Cytotoxic and Anti-inflammatory Constituents from *Juncus effusus*

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Abstract. A new sesquilignan effususin E (**1**) and a new long chain fatty enamide effususin F (**2**), together with eight known compounds were isolated from the medullae of *Juncus effusus*. The structures of **1** and **2** were elucidated by detailed spectroscopic analysis including ^{1}H and ^{13}C NMR, COSY, HSQC, HMBC, and HRMS, while the structures of the known compounds were deduced from comparison of their spectral data with those in the literature. The new compounds **1** and **2** were evaluated against five human cancer cell lines (SHSY-5Y, SMMC-7721, HepG-2, Hela, and MCF-7) by CCK-8 assay, but only **1** exhibited weak cytotoxic activity against SMMC-7721 with IC_{50} value of 57.5 μM. Inhibitory effects of **1** and **2** on nitric oxide (NO) production in lipopolysaccharide-induced RAW264.7 macrophages were also evaluated, and they were found to exhibit good inhibitory activities with IC_{50} values of 8.59 and 13.73 μM, respectively.
Key words: *Juncus effusus*; chemical constituent; cytotoxic activity; anti-inflammatory activity.

Resumen. Se elucidaron las estructuras de **1** y **2** mediante análisis espectrocópicos de RMN de ^{1}H y ^{13}C, COSY, HSQC, HMBC, y HRMS, mientras que las estructuras de los compuestos conocidos se dedujeron por comparación de sus datos espectrales con los de la literatura. Los nuevos compuestos **1** y **2** fueron evaluados contra cinco líneas celulares cancerígenas humanas (SHSY-5Y, SMMC-7721, HepG-2, Hela, and MCF-7) mediante el ensayo CCK-8. Solamente el compuesto **1** mostró una débil actividad citotóxica contra la línea SMMC-7721 con un valor de IC_{50} de 57.5 μM. También se evaluó el efecto inhibitorio de los compuestos **1** y **2** en la producción de óxido nítrico (NO) en macrófagos RAW264.7 inducidos por lipopolisacáridos. Ambos compuestos mostraron buena actividad inhibitoria con valores de IC_{50} de 8.59 y 13.73 μM, respectivamente.
Palabras clave: *Juncus effusus*; actividad citotóxica; actividad anti-inflamatoria.

Introduction

Juncus effusus L. (Juncaceae), a famous traditional Chinese medicine (TCM) herb, is often found in the wetlands and coastal marshes of South China and used as a sedative, anxiolytic, antipyretic, and detumescence agent [1]. The species is one of the traditional medicines recorded in the Chinese Pharmacopoeia and the medullae can be used in TCM for treatment of various diseases, such as fidgetiness and insomnia, which are anxiety-like psychiatric symptoms [2], and the traditional use of which in China and Japan as antipyretic and antiphlogistic [3], and anticancer agents [4]. Previous investigation of secondary metabolites from *J. effuses* resulted in the isolation of phenanthrene derivatives [5-7], benzocoumarins [8], triterpenes [9, 10], flavonoids [11], phenolic acid derivatives [12], and steroids [13, 14]. As a part of our continuous study aiming at the discovery of new bioactive compounds from *J. effuses* [15], this work reports the isolation and characterization of a new sesquilignan effususin E (**1**) and a new long chain fatty enamide effususin F (**2**), together with eight known compounds 2-*O*-*p*-coumaroyl glyceride (**3**) [12], 2-*O*-feruloyl glyceride (**4**) [16], balanophonin B (**5**) [17], β-sitosterol (**6**), stigmast-4-en-6β-ol-3-one (**7**) [18], 3β-hydroxy-5α,8α-epidiocyergosta-6*E*,22*E*-dienne (**8**) [19], 5-hydroxymethyl-2-furancarboxaldehyde (**9**) [20], and 3-oxo-α-ionol (**10**) [21] (Fig. 1). The cytotoxic

activities of the two new compounds were evaluated against SHSY-5Y, SMMC-7721, HepG-2, Hela, and MCF-7 cancer cell lines using the CCK-8 method [22]. In addition, the new isolates were evaluated for their inhibitory effects on NO production in lipopolysaccharide-activated murine macrophage RAW264.7 cells [23, 24].

Results and Discussion

Effususin E (**1**) was isolated as yellow gum with the formula as $C_{30}H_{32}O_8$ determined by HREIMS at *m/z* 520.2072 [M]+ (calcd 520.2097 for $C_{30}H_{32}O_8$). The ^{1}H NMR (Table 1) spectrum showed three singlets δ_H 3.72s, 3.73s, and 3.80s assignable to three methoxy groups. Three ABX systems were exhibited at δ_H 6.50 (d, *J* 1.8, H-2), 6.64 (d, *J* 8.0, H-5), 6.45 (dd, *J* 8.0, 1.8, H-6), 6.61 (d, *J* 1.8, H-2'), 6.65 (d, *J* 8.0, H-5'), 6.52 (dd, *J* 8.0, 1.8, H-6'), 6.90 (d, *J* 1.7, H-2"), 6.73 (d, *J* 8.1, H-5"), and 6.76 (dd, *J* 8.1, 1.7, H-6"). In the aliphatic region, the proton signals of four -CH and four -CH$_2$ groups appeared as two spin systems corresponding to -CH-CH-CH$_2$- and -CH$_2$-CH-CH-(CH$_2$)$_2$-, which were revealed by the ^{1}H-^{1}H COSY and HMBC experiments (Fig. 2). In addition, the ^{13}C NMR (Table 1) spectrum showed the characteristic signals due to a benzofuran unit resonating at δ_C 87.47d (C-7"), and 55.26d (C-8"),
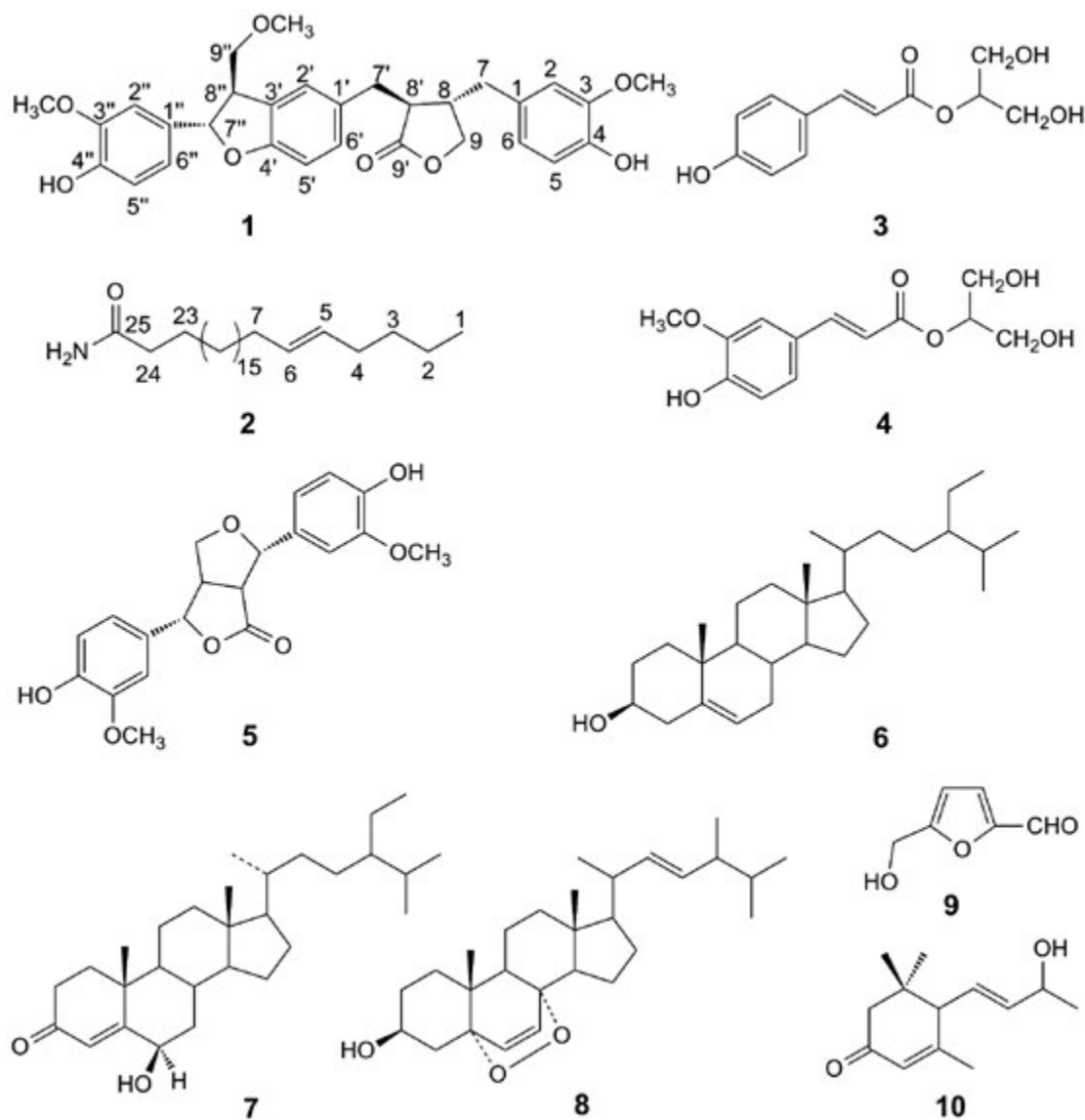
Fig. 1. Chemical structures of **1-10** isolated from the medullae of *Juncus effuses*.

and a furanone ring resonating at δ_C 42.48d (C-8), 72.96t (C-9), 47.71d (C-8'), and 181.76s (C-9'). Therefore compound **1** was sesquilignan. The assigned structure was further confirmed by analysis of HMBC and ^{1}H-^{1}H COSY correlations (Fig. 2). Furthermore, the correlations between H-7" and H-9", H_b-9 and H-8', H_b-9 and H-7, H-7' and H-8, H_a-9 and H-8, and H-2" and H-OCH$_3$ (3.80q), H-2' and H-OCH$_3$ (3.73q), H-2 and H-OCH$_3$ (3.72q) in the NOESY spectrum determined the configuration of **1** and the station of -OCH$_3$ (Fig. 3). The relative configuration of **1** was also assigned by comparison of NMR data and optical rotation with those previously described for compound 10 [25], the optical rotation for which was same as that of **1**, suggesting the same configuration. On the basis of these evidences, the structure of compound **1** was established as (3*S*,4*S*)-4-(4-hydroxy-3-methoxybenzyl)-3-(((2*S*,3*R*)-2-(4-hydroxy-3-methoxyphenyl)-3-(methoxymethyl)-2,3-dihydrobenzofuran-5-yl) methyl)dihydrofuran-2(3*H*)-one, named effususin E in Fig. 1.

Effususin F (**2**) was obtained as white powder. The molecular formula C$_{25}$H$_{49}$ON was determined by HREIMS at *m/z* 379.3821 [M]$^+$ (calcd 379.3814 for C$_{25}$H$_{49}$NO) from the molecular ion peak observed in the EIMS and HREIMS measurement. The IR spectrum showed double absorption bands for -NH$_2$ group at 3396 and 3188 cm^{-1}, and a broad overlap absorption band of a carbonyl group and a double bond at 1647 cm^{-1}. Its ^{1}H NMR showed one methyl at δ_H 0.88 (t, *J* 5.9 Hz, H-1), four methylenes at δ_H 2.00 (m, H-4 and H-7), δ_H 1.62 (m, H-23), δ_H 2.22 (t, *J* 7.6 Hz, H-24), a double bond at δ_H 5.33 (dt, *J* 15.5, 4.8 Hz, H-5), δ_H 5.36 (dt, *J* 15.5, 4.7 Hz, H-6), and two protons of an NH$_2$ group at δ_H 5.75 (s) and 5.49 (s), which is characteristic of the unsaturated long chain fatty amide derivative. The ^{13}C NMR and HREIMS showed that compound **2** has total 25 carbon atoms, and they are all located at the long chain moiety. In HMBC spectrum of **2**, appearance of key correlations between δ_H 2.00 (m, H-4 and H-7) and δ_C 130.03 d (C-5), δ_C
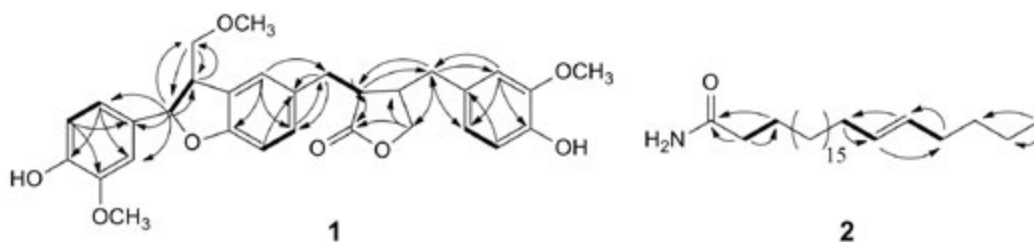
Fig. 2. Key COSY (■) and HMBC (→) correlations of compounds **1** and **2**.

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of **1** (in CD$_3$OD; δ in ppm, J in Hz).

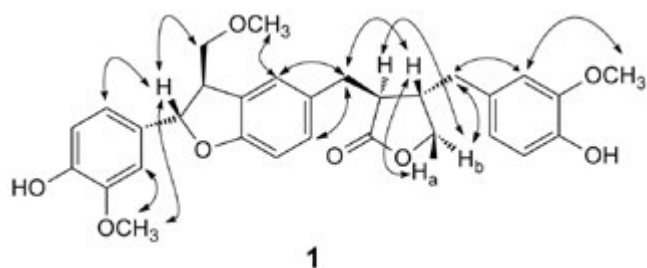| No.	δ_C	δ_H	No.	δ_C	δ_H
1	131.46 (s)		1"	133.72 (s)	
2	113.21 (d)	6.50 (d, J 1.8)	2"	110.94 (d)	6.90 (d, J 1.7)
3	148.96 (s)		3"	149.10 (s)	
4	146.08 (s)		4"	147.23 (s)	
5	116.08 (d)	6.64 (d, J 8.0)	5"	116.15 (d)	6.73 (d, J 8.1)
6	122.20 (d)	6.45 (dd, J 8.0, 1.8)	6"	120.05 (d)	6.76 (dd, J 8.1, 1.7)
7	38.83 (t)	2.46 (m)	7"	87.47 (d)	4.66 (d, J 4.3)
8	42.48 (d)	2.44 (m)	8"	55.26 (d)	3.09 (m)
9	72.96 (t)	a 4.10 (dd, J 8.8, 7.4)	9"	72.55 (t)	4.18 (dd, J 9.0, 6.8)
		b 3.87 (t, J 8.6)			3.78 (m)
1'	130.76 (s)		3-OCH$_3$	56.32 (q)	3.72 (s)
2'	113.84 (d)	6.61 (d, J 1.8)	3"-OCH$_3$	56.40 (q)	3.80 (s)
3'	149.00 (s)		9"-OCH$_3$	56.28 (q)	3.73 (s)
4'	146.27 (s)				
5'	116.07 (d)	6.65 (d, J 8.0)			
6'	123.01 (d)	6.52 (dd, J 8.0, 1.8)			
7'	35.31 (t)	2.79 (ddd, J 21.0, 14.0, 6.2)			
8'	47.71 (d)	2.60 (m)			
9'	181.76 (s)				

Fig. 3. Key ROESY (↔) correlations of compounds **1**.

130.00 d (C-6) suggested the double bond was located at C-5 and C-6, which was further confirmed by the fragment ion peak at m/z 336 [M - C$_3$H$_7$]$^+$ in the EIMS of **2** caused by Mclafferty rearrangement. The *E* configuration for the double bond was suggested by its coupling constant (J 15.5 Hz). The HMBC also showed the correlations of δ_H 0.88 (t, J 5.9 Hz, H-1) and δ_c 22.82t (C-2), 32.04t (C-3), δ_H 1.62 (m, H-23) and δ_c 175.97s

(C-25), δ_H 2.22 (t, 7.6 Hz, H-24) and δ_c 25.67t (C-23), 175.97s (C-25) (Fig. 2). Thus, the structure of **2** was elucidated as (*E*)-pentacos-20-enamide, named effususin F.

Compounds **1** and **2** were evaluated for their *in vitro* cytotoxicities against five human cancer cell lines (SHSY-5Y, SMMC-7721, HepG-2, Hela, and MCF-7) by using CCK-8 assay with paclitaxel as the positive control (Table 2), but only **1** exhibited weak cytotoxicity against SMMC-7721 cell line with IC$_{50}$ value of 57.5 μM. Inhibitory effects of **1** and **2** on NO production in lipopolysaccharide-induced RAW264.7 macrophages were also evaluated, and they were found to exhibit good inhibitory activities with IC$_{50}$ values of 8.59 and 13.73 μM, respectively (Table 2).

Juncusol and dehydrojuncusol, the main phenanthrene in *J. acutus*, were already reported in 2007 to show strong anti-inflammatory activities by effectively reducing the iNOS protein expression in LPS-activated RAW 264.7 cells [3]. Subsequent publication by our group reported that sixteen phenanthrenes,

Table 2. Cytotoxicity and Anti-inflammation Effect of Compounds **1** and **2** (IC$_{50}$ ± S.D. [μM]).

Compound	Cytotoxicity[a]					Anti-inflammation effect[b]
	SHSY-5Y	SMMC-7721	HepG-2	Hela	MCF-7	
1	>100	57.5±2.57	>100	>100	>100	8.59±0.62
2	>100	>100	>100	>100	>100	13.73±1.10
Paclitaxel[c]	>100	0.089±0.02	36.77±1.64	25.79±2.55	28.36±2.67	
Quercetin[c]						6.63±0.28

[a] The Cytotoxicity of the compounds was evaluated by CCK-8 assay against five human cancer cell lines.

[b] The anti-inflammatory effects on nitric oxide production in lipopolysaccharide activated murine macrophage RAW264.7 cells.

[c] Paclitaxel was used as positive controls in Cytotoxicity assay. Quercetin was used as positive control in Anti-inflammation assay.

the main constituents from *J. effusus*, displayed obvious anti-inflammatory effects by inhibition the NO production in LPS-activated murine macrophage RAW 264.7 cells [26]. The phenanthrenoids may be regarded as main anti-inflammatory constituents of the *J.* herbs on dysuria, pharyngitis, aphtha, and trauma infections. This study revealed two nonphenanthrenes, a new sesquilignan (**1**) and a new long chain fatty enamide (**2**), exhibited good anti-inflammatory activities. From the above observations, both phenanthrenes and nonphenanthrenes seem to be all important for the anti-inflammatory of the *J. effusus* herbs. The anti-inflammatory study provided new class of natural anti-inflammatory agents.

Conclusions

A new sesquilignan effususin E (**1**) and a new long chain fatty enamide effususin F (**2**), together with eight known compounds were isolated from the medullae of *J. effusus*. Compounds **4**, **5**, **9**, and **10** were reported from this genus for the first time. The cytotoxic activity of two new isolates **1** and **2** was evaluated against SHSY-5Y, SMMC-7721, HepG-2, Hela, and MCF-7 cancer cell lines, only **1** exhibited weak activity against SMMC-7721 with IC$_{50}$ value of 57.5 μM. Compounds **1** and **2** exhibited good inhibition of NO induced by lipopolysaccharide with IC$_{50}$ values of 8.59 and 13.73 μM, respectively. The results suggested that **1** and **2** were also the active compounds as phenanthrenoid compounds in *J. effuses* medullae on pyretic and phlogistic diseases [5-7]. This study further supported the traditional use of which in China and Japan as antipyretic and antiphlogistic [3].

Experimental

General experimental procedures

UV spectra were obtained using a Shimadzu UV-2401PC spectrophotometer. A Bruker Tensor 27 FT-IR spectrometer was used for scanning infrared (IR) spectroscopy with KBr pellets. 1D and 2D nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-400 and Avance III-600 spectrometer

with tetramethylsilane (TMS) as internal standard. Chemical shifts (δ) are expressed in ppm with reference to the solvent signals. EIMS and HREIMS were performed on a Waters Autospec Premier P776 spectrometer. Optical rotations were obtained using a Jasco P-1020 polarimeter. Column chromatography (CC) was carried out on Sephadex LH-20 gel (25-100 μm, Pharmacia Fine Chemical Co. Ltd.), RP-8 MB (100-40/75 μm, Fuji Silysia Chemical Co. Ltd.), and silica gel (200-300 mesh, Qingdao Haiyang Chemical Co. Ltd., Qingdao, P. R. China). Prep. TLC (PTLC) was precoated with silica-gel GF254 (Qingdao Haiyang Chemical Co. Ltd., Qingdao, P. R. China). Thin layer chromatography (TLC) was carried out on silica gel G precoated plates (Qingdao Haiyang Chemical Co. Ltd., Qingdao, P. R. China), and spots were detected by spraying with 5% H$_2$SO$_4$ in EtOH followed by heating.

Plant material

The medullae of *J. effuses* was purchased in October 2012 in Bozhou, in the Anhui province of China. The species was identified by Prof. Kai-Jin Wang from the School of Life Sciences, Anhui University. A voucher specimen (No. 20121001) has been deposited in our laboratory.

Extraction and isolation

The air-dried medullae of *J. effuses* (6 kg) were powdered and extracted with 95% ethanol (3 × 70 L) at room temperature. The extracts were combined and concentrated under vacuum to give a residue (180 g). The residue was subjected to column chromatography over a silica gel (200-300 mesh), eluted with petroleum ether-acetone (from 10:1 to 1:1) to generate fractions 1-5. Fraction 2 (2.8 g) was subjected to medium pressure silica gel column chromatography eluted with petroleum ether-ethyl ether-acetone (20:2:1) to yield three fractions 2-1 to 2-3. Fraction 2-2 (1.21 g) was subjected to medium pressure silica gel column chromatography eluted with petroleum ether-acetone (30:1) to afford six fractions 2-2-1 to 2-2-6. Fraction 2-2-2 (860 mg) was subjected to silica gel column chromatography eluted with petroleum ether-acetone (40:1) to yield five fractions 2-2-2-1 to 2-2-2-5. Fraction 2-2-2-5 (600 mg) was chromatographed on Sephadex LH-20 (petroleum ether-CHCl$_3$ -MeOH,

10:10:1) to afford compound **6** (300 mg). Fraction 3 (14.0 g) was subjected to chromatography over a silica gel (200-300 mesh) (petroleum ether-EtOAc, 5:1), and then Sephadex LH-20 (EtOH) to give fractions 3-1 to 3-3. Fraction 3-2 (412 mg) was chromatographed on Sephadex LH-20 (EtOH) to afford four fractions 3-2-1 to 3-2-4. Fraction 3-2-2 (260 mg) was chromatographed on Sephadex LH-20 (petroleum ether-CHCl$_3$-MeOH, 5:5:1) to give four fractions 3-2-2-1 to 3-2-2-4. Fraction 3-2-2-2 (180 mg) was subjected to silica gel column chromatography eluted with petroleum ether- ethyl acetate (10:1) to afford **8** (80 mg) and the rest of mixture by preparation of PTLC to obtain compound **7** (25 mg). Fraction 3-3 (75 mg) was repeated chromatographed on Sephadex LH-20 (EtOH) to afford compound **10** (10 mg). Fraction 4 (11 g) was subjected to column chromatography over a silica gel (200-300 mesh), eluted with petroleum ether-EtOAc (5:1) to give fractions 4-1 to 4-5. Fraction 4-4 (300 mg) was repeated chromatographed on Sephadex LH-20 (EtOH) to obtain compound **2** (150 mg). Fraction 5 (20.5 g) was subjected to column chromatography over a silica gel (200-300 mesh), eluted with ether-EtOAc (4:1) to generate fractions 5-1 to 5-4. Fraction 5-2 (3 g) was applied to Sephadex LH-20 eluted with EtOH-H$_2$O (from 20% to 100%) to generate fractions 5-2-1 to 5-2-3. Fraction 5-2-2 (1.2 g) was subjected to silica gel column chromatography (CHCl$_3$) to afford fractions 5-2-2-1 to 5-2-2-6. Fraction 5-2-2-3 (230 mg) was applied to Sephadex LH-20 (EtOH-H$_2$O, 40%) to obtain fractions 5-2-2-3-3 purified over Sephadex LH-20 (EtOH-H$_2$O, 40%) to afford compound **1** (10 mg) and fractions 5-2-2-3-4 purified by silica gel column chromatography (CHCl$_3$) to afford compound **5** (135 mg). Fraction 5-3 (2.8 g) was subjected to medium pressure silica gel column chromatography eluted with petroleum ether-acetone (5:1) to yield five fractions 5-3-1 to 5-3-5. Fraction 5-3-3 (115 mg) was chromatographed on Sephadex LH-20 (petroleum ether-CHCl$_3$-MeOH, 5:1:1) and then by preparation of silica gel column chromatography (petroleum ether-EtOAc, 5:2) to afford compound **9** (20 mg). Fraction 5-3-4 (750 mg) was applied to Sephadex LH-20 (EtOH) to generate fractions 5-3-4-1 to 5-3-4-4. Fraction 5-3-4-2 (120 mg) was chromatographed on RP-8 (EtOH-H$_2$O, 20%-80%) and then by preparation of silica gel column chromatography (CHCl$_3$-MeOH, 25:1) to obtain compounds **3** (50 mg) and **4** (35 mg).

Effususin E (1)

Yellow gum; [α]24D + 24.9 (*c* 1.95, MeOH); UV(MeOH) λ_{max} (log ε) 282 (3.71), 226 (4.11), 203 (4.72) nm; IR (KBr) v_{max} 3441, 3432, 2957, 2925, 2854, 1759, 1631, 1517, 1463, 1452, 1384, 1275, 1238, 1206, 1156, 1124, 1033, 578, 563 cm^{-1}; ^{1}H and ^{13}C NMR data (600 and 150 MHz, CD$_3$OD), see Table 1; ESIMS *m/z* 519 (100) [M-H]$^-$; HREIMS *m/z*, calcd. for C$_{30}$H$_{32}$O$_8$ [M]$^+$: 520.2097, found: 520.2072.

Effususin F (2)

White powder; [α]24D -11.0 (*c* 0.21, MeOH); UV(MeOH) λ_{max} (log ε) 201 (3.46) nm; IR (KBr) v_{max} 3396, 3188, 3011, 2955, 2921, 2849, 1647, 1469, 1419, 1378, 1343, 1325, 1301, 1273, 1246, 1216, 1119, 722, 647 cm^{-1}; ^{1}H NMR (CDCl$_3$, 400 MHz) δ 0.88 (3H, t, $J_{1,2}$ = 5.9 Hz, H-1), 1.20-1.38 (34H, m, H-2, 3, 8-22), 2.00 (4H, m, H-4, 7), 5.33 (1H, dt, $J_{5,6}$ = 15.5, $J_{4,5}$ = 4.8 Hz, H-5), 5.36 (1H, dt, $J_{5,6}$ = 15.5, $J_{6,7}$ = 4.7 Hz, H-6), 1.62 (2H, m, H-23), 2.22 (2H, t, $J_{23,24}$ = 7.6 Hz, H-24), 5.49 (1H, s, Ha-N) and 5.75 (1H, s, Hb-N); ^{13}C NMR (CDCl$_3$, 100 MHz) δ 14.27q (C-1), 22.82t (C-2), 32.04t (C-3), 27.33t (C-4, C-7), 130.03d (C-5), 130.00d (C-6), 29.37-29.90t (C-8-C-22), 25.67t (C-23), 36.10t (C-24), 175.97s (C-25); HREIMS *m/z*, calcd. for C$_{25}$H$_{49}$NO [M]$^+$: 379.3814, found: 379.3821.

Cytotoxicity assay

The effects of compounds on cell proliferation and the viability of the SHSY-5Y, SMMC-7721, HepG-2, Hela, and MCF-7 cells were measured by using Cell Counting Kit-8 (CCK-8, Best Bio China) assay. Cells were seeded in 96-well plates at 5.0×10^3 cells/well in a final volume of 100 μl and treated with different concentrations (6.25, 12.5, 25, 50, 100 μM) of compounds or without tested compounds at given concentrations. After treatment for 48 h, CCK-8 (10 μl) was added to each well containing 100 μl culture medium, and the plate was incubated for 4 h at 37 °C. The absorbance of each well was measured at 450 nm with a microplate reader. The experiments were performed in triplicate, and the data are expressed as the means ± SD of three independent experiments.

Anti-inflammatory assay

The cytotoxicity of the tested compounds was evaluated by MTT (Sigma, USA) assays. RAW264.7 cells at 5×10^3 per well were seeded in 96-well plates. After 12 h incubation, the cells were treated with or without tested compounds for 24 h. Then, 20 μl MTT solutions (5 mg ml^{-1}) were added to each well for another 4 h and the resulting crystals were dissolved in dimethyl sulfoxide (DMSO). The optical density was measured at 490 nm. The cytotoxicity was calculated from the plotted results using untreated cells at 100%.

The nitrite concentration was measured in supernatants of cultured RAW264.7 cells according to the Griess reaction as an indicator of NO production using an NO assay kit (Beyotime Insititute of Biotechnolgy, China). Cells were seeded in 96-well culture plates for 12 h incubation, and then pretreated with tested compounds (3 to 50 μM) and LPS (Sigma, USA, 1 μg mL^{-1} in PBS) for 24 h. 50 μl of cell-free supernatant was mixed with 100 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) and incubated at room temperature for 5 min. The concentration of nitrite was measured at 540 nm. Sodium nitrite (NaNO$_2$) was used as a standard curve. The experiments were performed in triplicate, and the data are expressed as the means ± SD of three independent experiments.

Statistics

The data presented are means ± SD of three determinations.

Acknowledgements

References

1. Su, X. H.; Yuan, Z. P.; Li, C. Y.; Zhong, Y. J.; Du, H. J.; Wen, Y.-Y.; Li, Y. F.; Liang, B. *Planta Med*. **2013**, *79*, 1447-1452.
2. Wang, Y. G.; Wang, Y. L.; Zhai, H. F.; Liao, Y. J.; Zhang, B.; Huang, J. M. *Nat. Prod. Res.* **2012**, *26*, 1234-1239.
3. Beherya, F. A. A.; Naeema, Z. E. M.; Maatooqa, G. T.; Amera, M. M. A.; Wen, Z. H.; Sheu, J. H.; Ahmedb, A. F. *Chem. Pharm. Bull*. **2007**, *55*, 1264-1266.
4. Jin, D. Z.; Min, Z. D.; Kong, L. Y.; Qiu, C. Y. *World Phytomed.* **1995**, *10*, 208-211.
5. DellaGreca, M.; Fiorentino, A.; Isidori, M.; Lavorgna, M., Monaco, P.; Previtera, L.; Zarrelli, A. *Phytochemistry* **2002**, *60*, 633-638.
6. DellaGreca, M.; Isidori, M.; Lavorgna, M.; Monaco, P.; Previtera, L.; Zarrelli, A. *J. Chem. Ecol.* **2004**, *30*, 867-879.
7. DellaGreca, M.; Previtera, L.; Zarrelli, A. *Nat. Prod. Res.* **2005**, *19*, 69-74.
8. DellaGreca, M.; Fiorentino, A.; Isidori, M.; Previtera, L.; Fabio, T.; Zarrellia, A. *Tetrahedron* **2003**, *59*, 4821-4825.
9. Corsaro, M. M.; Dellagreca, M.; Fiorentino, A.; Monaco, P.; Previtera, L. *Phytochemistry* **1994**, *37*, 515-519.
10. Dellagreca, M.; Fiorentino, A.; Monaco, P.; Previtera, L. *Phytochemistry* **1994**, *35*, 1017-1022.
11. Park, S.; Yang, S.; Ahn, D.; Yang, J. H.; Kim, D. K. *J. Korean Soc. Appl. Biol. Chem.* **2011**, *54*, 685-692.
12. Dellagreca, M.; Fiorentino, A.; Monaco, P.; Previtera, L.; Sorrentino, M. *Nat. Prod. Lett.* **1998**, *12*, 263-270.
13. Yang, G. Z.; Li, H. X.; Song, F. J.; Chen, Y. *Helv. Chim. Acta* **2007**, *90*, 1289-1295.
14. Wang, J.; Liu, J.; Wen, Q. F.; Li, C. Y.; Li, Y. F.; Lian, B.; Tang, H.; Yao, T. *Biochem. Syst. Ecol.* **2010**, *38*, 1039-1041.
15. Ma, W.; Liu, F.; Ding, Y. Y.; Zhang, Y.; Li, N. *Fitoterapia* **2015**, *105*, 83-88.
16. Rocha, F. D.; Yano, M.; da Cunha, M. R.; Gabriel, F. T.; Cordeiro, R. S. B.; Menezes, F. S.; Kaplan, M. A. C. *Rev. Bras. Farmacogn. Braz. J. Pharmacogn.* **2010**, *20*, 240-245.
17. Ma, G. X.; Wu, H. F.; Yuan, J. Q.; Wu, L. Z.; Zheng, Q. X.; Sun, Z. C.; Fan, X. R.; Wei, H.; Yang, J. S.; Xu, X. D. *Phytochem. Lett.* **2013**, *6*, 152-155.
18. Dellagreca, M.; Monaco, P.; Previtera, L. *J. Nat. Prod.* **1990**, *53*, 1430-1435.
19. Feng, L.; Shen, Y. M. *Nat. Prod. Res. Dev.* **2005**, *17*, 294-297.
20. Zuo, W. J.; Jin, P. F.; Dong, W. H.; Dai, H. F.; Mei, W. L. *Chin. J. Nat. Med.* **2014**, *12*, 151-153.
21. Aasen, A. J.; Kilmand, B.; Enzell, C. R. *Acta. Chem. Scand*. **1971**, *25*, 1481-1482.
22. Li, M. Y.; Yu, X. J.; Guo, H.; Sun, L. M.; Wang, A. J.; Liu, Q. J.; Wang, X. W.; Li, J. S. *Tumor Biol.* **2014**, *35*, 2461-2471.
23. Zhao, J. H.; Shen, T.; Yang, X.; Zhao, H.; Li, X.; Xie, W. D. *Arch. Pharm. Res.* **2012**, *35*, 1153-1158.
24. Liu, Z. G.; Bai J, Z. L.; D. Meng, L.; Li, N., Pei, Y. H.; Zhao, F.; Hua, H. M. *J. Nat. Prod.* **2014**, *77*, 792-799.
25. Xu, J. J.; Huang, H. Q.; Zeng, G. Z.; Tan, N. H. *Fitoterapia* **2012**, *83*, 1125-1130.
26. Ma, W.; Zhang, Y.; Ding, Y. Y.; Liu, F.; Li, N. *Arch. Pharm. Res*. **2016**, *39*, 154-160.