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CHEMICAL STRUCTURES AND CYTOTOXIC ACTIVITIES OF THE CONSTITUENTS ISOLATED FROM *CITRUS SPHAEROCARPA*

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Abstract – From the peels of *Citrus sphaerocarpa*, two new limonoids, sphaerocarpain I and II were isolated together with five known compounds including methyl deacetylnomilinate and ichangin. The chemical structures of the new compounds were elucidated based on chemical/physicochemical evidence. For sphaerocarpain I and II, the absolute configuration was established by comparison of experimental and predicted electronic circular dichroism (ECD) data. Among the isolated compounds, sphaerocarpain I, II, methyl deacetylnomilinate and ichangin showed cytotoxic activity against both human neuroblastoma cells (SH-SY5Y) and human glioblastoma cells (U-251 MG).

INTRODUCTION

Citrus fruits are commonly grown fruits all over the world.[1] *C. sphaerocarpa* is a relative of *C. junos* and is mainly cultivated in Oita Prefecture, Japan.[2] The branches have sharp thorns, and the fruits are harvested while they are green, but turn yellow when ripe.[2] The flesh is yellowish white, and the juice has a strong acidity and a unique aroma.[3,4] They are also edible as seasonings, juices, sweets, and alcoholic beverages.[3,4] The peels of *C. sphaerocarpa* are rich in pectins, flavonoids, carotenoids, limonoids and coumarins, which are valuable and functional nutritional components for human health, but are usually wasted.[5-7] Therefore, research into the utilization of this hide waste has received much attention in the recent literature. Previous studies have shown that sufficient amounts of orange peels extract may have chemoprotective properties and reduce cancer risk.[8] In addition, limonoids are a typical component found in citrus peels, and limonoids have been reported to have anticancer effects.[9] Limonin, norimin, and ichangin have been reported as limonoids contained in *C. sphaerocarpa*.[2]

Glioblastoma is the most malignant disease among gliomas, accounting for approximately 30% of primary brain tumors.[10] Glioblastoma is treated with a combination of radiation therapy and chemotherapy after surgery. The robust DNA repair and self-renewing capabilities of glioblastoma, promote resistance against all current treatment modalities.[11] Therefore, there is a clearneed for novel therapeutic agents.[11] To evaluate the anticancer properties of these limonoids, we isolated the constituents of *C. sphaerocarpa* and investigated their effects on cancer cells. This paper reports the structural elucidation of sphaerocarpain I and II (**1** and **5**) and their cytotoxic activities.

RESULTS AND DISCUSSION

Isolation of limonoids from Citrus sphaerocarpa

The methanol (MeOH) extract of the peels of *C. sphaerocarpa* was partitioned in ethyl acetate (EtOAc)–H_2O (1:1, v/v) to furnish an EtOAc (0.094%) soluble fraction and aqueous layer. Aqueous layer was further extracted with *n*-butanol (BuOH) to give *n*-BuOH (0.19%) and H_2O (1.61%) soluble fractions. The EtOAc soluble fraction was subjected to normal- and reversed-phase silica gel column chromatography and repeated high-performance liquid chromatography (HPLC) to give two new compounds: sphaerocarpain I (**1**, 0.00018%) and sphaerocarpain II (**5**, 0.000032%), together with five known compounds: deacetylnomilin (**2**, 0.010%),[12] methyl deacetylnomilinate (**3**, 0.00051%),[13,14] methyl nomilinate (**4**, 0.00018%),[14] limonin (**6**, 0.011%),[15] and ichangin (**7**, 0.00056%)[12] (Figure 1).

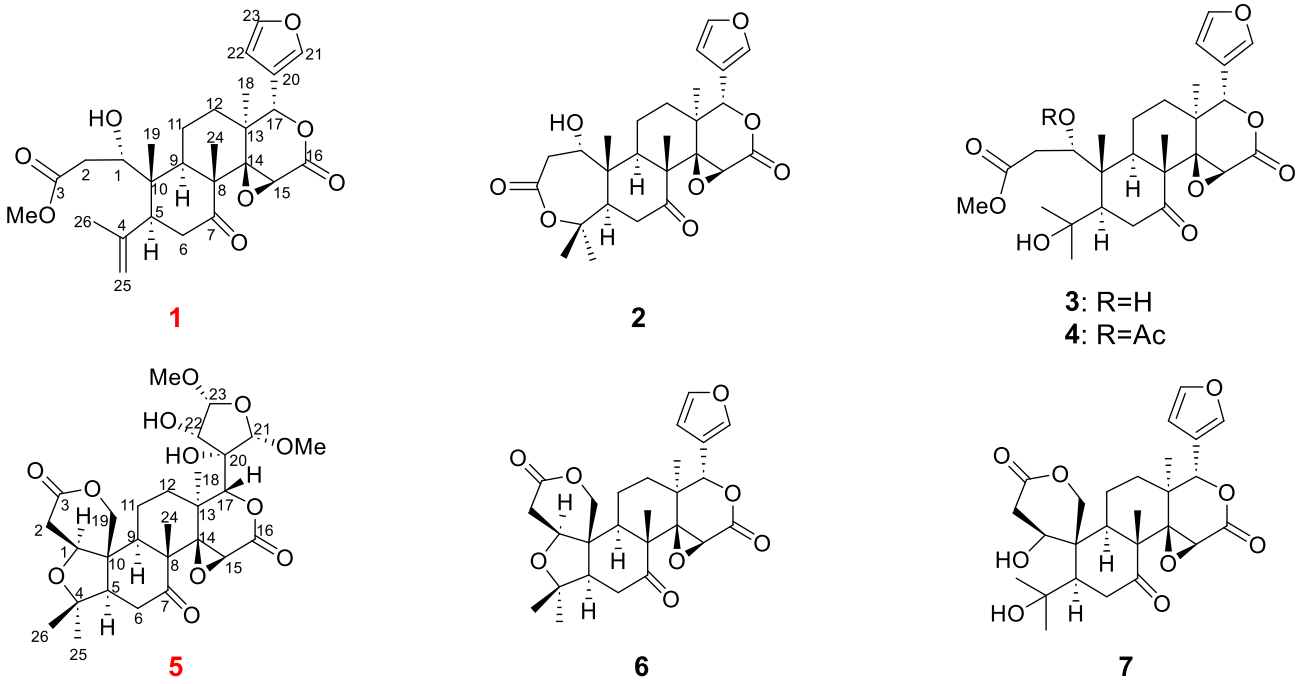

Figure 1. The chemical structures of isolated compounds from the peels of *C. sphaerocarpa*

Structures of sphaerocarpain I and II (1, 5)

Sphaerocarpain I (**1**) was obtained as a white amorphous powder with negative optical rotations ([α]$^{25}_{D}$

-65.32 in MeOH). The molecular formula ($C_{27}H_{34}O_8$) was determined using high-resolution electrospray-ionization mass spectrometry (HRESIMS) and ^{13}C NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra (chloroform-*d*) exhibited characteristic signals of a limonoid moiety (Table 1), including four methyl groups [δ_H 1.13 (s, H-18), 1.21 (s, H-19), 1.17 (s, H-24), and 1.77 (s, H-26), each 3H], a methoxy group [δ_H 3.72 (s)], four methylene groups [δ_C 35.8 (C-2), 41.9 (C-6), 18.4 (C-11), 32.4 (C-12)], two methine groups bearing an oxygen function [δ_C 73.9 (C-1), 53.0 (C-15)], furan ring [δ_H 7.41 (br s, H-21), 6.37 (br s, H-22), and 7.39 (br s, H-23), each 1H], two ester carbonyl groups [δ_C 173.8 (C-3), 167.2 (C-16)], and a ketone group [δ_C 210.0 (C-7)]. The positions of each function groups described above were determined by correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) NMR spectroscopy (Figure 2). Namely, long-range correlations were observed between the following proton and carbon pairs: H-1/C-2, 3, 5, 10, H-2/C-1, 3, 10, H-5/C-4, 10, 19, 25, 26, H-6/C-4, 5, 7, 10, H-9/C-10, 12, 19, 24, H-11/C-12, 13, H-12/C-11, 13, 18, H-15/C-14, 16, H-17/C-18, 20, H-18/C-12, 13, 14, 17, H-19/C-1, 5, 9, 10, H-21/C-20, 22, 23, H-23/C-21, H-24/C-7, 8, 9, 14, H-25/C-4, 5, 26, H-26/C-4, 25 and 3-OMe/C-3. Sphaerocarpain I (**1**) is a compound in which the A ring of a limonoid is cleaved and has an isopropenyl group in the C-5 side chain. The ^{1}H NMR signals of the 1,1-disubstituted olefinic moiety (H-25, δH 4.97 and 4.76) in sphaerocarpain I (**1**) exhibited HMBC correlations between C-4 (δC 144.9) and C-26 (δC 22.8), which is characteristic of an isopropenyl group (Table 1, Figure 1). Several limonoids with the same functional group, such as polystanin A,[16] and atalantolide derivatives,[17] have been reported. The relative configuration of **1** was determined via analysis of their NOESY spectra (Figure 2). The NOESY cross peaks of H-1/H-2β, 19, H-2α/H-9, H-5/H-6α, 9, 25α, H-6β/H-24, 26, H-9/H-18, H-11α/H-12α, H-11β/H-12β, H-12α/H-18, H-12β/H-17, 24, H-15/H-18, H-19/H-24 and H-25β/H-26 indicated that 1-OH, H-2α, 5, 6α, 9, 11α, 12α, 15, 18 and 25α were on one side of each molecule and that H-1, 2β, 6β, 11β, 12β, 17, 19, 24, 25β and 26 were on the opposite side. Finally, the absolute configuration of **1** was elucidated from the calculated ECD curve (Figure 3A). The calculated ECD spectra of **1** was identical with that of experimental data, while the calculated ECD spectra of *ent*-**1** had the opposite sign to the experimental data. These data suggested that the absolute configuration was 1*S*, 5*S*, 8*R*, 9*R*, 10*S*, 13*S*, 14*R*, 15*S*, 17*S*. Moreover, 1-OH, H-5, H-9, H-15, and H-18 were established as α-oriented, in accordance with those of similar compound, e.g. clauemargine I.[18] On the basis of all this evidence, the chemical structure of sphaerocarpain I (**1**) was established as shown (Figure 1).

Sphaerocarpain II (**5**) was obtained as a white amorphous powder with negative optical rotations ($[\alpha]^{25}_D$ -55.73 in MeOH). The molecular formula ($C_{28}H_{38}O_{12}$) was determined using HRESIMS and ^{13}C NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra (chloroform-*d*) exhibited characteristic signals of a limonoid moiety (Table 1), including four methyl groups [δ_H 1.33 (s, H-18), 0.98 (s, H-24), 1.29 (s, H-25), and 1.18 (s, H-26), each 3H], five methylene groups [δ_C 35.7 (C-2), 36.4 (C-6), 19.0 (C-11), 29.7 (C-12), 65.5 (C-19)], two methine groups bearing an oxygen function [δ_C 79.2 (C-1), 81.5 (C-17)], substituted furan ring [δ_H 4.79

(s, H-21), 4.12 (d, *J* = 3.6, H-22), and 4.96 (d, *J* = 3.6, H-23), each 1H], two ester carbonyl groups [δ_C 169.3 (C-3), 166.7 (C-16)], and a ketone group [δ_C 206.3 (C-7)]. The positions of each function groups described above were determined by COSY and HMBC NMR spectroscopy (Figure 2). Namely, long-range correlations were observed between the following proton and carbon pairs: H-1/C-3, 10, 19, H-2/C-1, 3, H-5/C-4, 6, 9, 10, 19, 25, 26, H-6/C-5, 7, 10, H-9/C-5, 8, 10, 11, 12, 24, H-11/C-9, 12, H-12/C-11, 13, 18, H-15/C-14, 16, H-17/C-13, 14, 18, H-18/C-12, 13, 14, 17, H-19/C-1, 3, 5, 10, H-21/C-17, 20, 21-OMe, 22, 23, H-22/C-21, 23, H-23/C-21, 23-OMe, H-24/C-7, 8, 9, 14, H-25/C-4, 5, 26, H-26/C-4, 5, 25, 21-OMe/C-21 and 23-OMe/C-23. It is worth noting that such a side chain as ring *E* is rare in the family of naturally occurring limonoids, and it have been reported kihadanin C,[19] isolated from *Dictamnus dasycarpus*, and yunnanol A,[20] isolated from *Walsura yunnanensis*. The relative configuration of **5** were determined via analysis of their NOESY spectra (Figure 2). The NOESY cross peaks of H-1/H-2α, 5, 9, H-5/H-6α, 9, 25, H-6α/H-25, H-6β/H-19β, 24, 26, H-9/H-18, H-12α/H-18, H-12β/H-17, 22, 24, H-15/H-18, H-17/H-21, 22, H-19β/H-24, 26, H-21/H-22, 23 and H-22/H-23 indicated that H-1, 2α, 5, 6α, 9, 12α, 15, 18, 19α, 25, 20-OH, 21-OMe, 22-OH and 23-OMe were on one side of each molecule and that H-2β, 6β, 12β, 17, 19β, 21, 22, 23, 24 and 26 were on the opposite side. Finally, the absolute configuration of **5** was elucidated from the calculated ECD curve (Figure 3B). The calculated ECD spectra of **5** was identical with that of experimental data, while the calculated ECD spectra of *ent*-**5** had the opposite sign to the experimental data. These data suggested that the absolute configuration was 1*S*, 5*R*, 8*R*, 9*R*, 10*R*, 13*S*, 14*R*, 15*S*, 17*R*, 20*S*, 21*S*, 22*R*, 23*S*. On the basis of all this evidence, the chemical structure of sphaerocarpain II (**5**) was established as shown (Figure 1).

Table 1. ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR data of sphaerocarpain I (**1**) and II (**5**) in chloroform-*d*

Position	**1**		**5**	
	δC	δH (*J* in Hz)	δC	δH (*J* in Hz)
1	73.9	4.05 (d, 10.8)	79.2	4.09 (br d, 3.0)
2	35.8	α 2.42 (dd, 10.8, 16.8)	35.7	α 2.71 (dd, 1.2, 16.8)
2		β 2.65 (m)		β 3.01 (dd, 3.6, 16.8)
3	173.8		169.3	
4	144.9		80.2	
5	50.1	2.63 (m)	61.0	2.21 (dd, 3.6, 15.6)
6	41.9	α 2.35 (dd, 4.8, 14.4)	36.4	α 2.45 (dd, 3.6, 14.4)
		β 2.98 (dd, 11.4, 14.4)		β 2.87 (br t, 14.4)
7	210.0		206.3	
8	52.8		51.9	
9	43.4	2.48 (br d, 12.0)	47.9	2.59 (t, 7.2)
10	43.8		45.9	
11	18.4	α 1.58–1.64 (m)	19.0	1.77–1.81 (m)
		β 2.29 (dd, 8.4, 13.8)		1.77–1.81 (m)
12	32.4	α 1.41–1.47 (m)	29.7	α 2.32-2.37 (m)
		β 1.75 (m)		β 1.83–1.87 (m)
13	37.3		38.1	

14	65.6		65.1	
15	53.0	3.76 (s)	52.7	3.74 (s)
16	167.2		166.7	
17	78.2	5.44 (s)	81.5	4.90 (s)
18	20.8	1.13 (s)	21.5	1.33 (s)
19	16.7	1.21 (s)	65.5	α 4.42 (d, 13.2)
				β 4.76 (d, 13.2)
20	120.4		81.5	
21	140.9	7.41 (br s)	108.9	4.79 (s)
22	109.9	6.37 (br s)	76.3	4.12 (d, 3.6)
23	143.0	7.39 (br s)	112.0	4.96 (d, 3.6)
24	16.9	1.17 (s)	17.0	0.98 (s)
25	116.4	α 4.76 (br s)	30.1	1.29 (s)
		β 4.97 (br s)		
26	22.8	1.77 (s)	21.3	1.18 (s)
3-OMe	52.1	3.72 (s)		
21-OMe			55.1	3.37 (s)
23-OMe			56.5	3.47 (s)

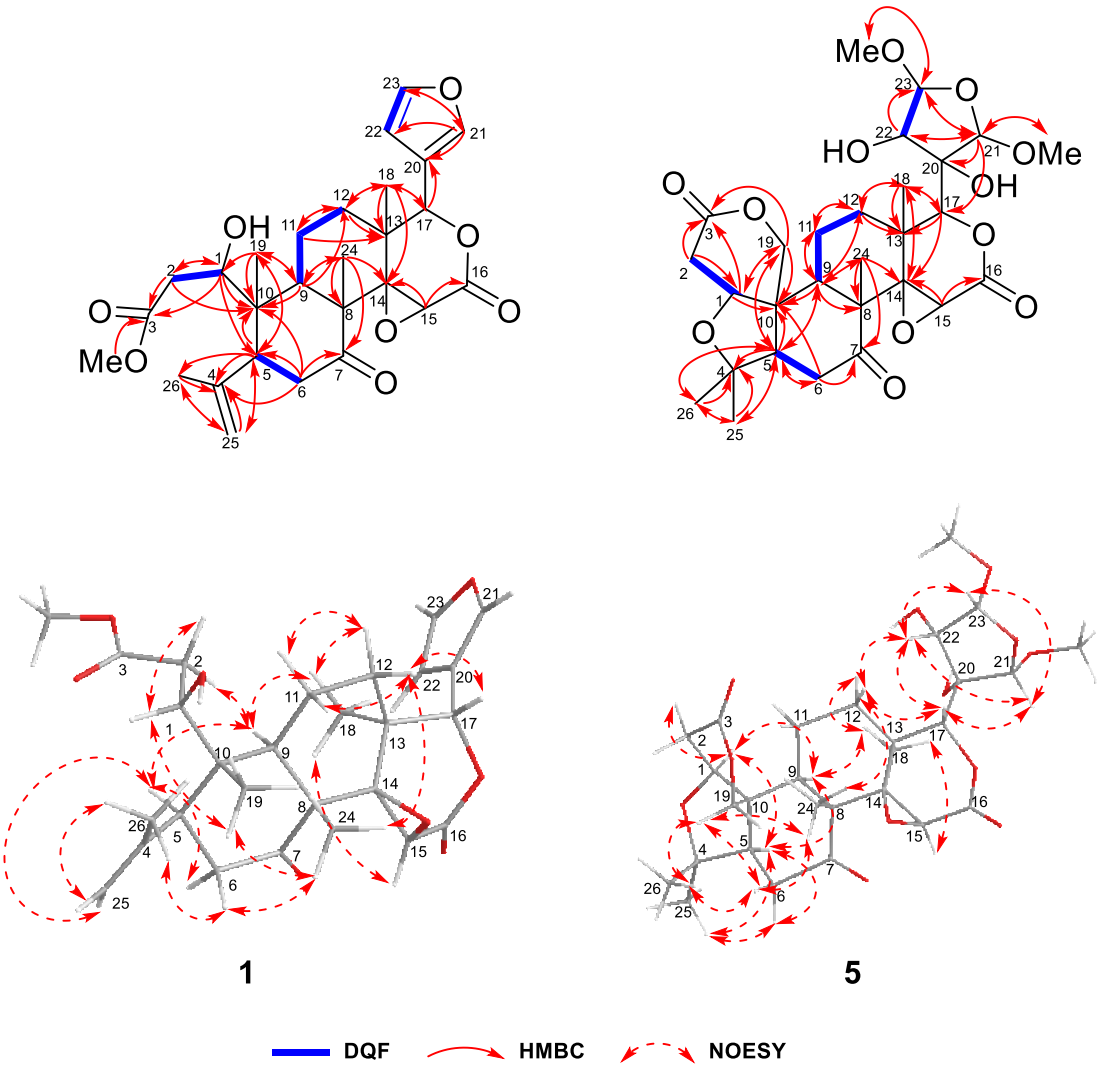

Figure 2. Important 2D NMR and NOESY correlations observed in sphaerocarpain I (**1**) and II (**5**)

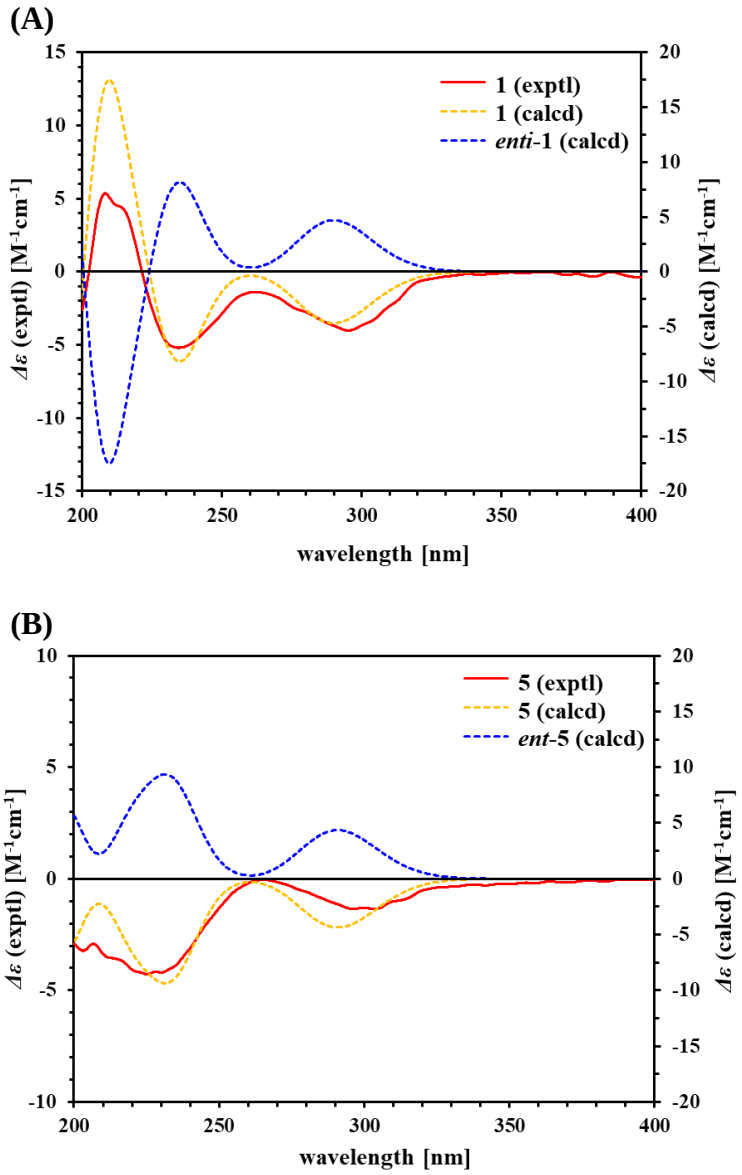

Figure 3. A comparison of the experimental ECD spectra of sphaerocarpain I (**1**, A) and II (**5**, B) with the calculated curves of **1**, **5** and its enantiomer

Evaluation cytotoxicity

The cytotoxicity of isolated compounds (**1**–**7**) was evaluated in human neuroblastoma cells (SH-SY5Y) and human glioma cells (U-251 MG) using adriamycin as a positive control. Cell proliferation was examined by the WST-8 assay. WST-8 is reduced by an NADH-dependent cellular oxidoreductase to form formazan possessing maximum absorbance at 450 nm, which is directly proportional to the number of viable cells.[21] Out of all compounds (30 µM) screened and when compared to control cells, only compound **1**, **3**, **5**, and **7** significantly decreased SH-SY5Y and U-251 MG cell proliferation (Figures 4A, B).

In SH-SY5Y cells, the cell proliferation rates of compounds **1**, **3**, **5**, and **7** were decreased to 70.6 ± 13.0%, 81.9 ± 2.8%, 76.2 ± 7.4%, and 67.0 ± 8.2%, respectively (Figure 4A). In U-251 MG cells, the cell proliferation rate of **1**, **3**, **5**, and **7** were decreased to 75.3 ± 6.1%, 80.4 ± 3.2%, 81.0 ± 5.4%, and 66.4 ±

14.7%, respectively (Figure 4B). The cell proliferation rates of SH-SY5Y and U-251MG cells for adriamycin (1.7 μM) were 17.3 ± 1.65% and 46.6 ± 3.45%, respectively. To date, research has been carried out on the cytotoxicity and mechanism of action of limonoids on various cancer cells.[22,23] These results suggested that compounds **1**, **3**, **5**, and **7** may be useful anticancer agents against brain tumors.

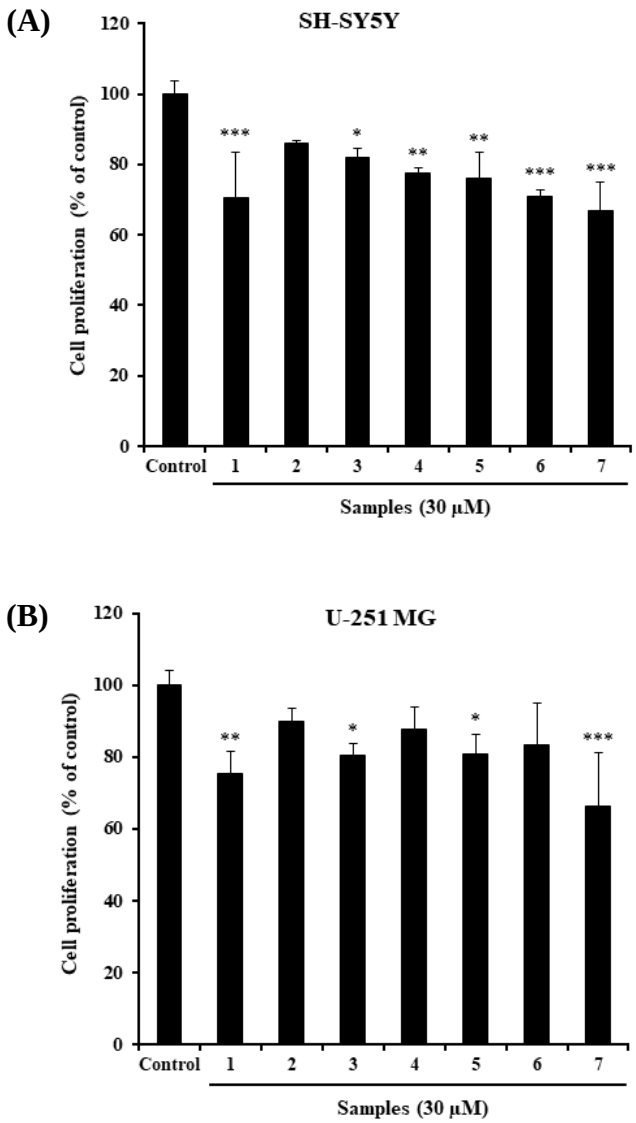

Figure 4. Cytotoxic evaluation of isolated compounds (**1**–**7**) by WST-8 assay

(A and B) SH-SY5Y or U-251 MG cells were seeded into 96-well plates (2,500 cells/well) and cultured at 37 °C. After 24 h, isolated compounds (**1**–**7**) were added to the culture medium at concentrations of 30 μM, and cells were cultured at 37 °C for 24 h. Surviving cells were detected by WST-8 assay. Data reported as the mean ± SD of three independent experiments. Statistical significance was analyzed using the Dunnett's multiple comparisons test ($^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ compared with DMSO-treated cells). Adriamycin was used as a positive control. The cell proliferation rates of SH-SY5Y and U-251MG cells for adriamycin (1.7 μM) were 17.3 ± 1.65% and 46.6 ± 3.45%, respectively.

CONCLUSION

In summary, two new limonoids, sphaerocarpain I (**1**) and sphaerocarpain II (**5**) were isolated from the peels of *C. sphaerocarpa*. The chemical structures of the new compounds were elucidated based on chemical/physicochemical evidences. For sphaerocarpain I and II, the absolute configuration was established by comparison of experimental and predicted ECD data. Compounds **1**, **3**, **5**, and **7** inhibited the cell proliferation of SH-SY5Y cells and U-251 MG cells. Therefore, we concluded that compounds **1**, **3**, **5**, and **7** may be useful anticancer agents against for brain tumors.

EXPERIMENTAL

General experimental procedures

Specific rotations were obtained on a model P-2200 digital polarimeter (l = 5 cm; JASCO, Tokyo, Japan). FTIR spectra were recorded on a JASCO FT/IR-4600 Fourier transform infrared spectrometer. UV spectra were measured using a Shimadzu UV-1850 UV/vis spectrophotometer (Shimadzu, Kyoto, Japan). ECD spectroscopy was recorded on a JASCO J-500CH spectrometer. ESIMS was recorded on a 6470 Triple Quad LC/MS (Agilent Technologies, CA, USA), HRESIMS was recorded on a JMS-T100LP AccuTOFLC-plus 4G (JEOL, Tokyo, Japan). ^{1}H and 2D NMR spectroscopy was recorded on JEOL JNM-ECZ 600R (600 MHz) spectrometers. ^{13}C NMR spectroscopy was recorded on JEOL JNM-ECZ 600R (150 MHz) spectrometers. Normal phase silica gel column chromatography was performed on Wakogel® 60N (FUJIFILM Wako Pure Chemical, Osaka, 63–212 mm). Reversed phase silica gel column chromatography was performed on C_{18}-OPN (Nacalai Tesque, Kyoto, Japan, 75 mm). TLC was performed using TLC plates pre-coated with $60F_{254}$ silica gel (Merck, Darmstadt, Germany; 0.25mm, ordinary phase) and Merck RP-18 $F_{254}S$ silica gel (0.25 mm, reversed phase). HPLC was performed on SPD-10Avp UV-vis detector (Shimadzu, Kyoto, Japan). COSMOSIL $5C_{18}$-AR-II (Nacalai Tesque, 250 × 4.6 mm i.d., 250 × 10 mm i.d.), COSMOSIL Cholester (Nacalai Tesque, 250 × 4.6 mm i.d. and 250 × 10 mm i.d.), and YMC-Triart PFP (YMC, Kyoto, Japan, 250 × 4.6 mm i.d. and 250 × 10 mm i.d.) columns were used for analytical and preparative purposes.

Plant material

C. sphaerocarpa peels were collected in Oita Prefecture, Japan in August 2022 (SOCU-CS-11).

Extraction and isolation

Dried peels of *C. sphaerocarpa* (22.0 kg) were extracted three times with MeOH under reflux for 3 h. Evaporation of the solvent provided a MeOH extract (4155.5 g, 1.89%). Additionally, an EtOAc soluble fraction (207.8 g, 0.094%) and aqueous layer were obtained from the MeOH extract described previously. Aqueous layer was further extracted with *n*-BuOH to give *n*-BuOH (415.3 g, 0.19%) and H_2O (3532.4 g,

1.61%) soluble fraction. The EtOAc soluble fraction was subjected to normal phase silica gel column chromatography [207.8 g, hexacene – $CHCl_3$ (20:1 → 5:1 → 1:1 → 1:5 v/v) → $CHCl_3$ → $CHCl_3$ – MeOH (1:0 → 200:1 → 100:1 → 50:1 → 10:1 → 7:1 → 5:1 → 1:1 v/v)] to give ten fractions [Fr. CSEA1–10], fractions CSEA7 and CSEA8 precipitated **2** (1301.9 mg) and **6** (1175.8 mg) as white crystals. Fraction CSEA8 (17.0 g) was further separated via reversed phase silica gel column chromatography to give twelve fractions [Fr. CSEA8-1–8-12]. Fraction CSEA8-6 (611.4 mg) was purified via HPLC {H_2O–MeCN–AcOH (80:20:0.3, v/v/v)} to give seventeen fractions. Fraction CSEA8-6-15 (19.9 mg) was purified via HPLC {H_2O–MeCN–AcOH (87:13:0.3 v/v/v)} to give **5** (7.1 mg). Fraction CSEA8-10 (947.5 mg) was purified via HPLC {H_2O–MeCN–AcOH (65:35:0.3, v/v/v)} to give sixteen fractions. Fraction CSEA8-10-14 (190.6 mg) was purified via HPLC {H_2O–MeOH–AcOH (45:55:0.3, v/v/v)} to give **3** (79.3 mg). Fraction CSEA8-11 (1433.9 mg) was purified via HPLC {H_2O–MeCN–AcOH (60:40:0.3, v/v/v)} to give sixteen fractions, fraction CSEA8-11-16 was **1** (40.0 mg). Fraction CSEA8-11-12 (65.9 mg) was purified via HPLC {H_2O–MeOH–AcOH (40:60:0.3, v/v/v)} to give **4** (39.0 mg). Fraction CSEA9 (10.3 g) was further separated via reversed phase silica gel column chromatography to give eight fractions [Fr. CSEA9-1–9-8]. Fraction CSEA9-4 (1644.0 mg) was purified via HPLC {H_2O–MeCN–AcOH (75:25:0.3, v/v/v)} to give twenty fractions, fraction CSEA9-4-4 was **7** (123.5 mg).

Sphaerocarpain I (1)

Amorphous white powder; $[\alpha]_D^{25}$ -65.32 (*c* 0.1, MeOH); IR(KBr): ν_{max} 1033, 1346, 2865, 2973 and 3680 cm^{-1}; For ^1H NMR (chloroform-*d*, 600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data, see Table 1; UV (MeOH) λ_{max} 203.5 nm (log ε 4.30) and 280.5 nm (log ε 3.37) ; ECD : $\Delta\varepsilon$ (nm) -5.4 (236), -5.8 (296) (MeOH); ESIMS; *m/z* 487.2 $[M+H]^+$; HRESIMS; *m/z* 509.21769 (Calcd for $C_{27}H_{34}O_8$ $[M+Na]^+$: *m/z* 509.21459).

Sphaerocarpain II (5)

Amorphous white powder; $[\alpha]_D^{25}$ -55.73 (*c* 0.1, MeOH); IR(KBr): ν_{max} 1033, 1346, 2866, 2971 and 3681 cm^{-1}; For ^1H NMR (chloroform-*d*, 600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data, see Table 1; UV (MeOH) λ_{max} 220.5 nm (log ε 4.04) and 279.0 nm (log ε 3.74) ; ECD : $\Delta\varepsilon$ (nm) -59.8 (222), -60.7 (293) (MeOH); ESIMS; *m/z* 583.3 $[M+H_2O]^+$; HRESIMS; *m/z* 589.22891 (Calcd for $C_{28}H_{38}O_{12}$ $[M+Na]^+$: *m/z* 589.22555).

Calculation of theoretical ECD spectra

The initial geometries of the conformers of each 1*S*, 5*S*, 8*R*, 9*R*, 10*S*, 13*S*, 14*R*, 15*S*, 17*S* -**1** and 1*S*, 5*R*, 8*R*, 9*R*, 10*R*, 13*S*, 14*R*, 15*S*, 17*R*, 20*S*, 21*S*, 22*R*, 23*S* -**5** were generated and then geometrically optimized in vacuum by using the Merck molecular force field (MMFF) as implemented in Spartan '18 program.[24] The initial low-energy conformers for each compound with Boltzmann distributions over 1% were further

optimized at the *ω*B97X-D/def2-TZVP level of density functional theory (DFT). In order to confirm none of the conformers showed imaginary frequencies and to obtain the enthalpies (*H*) including the zero-point energy (ZPE) correction, the normal mode analysis was done at the same level.[25,26] The distinctive low-energy conformers for each compound (Figure S3 and S4) with Boltzmann distributions over 1% were subjected to the ECD calculations using time-dependent density functional theory (TD-DFT) at the *ω*B97X-D/ma-TZVPP (the def2-TZVPP basis set with the *s* and *p* diffuse basis functions on non-hydrogen atoms level.[27,28] All of the DFT and TD-DFT calculations were performed using an integral equation formalism polarizable continuum model (IEFPCM) in MeOH using Gaussian 16.[29] The resultant rotatory strengths of the lowest 30 excited states for each conformer were converted into Gaussian-type curves with half-bands using SpecDis v1.71.[30] The calculated ECD spectra were composed after correction based on the Boltzmann distribution of conformers and their relative enthalpies including the ZPE correction (*ΔH*).

Cell culture

SH-SY5Y (European Collection of Authenticated Cell Cultures, Porton Down, UK) cells were cultured in Earl's balanced salt solution (EBSS) medium with 1:1 mixture of Ham's F-12 (FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) and Eagle's minimum essential medium (FUJIFILM Wako Pure Chemical Industries) supplemented with 15% fetal bovine serum (FBS: Wako Pure Chemical Industries), 1% non-essential amino acids solution (FUJIFILM Wako Pure Chemical Industries) and 5% penicillin-streptomycin solution (FUJIFILM Wako Pure Chemical Industries) under a 5% CO_2 atmosphere at 37 ℃.
U-251 MG (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with low glucose (FUJIFILM Wako Pure Chemical Industries) supplemented with 10% FBS (FUJIFILM Wako Pure Chemical Industries) and 5% penicillin-streptomycin solution under a 5% CO_2 atmosphere at 37 ℃.

WST-8 Assay

Cell proliferation was determined using a cell counting kit 8 (CCK-8: Wako Pure Chemical Industries) according to the manufacturer's instructions. Cells were seeded at a density of 2.5×10^3 cells/100 µL per well in 96-well cell culture plates (Coster 3596; Corning, NY, USA). After approximately 24 h, the cells were treated with adriamycin (Wako Pure Chemical Industries) or isolated compounds (30 µM) for 24 h. CCK-8 solution containing WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4disulfophenyl)-2*H*-tetrazolium, monosodium salt] (10 µL) was added to the plates and incubated in a CO_2 incubator for 3 h. The absorbance was measured at 450 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific, MA, USA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.43 software. The statistical analysis was conducted using a Dunnett's test to analyze the differences between the treatment groups. The differences were considered significant when $^{*}P$ <0.05, $^{**}P$ <0.01, or $^{***}P$ <0.001 compared with DMSO-treated cells.

SUPPORTING INFORMATION

Experimental details; ^{1}H, ^{13}C NMR spectra and optimized geometries, the minimum value of frequency, relative enthalpies including the ZPE correction, and Boltzmann distributions of conformers of new compounds.

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