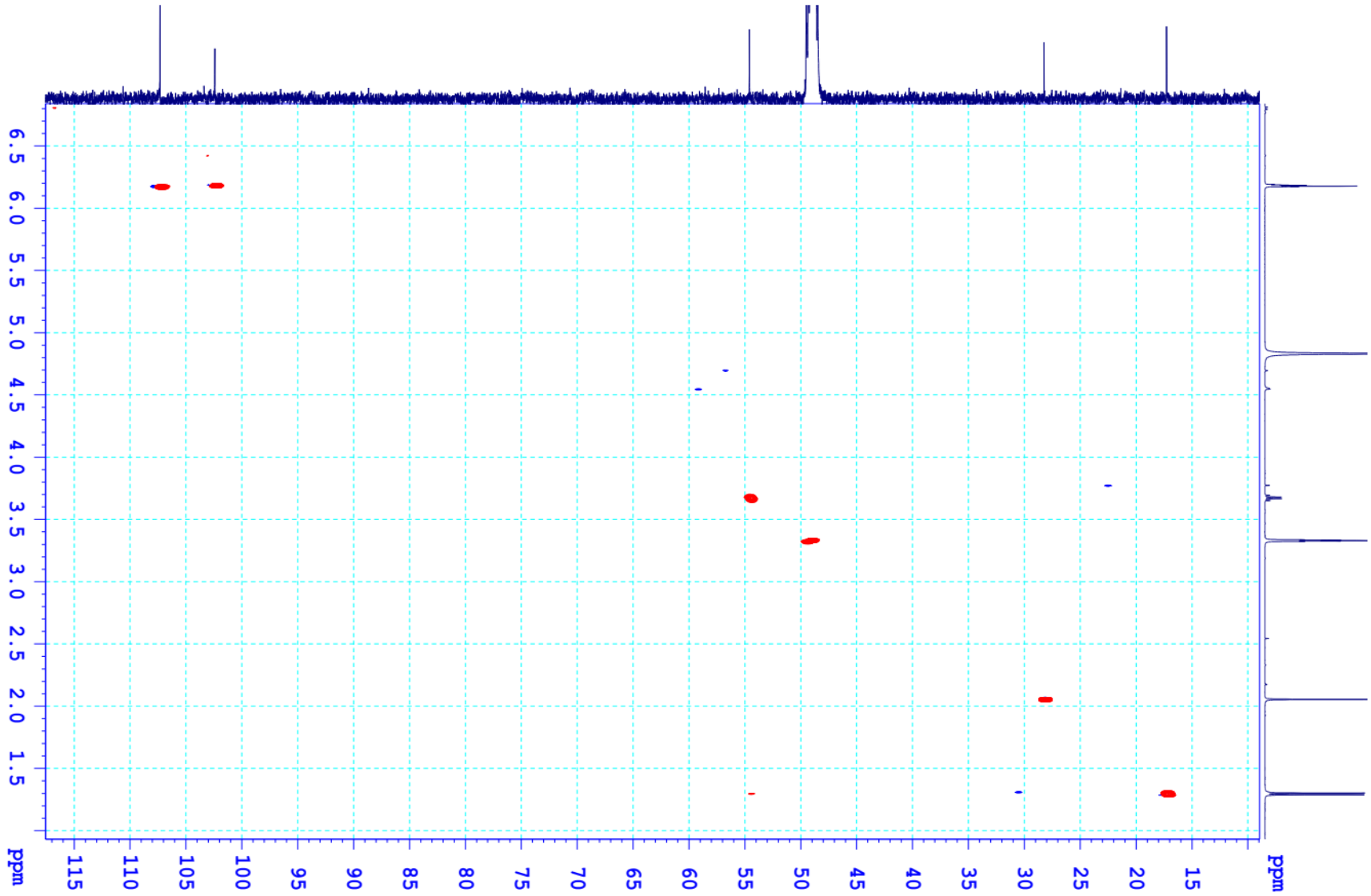


Figure S8. HSQC (CD_3OD, 500 MHz) spectrum of compound **1**

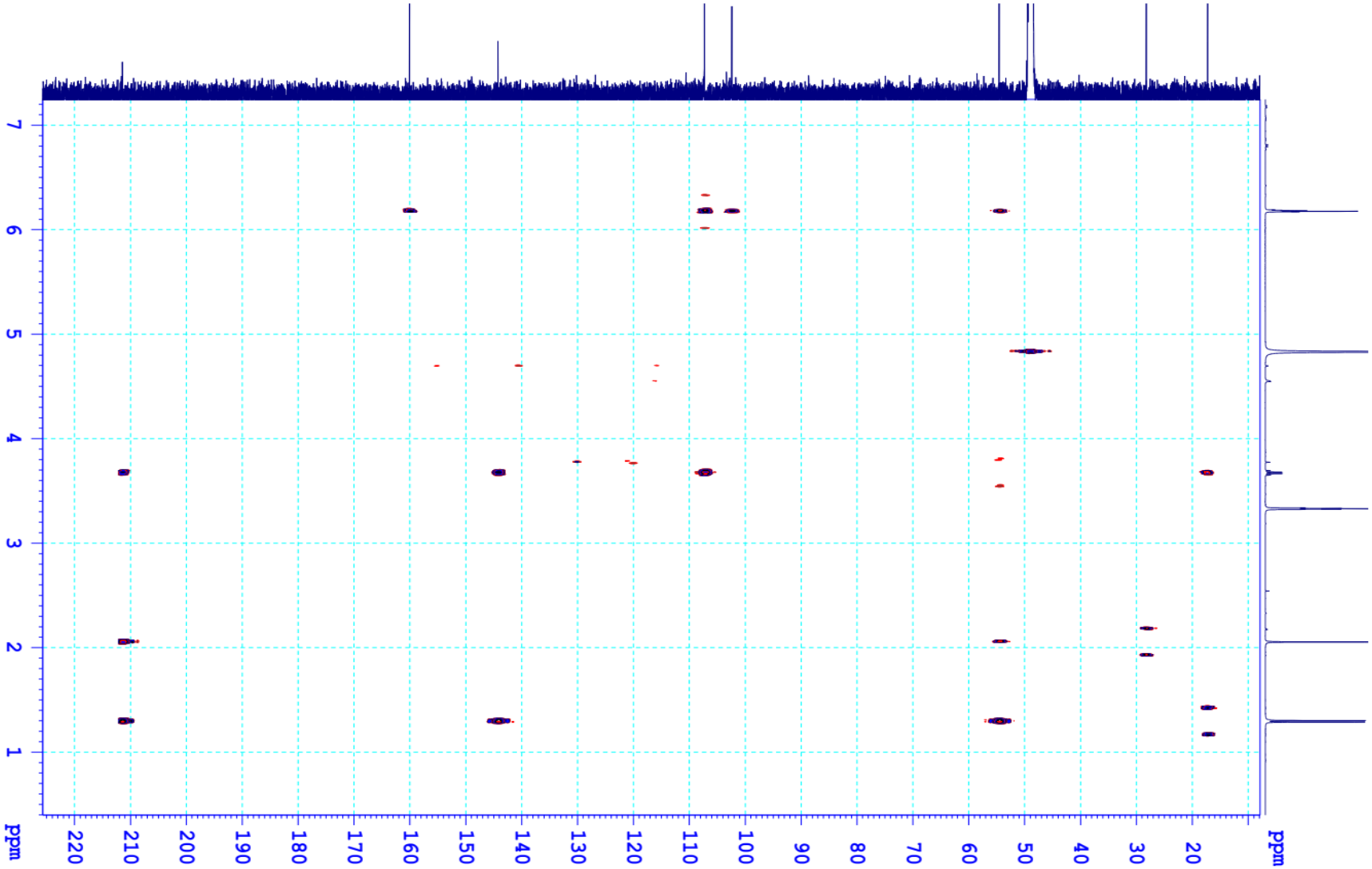


Figure S9. HMBC (CD_3OD, 500 MHz) spectrum of compound **1**

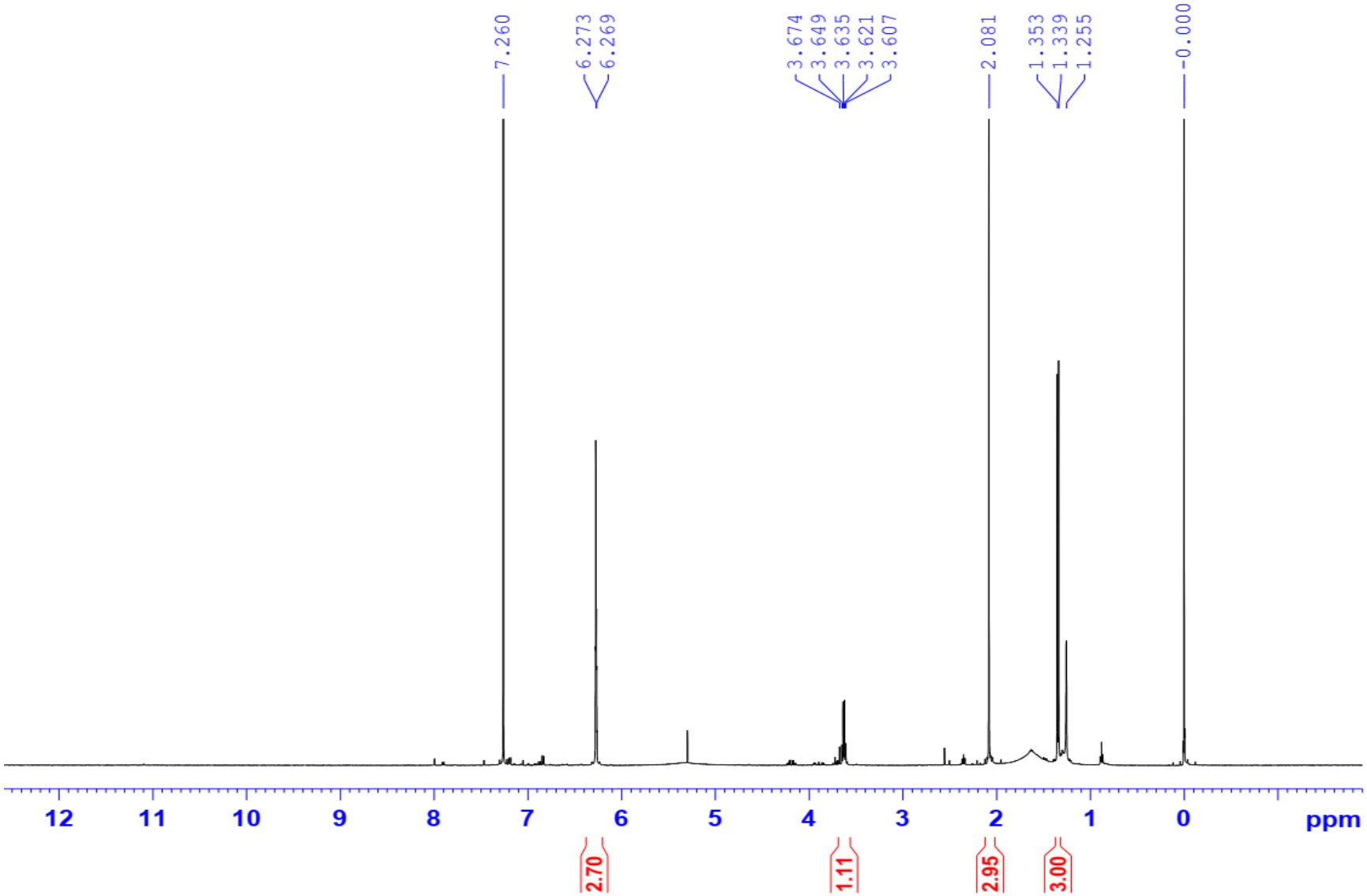


Figure S10. ^{1}H NMR ($CDCl_3$, 500 MHz) spectrum of compound **1**

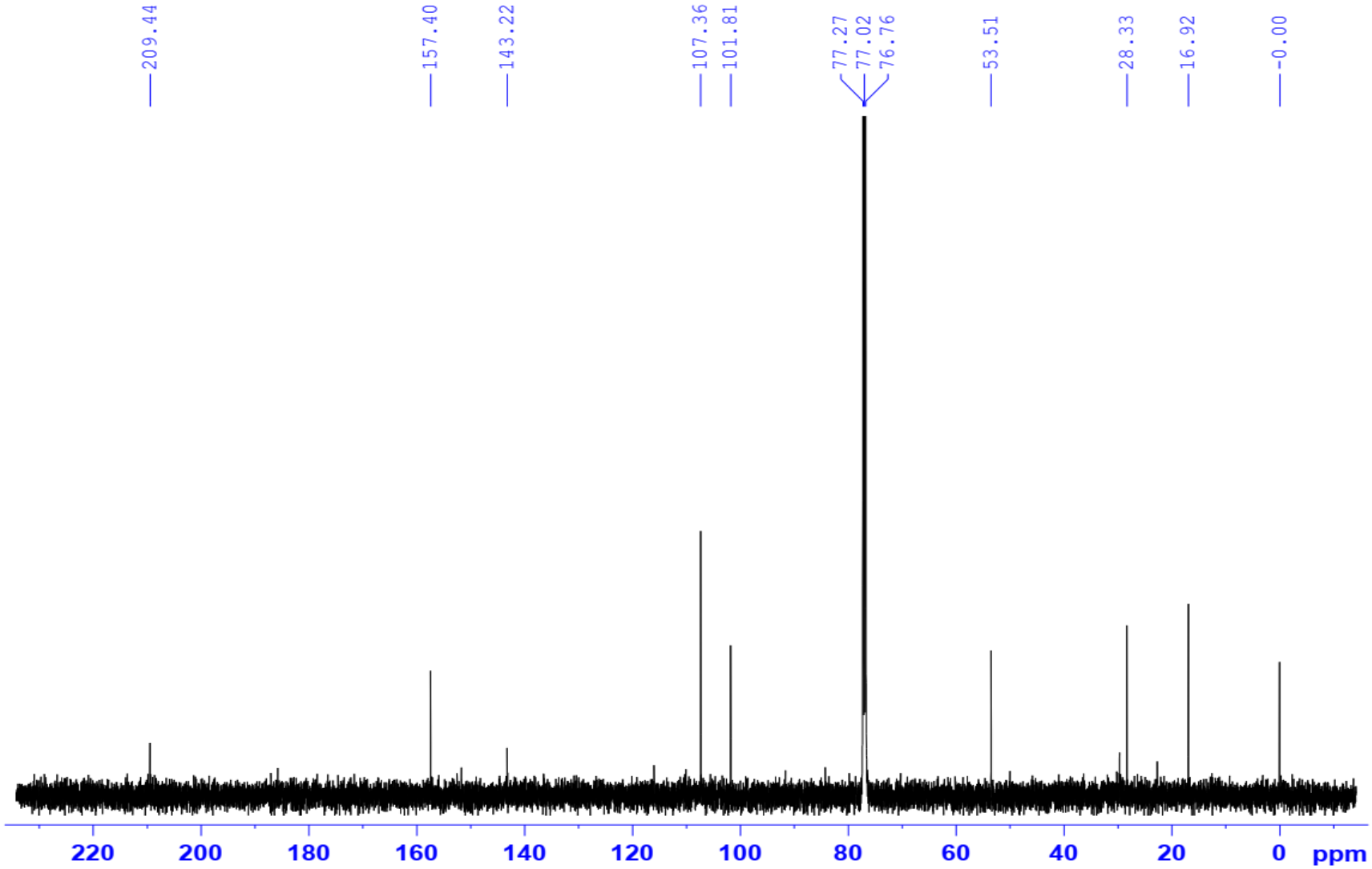


Figure S11. ^{13}C NMR ($CDCl_3$, 125 MHz) spectrum of compound **1**

References

Hehre W, Klunzinger P, Deppmeier B, Driessen A, Uchida N, Hashimoto M, Fukushi E, Takata Y. 2019. Efficient Protocol for Accurately Calculating 13C Chemical Shifts of Conformationally Flexible Natural Products: Scope, Assessment, and Limitations. J Nat Prod. 82(8):2299-2306.

Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, et al. 1991. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst. 83(11):757-766.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65(1-2):55-63.

Quang TH, Cong PT, Yen DTH, Nhiem NX, Tai BH, Yen PH, Ngan NTT, Kim DC, Kim YC, Oh H, et al. 2018. Triterpenoid saponins and phenylpropanoid glycosides from the roots of *Polygala japonica* Houtt. with anti-inflammatory activity. Phytochem Lett. 24:60-66.