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Hasubanan alkaloids with anti-inflammatory activity from *Stephania longa*

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ABSTRACT

Two new hasubanan alkaloids, stephalonester A (**1**) and stephalonester B (**2**), together with four known compounds, stephalonine E (**3**), longanone (**4**), cephatonine (**5**), and prostephabyssine (**6**) were isolated from the whole plant of *Stephania longa*. Their structures were determined by HR-ESI-MS, 1 D and 2 D NMR, ECD calculations, as well as by comparison with literature values. All compounds were evaluated for their anti-inflammatory activity in vitro. Compounds **4**, **5**, and **6** exhibited significantly inhibitory effects on TNF-α and IL-6 production with IC_{50} values range from 6.54 to 30.44 μM.

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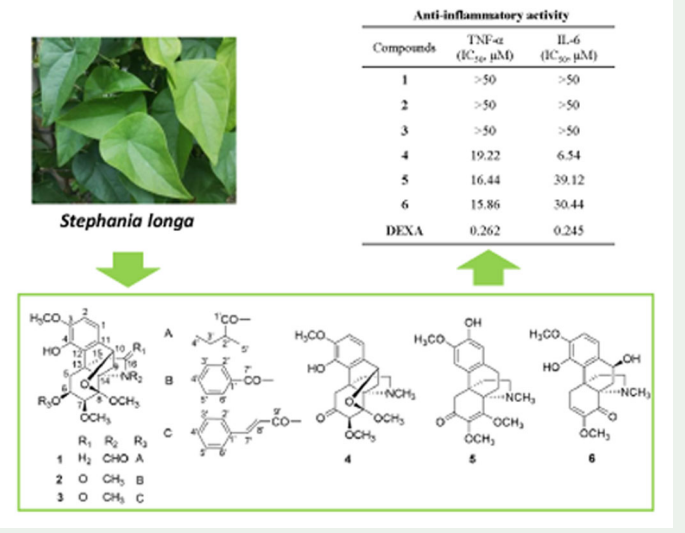

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1. Introduction

The hasubanan alkaloids represent a group of naturally occurring minor compounds, which are distributed mainly in genera *Stephania, Pericampylus,* and *Sinomenium* (King and Herzon 2014). Hasubanan alkaloids have been reported to possess a variety of biological activities, including opioid activity (Hsin et al. 2010), anti-HBV activity (Yan et al. 2008), and anti-microbial activity (Semwal and Rawat 2009). *Stephania longa* Lour., belonging to the genus *Stephania* (Menispermaceae), distributed mainly in the south of China, especially in Yunnan and Guangxi Provinces (Zhang and Yue 2005). *S. longa* is a well-known plant in the traditional Chinese medicine (TCM) and has been widely used for the treatment of fever, inflammation, and dysentery (Zhang and Yue 2006). Previous studies of this plant led to the isolation of some alkaloids (Zhang et al. 2006), sterols, and coumarins (Deng & Zhao 1993). To further explore the bioactive constituents of *S. longa*, the phytochemical study was conducted and two new hasubanan alkaloids and four known ones were isolated (Figure 1). Their structures were identified by various spectroscopic techniques, including HR-ESI-MS and NMR methods. In addition, compounds **4**, **5**, and **6** showed significant inhibition against TNF-α and IL-6 production with IC_{50} values range from 6.54 to 30.44 μM.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder. The UV spectrum of **1** (Figure S1) showed the absorption maxima at 205 and 280 nm. The IR spectrum of **1** (Figure S2) showed the presence of hydroxy group (3414 cm^{-1}), carbonyl (1724 and 1681 cm^{-1}), and aromatic ring (1618 and 1489 cm^{-1}) absorbances. The molecular formula was determined to be $C_{25}H_{33}NO_8$ on the basis of the HR-ESI-MS data at *m/z* 498.2098 $[M+Na]^+$ (calcd 498.2099), implying the presence of ten degrees of

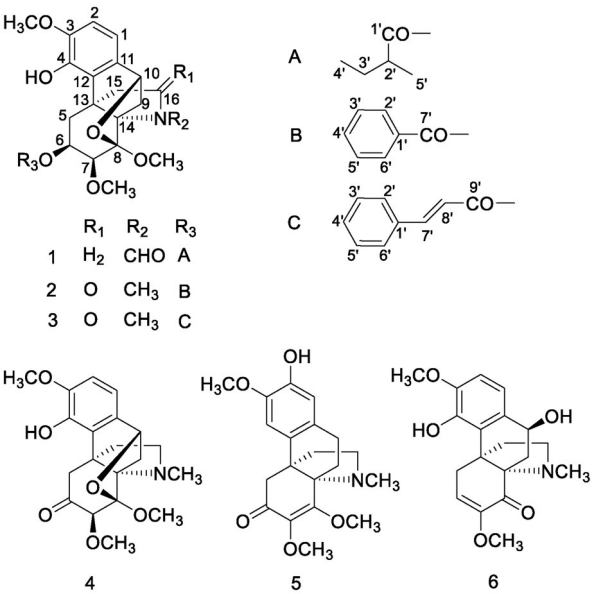

Figure 1. Structures of compounds **1**–**6** isolated from *S. longa*.

unsaturation (Figure S3). The NMR data (Figures S4–S6) showed the presence of two ester carbonyls (δ_C 178.2 and 176.9), four aromatic quaternary carbons (δ_C 149.8, 146.1, 135.1, and 126.1), two aromatic methines [δ_H 6.72 (1H, d, $J = 8.0$), 6.64 (1H, d, $J = 8.0$); δ_C 116.9 and 109.3], three oxygenated methines [δ_H 5.30 (1H, d, $J = 4.1$), 4.92 (1H, d, $J = 6.1$), and 3.60 (1H, d, $J = 4.1$); δ_C 83.2, 77.6, and 67.4], three O-methyls [δ_H 3.83 (1H, s), 3.55 (1H, s), and 3.32 (1H, s); δ_C 57.9, 56.5, and 52.2], and two aliphatic methyls [δ_H 0.71 (3H, t, $J = 7.4$), and 0.66 (3H, d, $J = 6.9$); δ_C 15.2 and 11.1]. The $^1H-^1H$ COSY spectrum of **1** revealed the existence of four spin-coupling systems (Figure S21). These data indicated that **1** was a hasubanan type alkaloid. Carefully analysis of the NMR data of **1** with those of the known stephalonine A (Zhang and Yue 2005) showed that their structures were similar, except for the presence of an additional amide carbonyls (δ_C 178.2), and the absence of an typical N-methyl group in **1**, which was supported by the HMBC correlations from δ_H 3.02 (H-16a) and 2.50 (H-16b) to δ_C 178.2 (C-17) (Figure S21), and obvious upfield shift of C-16 (δ_C 45.1).

The *cis* configuration of H-6 and H-7 was deduced by a small coupling constant value ($J = 4.1$ Hz). The NOE correlations between H-10 (4.92) and 8-OCH_3 (3.55), and between H-7 (3.60) and H-16a (3.58) (Figure S22) assigned the relative configuration of **1**. Experimental CD spectrum of **1** showed negative Cotton effect at 275 nm, which confirmed the absolute configuration to be 6*S*, 7*S*, 8*R*, 10*S*, 13*S*, 14*S*. (Carroll et al. 2010) (Figure S23). The 2′ absolute configuration of the 2-methylbutyryl moiety was further determined by comparison of the experimental and calculated ECD spectra. The calculated and experimental ECD spectra (Figure S23) demonstrated the 6*S*, 7*S*, 8*R*, 10*R*, 13*R*, 14*S*, 2′*S* absolute configuration of **1**. Therefore, the structure of compound **1** was established and named as stephalonester A.

Compound **2** was obtained as a white amorphous powder, which was assigned the molecular formula to be $C_{27}H_{29}NO_8$ according to the HR-ESI-MS data at *m/z* 518.1786 $[M + Na]^+$ (calcd 518.1785) (Figure S13). The UV spectrum of **2** (Figure S11) showed the absorption maxima at 203 and 276 nm. The IR spectra of **2** (Figure S12) showed the existence of hydroxy group (3477 cm^{-1}), carbonyl (1722 and 1681 cm^{-1}), and aromatic ring (1616 and 1489 cm^{-1}) absorptions. The ^{13}C NMR and DEPT NMR data (Figures S15 and S16) revealed the presence of four methyls, three methylenes, eleven methines, and eight quaternary carbons in **2**. The NMR data of **2** (Table S1) were shown to be quite similar to those of the known alkaloid, stephalonine F (Zhang et al. 2005), expect the difference of an additional carbonyl at C-16 (δ_C 176.9) appeared in **2**. This deduction was supported by obvious down-field shift of C-15 (δ_C 45.1), and upfield shift of C-17 (δ_C 28.6), which were further confirmed by the HMBC correlations from δ_H 3.10 (H-17), 3.07 (H-15a), and 2.56 (H-15b) to δ_C 176.9 (C-16) (Figure S21).

The coupling constant value ($J_{H6,\ H7} = 4.2$ Hz) along with NOE correlations of H-7/H-17 (Figure S22) suggested that **2** had the same relative configurations as **1**. Experimental CD spectrum of **2** showed negative Cotton effect at 274 nm, which confirmed the absolute configuration to be 6*S*, 7*S*, 8*R*, 10*S*, 13*S*, 14*S*. (Carroll et al. 2010) (Figure S23). Therefore, the structure of compound **2** was identified and named as stephalonester B.

The known compounds were identified as stephalonine E (**3**), longanone (**4**), cephatonine (**5**), and prostephabyssine (**6**), by comparing their spectral data with those reported in previous literatures (Zhang and Yue 2005, Kashiwaba et al. 1996).

All isolated compounds were evaluated for their anti-inflammatory activity in RAW264.7 macrophages stimulated by LPS in vitro. The results showed that compounds **4**, **5,** and **6** exhibited significant inhibition against TNF-α and IL-6 production (Table S2). Compounds **4**, **5,** and **6** suppressed TNF-α production with IC_{50} values of 19.22 μM, 16.44 μM, and 15.86 μM, respectively. Compounds **4**, **5,** and **6** showed inhibitory effects on IL-6 production with IC_{50} values of 6.54 μM, 39.12 μM, and 30.44 μM, respectively. In addition, compounds **1**–**6** did not have significant toxicity towards RAW264.7 cells at 50 μM (Figure S30).

3. Experimental section

3.1. General experimental procedures

Optical rotations were measured on an Autopol IV automatic polarimeter (Rudolph, New Jersey, USA). UV spectra were recorded on a Shimadzu UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan). IR (KBr) spectra were acquired on an IRTracer-100 spectrometer (Shimadzu, Kyoto, Japan). HR-ESI-MS were obtained on a Thermo Scientific Q Exactive mass spectrometer (Thermo Electron, Bremen, Germany). ECD spectra were obtained on a Chirascan-plus CD spectrometer (Applied Photophysics Ltd., UK). NMR spectral data were measured on a Bruker AV 400 spectrometer (Fallanden, Switzerland). Silica gel (200–300 mesh, Qingdao Haiyang Chemical Co. Ltd., China) and octadecylsilyl silica gel (ODS, 50 μm, YMC, Japan) were used for column chromatography. Preparative HPLC was performed on a Shimadzu LC-20A (Shimadzu Crop., Japan) with an ODS silica column (YMC-Pack ODS-A, 5 μm, 10 × 250 mm).

3.2. Plant material

Stephania longa were collected from Wuzhou, Guangxi, China, and were identified by Prof. An-ping Yang (Foshan University). A voucher specimen (FJD-201907) was stored in the Department of Pharmacy, School of Medicine, Foshan University.

3.3. Extraction and isolation

Dried and powdered of *S. longa* (5.0 kg) were extracted with 95% EtOH for 3 times. The EtOH extract was concentrated under vacuum to yield a crude extract (540 g), which was suspended in water and then partitioned successively with petroleum ether, chloroform, and EtOAc. The EtOAc fraction (130 g) was subjected to passage over a silica gel column using gradient mixtures of petroleum ether – EtOAc (1:0 – 2:1, v/v) as eluents, to afford six major fractions (Fr.1 – Fr.6). Fr. 2 (22 g) was chromatographed over an ODS column using gradient mixtures of MeOH – H_2O (2:8 – 1:0, v/v) as eluents, to afford fractions Fr.2-1 – Fr.2-5. Fr.2-2 (0.9 g) was purified through preparative HPLC (MeOH – H_2O, 65:35, v/v) to afford compound **4** (13.4 mg). Purification of Fr.2-3 (2.2 mg) using a preparative HPLC column (MeOH – H_2O, 70:30, v/v) gave compounds **5** (18.4 mg) and **6** (12.5 mg).

Fr.3 (18 g) was chromatographed over an ODS column using gradient mixtures of methanol – water (1:9 – 1:0, v/v) as eluents, to afford fractions Fr.3-1 – Fr.3-6. Fr.3-1

(1.3 g) was purified through preparative HPLC (ACN – H_2O, 35:65, v/v), yielding compounds **1** (37 mg) and **2** (6.3 mg). Fr.2-2 (2.2 g) was purified through preparative HPLC (ACN – H_2O, 35:65, v/v), yielding compound **3** (100.1 mg).

3.3.1. Stephalonester A (1)

Yellow amorphous powder; [α]25 D + 270 (c 0.67, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.91), 280 (3.83) nm; IR (KBr) ν_{max} 3414, 2964, 1724, 1681, 1618, 1489, 1406, 1392, 1130, 1089, 815 cm^{-1}; ECD (MeOH, Δε) λ_{max} 196 (+5.92), 201 (−1.46), 213 (+10.61), 275 (−0.20) nm; ^1H and ^{13}C-NMR spectral data, see Table S1; HR-ESI-MS at *m/z* 498.2098 $[M + Na]^+$ (calcd for $C_{25}H_{33}NO_8Na$, 498.2099).

3.3.2. Stephalonester B (2)

Yellow amorphous powder; [α]25 D − 7.0 (c 0.014, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.84), 276 (3.82) nm; IR (KBr) ν_{max} 3477, 1722, 1681, 1616, 1489, 1454, 1392, 1118, 1099, 711, 617 cm^{-1}; ECD (MeOH, Δε) λ_{max} 204 (−9.59), 214 (+7.66), 232 (−2.00), 274 (−1.00) nm; ^1H and ^{13}C-NMR spectral data, see Table S1; HR-ESI-MS at *m/z* 518.1786 $[M + Na]^+$ (calcd for $C_{27}H_{29}NO_8Na$, 518.1785).

3.4. Mtt assay and anti-inflammatory assay

MTT assay (Guo et al. 2021) was carried out to evaluate the non-cytotoxic concentrations of compounds **1-6** on RAW264.7 cells. RAW264.7 macrophages were seeded in 96-well plates (2×10^6 cells/well). Cells were incubated with compounds at the indicated concentrations for 24 h. Then cells were stimulated with 1 μg/ml of LPS for 4 h. The production of TNF-α and IL-6 were measured using the ELISA kit (Meimian biology, Jiangsu, China) according to the manufacturer's protocol (Sun et al. 2019).

4. Conclusions

In this study, two new hasubanan alkaloids, stephalonester A–B (**1**–**2**), and four known ones, stephalonine E (**3**), longanone (**4**), cephatonine (**5**), and prostephabyssine (**6**) were isolated from *S. longa* and their structures were identified. Moreover, we reported for the first time that compounds **4**, **5**, and **6** had significant anti-inflammatory activity against the production of TNF-α and IL-6.

Disclosure statement

No potential conflict of interest was reported by the authors.

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