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Localisation of Two Bioactive Labdane Diterpenoids in the Peltate Glandular Trichomes of *Leonurus japonicus* by Laser Microdissection Coupled with UPLC-MS/MS

Chao-Jiang Xiao,[a,b,c] Yan-Chun Liu,[a,b] Shi-Hong Luo,[a,b] Juan Hua,[a,b,c] Yan Liu[a,b]* and Sheng-Hong Li[a,b]*

ABSTRACT:

Introduction – Glandular trichomes of plants are biochemical factories that could produce, store and secrete copious pharmaceutically important natural products. The Labiatae plant *Leonurus japonicus* is an important traditional Chinese medicine used to treat gynecological diseases, and has abundant peltate glandular trichomes (PGTs), in which the secondary metabolites accumulated are still unknown.

Objective – To study the secondary metabolites specifically accumulated in the PGTs of *L. japonicus* and their biological activities, and provide a new way to pinpoint bioactive natural products from plants.

Methodology – Morphology of the trichomes on *L. japonicus* were observed under a scanning electron microscope. The PGTs of *L. japonicus* were precisely collected using laser microdissection (LMD) and analysed for their secondary metabolites with ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Targeted compounds were isolated with classical phytochemical methods, and their structures were elucidated by spectroscopic analysis. Biological activities were evaluated by *in vitro* assays.

Results – Two labdane diterpenoids, leoheterin (1) and galeopsin (2), were localised in the PGTs of *L. japonicus*. Antithrombotic activity of 1 in anti-platelet aggregation assay induced by arachidonic acid was observed. Both compounds showed potential anti-inflammatory activity by inhibiting proinflammatory cytokine TNF-α. In addition, anti-proliferative effect of both compounds on several cancer cell lines was also detected.

Conclusion – Two bioactive labdane diterpenoids were localised in the PGTs of *L. japonicus*. The findings suggested that it might be an efficient approach to explore bioactive natural products from the glandular trichomes of medicinal plants with LMD-UPLC/MS/MS. Copyright © 2017 John Wiley & Sons, Ltd.

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Keywords: *Leonurus japonicus*; glandular trichomes; laser microdissection; labdane diterpenoids; biological activities

Introduction

Plant trichomes (glandular and non-glandular) are epidermal protuberances widely distributed on the aerial organs of most terrestrial plants (Mccaskill and Croteau, 1999). Glandular trichomes (GTs) mainly include capitate glandular trichomes (CGTs) and peltate glandular trichomes (PGTs), having high capacity to synthesise, secrete and accumulate copious secondary metabolites with diverse functions, which therefore have been regarded as biochemical factories (Mccaskill and Croteau, 1999; Schilmiller *et al.*, 2008). For example, the most prestigious antimalarial agent, artemisinin, has been found to be synthesised in the PGTs of *Artemisia annua* (Lu *et al.*, 2013). Δ^9-Tetrahydrocannabinol (THC), a well-known psychoactive substance, was reported to be stored in the CGTs of *Cannabis sativa* (Happyana *et al.*, 2013). Another psychoactive compound, salvinorin A, was also found in the PGTs of *Salvia divinorum* (Siebert, 2004). *Marrubium vulgare* has been used to solve cough and respiratory problems worldwide, and its GTs produced a diterpenoid marrubiin which is the major active compound responsible for the therapeutic properties of the plant (Piccoli and Bottini, 2008). Parthenolide has drawn wide attention due to its medicinal value and pharmacological activities, especially against migraine and as an antineoplastic agent. Recently, its biosynthesis and accumulation were reported to occur in the GTs of *Tanacetum parthenium* (Majdi *et al.*, 2011). In our previous work on the chemistry and biological functions of plant GTs, we found that the PGTs of *Leucosceptrum canum*, *Colquhounia coccinea* var. *mollis*, and *Colquhounia seguinii* harboured unique

* Correspondence to: Sheng-Hong Li and Yan Liu, State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Lanhei Road 132, Heilongtan, Kunming 650201, P. R. China.
Email: shli@mail.kib.ac.cn; liuyan@mail.kib.ac.cn

[a] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, P. R. China

[b] Yunnan Key Laboratory of Natural Medicinal Chemistry, Kunming 650201, P. R. China

[c] University of Chinese Academy of Sciences, Beijing 100049, P. R. China

defensive leucosceptrane sesterterpenoids (= leucosceptroids), colquhounane sesterterpenoids (= colquhounoids), and clerodane diterpenoids, respectively (Li *et al.*, 2013; Li *et al.*, 2014; Luo *et al.*, 2010), and the CGTs of *Paragutzlaffia henryi* produced phytotoxic labdane diterpenoids (Wang *et al.*, 2015).

Leonurus japonicus Houtt. (synonyms: *L. artemisia* or *L. heterophyllus*; family: Labiatae or Lamiaceae), an annual or biennial herbaceous flowering plant, is an important traditional Chinese medicine that has been used for thousands of years in China (Editorial Board of China Herbal and State Administration of Traditional Chinese Medicine, 1999). It has another name "Yi-Mu-Cao", meaning "beneficial herb for mothers", because its primary use is to treat abnormal menstruation, dysmenorrhea, amenorrhea, lochia, postpartum hemorrhagic syncope, abdominalgia with blood stasis, edema of body, oliguria, sores, ulcerations and other women's diseases (Chinese Pharmacopeia Commission, 2015; Editorial Board of China Herbal and State Administration of Traditional Chinese Medicine, 1999). The alkaloids stachydrine and leonurine are the officially designated marker compounds to monitor the quality of *L. japonicus* according to the *Chinese Pharmacopoeia* (Chinese Pharmacopeia Commission, 2015), so the biological activities of these two compounds have been extensively investigated, and their effects on uterus contraction, cardioprotection, neuroprotection, diuresis, inflammation, cancer and other aspects have been disclosed (Shang *et al.*, 2014). In addition, approximately 140 other pharmacodynamic compounds have been isolated and identified from this medicinal plant hitherto. However, their biological activities have not covered all traditional medicinal applications of *L. japonicus* (Shang *et al.*, 2014). According to the traditional usage, both dry and fresh aerial parts of *L. japonicus* have been used to treat gynecological diseases in China. In the case of fresh *L. japonicus*, the abaxial surface of leaves was densely populated by intact GTs. However, so far the secondary metabolites in the GTs of *L. japonicus* still remain unknown. In this paper, the PGTs of *L. japonicus* were precisely collected using laser microdissection (LMD) and analysed for their secondary metabolites with ultra performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS). Two bioactive furan-containing labdane diterpenoids, leoheterin (**1**) and galeopsin (**2**), were identified in the PGTs of *L. japonicus*.

Experimental

General

Morphology of trichomes was observed by a Hitachi S-4800 scanning electron microscope (Hitachi Ltd., Tokyo, Japan). PGTs were collected through LMD on a Leica LMD7000 system (Leica, Wetzlar, Germany). NMR spectra were recorded on a Bruker Avance III-400 instrument (Bruker, Faellanden, Switzerland) with tetramethylsilane (TMS) as an internal reference. UPLC-MS/MS analysis was carried out on a Waters Xevo TQ-S spectrometer (Waters Corp., Milford, MA, USA) equipped with a turbo ion spray (electrospray) source and a triple quadrupole ion path. Silica gel (Qingdao Marine Chemical Ltd, Qingdao, China) and MCI gel (Mitsubishi Chemical Corp., Tokyo, Japan) were used for open column chromatography. Silica gel GF_{254} plates (Qingdao Marine Chemical Ltd) were used for thin layer chromatography (TLC) detection.

Plant material

Leaves of *Leonurus japonicus* Houtt. were collected from the plants growing in Kunming Botanical Garden in October of 2014, and were identified by Dr Jian Liu at Kunming Institute of Botany, Chinese Academy of Sciences. An authentic sample (No. LJ20141021) was kept in the State Key Laboratory of Phytochemistry and Plant Resources in West China.

Scanning electron microscopy (SEM)

Fresh leaves were cut into small pieces of approximately 4 mm^2 fixed in FAA fixative (5 mL of 38% formaldehyde, 5 mL of acetic acid, and 90 mL of 50% ethanol) for 12 h, followed by dehydration in ethanol series (70%, 80%, and 95% for 45 min, 45 min, and 12 h, respectively), and then stepwise rinsed with ethanol-isoamyl acetate (3:1, 1:1, 1:3, and 0:1, each 20 min). Subsequently, these specimens were critical point dried from carbon dioxide, and then coated by gold-powder using a sputter coater. Finally, the specimens were observed for trichomes in selected regions using a Hitachi S-4800 scanning electron microscope operated at 10.0 keV accelerating potential. Magnifications ranged from 180× to 2000×.

Laser microdissection (LMD) of PGTs

PGTs of *L. japonicus* were collected with LMD using a procedure similar to that previously described (Li *et al.*, 2013; Li *et al.*, 2014). Briefly, freshly-harvested leaves were fixed onto a specially manufactured metal frame (25 mm × 75 mm), which was then mounted on a Leica LMD7000 system. PGTs and other leaf tissues (LTs) that did not contain PGTs were carefully examined and targeted under a microscope. Cuttings were carried out with the following settings: TL-BF mode, 20× magnification, aperture of 15, intensity of 154, and speed of 10. The microdissected PGTs and LTs were separately collected in the caps of 0.5 mL Eppendorf microcentrifuge tubes.

Metabolite analysis of microdissected PGTs and LTs with UPLC-MS/MS

The microtube containing microdissected samples was centrifuged at 4°C (5000×*g*, 15 min) to settle the contents. Acetone (300 μL) was added and the mixture was sonicated for 10 min, and then centrifuged at 5000×*g* for 10 min at 4°C. The supernatant was directly analysed with UPLC-MS/MS. The liquid chromatography (LC) conditions were repeatedly optimised based on the separations of the major trichome metabolites (column: Waters Acquity UPLC BEH C_{18}, 1.7 μm, 2.1 mm × 100 mm; mobile phase: acetonitrile in water, 0–9 min, gradient 40–80%, 9–10 min, gradient 80–100%, *v/v*; injection volume: 5 μL; column temperature: 25°C; flow rate: 0.2 mL/min). Positive ionisation was obtained with the following parameters: mass 2–2048 Da, capillary voltage 2.0 kV, cone voltage 50 V, source temperature 150°C, desolvation temperature 350°C, desolvation gas flow 800 L/h, cone gas flow 150 L/h.

Extraction and isolation of compounds 1 and 2

Freshly-harvested leaves (2.2 kg) of *L. japonicus* were frozen in liquid nitrogen and immediately ground into small pieces, which were soaked three times with acetone (3 × 14 L, each for 24 h) at room temperature. The acetone extract was filtered and evaporated to dryness under reduced pressure. The resulting residue (90 g) was adsorbed onto 90 g of silica gel (200–300 mesh) and chromatographed on a silica gel (500 g, 200–300 mesh) column (diameter × height: 6.5 cm × 80 cm) eluting with a gradient of chloroform/acetone (1:0 to 0:1, 40 L) to yield eight fractions (A-H). Fraction C (5.5 g) was purified by MCI gel column chromatography (75–150 μm, 3 × 50 cm) eluting with methanol/water (80–100%, 8 L) to yield four sub-fractions (C_1-C_4). Sub-fraction C_1 (1.5 g) was subjected to repeated column chromatography over silica gel (200–300 mesh, 3 × 50 cm) eluting with petroleum ether/acetone (30:1, 2 L) to afford compounds **1** (392.0 mg) and **2** (200.4 mg).

Leoheterin (**1**): $C_{20}H_{30}O_4$, colourless needle crystals (acetone). ^{1}H–NMR (400 MHz, acetone-d_6) δ: 7.45 (1H, brs, H-15), 7.39 (1H, brs, H-16), 6.39 (1H, brs, H-14), 3.88 (1H, dd, J = 10.6, 3.1 Hz, H-7α), 3.80 (1H, brs, 9-OH), 3.76 (1H, d, J = 3.1 Hz, 7-OH), 3.08 (1H, s, H-5α), 1.31 (3H, s, Me-20), 1.26 (3H, d,

J = 6.5 Hz, Me-17), 0.95 (3H, s, Me-19), 0.93 (3H, s, Me-18); ^{13}C–NMR (100 MHz, acetone-d_6) δ: 35.9 (t, C-1), 18.9 (t, C-2), 42.9 (t, C-3), 32.8 (s, C-4), 56.6 (d, C-5), 213.0 (s, C-6), 78.1 (d, C-7), 48.0 (d, C-8), 77.6 (s, C-9), 49.7 (s, C-10), 32.2 (t, C-11), 22.1 (t, C-12), 126.3 (s, C-13), 111.7 (d, C-14), 143.8 (d, C-15), 139.5 (d, C-16), 12.6 (q, C-17), 33.0 (q, C-18), 22.6 (q, C-19), 18.7 (q, C-20).

Galeopsin (**2**): $C_{22}H_{32}O_5$, colourless prismatic crystals (acetone). ^{1}H–NMR (400 MHz, acetone-d_6) δ: 7.47 (1H, brs, H-15), 7.41 (1H, brs, H-16), 6.41 (1H, brs, H-14), 3.55 (1H, brs, 9-OH), 2.07 (3H, s, −OAc), 1.49 (3H, s, Me-17), 1.29 (3H, s, Me-20), 0.89 (3H, s, Me-19), 0.88 (3H, s, Me-18); ^{13}C–NMR (100 MHz, acetone-d_6) δ: 32.8 (t, C-1), 18.7 (t, C-2), 42.0 (t, C-3), 33.4 (s, C-4), 49.7 (d, C-5), 36.5 (t, C-6), 205.7 (s, C-7), 89.2 (s, C-8), 82.5 (s, C-9), 45.5 (s, C-10), 32.4 (t, C-11), 22.0 (t, C-12), 126.2 (s, C-13), 111.8 (d, C-14), 143.8 (d, C-15), 139.6 (d, C-16), 15.4 (q, C-17), 33.4 (q, C-18), 21.8 (q, C-19), 17.3 (q, C-20), 169.7 (s, −OAc), 21.4 (q, −OAc).

Anti-platelet aggregation assay of compounds 1 and 2

Anti-platelet aggregation activity was evaluated as previously described (Born, 1962) (for detailed procedure see Supporting Information).

Anti-inflammatory assay of compounds 1 and 2

Anti-inflammatory activity was tested with a method as previously described (Wu *et al.*, 2015) (for detailed procedure see Supporting Information).

Cytotoxicity assay of compounds 1 and 2

Cytotoxic activity was tested by a MTS method as previously described (García Giménez *et al.*, 2010) (for detailed procedure see Supporting Information).

Results and discussion

Morphology and distribution of trichomes

To study the morphology and distribution of the GTs on *L. japonicus*, slices of the leaves, buds and stems were observed under scanning electron microscopy (SEM). The non-glandular trichomes (NTs) were found on the leaves and young stems, and appeared as unicellular spears [Fig. 1(B)]. The PGTs were embedded into the epidermal cells and had a broad storage cavity of 40 to 50 μm in diameter, and were particularly abundant on the abaxial leaf surfaces and buds, but rarely distributed on the adaxial leaf surfaces [Fig. 1(C)].

Micro-collection of PGTs with LMD and metabolite analysis with UPLC-MS/MS

For analysis of secondary metabolites in PGTs, the intact leaf PGTs of *L. japonicus* were carefully selected under a microscope and dissected using LMD, as illustrated in Figs 1(D)–(F). Approximately 3000 PGTs with total area of ca. 15 mm^2 were collected. To avoid transformation and degradation of secondary metabolites, the dissected specimens were stored at −80°C immediately after LMD. In parallel studies, LTs were also carefully selected and dissected with similar total area as that of PGTs. The earlier collected PGTs and LTs samples were, respectively, extracted with acetone in an ultrasonic bath for 10 min. After centrifugation, the supernatant was directly analysed with UPLC-MS/MS. Both positive and negative electrospray ionisations (ESIs) were attempted. The positive ion mode gave a much better response than the negative one and was therefore selected for subsequent analysis. Two major secondary metabolites (peaks 1 and 2) with retention times of 7.51 and 8.27 min, respectively, were detected in the total ion chromatogram (TIC) of PGT extract [Figs 2(A) and (B)]. In their positive ESI mass spectra, the pseudo-molecular ion peaks ([M + Na]$^+$) at *m/z* 357 and 399 were found for peaks 1 and 2, respectively [Figs 2(G) and (K)]. The MS/MS spectrum of peak 1 displayed two major fragmental ion peaks at *m/z* 145 and 129 (base peak), while the MS/MS spectrum of peak 2 showed a completely different fragmentation pattern, with a series of fragmental ion peaks between *m/z* 95 and *m/z* 223. In the parallel study, much lower or trace amounts of these two compounds were detected in the LTs [Figs 2(C) and (D)]. In addition, six other major peaks with retention times at 5.27, 5.55, 5.87, 10.89, 11.26, and 11.67 min were detected in the TICs of the extracts of both PGTs and LTs. Their positive ESI mass spectra (Supporting Information Fig. S1) showed pseudo-molecular ion peaks

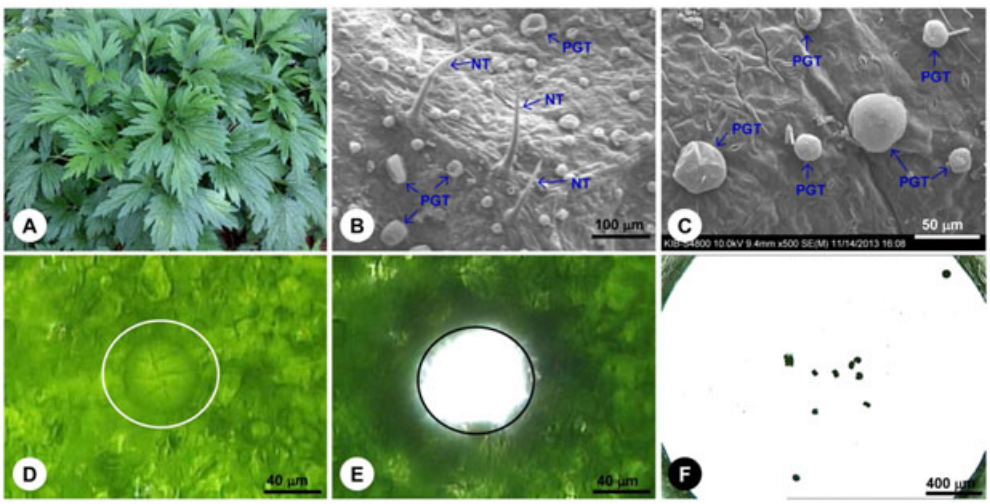

Figure 1. Morphology and distribution of trichomes of *Leonurus japonicus* and laser microdissection of peltate glandular trichomes (PGTs). (A) *Leonurus japonicus* growing in natural habitat. (B) Two major types of trichomes, non-glandular trichomes (NTs), and PGTs on the abaxial leaf surface. (C) PGTs on the abaxial leaf surface. (D) Intact PGTs before dissection. (E) The remaining leaf tissue after dissection. (F) Collected PGTs. [Colour figure can be viewed at wileyonlinelibrary.com]

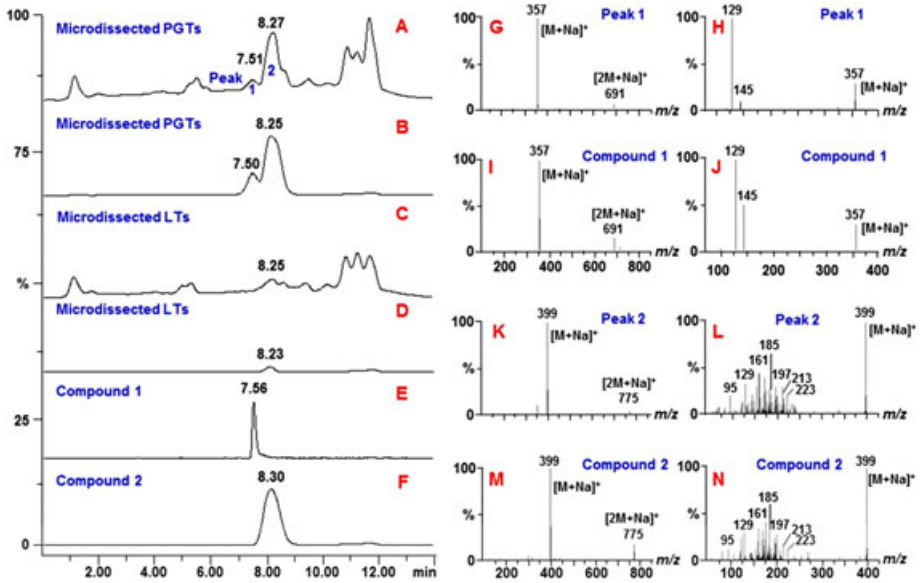

Figure 2. UPLC-MS/MS analysis of the secondary metabolites in microdissected peltate glandular trichomes (PGTs) and other leaf tissues (LTs) of *Leonurus japonicus*. (A–B) Total ion chromatogram and extracted ion chromatogram (*m/z* 357 and 399) of the acetone extract of the microdissected PGTs. (C–D) Total ion chromatogram and extracted ion chromatogram (*m/z* 357 and 399) of the acetone extract of the microdissected LTs. (E–F) Total ion chromatograms of the authentic isolated compounds **1** and **2**. (G–J) MS and MS/MS spectra of peak 1 and compound **1**. (K–N) MS and MS/MS spectra of peak 2 and compound **2**. [Colour figure can be viewed at wileyonlinelibrary.com]

([M + Na]$^+$) at *m/z* 437, 437, 451, 345, 494 and 504, respectively. However, further identification of these metabolites was not achieved due to lack of authentic samples.

Extraction, isolation and identification of compounds 1 and 2

The amount of PGTs collected by LMD was insufficient for direct isolation and identification of the two major compounds and for the subsequent biological tests. Therefore the acetone extract of the whole leaves of *L. japonicus* rich in PGTs was used to trace and isolate the targeted compounds. Consequently, compounds **1** and **2** (Fig. 3) were successfully purified as colourless needle crystals (acetone) and prismatic crystals (acetone), respectively. The ^{1}H–NMR spectrum of **1** (Fig. S2) showed three tertiary methyls at δ_H 0.93, 0.95 and 1.31, a secondary methyl at δ_H 1.26 (3H, J = 6.5 Hz), three oxygenated methines or free hydroxyl groups at δ_H 3.76 (d, J = 3.1 Hz), 3.80 (brs) and 3.88 (dd, J = 10.6, 3.1 Hz), and three olefinic protons at δ_H 6.39 (brs), 7.39 (brs), and 7.45 (brs) which were resonated from a typical furan-3-yl moiety. Analysis of the ^{13}C–NMR spectrum of **1** with the aid of the DEPT-90 and -135 spectra (Fig. S3) revealed the existence of 20 carbon resonances including a keto (δ_C 213.0), a furan-3-yl moiety (δ_C 111.7, 126.3, 139.5, and 143.8), four methyls, five sp^3 methylenes, three sp^3 methines (including an oxy-methine at δ_C 78.1), and three sp^3 quaternary carbons (including an oxygenated one at δ_C 77.6). These spectroscopic data obviously indicated a furan-containing labdane diterpenoid for **1**. Accordingly, compound **1** was straightforwardly identified as leoheterin through comparison of its spectroscopic data with those reported in the literature (Hon *et al.*, 1993). The ^{1}H- and ^{13}C–NMR spectral data of **2** (Figs. S4 and S5) were very similar to those of **1**, indicating that **2** was also a furan-containing labdane diterpenoid. Besides the existence of an additional acetoxy group (δ_C 169.7 and 21.4) in **2**, the obvious difference between the two compounds was that the secondary methyl (Me-17) in **1** changed to a tertiary methyl in **2**. In addition, two methines at δ_C 48.0 and 78.1 in **1** were replaced by a methylene and an oxygen-bearing quaternary carbon (δ_C 36.5 and 89.2) in **2**. Similarly, compound **2** was characterised as galeopsin through comparison of its spectroscopic data with those reported in the literature (Tasdemir *et al.*, 1995). Through further comparison of their retention times, MS, and MS/MS spectra (Fig. 2), the identities of peaks 1 and 2 in the TIC of PGT extract of *L. japonicus* were finally validated to be leoheterin (**1**) and galeopsin (**2**), respectively.

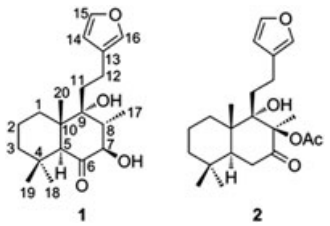

Figure 3. Structures of leoheterin (**1**) and galeopsin (**2**) identified in the peltate glandular trichomes of *Leonurus japonicus*.

Anti-platelet aggregation assay of compounds 1 and 2

Since *L. japonicus* is a traditional Chinese medicinal herb used to invigorate blood circulation (including anti-clotting), the anti-platelet aggregation effect of compounds **1** and **2** was tested. As shown in Fig. 4, compound **1** showed potential antithrombotic activity in arachidonic acid (AA) induced rabbit platelet aggregation, with inhibition rate of 41.9 ± 10.6% ($P < 0.01$) at 0.30 mg/mL. In contrast, compound **2** only showed a weak effect, with inhibition rate of 9.7 ± 1.2% ($P < 0.05$) at the same concentration. In another anti-platelet aggregation assay induced by adenosine diphosphate (ADP), compound **1** also exhibited a

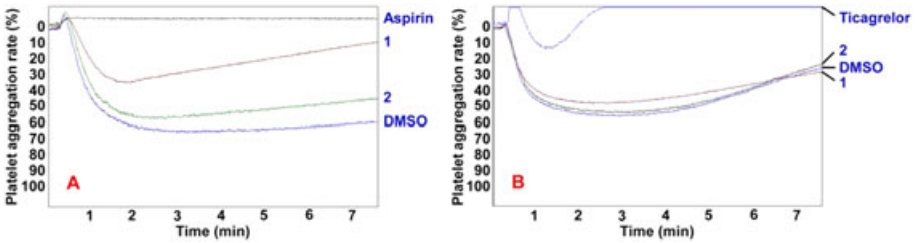

Figure 4. Effect of compounds **1** and **2** on rabbit platelet aggregation. (A) 0.5 mM arachidonic acid (AA)-induced rabbit platelet aggregation. (B) 10 μM adenosine diphosphate (ADP)-induced rabbit platelet aggregation. Aspirin: 0.04 mg/mL; Ticagrelor: 5 μg/mL; **1** and **2**: each 0.30 mg/mL; dimethyl sulfoxide (DMSO): 1% (*v*/*v*). [Colour figure can be viewed at wileyonlinelibrary.com]

weak effect, with inhibition rate of 13.1 ± 3.6% ($P < 0.05$) at 0.30 mg/mL, while compound **2** was inactive.

Anti-inflammatory and cytotoxicity assays of compounds 1 and 2

Considering that the crude extract of *L. japonicus* has been reported to exhibit anti-inflammatory and anti-cancer activities (Shang *et al.*, 2014), the anti-inflammatory and cytotoxic activities of compounds **1** and **2** were also tested. The inflammation model on RAW264.7 cells induced by lipopolysaccharides (LPS) was adopted for anti-inflammatory assay. As shown in Table 1, both compounds **1** and **2** showed significant anti-inflammatory activity by inhibiting proinflammatory cytokine tumor necrosis factor-α (TNF-α), with 50% inhibitory concentration (IC_{50}) values of 18.65 ± 0.43 and 17.01 ± 1.56 μM, respectively. However, neither of them was active in inhibiting proinflammatory cytokine interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1). Cytotoxic activity against human lung adenocarcinoma cell line (NCI-H1975, PC9, and XLA-07), human hepatoma cell line (HepG2) and mouse mononuclear macrophage leukemia cell line (RAW264.7) was evaluated by MTS assay. It was found that compounds **1** and **2** exhibited moderate cytotoxicity against H1975 and RAW264.7 cells (IC_{50} ranging from 61.15 ± 3.17 to 76.89 ± 0.16 μM). Moreover, compound **1** also showed moderate cytotoxicity against XLA-07 cells (IC_{50} = 67.08 ± 3.50 μM). However, neither of them showed obvious cytotoxicity against PC9 and HepG2 cells (Table 2).

Leonurus plants are widely distributed in China, comprising of 12 species. The abaxial leaf surfaces of most species are covered with numerous GTs. However, to our knowledge, only *L. sibiricus* has had the morphology of its GTs described (Moyano *et al.*, 2003; Yu *et al.*, 1999), while none of them have been phytochemically investigated for the secondary metabolites accumulated in their GTs. On the leaves of *L. sibiricu*, both PGTs and CGTs have been found (Yu *et al.*, 1999). Interestingly, GTs were also observed on the ventral and the dorsal side of the anthers of *L. sibiricu* (Moyano *et al.*, 2003). Nevertheless, in the present study, only PGTs but no CGTs were found on the leaves and young stems of *L. japonicus*. Therefore, the morphology and distribution pattern of the GTs might vary among different *Leonurus* species.

Table 1. Inhibitory activity of compounds **1** and **2** on TNF-α, IL-6 and MCP-1 production (n = 3)

Compound	IC_{50} (μM)		
	TNF-α	IL-6	MCP-1
1	18.65 ± 0.43	>40	>40
2	17.01 ± 1.56	>40	>40
Dexamethasone (×10^{-3})	1.17 ± 0.18	9.42 ± 0.75	2.91 ± 0.23

Leoheterin (**1**) and galeopsin (**2**) were originally isolated from *L. japonicus* in 1993 (Hon *et al.* 1993). This is the first time that the two compounds were precisely localised to the PGTs of this plant, suggesting that they probably not only accumulated but are also biosynthesised in the GTs. Acetylcholinesterase (AChE) and nitric oxide (NO) inhibitory activities of compounds **1** and **2** have been previously reported (Girón *et al.*, 2008; Hung *et al.*, 2011). In this paper, anti-platelet aggregation, anti-inflammatory, and cytotoxic activities of compounds **1** and **2** were observed, indicating that the two compounds could have been involved in the traditional applications especially the use of fresh *L. japonicus* for invigorating blood circulation, anti-inflammation and cancer prevention. Platelets hyperfunction plays a central role in thromboembolic diseases. Compound **1** showed obviously stronger activity in AA-induced rabbit platelet aggregation than ADP-stimulated platelet aggregation, suggesting that the anti-platelet aggregation effect of **1** might be selective. In contrast to **1**, the reduced anti-platelet aggregation activity of compound **2** implied that the substitution pattern of ring B should be important for this type of compound to exert their activity. In addition, compounds **1** and **2** were almost equally active in inhibiting the TNF-α production, indicating that the labdane skeleton and furan functionality might be more important for their activity than the substituents at ring B. The earlier biological activities of leoheterin (**1**) and galeopsin (**2**), in combination with their high yields (0.01–0.02% in fresh leaves), suggests that they could be considered as part of the marker compounds to control the quality of the medicinal herb *L. japonicas*.

To confront the complicated and changing environment, plants often synthesise and highly accumulate secondary metabolites with unusual chemical structures and special biological functions in their GTs (Mccaskill and Croteau, 1999; Schilmiller *et al.*, 2008). While the discovery of bioactive compounds from the rising complex chemodiversity of plants through classic phytochemical method is costly, less efficient, and time-consuming, it might be a shortcut to explore bioactive compounds from the largely untapped secondary metabolite repository in the GTs of plants. LMD is a convenient and powerful sampling technique to harvest homogeneous cell types or microscopic tissue pieces from different plant organs (Fang and Schneider, 2014). This approach in combination with sensitive analytical techniques has already been applied to analyse the specific metabolites in targeted tissues of medicinal plants (Happyana *et al.*, 2013; Jaiswal *et al.*, 2015). Besides the high-resolution microanalysis of tissue-specific metabolites, the findings in this paper further proved that LMD-

Table 2. Cytotoxic activity of compounds **1** and **2** (n = 3)

Compound	IC_{50} (μM)				
	H1975	XLA-07	PC9	HepG2	RAW264.7
1	71.42 ± 5.37	67.08 ± 3.50	>80	>80	61.15 ± 3.17
2	68.73 ± 1.04	>80	>80	>80	76.89 ± 0.16
Taxol ($\times 10^{-3}$)	3.89 ± 0.50	80.18 ± 6.89	3.68 ± 0.80	10.28 ± 1.88	76.82 ± 4.38

UPLC-MS/MS should be a highly accurate and efficient technique to access the bioactive natural products harboured by the GTs of plants.

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References

Born GVR. 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* **194**: 927–929.
Chinese Pharmacopeia Commission. 2015. *Chinese Pharmacopoeia*. China Medical Science Press: Beijing; 290–291.
Editorial Board of China Herbal, State Administration of Traditional Chinese Medicine. 1999. *China Herbal*. Scientific and Technical Publishers: Shanghai; 61–66.
Fang J, Schneider B. 2014. Laser microdissection: A sample preparation technique for plant micrometabolic profiling. *Phytochem Anal* **25**: 307–313.
García Giménez D, García Prado E, Sáenz Rodríguez T, Fernández Arche A, De La Puerta R. 2010. Cytotoxic effect of the pentacyclic oxindole alkaloid mitraphylline isolated from *Uncaria tomentosa* bark on human Ewing's sarcoma and breast cancer cell lines. *Planta Med* **76**: 133–136.
Girón N, Través PG, Rodríguez B, López-Fontal R, Boscá L, Hortelano S, De Las Heras B. 2008. Suppression of inflammatory responses by labdane-type diterpenoids. *Toxicol Appl Pharmacol* **228**: 179–189.
Happyana N, Agnolet S, Muntendam R, Van Dam A, Schneider B, Kayser O. 2013. Analysis of cannabinoids in laser-microdissected trichomes of medicinal *Cannabis sativa* using LCMS and cryogenic NMR. *Phytochemistry* **87**: 51–59.
Hon PM, Wang ES, Lam SKM, Choy YM, Lee CM, Wong HNC. 1993. Preleoheterin and leoheterin, two labdane diterpenes from *Leonurus heterophyllus*. *Phytochemistry* **33**: 639–641.
Hung TM, Luan TC, Vinh BT, Cuong TD, Min BS. 2011. Labdane-type diterpenoids from *Leonurus heterophyllus* and their cholinesterase inhibitory activity. *Phytother Res* **25**: 611–614.
Jaiswal Y, Liang Z, Ho A, Chen H, Zhao Z. 2015. Metabolite profiling of tissues of *Acorus calamus* and *Acorus tatarinowii* rhizomes by using LMD, UHPLC-QTOF MS, and GC-MS. *Planta Med* **81**: 333–341.
Li CH, Jing SX, Luo SH, Shi W, Hua J, Liu Y, Li XN, Schneider B, Gershenzon J, Li SH. 2013. Peltate glandular trichomes of *Colquhounia coccinea* var. *mollis* harbor a new class of defensive sesterterpenoids. *Org Lett* **15**: 1694–1697.
Li CH, Liu Y, Hua J, Luo SH, Li SH. 2014. Peltate glandular trichomes of *Colquhounia seguinii* harbor new defensive clerodane diterpenoids. *J Integr Plant Biol* **56**: 928–940.
Lu X, Zhang L, Zhang FY, Jiang WM, Shen Q, Zhang LD, Lv ZY, Wang GF, Tang KX. 2013. AaORA, a trichome-specific AP2/ERF transcription factor of *Artemisia annua*, is a positive regulator in the artemisinin biosynthetic pathway and in disease resistance to *Botrytis cinerea*. *New Phytol* **198**: 1191–1202.
Luo SH, Luo Q, Niu XM, Xie MJ, Zhao X, Schneider B, Gershenzon J, Li SH. 2010. Glandular trichomes of *Leucosceptrum canum* harbor defensive sesterterpenoids. *Angew Chem Int Ed* **49**: 4471–4475.
Majdi M, Liu Q, Karimzadeh G, Malboobi MA, Beekwilder J, Cankar K, de Vos R, Todorovic S, Simonovic A, Bouwmeester H. 2011. Biosynthesis and localization of parthenolide in glandular trichomes of feverfew (*Tanacetum parthenium* L. Schulz Bip.). *Phytochemistry* **72**: 1739–1750.
Mccaskill D, Croteau R. 1999. Strategies for bioengineering the development and metabolism of glandular tissues in plants. *Nat Biotechnol* **17**: 31–36.
Moyano F, Cocucci A, Sérsic A. 2003. Accessory pollen adhesive from glandular trichomes on the anthers of *Leonurus sibiricus* L. (Lamiaceae). *Plant Biol* **5**: 411–418.
Piccoli PN, Bottini R. 2008. Accumulation of the labdane diterpene Marrubiin in glandular trichome cells along the ontogeny of *Marrubium vulgare* plants. *Plant Growth Regul* **56**: 71–76.
Schilmiller AL, Last RL, Pichersky E. 2008. Harnessing plant trichome biochemistry for the production of useful compounds. *Plant J* **54**: 702–711.
Shang XF, Pan H, Wang XZ, He H, Li MX. 2014. *Leonurus japonicus* Houtt.: Ethnopharmacology, phytochemistry and pharmacology of an important traditional Chinese medicine. *J Ethnopharmacol* **152**: 14–32.
Siebert DJ. 2004. Localization of salvinorin A and related compounds in glandular trichomes of the psychoactive sage, *Salvia divinorum*. *Ann Bot* **93**: 763–771.
Tasdemir D, Wright AD, Sticher O, Calis I, Linden A. 1995. Detailed ^{1}H- and ^{13}C-NMR investigations of some diterpenes isolated from *Leonurus persicus*. *J Nat Prod* **58**: 1543–1554.
Wang Y, Luo SH, Hua J, Liu Y, Jing SX, Li XN, Li SH. 2015. Capitate glandular trichomes of *Paragutzlaffia henryi* harbor new phytotoxic labdane diterpenoids. *J Agric Food Chem* **63**: 10004–10012.
Wu K, Mo CF, Xiao HY, Jiang Y, Ye BG, Wang S. 2015. Imperialine and verticinone from bulbs of *Fritillaria wabuensis* inhibit pro-inflammatory mediators in LPS-stimulated RAW 264.7 macrophages. *Planta Med* **81**: 821–829.
Yu LJ, Cui JZ, Zhang DW, Sun DW, Wang FC. 1999. The diversity and the developmental morphology of the glandular hairs on the leaf surface of *Leonurus sibiricus*. *Chin Bull Bot* **16**: 602–605.

Supporting information

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