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# Antinociceptive and anti-inflammatory activities of a standardizedextract of bis-iridoids from *Pterocephalus hookeri*

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ABSTRACT

*Ethnopharmacological relevance: Pterocephalus hookeri* (C.B. Clarke) Höeck, one of the most popular Tibetan herbs, has been widely applied in Tibetan medicine prescriptions. Chemical investigations have led to the isolation of many bis-iridoids. However, the pharmacological activities of bis-iridoid constituents of this plant have never been reported before.

*Aim of the study:* This study evaluated the anti-inflammatory and analgesic activities of afraction of bis-iridoid constituents of *P. hookeri* (BCPH) in order to provide experimental evidence for its traditional use, such as for cold, flu, and rheumatoid arthritis.

*Materials and methods:* The analgesic effects of BCPH were investigated using the hot-plate test and acetic acid-induced writhing test. The anti-inflammatory activities were observed using the following models: carrageenin-induced edema of the hind paw of rats and xylene-induced ear edema in mice. The effects of dexamethasone administration were also studied.

*Results:* BCPH significantly increased the hot-platepain threshold and reduced acetic acid-induced writhing response in mice. Moreover, BCPH remarkably inhibited xylene-induced ear edema and reduced the carrageenin-induced rat paw edema perimeter.

*Conclusion:* The results reveal that BCPH has central, peripheral analgesic activities as well as anti-inflammatory effects, supporting the traditional application of this herb in treating various diseases associated with inflammation and pain.

## 1. Introduction

*Pterocephalus hookeri* (C.B. Clarke) Höeck is aperennial herbaceous plateau plant widely spread in Tibet. This species, known as “pang-zi-duo-wo” in Tibetan, has been used in popular medicine to treat pain, fever, and inflammatory chronic diseases such rheumatoid arthritis (Wu et al., 2014b).

A preclinical study reported that ethanol and aqueous extracts of *P. hookeri* have both central and peripheral antinociceptive effects and anti-inflammatory activities(Shen et al., 2017). Research confirmed these results and also revealed that a hydroalcoholic extract of *P. Hookeri* significantly reduced acute and chronic inflammation in cotton pellet-induced granuloma and in formaldehyde-induced arthritis in rats. However, the mechanisms underlying these antinociceptive and anti-inflammatory effects of *P. hookeri* still remain unclear.

Phytochemical studies on this plant have led to the isolation of a wide range of chemical constituents including triterpenoids (Zhang et al., 2012), iridoids (Wu et al., 2014b), and bis-iridoids and lignans (Wu et al., 2014b).

Iridoids and bis-iridoids are abundant in *P. hookeri* and have been shown to display a broad spectrum of bioactivities including analgesic activities and anti-inflammatory activity. Specifically, Pterocenoids A–E, bis-iridoid constituents of *P. hookeri* (BCPH), demonstrated the capacity of reducing the production of nuclear factor κB (NF-κB) and proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), as well as down-regulating inducible nitric oxide synthase and cyclooxygenase-2 expression in lipopolysaccharide- stimulated cells(Wu et al., 2015).

Despite *P. hookeri* being commonly used, its antinociceptive and anti-inflammatory effects still remain unclear. Moreover, the presence of triterpenoid toxins in its extracts deserves caution, especially when it is used for long periods, such as when treating arthritis or rheumatism with oral ingestion of extracts (Tian et al., 1993; Zhang et al., 2012).

Considering the potential chronic toxicity of total extracts of *P. hookeri* and the biological relevance of bis-iridoids, the aim of this study was to prepare a standardized extract of bis-iridoids from *P. hookeri*,

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avoiding the toxicity of the triterpenoids.

## 2. Material and methods

### 2.1. Plant material and extraction

Underground parts of *P. hookeri* were collected in August 2016 from Tibet, China, and identified by Prof. Zhi-Li Zhao (Shanghai University of Traditional Chinese Medicine). A voucher specimen (No. 20160720) was deposited at the School of Pharmacy, Shanghai University of Traditional Chinese Medicine, Shanghai, P. R. China.

Air-dried and powdered underground parts of *P. hookeri*(10 kg) were extracted with 10 L of 95% EtOH under refluxed conditions three times. The EtOH extract was filtered and concentrated under reduced pressure to yield a crude extract (1.2 kg).The extract was suspended in $H_2O$ (4.0 L) and then successively partitioned with petroleum ether (3 × 4.0 L), $CHCl_3$ (3 × 4.0 L), and n-BuOH (3 × 4.0 L). The n-BuOH fraction was concentrated under reduced pressure to yield a crude extract (1.2 kg). The fractions were analyzed on thin-layer chromatography silica gel 60 plates and revealed with anisaldehyde–sulfuric acid, which demonstrated the presence of purple and blue spots in the BCPH fraction, characteristic of bis-iridoids. An aliquot of the BCPH fraction was solubilized in methanol and filtered through a 0.22-mm membraneprior to analyses.

### 2.2. Analytical standards and Chemicals

Eight compounds were quantified: cantleyoside(**1**), sylvestroside I (**2**), laciniatosideII(**3**), sylvestroside IV(**4**), sylvestroside III dimethyl acetal(**5**), Sylvestroside III (**6**), Laciniatoside I (**7**), and Sylvestroside IV dimethyl acetal(**8**). The compounds were isolated from the n-BuOH extract of underground parts of *P. hookeri* by semipreparative high-performance liquid chromatography(HPLC) and an isocratic elution system of $CH_3CN/H_2O$(2/5, v/v) at 3 mL/min. The compounds were identified by comparing $^1H$ and $^{13}C$ nuclear magnetic resonance (NMR) data with the data in the literature(Wu et al., 2014a). NMR analysis was performed using a Bruker ARX-600 (600 MHz for $^1H$, 125 MHz for $^{13}C$) (Bruker Co., Germany), and the data were processed using MestReNova software; spectra of the compounds are available as supplementary material. The purity of the standards was assessedusing HPLC–photodiode array detection, and the analysis demonstrated the purity to be > 97% for all the compounds. Sylvestroside III was used as internal standard and was purchased from Sigma-Aldrich. For stock standard solutions of compounds 1–8, 1.0 mg/g of each standard was prepared in methanol, stored at 4 °C, brought to room temperature, and filtered through a 0.22-mm membrane before injection.

### 2.3. Qualitative analyses of bis-iridoid extract

HPLC–high-resolution mass spectrometry analysis was performed with the hybrid mass spectrometer LQT-Orbitrap Discovery XL with an electrospray ionization source coupled with an HPLC system (Thermo Scientific, Germany). System control and data processing were conducted using Thermo Xcalibur 2.2 software. Chromatographic separation was achieved using an Agilent 1200 HPLC system with an ultraviolet detector operated at 238 nm and Kromasil C18 reversed-phase column (250 mm × 10 mm, 5 μm) at a flow rate of 1 mL/min with an injection volume of 10 μL. The mobilephase consisted of a gradient system combining water (A) and acetonitrile (B), both in solution with 0.1% formic acid, and is outlined as follows: 0–20 min from 95% A to 5% A. Electrospray ionization was applied in positive-ion mode under the following conditions: capillary temperature, 350 °C; capillary voltage, 40 V; tube lens, 80 V; source voltage, 3.5 kV; sheath gas flow, 40 arb. units; and aux gas flow, 20 arb. units. Analysis was performed using Fourier transform mass spectrometry in full-scan mode, with a resolution of 30,000 in the m/z range of 1201000, along with data-dependent scans performed using an ion trap mass spectrometer with a resolution of 7500 and collision-induced dissociation value of 35%.

### 2.4. Animals and treatments

All experiments were performed in accordance with the guidelines and regulations of the Ethical Committee of Shanghai University of Traditional Chinese Medicine. The protocols for the animal study were approved by the Institute Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Approved Number: 20160807). Male or female imprinting control region mice (20–22 g) and male Sprague–Dawley rats (180–200 g) were purchased from the SLAC Laboratory (Shanghai, China). All animals were kept in a room maintained under environmentally controlled conditions of 24 ± 1 °Cand 12-h light and 12-h dark cycle. All animals had free access to water and standard diet. They were acclimatized at least 1 week before the experiments were started. The mice were fasted for 10 h prior to the experiments, and the test substances were given orally with free access to water.

### 2.5. Drugs

The following substances were used: Tween 80(Merck, Darmstadt, Germany), carrageenan(Sigma Chemical Co., St. Louis, USA), TNF-α (R &D Systems, Minneapolis, MN, USA), xylene, and glacial acetic acid (Sinopharm Chemical Reagent Co., Ltd); and dexamethasone sodium phosphate for injection (BBCA Pharmaceutical Co., Ltd). All the drugs were dissolved in saline solution (0.9% NaCl). A standardized extract of BCPH was dissolved in saline plus Tween 80. The final concentration of Tween 80 did not exceed 5% and did not cause any effect perse. All the control animals received the vehicle.

### 2.6. Writhing reflex induced by acetic acid in mice

In the writhing test, male mice were divided into six groups. This was performed according to apreviously described method (Zhang et al., 2009). The mice received 0.6% acetic acid solution in normal saline injected intraperitoneally at a dose of 10 mL/kg. The number of writhes was counted starting 5 min after injection that lasted for 15 min. The response consisted of abdominal wall contractions and pelvic rotation, followed by hind limb stretches. The test samples, dexamethasone, and distilled water were administered orally 1 h prior to acetic acid injection. The percentage analgesic activity was calculated as follows:

$$\text{Percentage analgesic activity} = (Nc–Nt)/Nc \times 100\%$$

where Nc is the average number of stretches of the control group, and Nt is the average number of stretches of the test drug group.

### 2.7. Hot-plate test

A hot-plate test was performed according to a previously reported method(Li et al., 2011). The test was conducted at a fixed temperature of 55 ± 0.5 °C. The reaction involved paw licking and jumping. The time (in seconds) between the platform and reaction was recorded as the response latency. Mice exhibiting latency time greater than 30 s or less than 5 s were excluded. The latency time was determined at 30, 60, and 90 min after administration of the test samples, indomethacin, and distilled water. If the reaction time was more than 60 s, the latency was recorded as 60 s. A single intraperitoneal injection of Rotundine (20 mg/kg) was considered as the positive control (Kang et al., 2016; Laste et al., 2013).

### 2.8. Ear edema induced by xylene in mice

A xylene-induced ear edema test was performed as previously