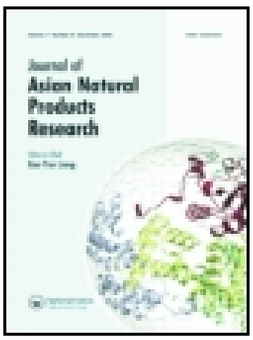

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Antioxidant and cytotoxic lignans from the roots of *Bupleurum chinense*

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ABSTRACT

In the search for biologically active compounds from the roots of *Bupleurum chinense* D C., phytochemical investigation of its ethanol extract led to the isolation and identification of a new 8-*O*-4′ neolignan glucoside, saikolignanoside A (**1**), along with eight known lignans (**2–9**). Their structures were determined on the basis of IR, UV, HRESIMS, and NMR spectroscopic analyses. The antioxidant and cytotoxic effects of isolated compounds were evaluated *in vitro*. The isolated compounds (IC_{50} > 200 μM) did not display 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. Whereas compounds **1–2**, **5**, **7,** and **9** exhibited potent 2, 2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging properties with IC_{50} values ranging from 8.34 to 15.24 μM, while compounds **3–4**, **6**, **8** showed moderate properties. In addition, all compounds were evaluated for cytotoxicities against A549, HepG2, U251, Bcap-37, and MCF-7 cell lines. Compounds **5** and **9** (IC_{50} < 51.62 μM) possessed stronger cytotoxic activities against all the tested tumor cell lines, compared with the positive control 5-Fluorouracil.

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1. Introduction

The roots of *Bupleurum chinense* D C. (Umbelliferae), first recorded in Shen Nong's Herbal Classic, has been used for more than 2000 years [1–3]. It also has been widely used as a key ingredient for many Chinese multi-herb remedies, such as Xiao-chaihu-tang, a famous hematopoietic remedy in oriental medicine [3]. According to the new Chinese Pharmacopoeia, the roots of *B. chinense* have been widely used to treat influenza, fever, inflammation, malaria, cancer and menstrual disorders [4,5]. Previous phytochemical studies on *B. chinense* have led to a series of highly functionalized saikosaponins, flavonoids, lignans, coumarins, polysaccharides, polyacetylenes, and volatile oil [6,7]. Nowadays, most of researches have tended to focus on the activities of saikosaponins, which have been

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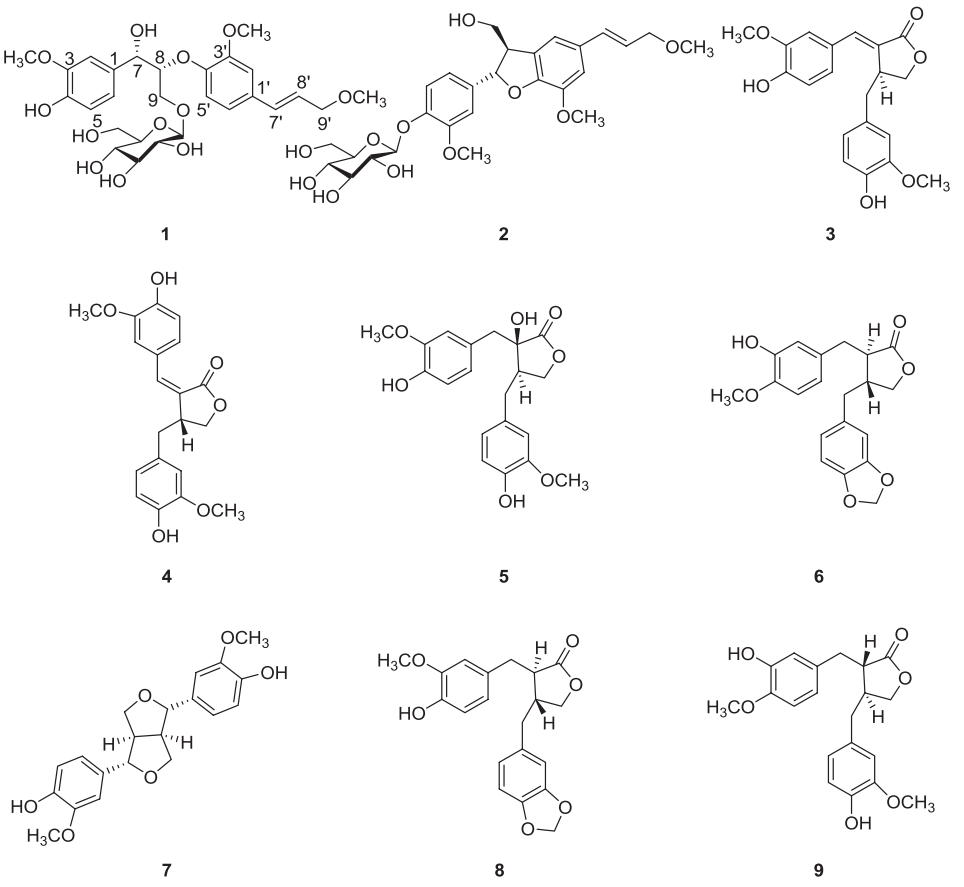

Figure 1. Structures of compounds **1–9**.

reported as the major biologically active components with antiviral, anticancer, anti-inflammatory activities, and so on [3–6]. However, there is relatively little information pertaining to isolation and pharmacological activities of the lignans from *B. chinense*.

As part of a program to search for more constituents with potential antioxidant and antitumor activities, a new 8-*O*-4′ neolignan glucoside saikolignanoside A (Figure 1), together with eight known lignans (7*S*, 8*R*)-9′-methoxyl-dehydrodi-coniferyl alcohol 4-*O*-*β*-D-glucopyranoside (**2**) [8], acutissimalignan B (**3**) [9], salicifoline (**4**) [10], (+)-nortrachelogenin (**5**) [11], guamarolin (**6**) [12], (+)-pinoresinol (**7**) [13], pluviatolide (**8**) [14], and 2-(3″-methoxy-4″-hydroxybenzyl)-3-(3′-methoxy-4′- hydroxylbenzyl)-*γ*-butyrolactone (**9**) [15] were isolated from the roots of *B. chinense.* Herein, we describe the isolation, structural elucidation of the new compound on the basis of the spectroscopic analysis, including 1D and 2D NMR, HRESIMS, CD, and the hydrolytic reaction. The antioxidant and cytotoxic activities of the nine lignans were also tested.

2. Results and discussion

Compound **1** was obtained as yellow oil and it showed a quasi-molecular ion peak at *m/z* 575.2099 $[M + Na]^+$ in the positive HRESIMS, indicating a molecular formula of $C_{27}H_{36}O_{12}$ with 10 degrees of unsaturation. The strong IR absorption bands indicated the presence

Table 1. ^{1}H NMR and ^{13}C NMR spectroscopic data for compound **1**.

Position	δ_H (600 MHz)[a]	δ_C (150 MHz)[a]
1		132.6
2	7.01 (1H, o)	111.2
3		146.9
4		145.5
5	6.68 (1H, d, *J* = 8.2 Hz)	114.7
6	6.82 (1H, dd, *J* = 8.2, 1.6 Hz)	119.0
7	4.83 (1H, d, *J* = 5.2 Hz)	70.6
8	4.44 (1H, dt, *J* = 11.0, 5.2 Hz)	82.3
9	3.96 (1H, dd, *J* = 11.0, 3.2 Hz)3.22 (1H, dd, *J* = 11.0, 3.2 Hz)	67.8
1′		131.5
2′	7.07 (1H, d, *J* = 1.8 Hz)	109.9
3′		149.6
4′		147.8
5′	7.01 (1H, o)	115.6
6′	6.89 (1H, dd, *J* = 8.4, 1.8 Hz)	119.4
7′	6.51 (1H, d, *J* = 15.9 Hz)	129.7
8′	6.22 (1H, dt, *J* = 15.9, 5.3 Hz)	124.3
9′	4.01 (2H, dd, *J* = 5.3, 1.4 Hz)	72.3
1″	4.05 (1H, d, *J* = 7.8 Hz)	103.4
2″	2.93–2.97 (1H, m)	73.4
3″	3.03 (1H, o)	76.8
4″	3.02 (1H, o)	69.9
5″	3.08–3.11 (1H, m)	76.6
6″	3.61–3.64 (1H, m)3.40 (1H, o)	60.9
3-OCH_3	3.73 (3H, s)	55.4
3′-OCH_3	3.79 (3H, s)	55.6
9′-OCH_3	3.26 (3H, s)	57.2

Note: NMR spectroscopic data were recorded in DMSO-d_6 at 600 MHz (^{1}H NMR) and 150 MHz (^{13}C NMR).
[a]Assignments are based on ^{1}H, ^{13}C NMR, HMBC, and HSQC experiments; o: The abbreviation for overlapped.

of hydroxyl group (3426 cm^{-1}) and aromatic rings (1639, 1461 and 1384 cm^{-1}). ^{1}H NMR spectrum (Table 1) of **1** displayed signals of a trans double bond [δ_H 6.51 (1H, d, *J* = 15.9 Hz, H-7′), 6.22 (1H, dt, *J* = 15.9, 5.3 Hz, H-8′)], two sets of oxygenated methylene protons [δ_H 3.96 (1H, dd, *J* = 11.0, 3.2 Hz, H-9a), 3.22 (1H, dd, *J* = 11.0, 3.2 Hz, H-9b); 4.01(2H, dd, *J* = 5.3, 1.4 Hz, H-9′)], one anomeric proton [δ_H 4.05 (1H, d, *J* = 7.8 Hz, H-1″)], two separate ABX proton signals [δ_H 7.01 (1H, o, H-2), 6.82 (1H, dd, *J* = 8.2, 1.6 Hz, H-6), 6.68 (1H, d, *J* = 8.2 Hz, H-5) and 7.07 (1H, d, *J* = 1.8 Hz, H-2′), 7.01 (1H, o, H-5′), 6.89 (1H, dd, *J* = 8.4, 1.8 Hz, H-6′)], together with three methoxy groups at δ_H 3.79 (3H, s), 3.73 (3H, s) and 3.26 (3H, s). The functional group signals appearing in the ^{13}C NMR spectrum also included a double bond, two aromatic rings, and three methoxy groups. The HMBC correlations from H-7 at δ_H 4.83 (1H, d, *J* = 5.2 Hz) to C-1 at δ_C 132.6, C-6 at δ_C 119.0, C-2 at δ_C 111.2, C-8 at δ_C 82.3 and C-9 at δ_C 67.8, as well as from H-7′ at δ_H 6.51 (1H, d, *J* = 15.9 Hz) to C-2′ at δ_C 109.9 and C-6′ at δ_C 119.4, evidenced the linkage positions of two phenyl propanoid units (Figure 2). The NOESY correlation between H-8 at δ_H 4.44 (1H, dt, *J* = 11.0, 5.2 Hz) and H-5′ at δ_H 7.01 (1H, o) was also observed. In addition, with the help of ^{1}H-^{1}H COSY spectrum (Figure 2), the correlations between H-7 at δ_H 4.83 (1H, d, *J* = 5.2 Hz) and H-8 at δ_H 4.44 (1H, dt, *J* = 11.0, 5.2 Hz), between H-8 at δ_H 4.44 (1H, dt, *J* = 11.0, 5.2 Hz) and H-9 at δ_H 3.22 (1H, dd, *J* = 11.0, 3.2 Hz) established that compound **1** had an 8-*O*-4′ neolignan system. Two methoxy groups were assigned to C-3 and C-3′ positions by the HMBC correlations from δ_H 3.73 (3H, s) and 3.79 (3H, s) to the carbons at δ_C 146.9 (C-3) and δ_C 149.6 (C-3′), respectively. In addition, a methoxy group was attached to C-9′ by the HMBC correlations between δ_H 3.26 (3H, s) and δ_C 72.3 (C-9′) (Figure 2).

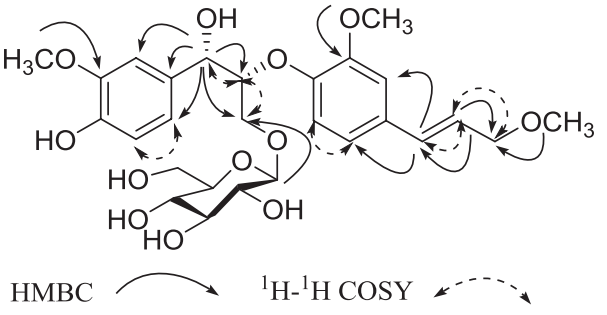

Figure 2. Key HMBC and ^{1}H-^{1}H COSY correlations of compound **1**.

The ^{1}H NMR and ^{13}C NMR spectra also displayed a set of sugar moiety signals [δ_H 4.05 (1H, d, J = 7.8 Hz, H-1″), 3.61–3.64 (1H, m, H-6a″), 3.40 (1H, o, H-6b″), 3.08–3.11 (1H, m, H-5″), 3.03 (1H, o, H-3″), 3.02 (1H, o, H-4″) and 2.93–2.97 (1H, m, H-2″), as well as δ_C 103.4 (C-1″), 60.9 (C-6″), 76.6 (C-5″), 76.8 (C-3″), 69.9 (C-4″), and 73.4 (C-2″)]. Initially, we assigned the sugar as glucose by comparison of its data with literature. To determine the absolute configuration, compound **1** was subjected to acidic hydrolysis, followed by GC analysis in comparison with authentic D-glucose [t_R (D-glucose) = 13.83 min]. The results confirmed the presence of D-glucose moiety in **1**. In addition, the β-oriented anomeric configuration was determined based on the J_{H-H} values (7.8 Hz). The linkage position of the glucosyl group was assigned by HMBC correlations between the anomeric proton at δ_H 4.05 (1H, d, J = 7.8 Hz, H-1″) and C-9 at δ_C 67.8 (Figure 2). Detailed analysis of the 2D NMR of **1** (Figure 2), along with comparison of the NMR data revealed the similar structure of **1** with those of the debilignanoside [16]. However, one methoxy group at C-9′ (δ_C 72.3) in compound **1** appeared instead of a hydroxyl group for debilignanoside. The stereochemistry of **1** was determined by a comprehensive analysis of the NMR and CD data. The $\Delta\delta_{C8-C7}$ values (11.7 ppm) of **1** were smaller than those of the *threo* isomer (13.4 or 13.0 ppm) in the literatures and suggested a 7, 8-*erythro* relative configuration [16,17]. The CD spectrum showed a negative Cotton effect at 248 nm, indicating 8*R* configuration for compound **1**. So, it was found that the new compound had 7*S*, 8*R*-configuration [18,19]. Thus, the structure of **1** was determined and named saikolignanoside A.

Reactive oxygen species (ROS) increase the chance of occurrence of cancer, atherosclerosis, neurodegenerative diseases, and inflammation [20]. Several studies demonstrated that lignans can decrease the incidence of oxidative stress and associated diseases [21,22]. Compounds **1–9** were evaluated for antioxidant effects using the DPPH and ABTS assays (Table 2). Trolox served as a positive control, with IC_{50} values of 5.70 and 12.21 μM, respectively. As shown in Table 2, all compounds were inactive (IC_{50} > 200 μM) in the DPPH assay. But in the ABTS assay, compounds **1–2**, **5**, **7,** and **9** display significant ABTS radical-scavenging activities, whereas the other compounds showed moderate activities comparable with that of the standard compound trolox (IC_{50} = 5.70 μM).

It has been reported that antioxidants play an important role in protecting the body from oxidative stress by scavenging free radicals and preventing cancer invasion [23]. In order to evaluate the potential cytotoxic activities, all isolated lignans were tested for their cytotoxic activities against the five selected human tumor cell lines by the MTT method (Table 3). The results obtained are expressed as IC_{50} values in μM. 5-Fluorouracil was used

Table 2. Free radical scavenging activity of compounds **1–9**.

Compounds	DPPH (IC_{50}, μM)	ABTS (IC_{50}, μM)
1	>200	8.34
2	>200	15.24
3	>200	32.70
4	>200	25.62
5	>200	15.13
6	>200	19.87
7	>200	13.43
8	88.52	23.20
9	>200	10.62
Trolox[a]	12.21	5.70

[a]Trolox was used as the positive control.

Table 3. Cytotoxic activities of compounds **1–9** *in vitro* (IC_{50}, μM).

	IC_{50} (μM)[a] Cell line				
Compounds	A549	HepG2	U251	Bcap-37	MCF-7
1	20.11	32.021	>80	>80	50.63
2	>80	>80	>80	>80	>80
3	30.77	>80	>80	>80	>80
4	>80	29.18	>80	>80	>80
5	19.61	17.62	39.10	51.62	45.64
6	20.11	32.02	>80	>80	50.63
7	29.35	62.35	>80	>80	75.32
8	>80	>80	>80	>80	>80
9	17.05	15.14	23.92	50.32	25.34
5-Fu[b]	34.27	49.72	48.60	47.09	42.78

[a]IC_{50} (μM) represents means ± SD of three independent replicates. The IC_{50} greater than 80 μM was considered to indicate no cytotoxicity.
[b]5-Fu (5-Fluorouracil) was used as positive control.

as positive control. In Table 3, compounds **5**, **9** possessed stronger cytotoxic activities against all the selected tumor cell lines. In addition, compounds **1**, **6** possessed stronger cytotoxic activities against the A549 and HepG2 human tumor cell lines.

In this study, antioxidant and cytotoxic effects of the lignans isolated from *B. chinense* were reported for the first time. These findings were valuable for further investigation and optimization of the roots of *B. chinense*. On the basis of these promising results, compounds **5** and **9** which exhibited potent antioxidant and antitumor activities deserve further exploration for the deeper investigations into its mechanism of action, as well as their anticancer effects *in vivo*.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-370 digital polarimeter (Jasco Co., Tokyo, Japan). The UV spectra were obtained on a Shimadzu UV-1700 spectrometer (Shimadzu Corporation, Kyoto, Japan). IR spectra were recorded on a Bruker IFS-55 spectrometer (Bruker Co., Karlsruhe, Germany). HR-ESIMS data were measured on an Agilent G6520 Q-TOF LC/MS spectrometer (Bruker Co., Karlsruhe, Germany). CD spectra were obtained on MOS 450 detector in the 200–400 nm wavelength range (Bio-Logic Co., Claix, France).

NMR spectra were recorded on Bruker AV-400 and AV-600 spectrometers (Bruker Co., Billerica, MA, U.S.A) with TMS as internal standard. An Agilent 1100 HPLC (Agilent, Santa Clara, CA, U.S.A) was used for isolation and purification with YMC C-18 semi-preparative HPLC column (5 μm, 250 × 10 mm; YMC Co. Ltd., Kyoto, Japan). GC was carried out using an Agilent 7890A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, U.S.A) equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm; Agilent Technologies, Inc., Santa Clara, CA, U.S.A). Column chromatography (CC) was performed on macroporous adsorption resin D101 (Cangzhou Bon Adsorber Technology Co., Ltd., Cangzhou, China), Silica gel (200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, China), ODS silica gel (50 μm, YMC Co. Ltd., Kyoto, Japan) and Sephadex LH-20 (25–100 m, Green Herbs Science and Technology Development Co., Ltd., Beijing, China). The fractions were monitored by TLC (SiO_2 GF254; Qingdao Marine Chemical Inc., Qingdao, China). DPPH and ABTS assays were performed on Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA, U.S.A).

3.2. Plant material

The roots of *Bupleurum chinense* were purchased from Liaoning Tongren Pharmaceutical Co. Ltd (Shenyang, China), and identified by professor Jin-Cai Lu (Department of Natural Products Chemistry, Shenyang Pharmaceutical University). A voucher specimen (No.1012002) has been preserved in the Herbarium of Shenyang Pharmaceutical University.

3.3. Extraction and isolation

The dried plant material (9.5 kg) was extracted by homogenate extraction with 75% ethanol. Evaporation of the ethanol extract afforded 800.0 g dark gummy residue. The residue (800.0 g) was chromatographed on silica gel (200–300 mesh) with a gradient of CH_2Cl_2/MeOH of 100: 1, 50: 1, 30: 1, 20: 1, 10: 1 and 0: 1, to afford five fractions (Fr. 1-5). Fr. 2 (102.0 g) was further separated by silica gel CC eluting with a CH_2Cl_2/MeOH gradient system (v/v = 50: 1, 25: 1, 10: 1, 8: 1, 5: 1 and 0: 1) to yield 6 fractions (Fr. 2-1-Fr. 2-6), Fr. 2-2 (10.4 g) was separated by C_{18} column chromatography and eluted with MeOH–H_2O gradient (v/v = 30: 70, 60: 40 and 100: 0) to give 5 fractions (Fr. 2-2-1-Fr. 2-2-5). Fr. 2-2-2 (1.5 g) was purified by semi-preparative HPLC (solvent: 25% CH_3CN-H_2O) to give **1** (10.3 mg, t_R = 15.4 min) and **2** (14.5 mg, t_R = 21.3 min). Fr. 2-2-3 (2.5 g) was chromatographed on Sephadex LH-20 (MeOH) and further purified by semi-preparative HPLC (solvent: 35% MeOH–H_2O) to yield **3** (11.5 mg, t_R = 13.1 min), **9** (15.2 mg, t_R = 15.6 min), **5** (10.1 mg, t_R = 21.6 min) and **6** (7.3 mg, t_R = 30.6 min). Fr. 2-2-4 (2.3 g) was also chromatographed over Sephadex LH-20 and eluted with MeOH to give compound **8** (13.1 mg, t_R = 15.6 min) and four subfractions A-D. Compound **7** (14.1 mg, t_R = 17.8 min) and compound **4** (11.3 mg, t_R = 24.3 min) was purified from subfraction B by HPLC (solvent: 37% MeOH–H_2O).

3.3.1. Saikolignanoside A

Yellow oil, $[\alpha]_D^{20}$ -17.3 (*c* 0.1, CH_3OH); UV (MeOH) λ_{max} (log ε): 281.0 (2.96) nm; IR (KBr) ν_{max}: 3426, 2920, 2850, 1639, 1461, 1384, 1338, 1263 1130, 878 and 619 cm^{-1}; CD (*c* 0.10 mg/ml, MeOH): $\Delta\varepsilon_{248\ nm}$ −8.86; ^1H and ^{13}C NMR spectral data, see Table 1; HRESIMS: *m/z* 575.2099 $[M + Na]^+$ (calcd for $C_{27}H_{36}O_{12}Na$, 575.2098).

3.4. Acid hydrolysis of compound 1

A solution of compound **1** (2.0 mg) was heated in 1 M HCl (2.0 ml) at 95 °C for 4 h in a sealed ampoule and extracted with EtOAc (3 × 2.0 ml). The aqueous layer was concentrated to dryness, to yield a residue. This residue was dissolved in pyridine (1.0 ml), and then L-cysteine methyl ester hydrochloride (2.0 mg) was added to the solution. The mixture was heated at 60 °C for 2 h, and 0.1 ml N-(trimethylsily)-imidazole (TMSI) was added, followed by heating at 60 °C for 1 h. After drying the solution, the residue was partitioned between H_2O (2 ml) and n-hexane (2 ml). The n-hexane layer was subjected to GC analysis on Agilent 7890A (HP-5, 30 m × 320 mm × 0.25 μm) with flame ionization detection under the following conditions: The column temperature was set at 150–280 °C at the rate of 10 °C/min, and the carrier gas was N_2 (1.4 ml/min); the injection temperature was 250 °C and the injection volume, 1 μl. The absolute configurations of the monosaccharides were identified to be D-Glu by comparison of the retention times of its Me_3Si ethers with those of standard samples [t_R (D-glucose) = 13.83 min].

3.5. Antioxidant assays

3.5.1. DPPH radical scavenging assay

The DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical-scavenging effect was performed as previously described method with some modifications [24]. Trolox (Purity. 98%; Sigma, Los Angeles, CA, U.S.A) was used as the positive control. Tests were performed in triplicate. The samples with different concentrations (0.1–100 μM) in EtOH (100 μl) were added to 0.2 mM DPPH in EtOH (150 μl). The mixture was placed in a 96-well microplate and incubated at 37 °C for 30 min. The scavenging activity of samples was estimated by measuring the absorption of the mixture at 517 nm with a Varioskan Flash (Thermo Scientific, Waltham, MA, U.S.A) and the means of three readings were calculated. DPPH radical scavenging (capacity) (%) = $[1-(S-S_b)/(C-C_b)] \times 100\%$, where S sample is the absorbance of the test sample, S_b is the blank sample, C is the absorbance of the control, and C_b is the blank control, respectively. The scavenging activity was expressed as IC_{50}, the concentrations of samples required for scavenging 50% of DPPH radical in the solution.

3.5.2. ABTS radical scavenging activity assay

The radical scavenging activity of compounds (**1–9**) was tested using the improved ABTS decolorization assay with modification [25]. Trolox, a water-soluble analog of vitamin E, was used as a positive control. ABTS, 2, 2′-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS solution with 2.45 mM $K_2S_2O_8$ (final concentration) and allowing the mixture to store in the dark at room temperature for 12–16 h before use. For analysis, the $ABTS^{\bullet+}$ solution was diluted with ethanol so that the absorbance at 734 nm was 0.700 ± 0.020. The diluted $ABTS^{\bullet+}$ solution (150 μl) was mixed with ethanolic solution (50 μl) of samples at various concentrations, and then the absorbance was detected at 734 nm after 20 min. All experiments involving these samples were replicated three times. $ABTS^{\bullet+}$ radical scavenging activity of the isolated compounds was calculated by formula as below: $ABTS^{\bullet+}$ scavenging activity (%) = $[1 - (S-S_b)/(C-C_b)] \times 100\%$, where S, S_b, C and C_b are the absorbencies of the sample, the blank sample, the control, and the blank control, respectively.

3.6. In vitro *cytotoxicity assay*

All isolated compounds (**1–9**) were subjected to cytotoxic evaluation against A549, HepG2, U251, Bcap-37and MCF-7 cell lines employing the MTT (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) method with 5-Fluorouracil as the positive control [24]. Briefly, cells were seeded into each well of a 96-well cell culture plate and incubated at 37 °C for 24 h before drug treatment. Compounds (**1–9**) and 5-fluorouracil were then added to make various final concentrations (6.25, 12.5, 25, 50, 100 μM). The cells were then exposed to 20 μl of the test compounds at various concentrations in triplicate and incubated for 48 h. After the incubation, MTT solution (0.5 mg/ml) was added to each well, and the incubation continued for 4 h at 37 °C. The supernatant was decanted, and DMSO (100 μl/well) was added to allow formazan solubilization. The level of MTT formazan was determined by measuring its absorbance at 492 nm with the Spectra Shell reader. The percentage of cell inhibition was calculated as follows: Cell death (%) = [A492 (control)−A492 (test)]/A492 (control) × 100%. IC_{50} values were calculated with the LOGIT method.

Disclosure statement

No potential conflict of interest was reported by the authors.

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