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Monoterpenoid indole alkaloids from the stems of *Kopsia officinalis*

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ABSTRACT

Five new indole alkaloids, kopsiofficines H-L (**1–5**), along with fourteen known alkaloids (**6–19**) were isolated from the stems of *Kopsia officinalis*. Their structures were elucidated by extensive NMR, mass spectroscopic analyses and comparison to the reported data. All the isolated compounds were evaluated their anti-inflammatory activities by inhibiting IL-1β, PGE2 and TNF-α secretion in lipopolysaccharide (LPS)-activated RAW264.7 cells. Compounds **2**, **3**, **6**, **7**, **11**, **12**, **15**, and **17** show significant anti-inflammatory activities. These results demonstrate pharmacodynamic substance basis of these folkloric claims.

1. Introduction

Monoterpenoid indole alkaloids (MIAs) always fascinated pharmacists and chemists for their complicated architectural as well as potent biological activities [1–8]. MIAs have long been one of the key directions in global medicinal chemistry research. Many of them, such as vinblastine [9], camptothecin [10], strychnine [11] have also been used as clinical first-line drugs. Previous studies of the genus Kopsia have been proven to be rich resources of MIAs with novel and complex carbon skeletons [12–16]. Many of these alkaloids have been reported to possess cytotoxic effects [17], antibacterial and antifungal [18], antitumor [19,20], inhibitory effects on α-glucosidase [21]. As one of the good resources of MIAs, *K. officinalis* was cultivated in Yunnan province, China, which is traditionally used for the treatment of pharyngitis, rheumatoid arthritis, tonsillitis and edema [22]. To demonstrate these folkloric claims from this plant, the chemical studies on its alkaloidal constituents have been investigated. Our previous studies reported the isolation and anti-inflammatory activities of kopsiofficines A-G from *K. officinalis* [23]. As part of our ongoing research on bioactive indole alkaloids has led to the isolated of five new indole alkaloids, kopsiofficines H-L (**1–5**), and fourteen known alkaloids (**6–19**), namely (+)-*O*-methyleburnamine (**6**) [24], (−)-*O*-methylisoeburnamine (**7**) [25], 16-isoeburnamine (**8**) [26], 20-oxoeburnamenine (**9**) [27], (−)-19(*R*)-hydroxyeburnamenine (**10**) [28], methyl 11, 12-methylenedioxychanof ruticosinate (**11**) [29], methyl demethoxycarbonylcha nofruticosinate (**12**) [30], methyl chanofruticosinate (**13**) [30], methyl N-(decarbomethoxy)-11, 12-(methylenedioxy) chanofruticosinate (**14**) [31], methyl 12-methoxychanofruticos inate (**15**) [32], *O*-methylleuconolamm (**16**) [33], leuconodine D (**17**) [34], oxayohimban-16-carboxylic acid (**18**) [35], 19, 20-dihydroisositsirikine (**19**) [36]. This paper described the isolated, structure elucidation of the indole alkaloids, and tested for their anti-inflammatory activities toward LPS-induced RAW264.7 cells.

2. Experimental

2.1. General experimental procedures

Optical rotations were determined using a JASCO P-1020 polarimeter and an Autopol IV polarimeter (Rudolph, Hackettstown, USA). UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer (Shimadzu Corp., Kyoto, Japan). IR spectra were obtained on a Bruker FT-IR Tensor 27 and Nicolet iS10 spectrophotometer with KBr pellets. UPLC-IT-TOF were performed on Agilent 1290 UPLC/6540 Q-TOF mass spectrometer. 1D and 2D NMR spectra were obtained on a Bruker DRX-400 and DRX-500 spectrometers with tetramethylsilane as an internal standard. Coupling constants were expressed in Hz and chemical shifts (δ) were given on a ppm scale. The extracts were

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chromatographed on silica gel (200–300 mesh, Qingdao Marien Chemical Ltd., People's Republic of China), Sephadex LH-20 (Pharmacia Fine Chemical Co., Ltd., Sweden), and RP-18 gel (20–45 μm, Fuji Silysia Chemical Ltd., Japan). Fractions were monitored by TLC on silica gel plates (GF254, Qing-dao Haiyang Chemical Co., Ltd.), and spots were visualized with iodine vapor and Dragendorff's reagent. High performance liquid chromatography (HPLC) was performed using a Waters 600 pump (Waters Corp., Milford, MA, USA).

2.2. Plant material

The stems of *K. officinalis* were collected from Xishuangbanna, Yunnan Province, People's Republic of China, and identified by Dr. Jing-Yun Cui. A voucher specimen (No. Cui121113) has been deposited in the Kunming Institute of Botany, Chinese Academy of Science.

2.3. Extraction and isolation

The air dried and powdered stems of *K. officinalis* (13 kg) were extracted with 90% MeOH (30 L, 48 h) four times at room temperature. After filtration, all the solvent was evaporated under reduced pressure at 55 °C to yield the extract. The crude extract was acidified with 0.5% aqueous hydrochloric acid (v/v), and then filtered to give nonalkaloid part and acidic aqueous. The acidic water-soluble material, adjusted to PH = 9–10 with 10% ammonia solution, was extracted with EtOAc to give an alkaloidal extract (98 g). The extract was subjected to CC over silica gel (200–300 mesh, 3.0 kg) and eluted with a $CHCl_3$-MeOH gradient (1:0–0:1, v/v) to afford six fractions (Fr.A-Fr.F). Fr.A (1.5 g) was separated by silica gel CC, using a petroleum ether-acetone gradient eluent (4:1, v/v) to yield alkaloids **12** (16 mg), **15** (31 mg) and subfraction A1, which was purified on a Sephadex LH-20 ($CHCl_3$-MeOH, 1:1, v/v) to yield **18** (38 mg). Fr.B (2.7 g) was subjected to MPLC with RP-18 CC (MeOH-H_2O, 40:60–70:30, v/v), and then purified by silica gel CC (petroleum ether-EtOAc, 2:1, v/v) to yield **13** (14.4 mg). Fr.C (18 g) was purified by C_{18} preparative column with a MeOH-H_2O gradient eluent (20:80–80:20, v/v) to produce subfractions C1-C3. Subfraction C1 was further separated on a semipreparative C_{18} HPLC column using a gradient of MeOH-H_2O (70:30, v/v) to yield **17** (57 mg), **14** (28 mg). Subfraction C3 was further separated by silica gel CC with $CHCl_3$-Me_2CO eluent (10:1–3:1, v/v) to obtain **1** (12.3 mg), **6** (56 mg), **11** (18.5 mg) and a mixture, which was chromatographed on a Sephadex LH-20 using a gradient MeOH to product **7** (25 mg). Fr.D (32.8 g) was subjected to MPLC with RP-18 CC (MeOH-H_2O, 30:70–80:20, v/v) to give subfractions D1-D2. Subfraction D1 was subjected to Sephadex LH-20 CC ($CHCl_3$-MeOH, 1:1, v/v), to yield **16** (48 mg). Subfraction D2 was further separated by silica gel CC ($CHCl_3$-MeOH, 12:1, 3:1, v/v) to afford **19** (56.9 mg) and **2** (8 mg). Fr.E (17 g) was separated by silica gel CC ($CHCl_3$-MeOH, 8:1, 2:1, v/v), to yield **4** (4.2 mg), and a mixture. The latter was purified by Sephadex LH-20 (MeOH), to give **3** (1.9 mg) and **8** (22 mg). Fr.F (15 g) was separated by RP-18 CC (MeOH-H_2O, 20:80–100:0) to yield **5** (2 mg), **10** (35 mg) and a mixture, which was further separated on a preparative C_{18} HPLC column with a gradient MeOH-H_2O (45:55–80:20, v/v) to afford **9** (27 mg).

2.3.1. Kopsiofficine H (1)

White amorphous powder; $[a]_D^{22.4}$ -83.4 (c 0.15, CH_3OH); UV (CH_3OH) λ_{max} (log ε): 207 (4.20), 227 (4.48), 275 (4.09) nm; IR (KBr) V_{max} 3440, 2925, 2851, 1703, 1619, 1457, 1440, 1363, 1306, 1213, 1073, and 741 cm^{-1}; ^1H (400 MHz) and ^{13}C NMR (100 MHz) data ($CDCl_3$), see Tables 1 and 2; HRESIMS *m/z* 325.1911 (calcd for $C_{20}H_{25}N_2O_2$ $[M + H]^+$, 325.1909).

2.3.2. Kopsiofficine I (2)

White amorphous powder; $[a]_D^{19.4}$ -62.5 (c 0.10, CH_3OH); UV (CH_3OH) λ_{max} (log ε): 242 (3.83), 295 (3.51) nm; IR (KBr) V_{max} 3437, 2929, 2852, 1703, 1617, 1456, 1353,1186, 1085, and 744 cm^{-1}; ^1H (500 MHz) and ^{13}C NMR (125 MHz) data ($CDCl_3$), see Tables 1 and 2; HRESIMS *m/z* 325.1911 (calcd for $C_{20}H_{25}N_2O_2$ $[M + H]^+$, 325.1913).

2.3.3. Kopsiofficine J (3)

White amorphous powder; $[a]_D^{22.4}$ +17.3 (c 0.18, CH_3OH); UV (CH_3OH) λ_{max} (log ε): 207 (4.45), 224 (3.97), 275 (3.30) nm; IR (KBr) V_{max} 3441, 2929, 2855, 1632, 1456, 1087, and 744 cm^{-1}; ^1H (500 MHz) and ^{13}C NMR (125 MHz) data (DMSO-d_6), see Tables 1 and 2; HRESIMS *m/z* 327.2067 (calcd for $C_{20}H_{27}N_2O_6$ $[M + H]^+$, 327.2065).

2.3.4. Kopsiofficine K (4)

Amorphous powder; $[a]_D^{23.2}$ +17.2 (c 0.11, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε): 202 (2.86), 233 (2.72), 377 (1.68) nm; IR (KBr) V_{max} 3442, 2920, 1637, 1465, 1342, 1027 and 891 cm^{-1}; ^1H (500 MHz) and ^{13}C NMR (125 MHz) data ($CDCl_3$), see Tables 1 and 2; HRESIMS *m/z* 309.1599 (calcd for $C_{19}H_{21}N_2O_2$ $[M + H]^+$, 309.1598).

2.3.5. Kopsiofficine L (5)

Amorphous powder; $[a]_D^{22.4}$ +14.8 (c 0.12, CH_3OH); UV (CH_3OH) λ_{max} (log ε): 213 (4.37); 289 (3.33); 344 (2.83) nm; IR (KBr) V_{max} 3440, 2929, 1751, 1626, 1456, 1243, 1087, and 878 cm^{-1}; ^1H (500 MHz) and ^{13}C NMR (125 MHz) data (DMSO-d_6), see Tables 1 and 2; HRESIMS m/z 383.1965 (calcd for $C_{22}H_{27}N_2O_4$ $[M + H]^+$, 383.1963).

2.4. Anti-inflammatory assay

The macrophage RAW 264.7 cells were cultivated in DMEM in a humidified atmosphere with 5% CO_2 at 37 °C, with 2×10^4 cells/well grown in 96-well plates and incubated for 24 h. After that, the same concentration of alkaloids (5 μg/mL) were added to each well for 2 h, and then stimulated with lipopolysaccharide (LPS) (20 μM) for 24 h, with dexamethasone (10 μg/mL) as a positive control. Cell supernatants were collected and then tested for interleukin (IL)-1β, prostaglandin E2 (PGE2), and tumor necrosis factor-α (TNF-α) by ELISA assay kits (Wuhan Huamei Biotechnology, Wuhan, China) according to the manufacturers' protocols. All experiments were performed within the concentration without cytotoxicity. Results are expressed as the mean ± SEM. Statistical significance was determined using the two tailed Student's *t*-test, with $^{**}p < 0.01$ or $^{*}p < 0.05$ accepted as significant.

3. Results and discussion

Compound **1** was obtained as white amorphous powder and showed positive reaction to the Dragendorff's reagent (BiI_3.KI) on TLC, characteristic for an alkaloid. The molecular formula of **1** was established as $C_{20}H_{24}N_2O_2$ by HRESIMS (*m/z* 325.1909 $[M + H]^+$) and ^{13}C NMR spectral data (Table 2), which was indicated ten indices of hydrogen deficiency. The UV spectrum showed absorption maxima characteristic of an indole chromophore (207, 227 and 275 nm) [37]. The IR spectrum suggested the presence of a -NH (3440 cm^{-1}), a carbonyl group (1703 cm^{-1}) and an aromatic ring (1619 and 1457 cm^{-1}). In the ^1H NMR spectrum, four aromatic protons at δ_H 7.41 (d, J = 7.8 Hz, H-9), 7.08 (t, J = 7.8 Hz, H-10), 7.13 (t, J = 7.8 Hz, H-11), and 7.20 (d, J = 7.8 Hz, H-12) were assigned to be the unsubstituted indole moiety in **1** (Fig. 1). A singlet at δ_H 4.72 (1H, s) was the characteristic resonance for H-21 linkage to a nitrogen group. Two singlets at δ_H 3.45 (3H, s, 16-OCH_3) and 2.30 (3H, s, Me-18) were assigned to a methoxyl group and a methyl connected to carbonyl groups, respectively. In the ^{13}C NMR spectrum, 20 carbons were observed (Table 2), including one methyl (δ_C 25.7), one methoxy (δ_C 55.9), six methylenes (δ_C 16.8, 23.1, 25.4, 34.0, 44.4, 51.1), six methines (δ_C 55.0, 82.8, 110.4, 118.3, 120.2, 121.3), and six quaternary carbons (δ_C 49.6, 106.2, 128.7, 130.1, 135.4,

Table 1
^{1}H NMR data of **1-5**[b] (δ in ppm and J in Hz).

Entry	1	2	3	4	5
3a	2.61 (m)	2.36 (td, 11.3, 3.8)	2.51 (m)	3.45 (m)	2.87 (dd, 11.1, 3.0)
3b	2.54[a]	2.52 (m)	2.43 (m)	3.14 (m)	
5a	3.31 (dd, 9.5, 3.6)	3.35 (m)	3.12 (m)	2.57 (m)	3.28 (m)
5b		2.54 (m)		3.47 (m)	2.93 (dd, 9.5, 5.1)
6a	2.93 (m)	2.98 (m)	2.84 (m)	2.38 (m)	2.18 (m)
6b	2.54[a]		2.41 (m)	2.02 (m)	
9	7.41 (d, 7.8)	7.50 (d, 7.3)	7.39 (d, 8.0)	7.64 (d, 7.7)	6.78 (d, 8.1)
10	7.08 (t, 7.8)	7.17 (t, 7.3)	7.04 (t, 8.0)	7.02 (t, 7.7)	6.29 (d, 8.1)
11	7.13 (t, 7.8)	7.21 (t, 7.3)	7.08 (t, 8.0)	7.54 (t, 7.7)	
12	7.20 (d, 7.8)	7.56 (d, 7.3)	7.44 (d, 8.0)	7.16 (d, 7.7)	
14a	1.46 (dt, 13.5, 3.2)	1.41 (m)	0.66 (td, 13.4, 4.1)	5.97 (ddd, 9.7, 4.8, 1.4)	1.77 (m)
14b	1.39 (t, 13.5)		1.76 (m)		1.10 (m)
15a	2.01 (m)	0.97 (td, 13.1, 4.5)	1.79 (m)	5.43 (d, 9.7)	1.29 (m)
15b	1.85 (dd, 14.1, 3.8)	2.07 (m)	1.20 (m)		1.23 (m)
16	5.42 (d, 4.1)	5.59 (dd, 9.0, 5.5)	5.44 (dd, 9.2, 5.3)	5.64 (d, 5.6)	
17a	2.23 (dd, 14.6, 1.5)	2.17 (dd, 7.7, 3.9)	2.19 (m)	2.07 (m)	1.41 (m)
17b	1.89 (dd, 14.6, 4.1)	2.02 (m)	1.69 (dd, 13.6, 9.3)		1.25[a]
18a	2.30 (s)	2.42 (s)	1.15 (d, 6.4)	0.87 (d, 6.6)	1.87 (dd, 11.1, 3.0)
18b					1.37 (m)
19a			4.24 (q, 6.4)	3.80 (q, 6.6)	1.24[a]
19b					2.09 (m)
21	4.72 (s)	4.80 (s)	4.05 (s)	2.89 (s)	3.12 (d, 1.3)
11-OCH_3					3.71 (s)
12-OCH_3					3.61 (s)
16-OCH_3	3.45 (s)	3.33 (s)	3.32 (s)		
OH					5.42 (s)
NH					4.95 (s)

[a] Overlap.
[b] Compounds **1, 2** and **4** were measured in $CDCl_3$, **3** and **5** were measured in DMSO-d_6.

Table 2
^{13}C NMR data of **1-5.**[a]

Entry	1	2	3	4	5
2	130.1, s	131.9, s	132.0, s	71.8, s	71.0, s
3	44.4, t	44.0, t	43.6, t	52.0, t	46.2, t
5	51.1, t	51.2, t	50.0, t	51.5, t	52.6, t
6	16.8, t	16.8, t	16.3, t	40.1, t	53.7, d
7	106.2, s	106.7, s	104.7, s	206.9, s	59.5, s
8	128.7, s	128.6, s	128.2, s	125.2, s	128.8, s
9	118.3, d	118.1, d	117.7, d	124.8, d	117.0, d
10	121.3, d	120.3, d	119.7, d	122.3, d	102.7, d
11	120.2, d	121.6, d	120.9, d	136.9, d	152.9, s
12	110.4, d	111.7, d	111.7, d	116.2, d	134.0, s
13	135.4, s	136.6, s	136.0, s	162.1, s	144.2, s
14	23.1, t	22.6, t	23.1, t	127.6, d	14.7, t
15	25.4, t	25.0, t	20.0, t	130.0, d	35.5, t
16	82.8, d	81.9, d	82.1, d	87.9, d	81.1, s
17	34.0, t	35.7, t	31.6, t	39.8, t	33.5, t
18	25.7, q	25.6, q	17.6, q	15.5, q	20.4, t
19	211.3, s	210.5, s	67.4, d	81.3, d	42.3, t
20	49.6, s	50.9, s	39.7, s	47.2, s	32.9, s
21	55.0, d	54.9, d	56.1, d	64.2, d	69.1, d
22					215.4, s
16-OCH_3	55.9, q	50.7, q	51.7, q		
11-OCH_3					55.8, q
12-OCH_3					59.7, q

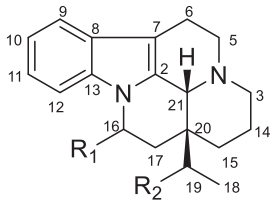
[a] Compounds **1, 2** and **4** were measured in $CDCl_3$, **3** and **5** were measured in DMSO-d_6.

211.3). Then, compound **1** was identified preferentially as an eburnan-type alkaloid with a methoxyl group and a carbonyl group [28]. The NMR spectral data of compound **1** was similar to those of *O*-methyleburnamine [38] except that one more carbonyl group substituted at C-19, which was supported by the HMBC correlations of δ_H 4.72 (1H, s, H-21), 2.30 (3H, s, Me-18), 2.23 (1H, dd, J = 14.6, 1.5 Hz, H-17a), 1.89 (1H, dd, J = 14.6, 4.1 Hz, H-17b), 2.01 (1H, m, H-15a), and 1.85 (1H, dd, J = 14.1, 3.8 Hz, H-15b) with δ_C 211.3 (s, C-19). Furthermore, the methoxyl group was placed at C-16, as evidenced by HMBC correlations of δ_H 3.45 (3H, s, 16-OCH_3) with δ_C 82.8 (d, C-16), and of δ_H 5.42 (1H, d, J = 4.1 Hz, H-16) with δ_C 55.9 (q, 16-OCH_3), 130.1 (s, C-2), and 49.6 (s, C-20) (Fig. 2).

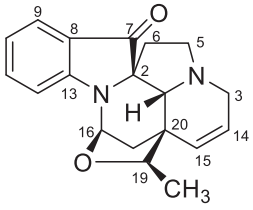
ROESY correlations of δ_H 4.72 (1H, s, H-21)/2.30 (3H, s, Me-18)/5.42 (1H, d, J = 4.1 Hz, H-16), indicated H-21, Me-18, and H-16 at the same side. Biogenetically, like the other eburnane alkaloids having the 20β, 21β orientations (or 20R, 21R configurations) [e.g., (+)-eburnamonine, (+)-isoeburnamine, (+)-*O*-methyleburnamine (**6**), (−)-*O*-methylisoeburnamine (**7**), 16-isoeburnamine (**8**), 20-oxoeburnamenine (**9**) and (−)-19(*R*)-hydroxyeburnamenine (**10**)] in this plant, the configurations at the C-20 and C-21 of compound **1** were possessed 20β, 21β orientations [27–29,38,39]. Then, the configuration of H-16 was assigned to be β orientation and the 16-OCH_3 was assigned to be α orientation (Fig. 2), which was also supported by the peak shape and coupling constant of H-16 (J = 4.1 Hz) [40,41]. So compound **1** is therefore readily identified as (−)-19-oxo-*O*-methylisoeburnamine, and named kopsiofficine H.

The HRESIMS of **2** at m/z 325.1913 $[M + H]^+$ indicated a molecular formula of $C_{20}H_{24}N_2O_2$, which was confirmed by the analysis of its ^{13}C NMR and DEPT spectra, identical to that of compound **1**. They have almost the same physical data in the UV and IR spectra, indicating existence of the same functional groups. Detailed analysis of 1D and 2D NMR spectral data (Tables 1 and 2) suggested that the two compounds were isomers. The key difference was that the configuration of H-16 in **2** was α orientation, as supported by peak shape and coupling constants of δ_H 5.59 (1H, dd, J = 9.0, 5.5 Hz, H-16) [19]. The ROESY correlations of δ_H 5.59 (1H, dd, J = 9.0, 5.5 Hz, H-16)/0.97 (1H, td, J = 13.1, 4.5 Hz, H-15a)/2.36 (1H, td, J = 11.3, 3.8 Hz, H-3a), of δ_H 2.52 (1H, m, H-3b)/4.80 (1H, s, H-21), and of δ_H 4.80 (1H, s, H-21)/2.42 (3H, s, Me-18) indicated H-3b, H-21, and Me-18 at the same side, H-16, H-15a, and H-3a at the same side, while H-16 and H-21 at the opposite side. Basing on a common biosynthetic origin, the configurations at C-20 and C-21 of compound **2** were assumed to be 20β, 21β orientations. Thus, compound **2** was therefore readily identified as (+)-19-oxo-*O*-methyleburnamine, named as kopsiofficine I.

The molecular formula $C_{20}H_{26}N_2O_2$ for compound **3** was established



	R_1	R_2
1	α-OCH_3	O
2	β-OCH_3	O
3	β-OCH_3	OH
6	α-OCH_3	H
7	α-OCH_3	H
8	α-OCH_3	H



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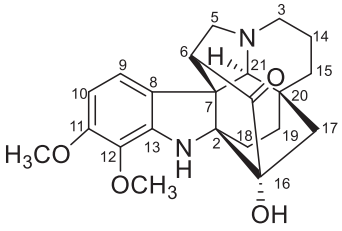

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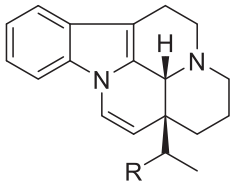
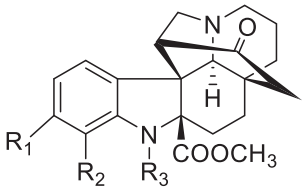
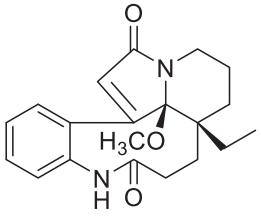


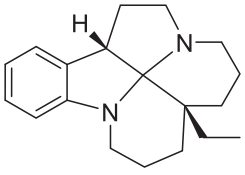
	R
9	O
10	OH



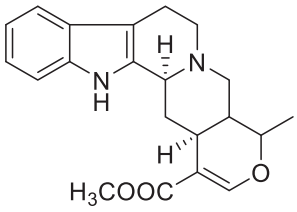
	R_1	R_2	R_3
11	H	H	H
12	OCH_2O		$COOCH_3$
13	H	H	$COOCH_3$
14	OCH_2O		H
15	H	OCH_3	$COOCH_3$



16



17



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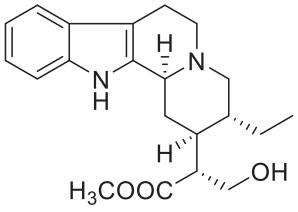


19

Fig. 1. Structure of compounds **1**–**19**.

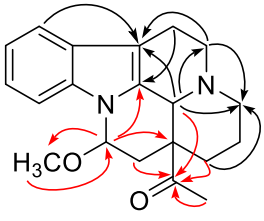
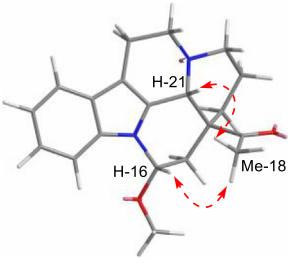
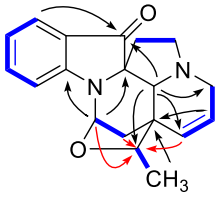


Fig. 2. Key HMBC (↷) and ROESY (⋯) correlations of **1**.





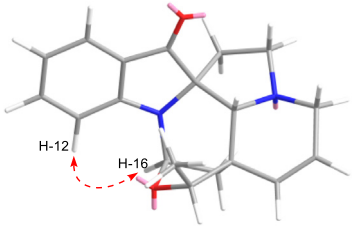


Fig. 3. Key HMBC (→), $^1H-^1H$ COSY (—), and ROESY (⋯) correlations of **4**.

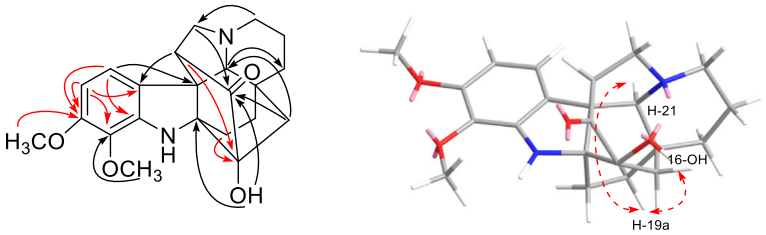


Fig. 4. Key HMBC (↷) and ROESY (↷) correlations of **5**.

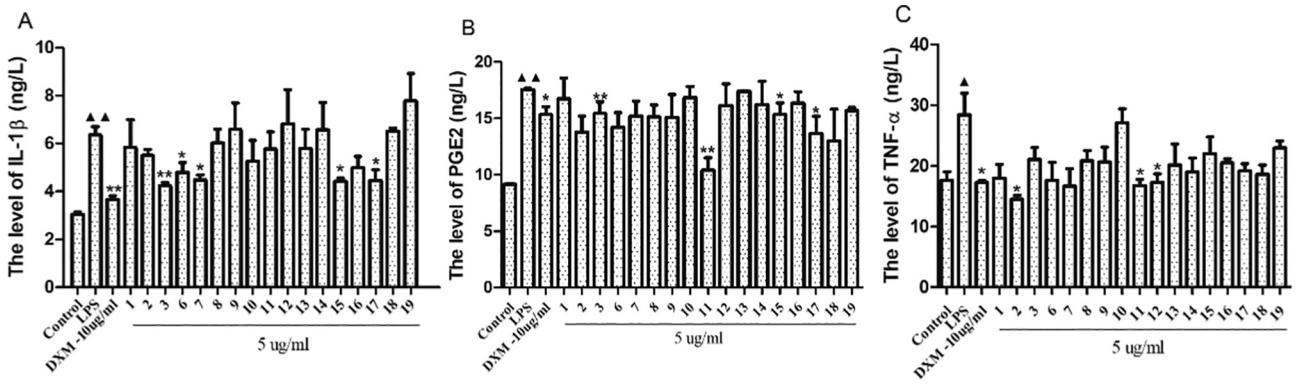


Fig. 5. The anti-inflammatory activities of the alkaloids for LPS-activated RAW 264.7 cells. DXM, Deametasona, as the positive control; data are expressed as mean ± SEM; statistical differences are represented as ▲▲$p < 0.01$ vs. control, *$p < 0.05$, **$p < 0.01$ vs. LPS. (A) The level of IL-1β; (B) The level of PGE2; (C) The level of TNF-α.

from the HRESIMS data (*m/z* 327.2065 $[M + H]^+$). The UV spectrum showed absorption indole chromophore at 207, 224 and 275 nm [19], while IR spectrum indicated the presence of a nitrogen hydrogen group (-NH) at 3441 nm. Its ^{13}C and DEPT spectra of compound **3** displayed 20 carbon resonances ascribed to one methyl, one methoxy, six methylenes, six methines, six quaternarys (Table 2). The 1D-NMR data (Tables 1 and 2) of **3** were similar to those of (+)-19(*R*)-hydroxyeburnamine [38], except for a methoxyl at C-16 in **3** instead of a hydroxyl in latter, as indicated by the HMBC correlations of δ_H 3.32 (3H, s, 16-OCH_3) with δ_C 82.1 (d, C-16), of δ_H 5.44 (1H, dd, J = 9.2, 5.3 Hz, H-16) with δ_C 51.7 (q, 16-OCH_3), and a downfield signal characteristic of C-16 (δ_C 82.1, d). ROESY correlations of δ_H 5.44 (1H, dd, J = 9.2, 5.3 Hz, H-16)/0.66 (1H, td, J = 13.4, 4.1 Hz, H-14a), of δ_H 1.76 (1H, m, H-14b)/4.24 (1H, q, J = 6.4 Hz, H-19)/ 4.05 (1H, s, H-21) indicated H-16 and H-21 at the opposite side. The H-16 signal of **3** appeared as a doublet of doublets with coupling constants of 9.2 and 5.3 Hz, which also suggested that the H-16 of configuration was *α* orientation [19]. Detailed analysis of HSQC, HMBC, ROESY established the structure of **3** to be as shown.

Compound **4** possessed a molecular formula of $C_{19}H_{20}N_2O_2$ as established by the HRESIMS ion at *m/z* 309.1599 (calcd 309.1598). The UV spectrum showed absorption maxima at 202, 233, and 377 nm, characteristic of indole and pseudoindoxyl chromophores [42]. The ^1H NMR spectrum showed the presence of an unsubstituted indole moiety (δ_H 7.02, 7.16, 7.54, and 7.64), a double bond protons signal (δ_H 5.43, 5.97), one singlet methyl signal (δ_H 0.87). The ^{13}C NMR spectrum of compound **4** displayed 19 carbons resonances ascribed one methyl, four methylenes, nine methines, five quaternary carbons (Table 2). The spectroscopic data of **4** were similar to those of larutienine B [42], except for one more double bond at C-14/15 in **4**, which were supported by the $^1H-^1H$ COSY corss-peaks of δ_H 5.43 (1H, d, J = 9.7 Hz, H-15)/5.97 (1H, ddd, J = 9.7, 4.8, 1.4 Hz, H-14)/3.45 (1H, m, H-3a) and 3.14 (1H, m, H-3b) (Fig. 3). The HMBC corrletions of δ_H 5.64 (1H, d, J = 5.6 Hz, H-16), 2.07 (2H, m, H-17), and 5.43 (1H, d, J = 9.7 Hz, H-15) with δ_C 81.3 (d, C-19) suggested a five-membered ring was formed between C-19 and C-16 via an oxygen atom. The configuration of C-2 was determined as R by the rigid architecture of the doubly spirocyclic molecule [42]. Sharing a common biogenetic origin in eburnane-type alkaloids in this plant, the configurations at C-20 and C-21 of **4** were the same to that of **1–3**. The configuration of H-16 was *α* orientation, which was supported by the ROESY correlation of δ_H 7.16 (1H, d, J = 7.7 Hz, H-12)/5.64 (1H, d, J = 5.6 Hz, H-16) (Fig. 3). Complete analysis of 2D NMR data of **4** confirmed the other parts were the same to those of larutienine B.

Compound **5**, isolated as amorphous powder, had a molecular formula of $C_{22}H_{26}N_2O_4$ according to the HRESIMS data ($[M + H]^+$ at *m/z* 383.1963) and ^{13}C NMR spectra. Its IR spectrum indicated the presence of -NH (3440 cm^{-1}), a conjugated carbonyl functional group (1626 cm^{-1}). The ^1H and ^{13}C NMR spectral data exhibited *o*-position substituted indole alkaloid [(δ_H 6.29 (1H, d, J = 8.1 Hz), 6.78 (1H, d, J = 8.1 Hz) and δ_C 71.0 (s, C-2), 59.5 (s, C-7), 128.8 (s, C-8), 117.0 (d, C-9), 102.7 (d, C-10), 152.9 (s, C-11), 134.0 (s, C-12), 144.2 (s, C-13)]. The 1D-NMR data of **5** (Tables 1 and 2) were similar to those of *N*1-demethoxycarbonyl-12-methoxykopsine [25] except for one more methoxy substituted at C-11 in **5**, as supported by the HMBC cross peaks of δ_H 3.71 (3H, s, 11-OCH_3) with δ_C 152.9 (s, C-11), of δ_H 6.78 (1H, d, J = 8.1 Hz, H-9) with δ_C 152.9 (s, C-11) and 144.2 (s, C-13), and of δ_H 6.29 (1H, d, J = 8.1 Hz, H-10) with δ_C 152.9 (s, C-11), 134.0 (s, C-12), and 128.8 (s, C-8) (Fig. 4). The HMBC correlations of δ_H 2.18 (1H, m, H-6), 1.87 (1H, dd, J = 11.1, 3.0 Hz, H-18a) with δ_C 81.1 (s, C-16), and the chemical shift of C-16 (δ_C 81.1, s) supported that the OH group placed at C-16. Taking a common biogenetic origin in a kopsine-type alkaloid in this genus, the configuration of H-21 of **5** was *α* oriented [19,22,40]. The relative configuration of 16-OH was assigned to be *α* orientation, which was deduced from the observed ROESY correlations of δ_H 3.12 (1H, d, J = 1.3 Hz, H-21)/1.24 (1H, overlap, H-19a)/ 5.42 (1H, s, 16-OH) (Fig. 4). Detailed analysis of 2D NMR data (HSQC, HMBC, ROESY) established the structure of **5**, and named as kopsioficine L.

In the bioassay, the isolated compounds were evaluated for their anti-inflammatory activities in vitro by inhibiting IL-1β, PEG2, and TNF-α secretion in LPS-activated RAW264.7 cells. As a result, the anti-inflammatory effects of alkaloids **2**, **3**, **6**, **7**, **11**, **12**, **15**, and **17** at the concentration of 5 μg/mL showed close to that of positive control

(DXM) at the concentration of 10 μg/mL (Fig. 5). Simultaneously, compounds **3**, **6**, **7**, **15**, and **17** displayed stronger inhibitory effects on IL-1β level compared with LPS group ($p < 0.05/0.01$) (Fig. 5A), and compounds **3**, **11**, **15**, and **17** significantly inhibited the productions of PGE2 ($p < 0.05/0.01$) (Fig. 5B), while compounds **2**, **11**, and **12** significantly inhibited the productions of TNF-α ($p < 0.05/0.01$) (Fig. 5C). Furthermore, those compounds did not show cytotoxic against RAW 264.7 cell line at test concentrations evaluated by the MTT method, which suggested that their anti-inflammatory activity did not result from cytotoxicity. Summarily, the anti-inflammatory effects of compounds **2**, **3**, **6**, **7**, **11**, **12**, **15**, and **17** may be demonstrated by inhibiting the productions of IL-1β, PGE2 and TNF-α.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

1D and 2D NMR spectra, HRESIMS, UV, IR, and ORD spectra of compounds **1–5** are available as Supplementary materials. Supplementary data to this article can be found online at [https://doi.org/10.1016/j.fitote.2020.104547].

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