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Alkaloids and flavonoid glycosides from the aerial parts of *Leonurus japonicus* and their opposite effects on uterine smooth muscle

Juan Liu [a, b], Cheng Peng [a, b, **], Qin-Mei Zhou [a, b, d], Li Guo [a, b, d], Zhao-Hua Liu [c], Liang Xiong [a, b, d, *]

[a] State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine Resources, Chengdu University of Traditional Chinese Medicine, Chengdu, 611137, China
[b] School of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu, 611137, China
[c] Chengdu No.1 Pharmaceutical Co. Ltd., Chengdu, 610031, China
[d] Institute of Innovative Medicine Ingredients of Southwest Specialty Medicinal Materials, Chengdu University of Traditional Chinese Medicine, Chengdu, 611137, China

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ABSTRACT

The crude extract and some Chinese patented medicines of *Leonurus japonicus* Houtt. have been proven to affect the uterine smooth muscle. *L. japonicus* injection is widely used in obstetric departments in China for treating postpartum hemorrhage caused by uterine inertia. Bioassay-guided isolation of the 95% EtOH extract of *L. japonicus* yielded four cyclopeptides, nine alkaloids, and three flavonoid glycosides, including two previously undescribed cyclopeptides, namely, cycloleonuripeptide G and cycloleonuripeptide H. The structures of the cyclopeptides were elucidated to be *cyclo*-(L-Phe-L-Phe-Gly-L-Pro-Gly-L-Pro) and *cyclo*-(L-Phe-L-Ala-L-Pro-L-Ile-L-His-Gly-L-Ala-L-Pro), respectively, via spectroscopic and chemical methods. Cyclopeptides (cycloleonuripeptides C and D) and alkaloids (imperialine-3β-D-glucoside and leonurine) promoted contraction of uterine smooth muscle strips isolated from normal rats. However, it was observed that flavonoid glycosides (spinosin, linarin, and apigenin-7-*O*-β-D-glucopyranoside) significantly inhibited contraction of the uterine smooth muscle strips.

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1. Introduction

Postpartum hemorrhage (PPH) is a major cause of mortality among pregnant women. It accounts for more than 100,000 maternal deaths per year. It can also result in long-term uterine recovery difficulties after normal vaginal delivery or cesarean section (Khan et al., 2006). The main cause of PPH is uterine atony. Oxytocin, ergometrine, misoprostol, and other prostaglandins were recommended for preventing PPH in The World Health Organization (WHO) 2012 (WHO Guidelines Approved by the Guidelines Review Committee, 2012) and International Federation of Gynecology and Obstetrics (FIGO) 2012 guidelines (FIGO Safe Motherhood and Newborn Health Committee, 2012). In China, several Chinese patented medicines have been developed from the extract of *Leonurus japonicus* Houtt. (Labiatae) and are used in combination with oxytocin for treating PPH or uterine recovery difficulties. A number of clinical literature have indicated that *L. japonicus* injection has a notable curative effect on PPH and has gradually become a common drug for obstetric use in China (Gong et al., 2011; Lin et al., 2009; Liu et al., 2016; Su et al., 2016; Tan et al., 2017; Yuan and Chen, 2015).

The aerial parts of *L. japonicus*, a well-known traditional Chinese medicine, ranked as the top grade in “Sheng Nong Ben Cao Jing” and is called “Motherwort” or “Yi Mu Cao” (Chinese), meaning literally “beneficial herb for mothers”. It has been used to treat various gynecological blood disorders, particularly menstrual disturbances and PPH, for thousands of years (Peng, 2011). Modern pharmacological studies have demonstrated that the extract of *L. japonicus* promotes uterine muscle contraction in rats (Li et al., 2014b; Ma et al., 2000; Shang et al., 2014). Thus, *L. japonicus* is a good natural uterotonic.

* Corresponding author. State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine Resources, Chengdu University of Traditional Chinese Medicine, Chengdu, 611137, China.
** Corresponding author. State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine Resources, Chengdu University of Traditional Chinese Medicine, Chengdu, 611137, China.
E-mail addresses: pengchengchengdu@126.com (C. Peng), xiling0505@126.com (L. Xiong).

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Our previous investigations on *L. japonicus* have led to the isolation of diterpenoids (Xiong et al., 2015a; 2015b), sesquiterpenoids (Xiong et al., 2013), and steroids (Zhou et al., 2015) with vasorelaxant, anti-platelet aggregative, or antibacterial activity. Based on a previous report that the total alkaloid extract of *L. japonicus* has a strong effect on the uterine muscle (Shang et al., 2014), we further carried out experiments to study the alkaloids via TLC and ultra-performance liquid chromatography-mass spectrometry. The results showed that alkaloids mainly exist in the *n*-butanol extract of *L. japonicus*. Furthermore, we have previously shown that the *n*-butanol extract has significant uterotonic effect on isolated rat uterine smooth muscle strips (Li et al., 2014a). Therefore, in the present study, we investigated the components of the *n*-butanol extract of *L. japonicus* that are responsible for the uterotonic effects of the extract.

2. Results and discussion

Sixteen chemical compounds, including four cyclopeptides (**1**–**4**), four steroidal alkaloid saponins (**5**–**8**), two guanidines (**9** and **10**), two spermidines (**11** and **12**), an isoquinoline (**13**), and three flavonoid glycosides (**14**–**16**) were isolated from the extract (Fig. 1). Interestingly, two cyclopeptides (**3** and **4**) and two alkaloids (**8** and **9**) exhibited an excitatory effect on uterine smooth muscle strips, whereas three flavonoid glycosides (**14**–**16**) had an opposite effect.

Compound **1** was obtained as a white powder. Its molecular formula was determined to be $C_{32}H_{38}N_6O_6$ with 17 degrees of unsaturation based on positive HRESIMS data. The ^{13}C NMR and DEPT spectra of **1** displayed resonances attributable to 6 ester/amide carbonyl groups (δ_C 172.3, 171.3, 170.3, 169.3, 168.5, 167.6), 2 mono-substituted aromatic rings [δ_C 137.5 (C), 135.8 (C), 129.4 (CH × 4), 128.8 (CH × 2), 128.2 (CH × 2), 127.3 (CH), 126.4 (CH)], 10 aliphatic methylenes, and 4 aliphatic methines (Table 1). The ^1H NMR spectrum of **1** revealed 38 proton resonances (Table 1) corresponding to the above methylene and methine units and four exchangeable amide NH signals [δ_H 8.92 (1H, d, J = 9.0 Hz), 8.87 (1H, brs), 8.49 (1H, m), 7.09 (1H, brs)]. These spectroscopic data combined with the molecular formula suggested that **1** was a cyclohexapeptide (Jang et al., 2017; Morita et al., 1997, 2006). Detailed analysis of the 2D NMR data (HSQC, ^1H-^1H COSY, and HMBC) revealed the existence of six standard amino acid residues in **1**, including two phenylalanines (Phe^1 and Phe^2), two prolines (Pro^1 and Pro^2), and two glycines (Gly^1 and Gly^2) (Fig. 2).

Sequencing of these amino acid residues was accomplished via HMBC data analysis using correlations of the exchangeable amide protons and α-amino protons (Fig. 2). The linkage of Phe^1 to Phe^2 was established using HMBC correlations from Phe^1 NH (δ_H 8.87) to Phe^1 C-1 (δ_C 169.3), C-2, C-3, and Phe^2 C-1 (δ_C 170.3), and from Phe^1 H-2 to Phe^2 C-1. This dipeptide fragment was in turn linked to the Gly^1 unit through interpretation of the correlations from Phe^2 NH (δ_H 7.09) to Phe^2 C-1, C-2, and Gly^1 C-1 (δ_C 168.5), and from Phe^2 H-2 to Gly^1 C-1. Similarly, correlations from Gly^1 NH (δ_H 8.49) and H_2-2 to Pro^1 C-1 (δ_C 172.3) extended the sequence to Phe^1-Phe^2-Gly^1-Pro^1. The subsequent connection of Pro^1 to Gly^2 was achieved using correlations from Pro^1 H-2 and H_2-5 to Gly^2 C-1 (δ_C 167.6). Furthermore, HMBC correlations from Gly^2 NH (δ_H 8.92) and H_2-2 to Pro^2 C-1 (δ_C 171.3) led to the assignment of a Gly^2 to Pro^2 connection. Finally, the ring closure linkage was secured by correlations from Pro^2 H-2 and H_2-5 to Phe^1 C-1 (δ_C 169.3). Consequently, the amino acid sequence of **1** was elucidated as *cyclo*-(Phe^1-Phe^2-Gly^1-Pro^1-Gly^2-Pro^2).

The absolute configurations of the amino acid residues were established based on Marfey's method (Ashour et al., 2006). After acid hydrolysis of **1**, the amino acids were derivatized with 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (L-FDAA) and analyzed by RP-HPLC. The retention times of the derivatives were compared to those of authentic derivatized amino acid standards (Fig. 3), which revealed the L configuration for the Phe and Pro amino acids in **1**. Therefore, compound **1** was determined to be *cyclo*-(L-Phe-L-Phe-Gly-L-Pro-Gly-L-Pro) and named cycloleonuripeptide G.

The spectroscopic data of **2** indicated that it was another cyclopeptide. Its molecular formula, $C_{39}H_{54}N_{10}O_8$, was deduced from positive HRESIMS at m/z 813.4026 $[M + Na]^+$ (calcd for $C_{39}H_{54}N_{10}O_8Na$, 813.4024). The presence of eight amide carbonyl groups (δ_C 173.0, 172.6, 172.1, 171.3, 171.2, 170.9, 170.7, 167.5) in the ^{13}C NMR spectrum, together with the molecular formula indicated **2** to be a cyclooctapeptide. ^1H and ^{13}C NMR spectra showed characteristic signals for an imidazole ring [δ_H 12.25 (NH), 7.70 (s), 7.17 (s); δ_C 135.5, 134.5, 115.1] and a mono-substituted aromatic ring [δ_H 7.31 (2H, t, J = 7.2 Hz), 7.23 (2H, d, J = 7.2 Hz), 7.20 (1H, t, J = 7.2 Hz); δ_C 137.9, 129.1, 128.3, 126.5], which revealed the existence of a histidine (His) and a phenylalanine (Phe) in **2**. Detailed analysis of 2D NMR data and acid hydrolysis further verified that **2** contained two alanines (Ala^1 and Ala^2), two prolines (Pro^1 and Pro^2), an isoleucine (Ile), and a glycine (Gly), in addition to the above His and Phe. Applying Marfey's method, L configurations were assigned to all the amino acids (Fig. 3).

Using the same method as described for **1**, the sequence of the amino acid units in **2** was determined using HMBC correlations of the exchangeable amide protons and α-amino protons (Fig. 2). Particularly, correlations from Phe NH (δ_H 7.83) and H-2 (δ_H 4.71) to Ala^1 C-1 (δ_C 173.0), from Ala^1-NH (δ_H 10.31) and H-2 (δ_H 4.36) to Pro^1 C-1 (δ_C 170.9), from Pro^1 H-2 (δ_H 4.04) to Ile C-1 (δ_C 172.1), from Ile H-2 (δ_H 3.92) to His C-1 (δ_C 171.3), from His NH (δ_H 7.06) and H-2 (δ_H 4.81) to Gly C-1 (δ_C 167.5), from Gly NH (δ_H 8.39) and H_2-2 (δ_H 3.89 and 3.39) to Ala^2 C-1 (δ_C 172.6), from Ala^2 NH (δ_H 7.23) and H-2 (δ_H 4.42) to Pro^2 C-1 (δ_C 170.7), and from Pro^2 H-2 (δ_H 4.06) to Phe C-1 (δ_C 171.2) indicated the cyclic sequence of **2**. Therefore, compound **2** was determined to be *cyclo*-(L-Phe-L-Ala-L-Pro-L-Ile-L-His-Gly-L-Ala-L-Pro) and named cycloleonuripeptide H.

The remaining known compounds were identified as cycloleonuripeptide C (**3**) (Morita et al., 1996), cycloleonuripeptide D (**4**) (Morita et al., 1997), hapepunine 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**5**) (Kitajima et al., 1982), hapepunine 3-O-β-cellobioside (**6**) (Qian and Nohara, 1995), yibeinoside A (**7**) (Xu et al., 1990b), imperialine-3β-D-glucoside (**8**) (Xu et al., 1990a), leonurine (**9**) (Yeung et al., 1977), 4-hydroxy-3,5-dimethoxybenzoic acid 4-guanidinopentyl ester (**10**) (Langford et al., 1980), N^1,N^5,N^{10}-tri-*p*-(*ZZZ*)-coumaroylspermidine (**11**) (Jiang et al., 2008), N^1,N^5,N^{10}-tri-*p*-(*EEZ*)-coumaroylspermidine (**12**) (Jiang et al., 2008), juzirine (**13**) (Kimura et al., 1983), spinosin (**14**) (Lewis et al., 2000), linarin (**15**) (Quintin and Lewin, 2004), and apigenin-7-*O*-β-D-glucopyranoside (**16**) (He et al., 2014) by comparing their spectroscopic data with those reported in literature.

Since TCM preparations from *L. japonicus* are not administered orally but instead by injection for treating PPH, the isolates were evaluated for their *ex vivo* effect on uterine smooth muscle strips isolated from adult female Sprague-Dawley (SD) rats. However, compounds **6**, **7**, **10**, and **13** were not examined for this effect due to their limited quantities. Using a cumulative dosing regimen, the effects of the isolates on contractile activity (entire area under the curve, AUC, g*n/10 min), contractile tension (average force of contraction, g), and contractile frequency (numbers of contraction, n/10 min) of the uterine smooth muscle strips were evaluated. The difference values of contractile activity (ΔAUC), contractile tension (ΔT), and contractile frequency (ΔF) after and before each treatment were calculated for statistical analysis.

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