SUPPLEMENTARY MATERIAL

Flavonoids with antioxidant and tyrosinase inhibitory activity from Corn Silk (*Stigma Maydis*)

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Abstract: Corn silk (*Stigma Maydis*), being the styles and stigmas of maize, is a famous traditional medicine and functional tea in China. Research into the chemical composition of corn silk led to the identification of an unreported flavone (**1**, silkone A), accompanying with three known flavonoids (**2-4**). And their structures were elucidated through comprehensive spectroscopic analysis. Each obtained compound was evaluated for antioxidant capacity by DPPH, ABTS and FRAP assays. As a result, all tested compounds exhibited stronger radicals scavenging activities than Trolox in ABTS radical assay and displayed relatively weak antioxidant capacity in the other two experiments. Tyrosinase inhibitory activities of compounds **1-4** were also investigated, and compound **3** and **4** demonstrated moderate inhibitory activities to tyrosinase with IC_{50} values of 0.49 and 0.21 mM, respectively, which was further investigated through molecular docking calculation. These results may contribute to the development of new antioxidants and tyrosinase inhibitors from corn silk.

Keywords: corn silk, flavonoid, antioxidant activity, tyrosinase inhibitory activity

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1. Experimental

1.1 General Experimental Procedures

UV spectra were acquired by a Shimadzu UV-1700 spectrometer (Shimadzu, Tokyo, Japan). HRESIMS data were determined on a X500-Q-TOF mass spectrometer (SCIEX, Shanghai, China). ECD spectra were detected via a Bio-Logic Science MOS-450 spectrometer (Bio-Logic Science Instruments, France). The 1D and 2D NMR spectra were obtained using Bruker ARX-400 and AV-600 spectrometers (Bruker Corporation, Bremen, Germany) with chemical shifts being expressed by δ values (ppm) (measured in DMSO-d_6). The absorbances were measured using a Varioskan Flash (Thermo Fisher Scientific Rockford, LC, America). Column chromatography was applied on ODS gel (60-80 μm, Merck, Germany), and silica gel (200-300 and 100-200 mesh, Qingdao Hailang Silica gel desiccant Co., LTD, Qingdao, China) successively. Thin-layer chromatography (TLC) systems were performed on silica gel GF254 plates (Qingdao Marine Chemical, Ltd., Qingdao, China). Semi-preparative HPLC separations were carried out on a YMC Pack ODS-A column (250 × 10 mm, 5 μm, YMC Company, Kyoto, Japan), which is equipped with Shimadzu LC-20 pump system and an SPD-2 0A UV/VIS detector (Shimadzu, Kyoto, Japan). HRESIMS analysis were performed on a X500-Q-TOF mass spectrometer (SCIEX, Shanghai, China). DPPH was acquired from Macklin Biochemical Co., Ltd (Shanghai, China). ABTS and TPTZ was gained from Aladdin Industrial Corporation (Shanghai, China). Tyrosinase was got from Duly Biotech Co., Ltd (Nanjing, China).

1.2 Plant material

Corn silk was collected in March 2018 from Huaibei, Anhui Province, People's Republic of China, and was identified by Prof. Jin-Cai Lu of College of Traditional Chinese Medicine, Shenyang Pharmaceutical University. A voucher specimen (NO. 20160607) was deposited in the herbarium of the Department of Natural Products Chemistry, Shenyang Pharmaceutical University.

1.3 Extraction and isolation

Corn silk, the dried styles and stigmas of maize (100 kg) was refluxed with 75% EtOH for 3 times × 50L × 4 h. The solvent was removed under reduced pressure. After being suspended in H_2O, the crude extract (1.7 kg) was extracted with EtOAc and n-BuOH successively. Then, the EtOAc (350 g) and n-BuOH extracts (864 g) were fractionated over silica gel, which was eluted with CH_2Cl_2-MeOH (50:1 to 1:1) to yield six fractions (Fr. A to Fr. F). Then, Fr.B (150 g) was divided into three parts (Fr. 1 to Fr. 3) over HP-20 macro-porous resin using EtOH-H_2O (30% to 90%) as eluents. Then, Fr. B3 (38 g) was chromatographed on a silica gel column using EtOH/H_2O (70% to 90%) as mobile phase to afford two fractions (Fr. B2-1 to Fr. B2-2). Fr. B2-1 was further separated using silica gel, HPLC and semipreparative HPLC, to yield compound **1** (20.5 mg) and compound **2** (10.0 mg). Fr. A (27.6 g) was purified into six fractions (Fr. A1 to Fr. A6) over silica gel using PE-EA (100:0 to 1:1) and CH_2Cl_2-MeOH (10:1 to 1:1) as gradient eluents. Then, Fr. A5 (1.9 g) was separated over silica gel, HPLC and semipreparative HPLC to yield compound **3** (2.0 mg). Fr. A1 (6.4 g) was chromatographed over silica gel, HPLC and semipreparative HPLC to yield compound **4** (38.2 mg).

1.4 Physical and spectroscopic data of isolated compounds

silkone A (**1**): yellow powder, UV (MeOH) λmax (log ε): 344 (2.29), 272 (1.73); ^{1}H NMR (600 MHz, DMSO-d_6): δ_H 6.90 (1H, s, H-3), 6.56 (1H, s, H-8), 7.56 (1H, o, H-2'), 6.94 (1H, d, J = 8.1 Hz, H-5'), 7.56 (1H, o, H-6'), 3.55 (2H, s, H-1''), 3.89 (3H, s, 3'-OCH_3), 3.60 (3H, s, 2''-OCH_3), 13.32 (1H, s, 5-OH); ^{13}C NMR (150 MHz, DMSO-d_6): δ_C 163.6 (C-2), 103.1 (C-3), 181.1 (C-4), 158.7 (C-5), 104.9 (C-6), 162.6 (C-7), 93.3 (C-8), 155.9 (C- 9), 103.2 (C-10), 121.5 (C-1'), 110.2 (C-2'), 148.0 (C-3'), 150.8 (C-4'), 115.8 (C-5'), 120.4 (C-6'), 27.4 (C-1''), 171.2 (C-2''); HRESIMS (*m/z*): $[M + H]^+$ 373.0919 (calcd for $C_{19}H_{17}O_8^+$, 373.0918).

alternanthin (**2**): yellow powder, ^{1}H NMR (600 MHz, DMSO-d_6): δ_H 7.56 (1H, m, H-2'), 7.56 (1H, m, H-6'), 6.93 (1H, d, J = 8.9 Hz, H-5'), 6.89 (1H, s, H-8), 6.56 (1H, s, H-3), 5.33 (1H, d, J = 2.9 Hz, H-1''), 4.03 (1H, m, H-5''), 3.89 (3H, s, 3'-OCH_3), 3.85

(1H, q, *J* = 3.5 Hz, H-3"), 3.24 (1H, d, *J* = 3.5 Hz, H-4"), 2.24 (1H, t, *J* = 13.5 Hz, H-2e"), 1.48 (1H, dt, *J* = 13.5, 2.9 Hz, H-2a"), 1.15 (3H, d, *J* = 6.6 Hz, H-6"). ^{13}C NMR (150 MHz, DMSO-d_6): δ_C 182.0 (C-4), 163.7 (C-2), 162.9 (C-7), 157.4 (C-5), 156.2 (C-9), 151.0 (C-3'), 148.1 (C-4'), 121.3 (C-1'), 120.4 (C-6'), 115.8 (C-5'), 110.5 (C-6), 110.1 (C-2'), 103.3 (C-3), 103.0 (C-10), 94.8 (C-8), 70.6 (C-1"), 68.8 (C-4"), 67.4 (C-3"), 66.6 (C-5"), 55.9 (3'-OCH_3), 31.4 (C-2"), 17.2 (C-6").

[(2*S*)-7,4'-dihydroxy-3'-prenylflavan] (**3**): yellow oil, ECD (MeOH) 201 (Δε -7.75), 209 (Δε +4.77), 217 (Δε -7.87), 224 (Δε +0.05), 230 (Δε -3.16), 284 (Δε -1.92); ^{1}H NMR (600 MHz, DMSO-d_6): δ_H 7.04 (1H, d, *J* = 2.3 Hz, H-2'), 7.02 (1H, dd, *J* = 8.2, 2.3 Hz, H-6'), 6.84 (1H, d, *J* = 8.2 Hz, H-5'), 6.77 (1H, d, *J* = 8.2 Hz, H-5), 6.27 (1H, dd, *J* = 8.2, 2.4 Hz, H-6), 6.16 (1H, d, *J* = 2.4 Hz, H-8), 5.27 (1H, t, *J* = 7.4 Hz, H-2"), 4.86 (1H, dd, *J* = 10.2, 2.3 Hz, H-2), 3.21 (2H, d, *J* = 7.4 Hz, H-1"), 2.78 (1H, m, H-4a), 2.58 (1H, m, H-4b), 2.01 (1H, m, H-3a), 1.91 (1H, m, H-3b), 1.68 (3H, s, H3-5"), 1.66 (3H, s, H3-4"). ^{13}C NMR (150 MHz, DMSO-d_6): δ_C 156.4 (C-7), 155.5 (C-9), 154.5 (C-4'), 131.8 (C-3"), 131.2 (C-1'), 129.8 (C-5), 127.4 (C-2'), 127.2 (C-3'), 124.7 (C-6'), 122.8 (C-2"), 114.6 (C-5'), 112.2 (C-10), 107.9 (C-6), 102.7 (C-8), 77.0 (C-2), 29.3 (C-4), 28.1 (C-3), 25.5 (C-1"), 23.9 (C-5"), 17.7 (C-4").

[(2*S*)-7,3'-dihydroxy-4'-methoxy-flavan] (**4**): yellow oil, ECD (MeOH) 205 (Δε -3.29), 217 (Δε +11.94), 228 (Δε +2.69), 234 (Δε +4.83), 246 (Δε -3.02), 275 (Δε +2.89), 290 (Δε -11.42); ^{1}H NMR (600 MHz, DMSO-d_6): δ_H 6.89 (1H, d, *J* = 8.2 Hz, H-5'), 6.84 (1H, d, *J* = 8.2 Hz, H-5), 6.82 (1H, d, *J* = 2.1 Hz, H-2'), 6.77 (1H, dd, *J* = 8.2, 2.1 Hz, H-6'), 6.27 (1H, dd, *J* = 8.2, 2.4 Hz, H-6), 6.18 (1H, d, *J* = 2.4 Hz, H-8), 4.89 (1H, dd, *J* = 9.9, 2.3 Hz, H-2), 3.75 (3H, s, 4'-OCH_3), 2.78 (1H, m, H-4a), 2.58 (1H, m, H-4b), 2.03 (1H, m, H-3a), 1,88 (1H, m, H-3b). ^{13}C NMR (150 MHz, DMSO-d_6): δ_C 156.5 (C-9), 155.4 (C-7), 147.1 (C-4'), 146.4 (C-3'), 134.3 (C-1'), 129.8 (C-5), 116.8 (C-6'), 113.4 (C-10), 112.2 (C-5'), 112.0 (C-2'), 107.9 (C-6), 102.7 (C-8), 76.6 (C-2), 55.7 (4'-OCH_3), 29.4 (C-4), 23.7 (C-3).

1.5 Antioxidant assays

1.5.1 DPPH assay

The test compounds and the positive control Trolox were solubilized in absolute ethanol to prepare solutions of different concentrations. DPPH was solubilized in absolute ethanol and diluted to make its absorbance around 0.7 at 517 nm. 100 μL ethanol solution of DPPH and 100 μL ethanol solution of various concentrations of tested compounds were added to a clean 96-well plate and incubated for 30 minutes in the dark at room temperature. After being mixed well, the absorbances were measured using a microplate reader at 517 nm.

The scavenging rates of tested compounds against DPPH free radicals were calculated using the formula listed below, and then SPSS data analysis software (Graphpad prism 8.0.1) was used to obtain IC_{50} values:

DPPH inhibition rate (%) = (1- (S-SB)/(C-CB)) ×100%

(S: absorbance of sample, SB: absorbance of sample blank, C: absorbance of negative control, CB: absorbance of blank control)

1.5.2 ABTS assay

The test compounds and the positive control Trolox were dissolved in absolute ethanol to prepare solutions of different concentrations. Then, potassium persulfate solution (2.5 mmol/L) and ABTS PBS solution (7.0 mmol/L) were mixed with a volume ratio of 1:1. The $ABTS^{\bullet+}$ solution was generated after the mixture being placed in the dark for 12 to16 hours. Then, diluting the $ABTS^{\bullet+}$ solution with PBS to make its absorbance around 0.7 at 734 nm. It could be used after being incubated at room temperature, away from light for 30 minutes. 150 μL $ABTS^{\bullet+}$ solution and 100 μL ethanol solution of various concentrations of tested compounds were added to a clean 96-well plate and incubated in the dark for 30 minutes. After being mixed well, the absorbances were measured using a microplate reader at 734 nm.

The scavenging rates of tested compounds to ABTS free radicals were calculated using the formula listed in part 2.4.1, and then SPSS data analysis software (Graphpad prism 8.0.1) was used to obtain the IC_{50} values.

1.5.3 FRAP assay

The sodium acetate buffer (0.3mol/L, PH=3.6), TPTZ hydrochloric acid solution (40mmol/L), Fecl3 solution (20mmol/L) were mixed uniformly in a volume ratio of 10:1:1 to make FRAP working solution. Precisely weighed $FeSO_4 \cdot 7H_2O$ was dissolved with distilled water to make a solution of 100 mM, and then diluted it with distilled water to various concentrations. $FeSO_4 \cdot 7H_2O$ solution of different concentrations, sodium acetate buffer and TPTZ hydrochloric acid solution were mixed in a volume ratio of 10:1:1 uniformly. After the mixture being incubated for 30 minutes at 37°C, the absorbances were acquired at 593 nm by a microplate reader. Then, the standard curve was obtained by taking absorbances as abscissa and concentrations of $FeSO_4 \cdot 7H_2O$ solutions as the ordinate. 145 μL of FRAP working solution and 5 μL ethanol solution of various concentrations of tested compounds were added to a clean 96-well plate and incubated for 30 minutes at 37°C. After being mixed well, absorbance of each sample was acquired at 593 nm with a microplate reader. Then the obtained absorbances were inserted into the standard curve and the FRAP (mM Fe^{2+}/ mM of sample) values were calculated.

1.6 Tyrosinase inhibition activity

The test compounds and the positive control Arbutin were dissolved in 20% methanol aqueous solution to make solutions of different concentrations. L-tyrosine was dissolved with PBS to prepare a solution of 0.1 mg/ml. And tyrosinase was dissolved with PBS to make a solution of 0.2 mg/ml (100U/ml). 40 μL L-tyrosine PBS solution, 40 μL 20% methanol aqueous solutions of different concentrations of tested compounds and arbutin, 80 μL PBS and 40 μL tyrosinase PBS solution were added to a clean 96-well plate, and the absorbances were measured using a microplate reader at 492 nm. After being incubated at 37℃ for 30 minutes, the absorbances were acquired using a microplate reader at 492 nm again. The inhibition rates of tested compounds to tyrosinase were calculated using the formula listed below, and then SPSS data analysis software (Graphpad prism 8.0) was used to obtain IC_{50} values.

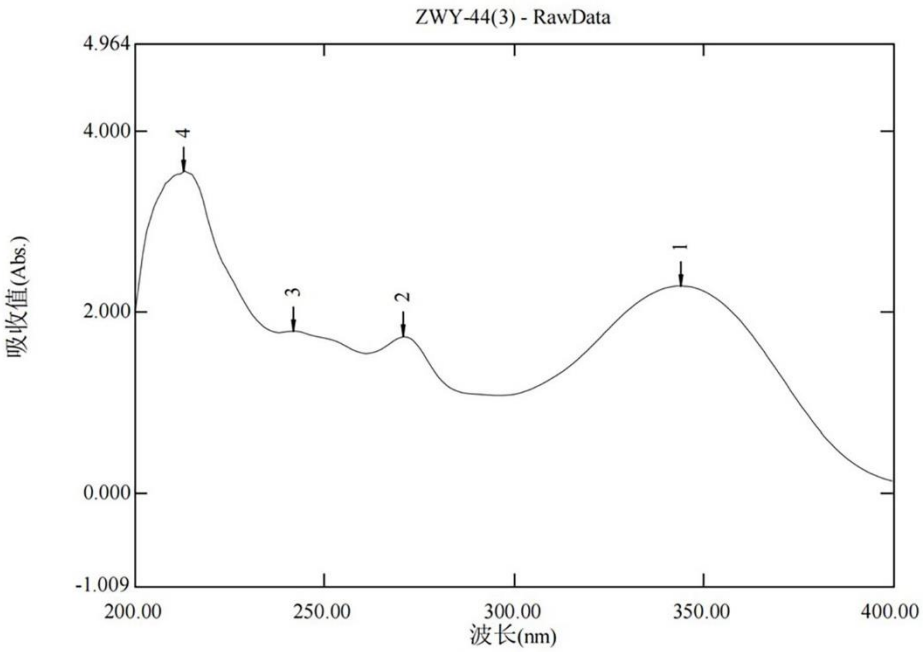
Tyrosinase inhibition rate (%) = [(A-B)- (C-D)]/(A-B) ×100%

(A: the absorbance of blank after incubation, B: the absorbance of blank before incubation, C: the absorbance of sample after incubation, D: the absorbance of sample before incubation)

1.7 Molecular docking calculation

Molecular docking calculation was performed on Schrödinger software suite. The crystal structure of tyrosinase in complex with Tropolone (PDB ID: 2Y9X) was obtained from the Protein Data Bank (http://www.rcsb.org/pdb). The protein file was preprocessed on the protein preparation wizard module of Schrödinger suite. Receptor grid was generated by using Glide, and the docking pocket was defined by the atomic coordinates of the native ligand (-11.114, -20.782, -47.518). Finally, the results of molecular docking were visualized by Discovery Studio 2020 Client.

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



阈值: 0.001000
点数: 4

No.	P/V	波长(nm)	吸收值(Abs.)	说明
1	↑	344.0	2.292	
2	↑	271.0	1.730	
3	↑	242.0	1.793	
4	↑	213.0	3.562	

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

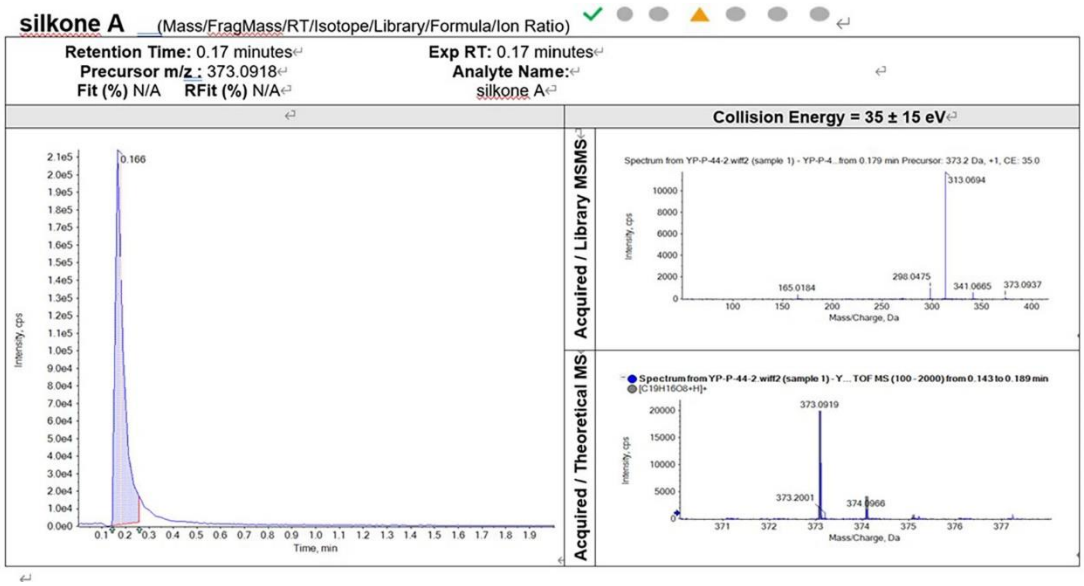


Table S1[1]H (600 MHz) and [13]C (150 MHz) data for compound **1** (DMSO-d_6)

No.	δ_H	δ_C	No.	δ_H	δ_C
2		163.6	2'	7.56 (1H, o)	110.2
3	6.90 (1H, s)	103.1	3'		148.0
4		181.8	4'		150.8
5		158.7	5'	6.94 (1H, dd, J = 8.1, 2.2 Hz)	115.8
6		104.9	6'	7.56 (1H, o)	120.4
7		162.6	1"	3.55 (2H, s)	27.4
8	6.56 (1H, s)	93.3	2"		171.2
9		155.9	5-OH	13.32 (1H, s)	
10		103.2	3'-OCH_3	3.89 (3H, s)	56.0
1'		121.5	2"-OCH_3	3.60 (3H, s)	51.6

Figure S3. [1]H NMR spectrum (600 MHz, DMSO-d_6) of compound **1**.

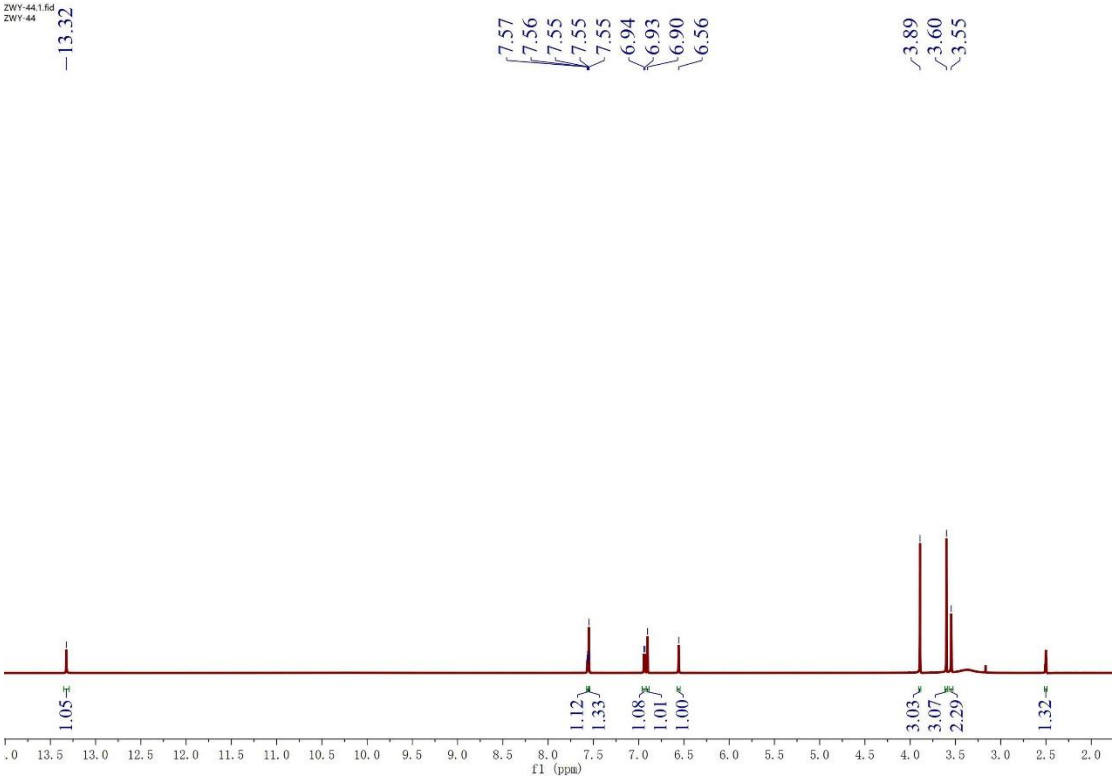


Figure S4. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of compound **1**.

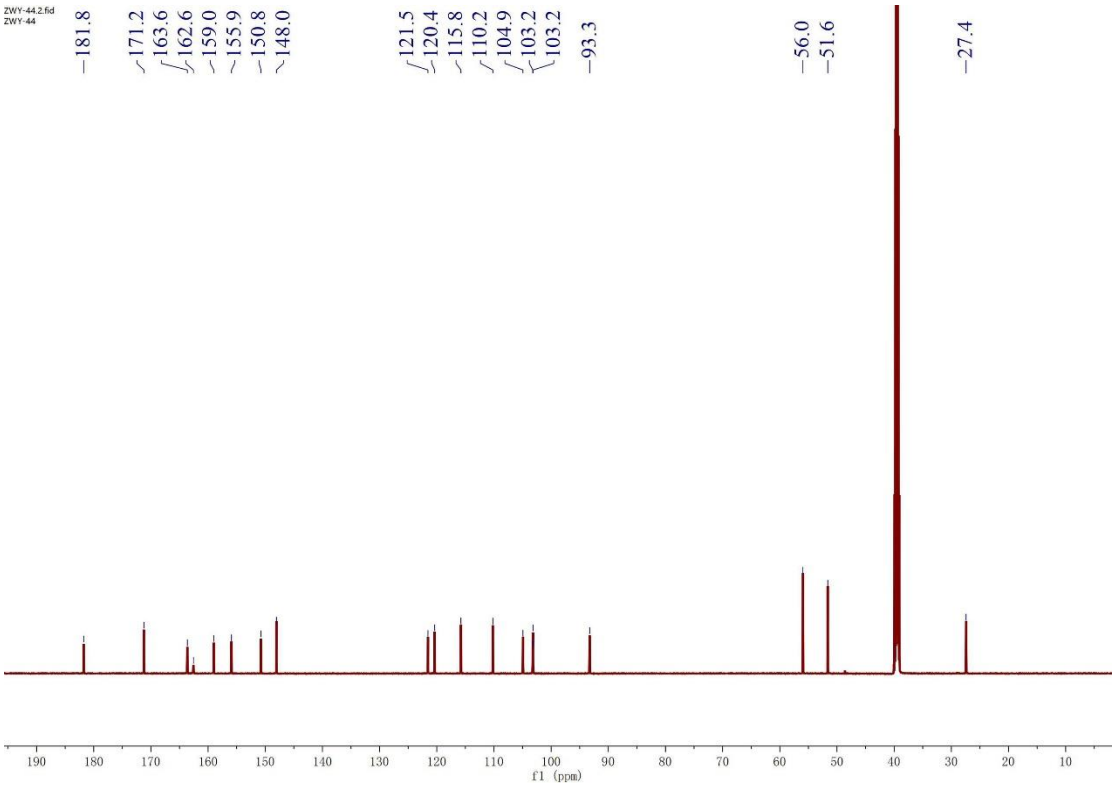


Figure S5. Key HMBC correlations of compound **1**.

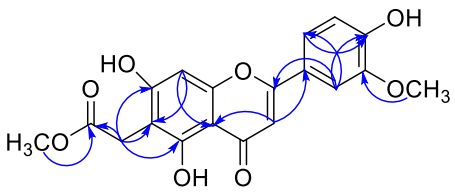


Figure S6. HSQC spectrum (600 MHz, DMSO-d_6) of compound **1**.

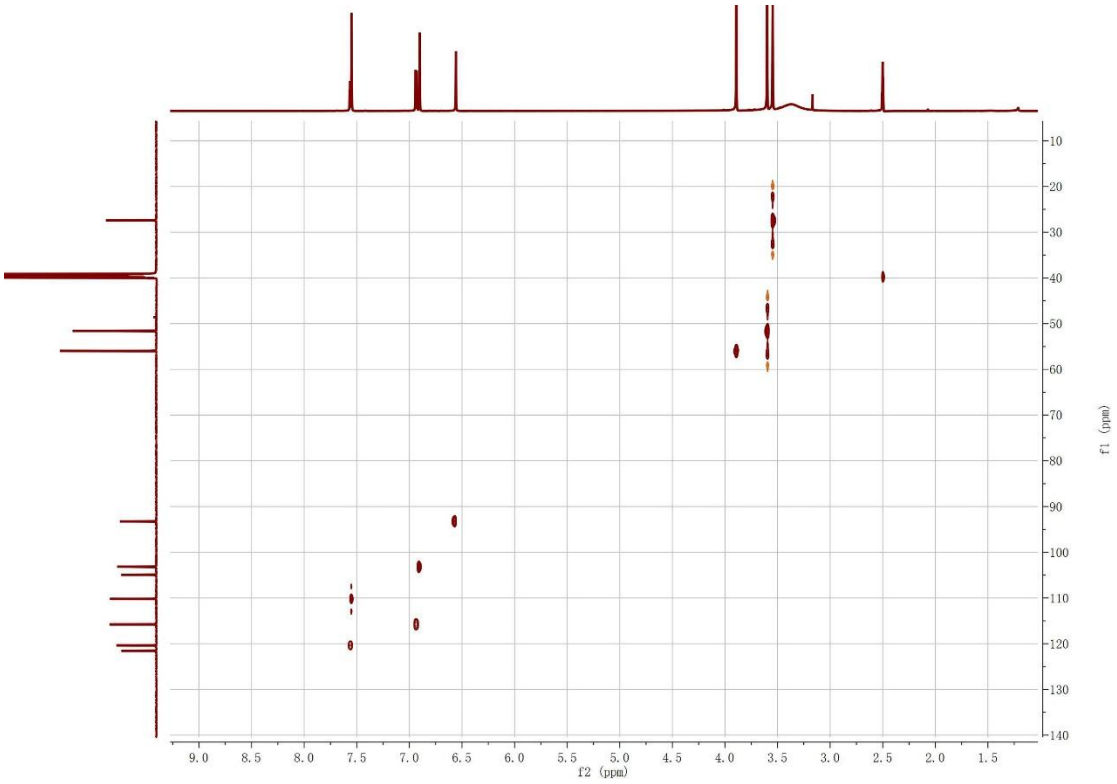


Figure S7. HMBC spectrum (600 MHz, DMSO-d_6) of compound **1**.

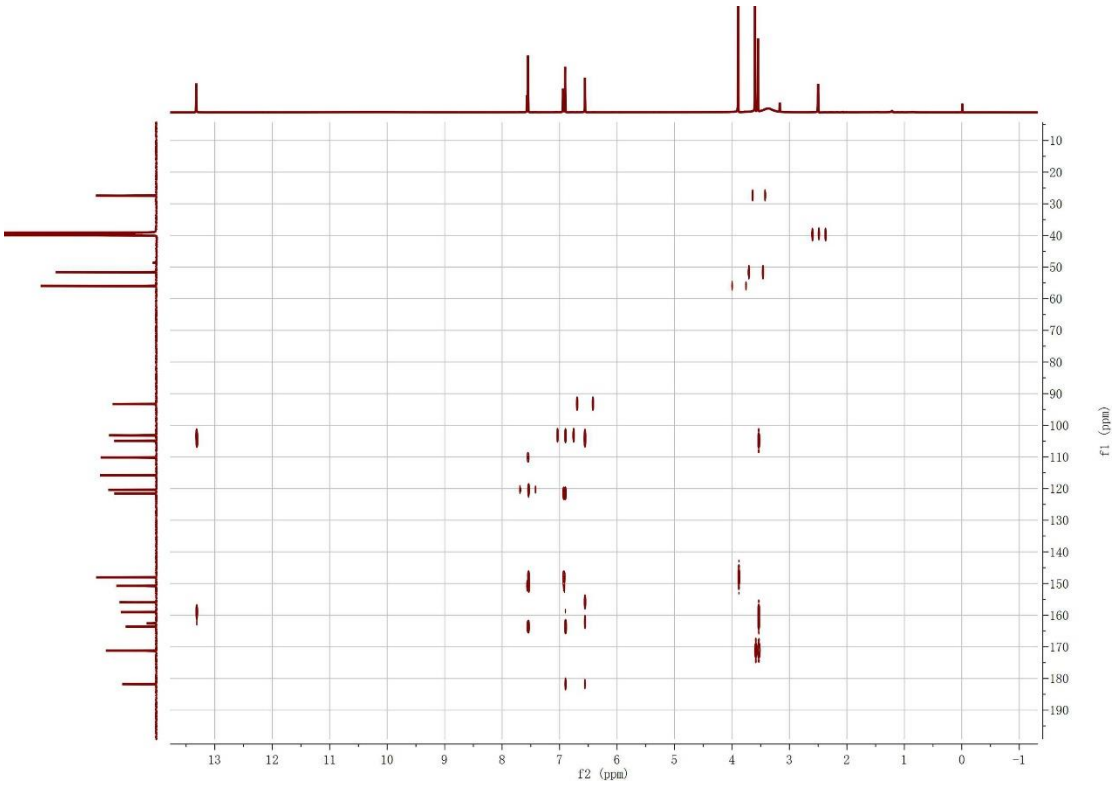


Figure S8. Antioxidant activity of compound **1-4**. All data are presented as the means ± SD of three independent experiments. Trolox was used as a positive control.

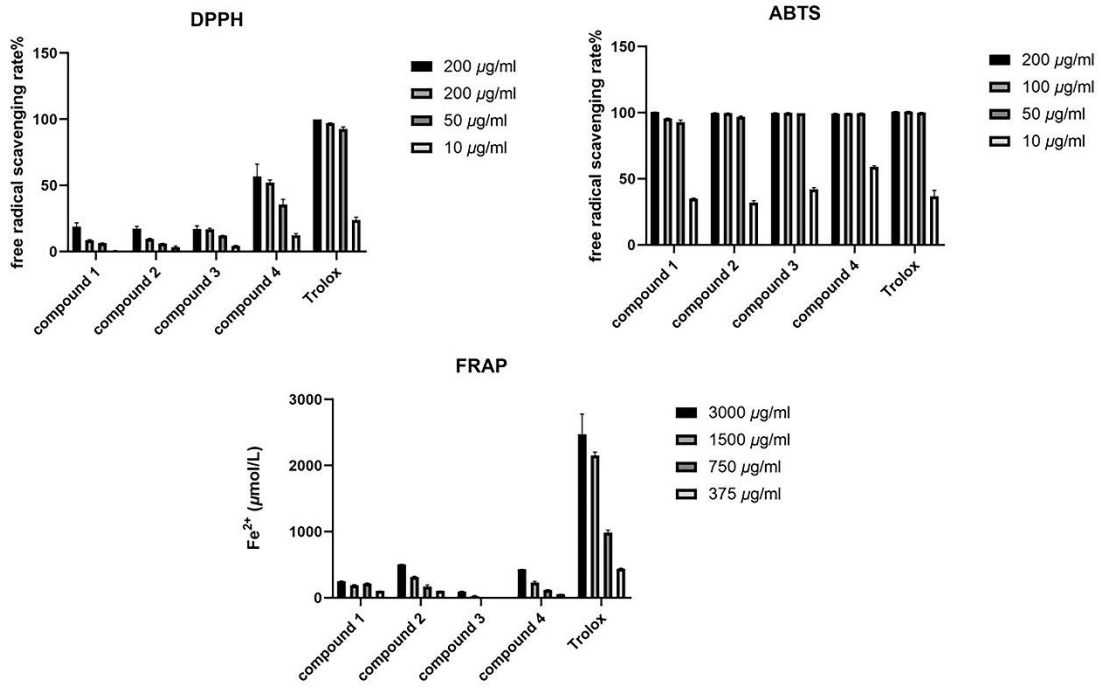


Figure S9. Tyrosinase inhibitory effects of compound **1-4**. All data are presented as the means ± SD of three independent experiments. Arbutin was used as a positive control.

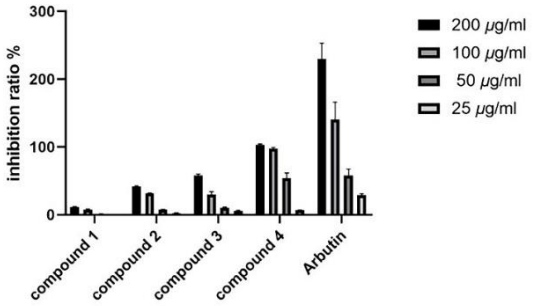
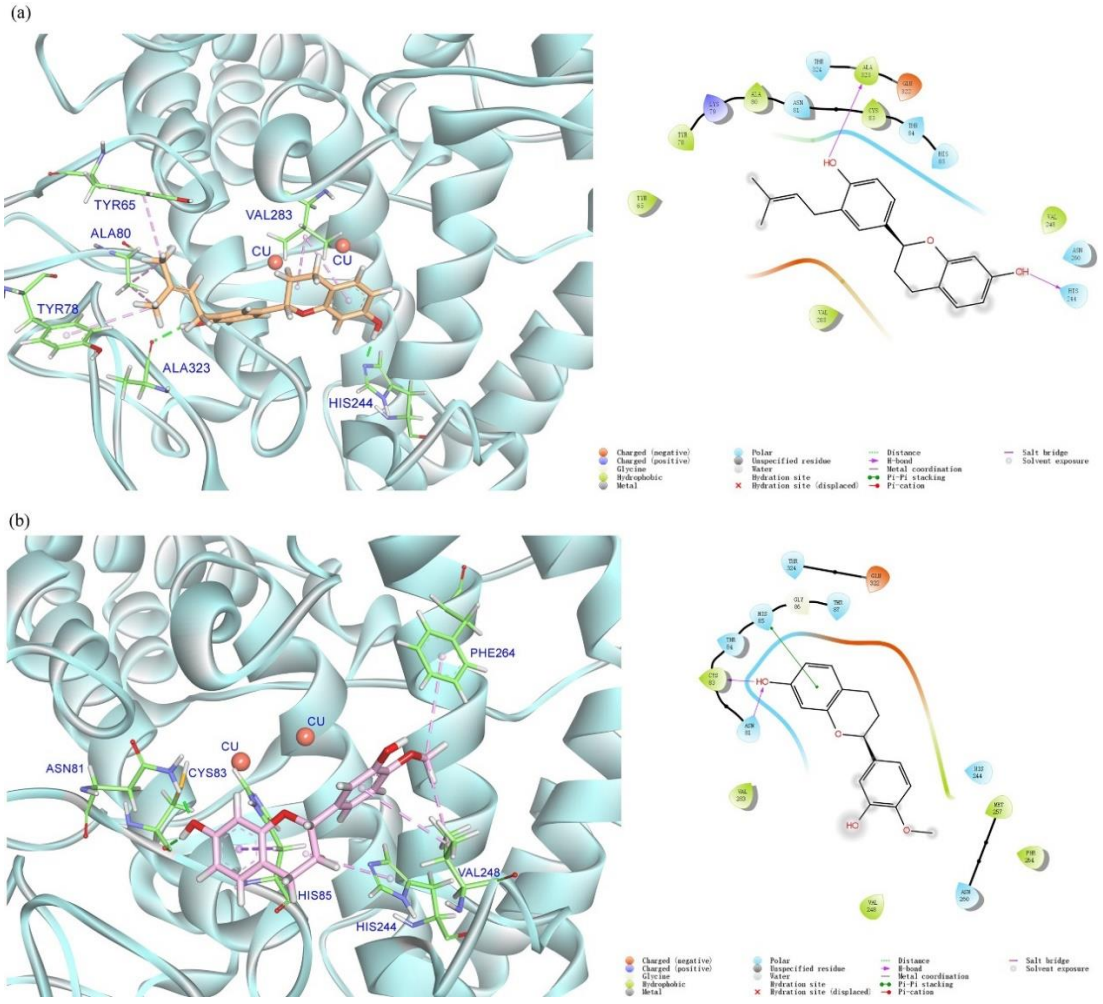


Table S2. Results of activity tests

Sample	DPPH (IC_{50}, μM)	ABTS (IC_{50}, μM)	FRAP (mM Fe^{2+}/ mM of sample)	Anti-tyrosinase activity (IC_{50}, mM)	Docking score
1	>100	37.04 ± 3.191	< 0.1	>1	nt†
2	>100	31.34 ± 2.142	0.15 ± 0.041	>1	nt†
3	>100	35.08 ± 1.871	< 0.1	0.49 ± 0.004	-5.301
4	>100	32.56 ± 2.173	0.14 ± 0.062	0.21 ± 0.004	-5.109
Trolox	66.24 ±	48.42 ± 2.452	1.32 ± 0.291	–	–
Arbutin	–	–	–	0.15 ± 0.002	-5.488

Note: IC_{50} values represent the means ± of three parallel measurements; †: not tested.

Figure S10. Results of molecular docking



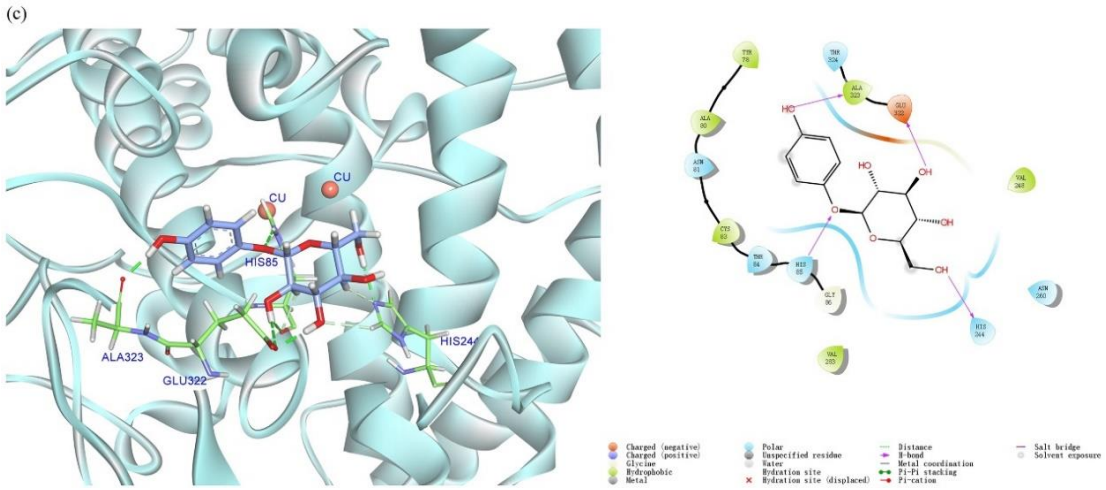


Figure S11. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of compound **2**.

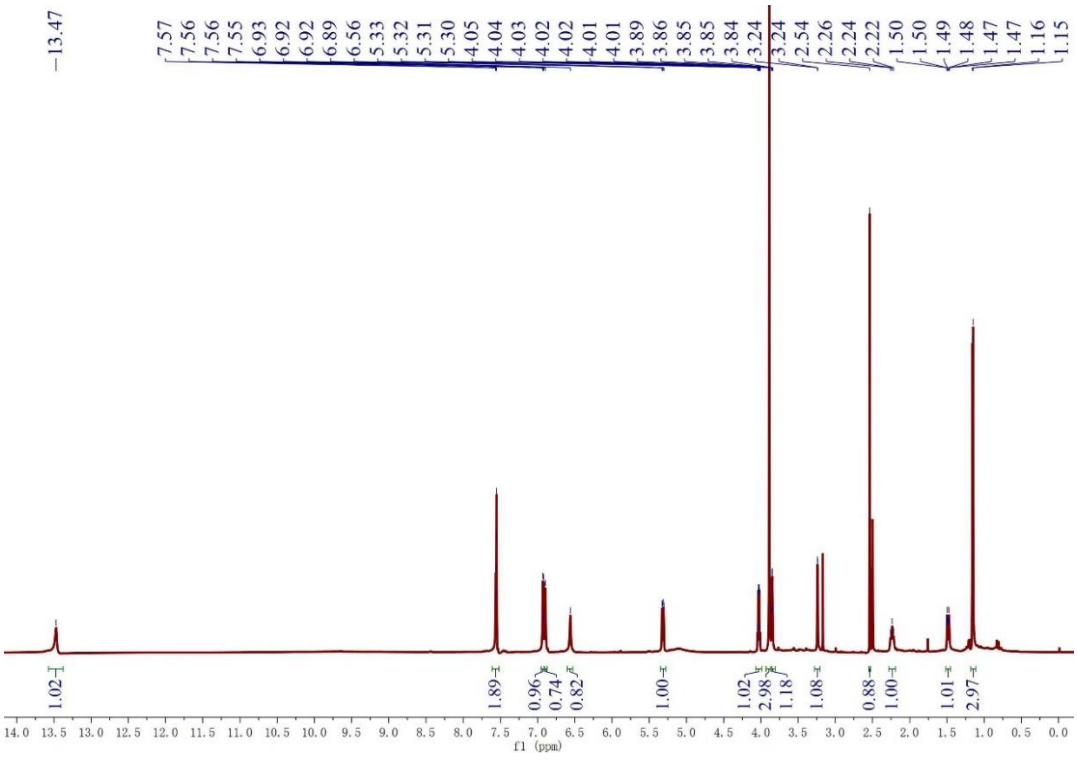


Figure S12. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of compound **2**.

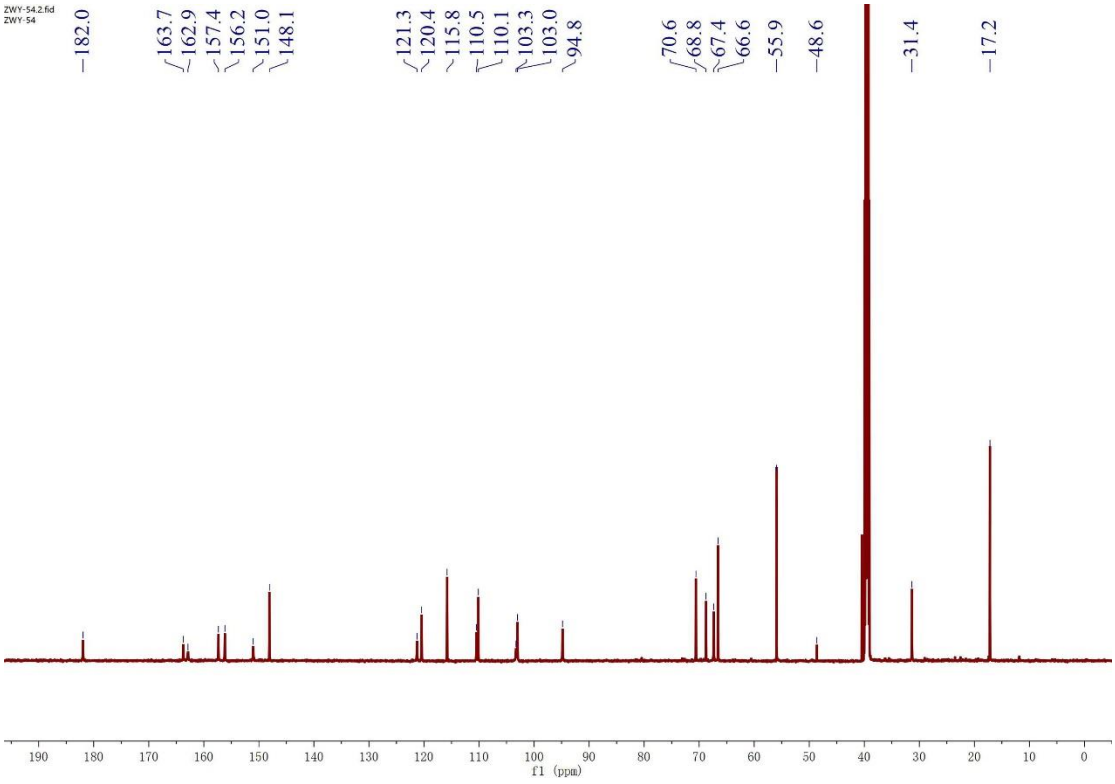


Figure S13. ^{1}H NMR spectrum (600 MHz, DMSO-*d*$_6$) of compound **3**.

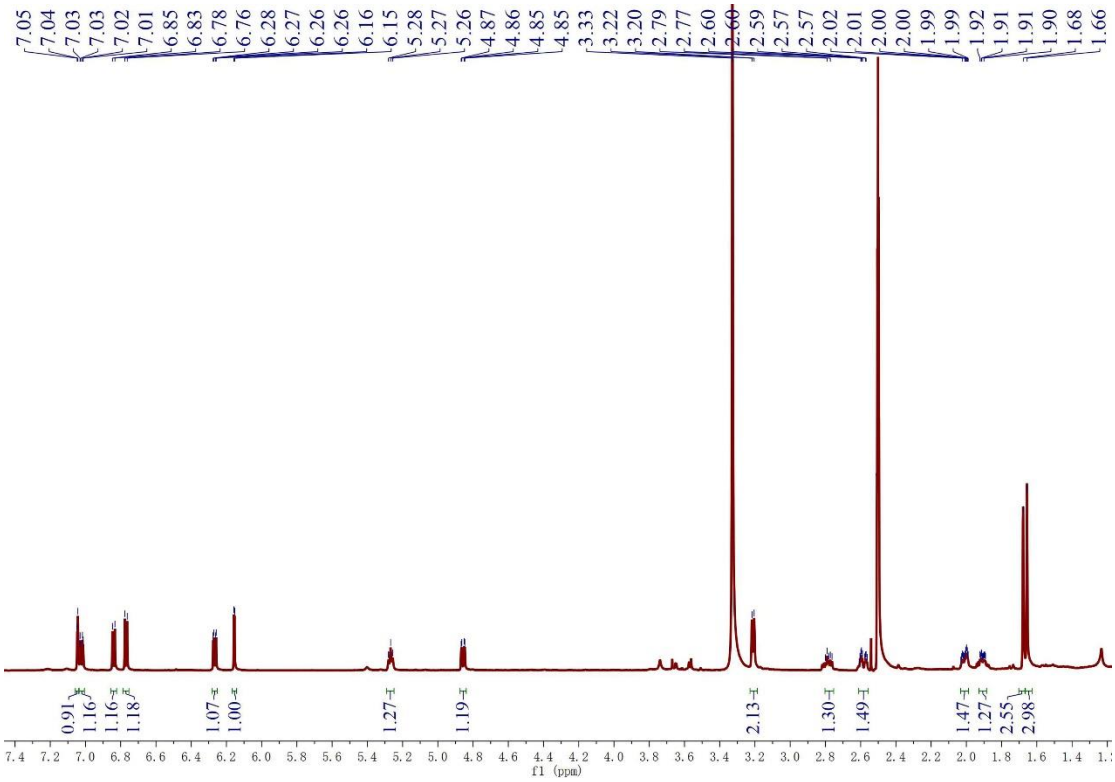


Figure S14. ^{13}C NMR spectrum (100 MHz, DMSO-*d*$_6$) of compound **3**.

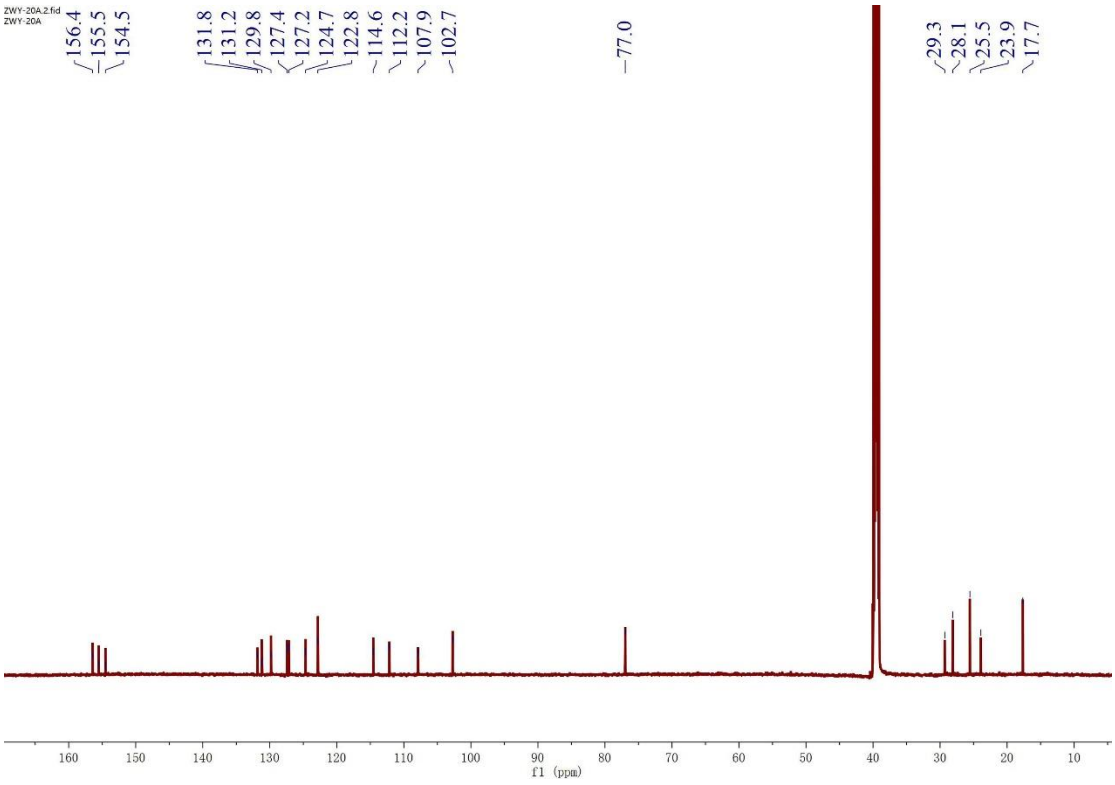


Figure S15. ^{1}H NMR spectrum (600 MHz, DMSO-d_6) of compound **4**.

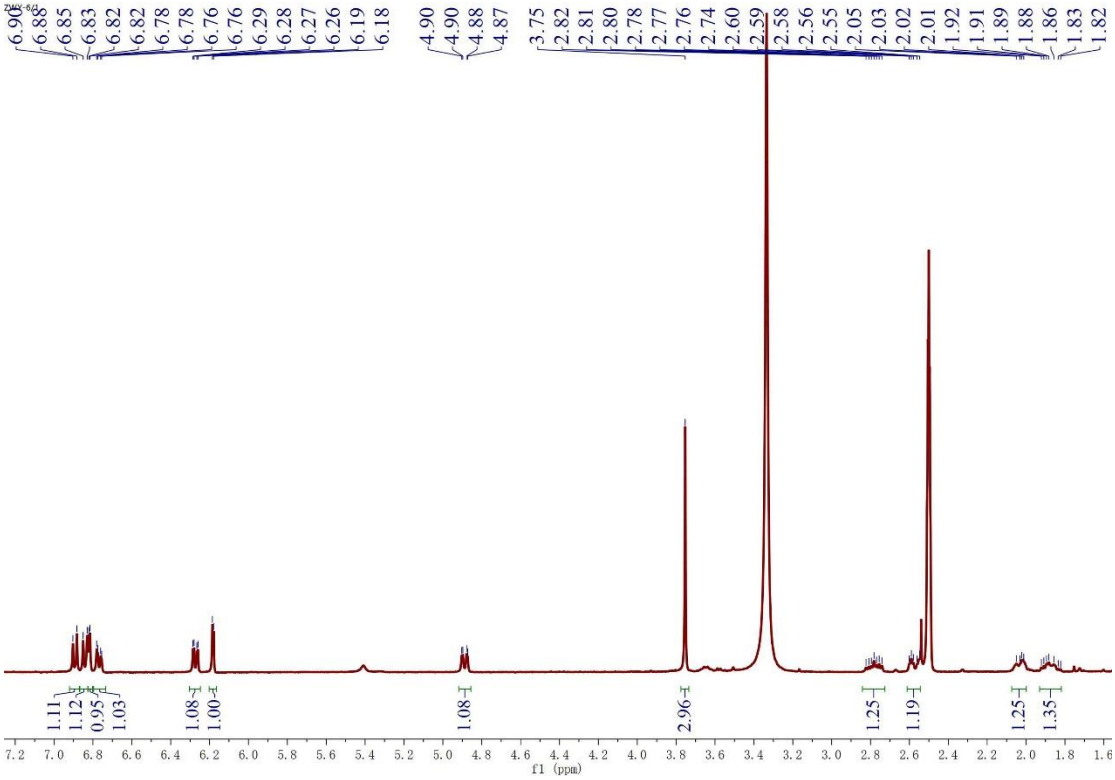


Figure S16. ^{13}C NMR spectrum (100 MHz, DMSO-d_6) of compound **4**.