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ORIGINAL ARTICLE

Anti-inflammatory Activity of Total Alkaloids from *Hypecoum leptocarpum* Hook. f. et Thoms

Huaixiu Wen[1,3†], Lei Jiang[1†], Defang Zhang[2], Xiang Yuan[1,3], Jun Dang[1], Lijuan Mei[1], Yun Shao[1,4], Yanduo Tao[1,4]

[1]Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China, [2]Research Institute of Forestry, Qinghai Academy of Agriculture and Forestry, Qinghai University, Xining 810016, China, [3]College of Life Science, University of Chinese Academy of Sciences, Beijing 100049, China, [4]Qinghai Key Laboratory of Tibetan Medicine Research, Xining 810008, China
†These authors contributed equally to this work

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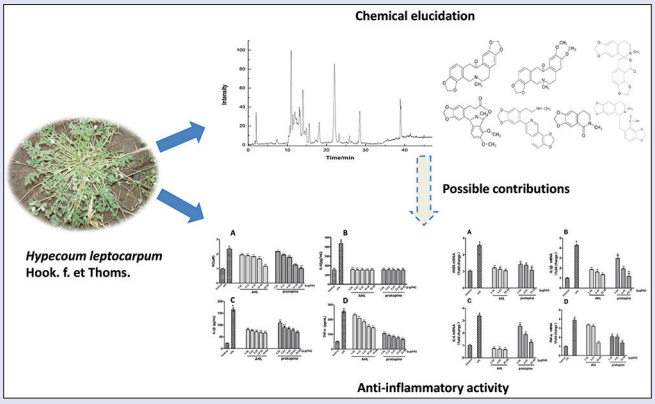
ABSTRACT

Background: *Hypecoum leptocarpum* Hook. f. et Thoms., which is used in traditional Tibetan medicine as an antipyretic, antitussive, analgesic, and anti-inflammatory agent, contains a variety of alkaloids that could be responsible for its analgesic and anti-inflammatory properties. **Objective:** The present study was designed to investigate the anti-inflammatory activity of the total alkaloids from *H. leptocarpum* (AHL) *in vitro* and to elucidate the chemical structure of the anti-inflammatory components in AHL. **Materials and Methods:** Chemical characterization was performed using liquid chromatography/quadrupole-time-of-flight mass and diode-array detector-high performance liquid chromatography. The anti-inflammatory effects of AHL were investigated by measuring the production of inflammatory cytokines using enzyme-linked immunosorbent assay and mRNA expression by real-time polymerase chain reaction in lipopolysaccharide-induced RAW 264.7 macrophages. **Results:** Chemical analysis of AHL revealed the presence of seven alkaloids, protopine (13.3%), cryptopine (1.5%), leptopidinine, leptocarpine, corydamine, dihydroleptopine, and oxohydrastinine. AHL significantly suppressed the production of nitric oxide (NO), interleukin-1 beta (IL-1 β), IL-6, and tumor necrosis factor-alpha (TNF-α) in LPS-induced RAW 264.7 cells. The maximum levels of suppression of NO, IL-1 β, IL-6, and TNF-α were 86.8% ± 2.2%, 70.1% ± 1.5%, 100.1% ± 2.5%, and 50.8% ± 3.6%, respectively. IC_{50} values of suppression of cytokine production by AHL were 7.47 ± 2.81 μg/mL (NO), 0.12 ± 0.28 μg/mL (IL-1 β), 0.56 ± 0.37 μg/mL (IL-6), and 18.95 ± 5.23 μg/mL (TNF-α). AHL was also shown to downregulate mRNA expression of inducible NO synthase, IL-1 β, IL-6, and TNF-α *in vitro*. **Conclusion:** The study provides convincing evidence that AHL has strong anti-inflammatory activity. The potent activity is likely a result of synergy between the different alkaloids.

Key words: Analgesic, anti-inflammatory, *Hypecoum leptocarpum* Hook. f. et Thoms, total alkaloids

SUMMARY

- The present study aims to investigate the anti-inflammatory activity of the total alkaloids from *H. leptocarpum* (AHL) in vitro, and to elucidate the chemical structure of the anti-inflammatory components in AHL. It was observed that AHL significantly suppressed the production of nitric oxide (NO), interleukin-1 beta (IL-1β), IL-6 and tumor necrosis factor-alpha (TNF-α) in LPS-induced RAW 264.7 cells. AHL was also shown to down-regulate mRNA expression of inducible nitric oxide synthase, IL-1β, IL-6 and TNF-α *in vitro*. The study provides convincing evidence that AHL has strong anti-inflammatory activity.



Abbreviations used: The total alkaloids from *H. leptocarpum*: AHL; Nitric oxide: NO; Interleukin-1 beta IL-1β; Interleukin-6: IL-6; Tumor necrosis factor-alpha: TNF-α; Prostaglandin E2: PGE2; Inducible nitric oxide synthase: iNOS; Nonsteroidal anti-inflammatory drugs: NSAIDs; lipopolysaccharide: LPS; The total ion chromatograms: TIC; The liquid chromatography/quadrupole-time of flight: LC/Q-TOF; Nuclear factor-kappa B: NF-κB; Janus kinase-signal transducers and activators of transcription: JAK-STAT.

Correspondence:

Prof. Yun Shao,
Key Laboratory of Tibetan Medicine Research,
Northwest Institute of Plateau Biology,
Chinese Academy of Sciences, No. 23, Xining Road,
Xinning 810008, China.
E-mail: shaoyun11@126.com
Prof. Yanduo Tao,
Key Laboratory of Tibetan Medicine Research,
Northwest Institute of Plateau Biology, Chinese
Academy of Sciences, No. 23, Xining Road,
Xinning 810008, China.
E-mail: 4576805@qq.com
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INTRODUCTION

Inflammation is an adaptive response to noxious stimuli, such as infection and tissue injury.[1] While inflammation plays an important role in host defense against infectious agents and injury, it also contributes to the pathophysiology of many chronic diseases.[2] During inflammation, macrophages secrete a variety of pro-inflammatory cytokines, including nitric oxide (NO), prostaglandin E2, tumor necrosis factor-α (TNF-α), and interleukins (ILs).[3] NO is synthesized in macrophages by the catalytic action of inducible NO synthase (iNOS), overexpression

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of which is involved in the pathogenesis of inflammation leading to tissue damage and even sepsis.[4,5] TNF-α, IL-6, and IL-1 β are major pro-inflammatory cytokines that stimulate production of other mediators involved in cellular recruitment, fever, release of acute-phase proteins, increased vascular permeability, and hyperalgesia.[6,7] Overproduction of these mediators is harmful to tissues and organisms and may cause inflammation-related diseases, such as rheumatoid arthritis, diabetes, cardiovascular diseases, and cancer.[8,9] Due to the critical role of cytokines during inflammation, many pharmacological studies have been carried out to evaluate the effects of regulation on inflammatory pathways.

At present, the most frequently used anti-inflammatory agents are nonsteroidal and steroidal anti-inflammatory drugs.[10] Nonsteroidal anti-inflammatory drugs (NSAIDs) are the frontline drugs and are effective for the treatment of various inflammatory diseases.[11,12] The use of NSAIDs may, however, cause gastric irritation, even bleeding and ulcers.[13,14] Steroidal anti-inflammatory drugs, typically glucocorticoids, also have adverse drug reactions on long-term use and can lead to cardiovascular, endocrine, metabolic, musculoskeletal, and ophthalmologic problems.[15] New, safe and effective therapies for chronic inflammation are thus needed and many studies have been carried out to discover new anti-inflammatory agents with novel structures and fewer adverse effects.[16] Compounds that inhibit the production of pro-inflammatory cytokines are likely to have anti-inflammatory properties, and natural products are a potentially rich source of such compounds.[17,18]

Hypecoum leptocarpum Hook. f. et Thoms. is an herbaceous plant of the Papaveraceae family that grows in South and Northwest China.[19] It is used in traditional Tibetan medicine as an antipyretic, antitussive, analgesic, and anti-inflammatory agents.[20] An ethanol extract of *H. leptocarpum* has been shown to suppress the systemic inflammatory response to lipopolysaccharide (LPS) by reducing inflammatory cell infiltration into lung tissue, decreasing peripheral white blood cell count, and downregulating TNF-α and IL-6.[21]

It is unclear which chemical components of the ethanol extract are responsible for the anti-inflammatory activity although *H. leptocaupum* has been shown to contain a variety of alkaloids, especially isoquinoline alkaloids, including protopine,[22] cryptopine,[23] oxyhydrastinine,[24] hypecoumine,[25] leptocarpine,[26] hyperectine,[27] and corydamine.[28] Members of this class of alkaloids have been shown to have anti-spasmodic,[29] anti-arrhythmic,[30] anti-cancer,[31] anti-bacterial,[32] anti-viral,[33] and anti-inflammatory effects.[34]

We recently reported that the total alkaloids from *H. leptocarpum* (AHL) not only reduced paw edema but also provided pain relief in formalin test *in vivo*.[35] The aim of the present study was to evaluate the anti-inflammatory effects of AHL on the LPS-induced RAW 264.7 macrophages *in vitro*. We set out to investigate the expression of mediators and cytokines involved in the inflammatory response and to explore the mechanism of the anti-inflammatory actions of AHL.

MATERIALS AND METHODS

Plant material

H. leptocarpum Hook. f. et Thoms. was collected from Datong County, Qinghai Province, China (GPS coordinates: E 100° 54′ 20”, N 37° 11’ 17”, 2350 m), in September 2014. The herb was identified by Professor Mei Lijuan of the Northwest Institute of Plateau Biology, Chinese Academy of Sciences, and a voucher specimen (HNWP-0310669) was deposited in the Herbarium of the Northwest Institute of Plateau Biology, Chinese Academy of Sciences.

Plant extraction and isolation

Air-dried whole *H. leptocarpum* plants were ground to a fine powder and sieved (40 mesh). A powdered sample (1.58 kg) was extracted twice with 95% ethanol under reflux at 60°C (3 h and each time) and the combined extracts were evaporated under reduced pressure. The crude ethanol extract (107.4 g) was then suspended in water and extracted with petroleum ether. The water layer was acidified to pH 1–2 with 2.5% HCl and extracted with ethyl acetate. The resulting aqueous solution was neutralized with concentrated ammonium hydroxide and evaporated. The residue (dried weight 63.4 g) was subjected to column chromatography on MCI® gel[36] (CHP-20P, 75–150 mm, Mitsubishi, Tokyo, Japan), eluting successively with water and methanol. The methanol fraction was evaporated to give AHL (43.9 g).

The AHL was further separated by chromatography on a silica-based strong cation exchange stationary phase[37] (SCX, 60 μm, Acchrom, Dalian, China), eluting successively with methanol and methanol containing 250 mM NH_4ClO_4. The latter fraction was collected and the NH_4ClO_4 was removed by chromatography on a C18 column (50 μm, Acchrom), eluting with water and methanol. After desalination, the methanol fraction (CMF, 13.5 g) was further separated by preparative chromatography on an XCharge C18 preparative high-performance liquid chromatography (HPLC) column (150 mm × 20 mm, 7 μm, Acchrom, Dalian, China), eluting with acetonitrile/0.1% aqueous formic acid (15: 85, v/v) from 0 to 25 min. Two known compounds, protopine and corydamine, were obtained by this procedure.

Phytochemical analysis

Qualitative analysis was performed on an Agilent 1290 Infinity LC system, coupled to a 6540 UHD accurate mass quadrupole-time-of-flight (Q-TOF) mass spectrometer (MS) (Agilent Technologies, Santa Clara, CA, USA). An XCharge C18 HPLC column (3 mm × 100 mm, 5 μm) was used for the separation. The mobile phase was acetonitrile and 0.1% aqueous formic acid and the gradient program was 5%–20% acetonitrile from 0 to 32 min, 20%–90% acetonitrile from 32 to 35 min, and 90% acetonitrile from 35 to 47 min. MS experiments were operated in positive electrospray ionization mode, with data collected between m/z 100 and 1700.

Quantitative analysis was performed on Agilent 1200 HPLC system (Agilent Technologies), using protopine and corydamine (isolated in this study) as reference substances. An XCharge C18 HPLC column (4.6 mm × 250 mm, 5 μm) was used for the separation. The mobile phase was acetonitrile and 0.1% aqueous formic acid, and the gradient program was 5%–35% acetonitrile from 0 to 50 min and 35%–90% acetonitrile from 50 to 80 min.

Cell lines and reagents

RAW 264.7 cells were obtained from the Chinese Academy of Medical Sciences Basic Medicine Cell Center (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone Laboratories, Inc. (Logan, UT, USA). Cytokine enzyme-linked immunosorbent assay (ELISA) kits were purchased from R and D Systems, Inc. (Minneapolis, MN, USA). LPS (*Escherichia coli* 055:B5), 3-(4, 5-dimethylthylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), trypsin, penicillin-streptomycin cocktail, phosphate-buffered saline, and N-(naphthyl) ethylenediamine dihydrochloride were purchased from Sigma–Aldrich Co. LLC (St. Louis, MO, USA). Samples for biological tests were dissolved in dimethyl sulfoxide (DMSO) to provide a stock solution prior to addition to cell culture medium. The final concentration of DMSO never exceeded 0.1% (v/v). TRIzol reagent was purchased from OMEGA Bio-Tek (Doraville, GA, USA). Reverse transcriptase and SYBR quantitative polymerase chain reaction (qPCR) kits were purchased from Takara Biotechnology (Shiga, Japan).

Cell culture

RAW 264.7 cells were cultured at 37°C in DMEM, supplemented with 10% FBS, 1% L-glutamine, 0.1% β-mercaptoethanol, 100 IU/mL penicillin G, and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability test

RAW264.7 cells were plated into a 96-well plate (4×10^5 cells/mL). The cells were cultured overnight and treated with medium containing AHL (0, 12.5, 25, 50, 100, or 200 μg/mL) or protopine (0, 12.5, 25, 50, 100, or 200 μg/mL, isolated from *H. leptocarpum* in this study). The cells were then incubated for a further 20 h at 37°C in a humidified atmosphere containing 5% CO_2. MTT solution (50 μL, 5 mg/mL in phosphate-buffered saline) was added to each well and the cells were incubated for 4 h. DMSO (150 μL) was then added to each well to solubilize the blue formazan crystalline product, and the absorbance of the solution was measured at 570 nm using a microplate reader. The amount of formazan is proportional to the number of functional mitochondria in viable cells. The percentage cell viability was expressed as (absorbance of treated well/absorbance of control well) × 100.

Determination of nitric oxide production

Nitrite is a stable oxidation product of NO, a major pro-inflammatory mediator involved in various inflammation-related diseases. Cells were plated into a 96-well plate and treated with AHL (1.56, 3.13, 6.25, 12.50, or 25 μg/mL) or protopine (25 μg/mL) in the presence of LPS (*E. coli* 055:B5) (1 μg/mL) for 24 h. Samples of culture medium (100 μL) were collected and mixed with an equal volume of Griess reagent (0.1% N-(1-naphthyl)-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 min. Absorbance at 540 nm was then measured using a microplate reader. The nitrite concentration was determined by reference to a standard curve for sodium nitrite solution.

Determination of interleukin-1 β, interleukin-6, and tumor necrosis factor-α concentrations

RAW264.7 cells were plated into a 96-well plate and treated with AHL (1.56, 3.13, 6.25, 12.50, or 25 μg/mL) or protopine (25 μg/mL) in the presence of LPS (1 μg/mL) for 24 h. The cell culture supernatants were then collected and stored at − 20°C for the ELISA. The concentrations of IL-1 β, IL-6, and TNF-α were measured using commercial ELISA kits, following the manufacturer's protocols.

Real-time reverse transcription-polymerase chain reaction for detecting mRNA of inducible nitric oxide synthase, interleukin-1 β, interleukin-6, and tumor necrosis factor-α

Similar protocols were used to evaluate the effects of AHL and protopine on the expression of genes for different cytokines involved in inflammation. Total cellular RNA was isolated with TRIzol reagent, following the manufacturer's instruction, and reversely transcribed into cDNA using M-MLV reverse transcriptase and oligo dT primers. Real-time PCR was performed using a SYBR qPCR kit, according to the manufacturer's instructions. The following primers were used in this experiment: [38] iNOS, 5′-CTC AGC CCA ACA ATA CAA G-3′ (f) and 5′-CTA CAG TTC CGA GCG TCA-3′ (r); IL-1 β, 5′-ATT GTG GCT GTG GAG AAG-3′ (f) and 5′-TTG TGA GGT GCT GAT GTA-3′ (r); IL-6, 5′-CGA TAG TCA ATT CCA GAA ACC GC-3′ (f) and 5′-TTG GGA GTG GTA TCC TCT GTG AAG-3′ (r); TNF-α, 5′-GCC TAT GTC TCA GCC TCT T-3′ (f) and 5′-GGT TGA CTT TCT CCT GGT AT-3′ (r); and β-actin, 5′-TGT TAC CAA CTG GGA CGA CA-3′ (f) and 5′-AAG GAA GGC TGG AAA AGA GC-3′ (r).

Statistical analysis

Results are reported as mean ± standard deviation and linear regression was used to establish associations between parameters. Differences between means were established using one-way analysis of variance followed by Bonferroni's posttest method using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Phytochemical elucidation

The yield of AHL was calculated to be 2.78% (*w*/*w*), based on dry plant material. Two compounds isolated from AHL were confirmed to be protopine (1) and corydamine (5) [Figure 1b], based on their NMR and MS spectra (supplementary data). Chemical analyses were performed to characterize the composition of AHL, and the total ion chromatograms (TIC) are shown in Figure 1a. The results of the liquid chromatography/Q-TOF (LC/Q-TOF) MS experiment are

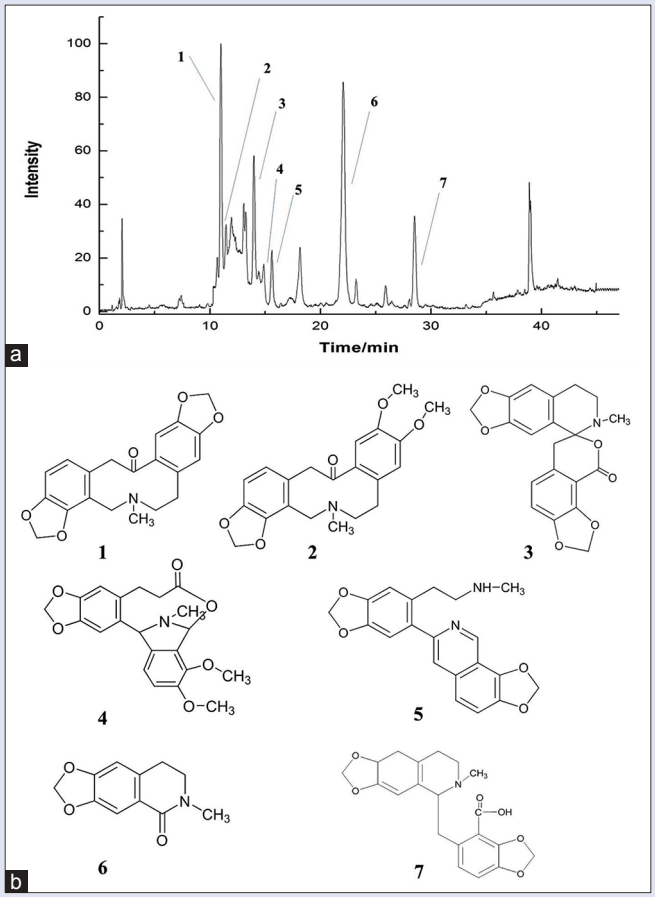

Figure 1: (a) Total ion chromatograms of alkaloids from *Hypecoum leptocarpum* by high-performance liquid chromatography-time-of-flight mass spectrometry. Identified peaks are marked as 1–7. (b) Putative structures of seven compounds corresponding to chromatographic peaks 1–7 in total ion chromatograms. Compounds: (1) protopine; (2) cryptopine; (3) leptopidinine; (4) leptocarpine; (5) corydamine; (6) dihydroleptopine; (7) oxohydrastinine

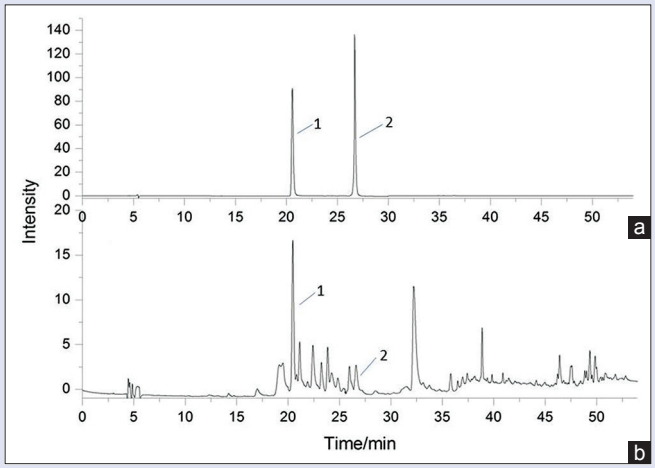

Figure 2: Diode-array detector high-performance liquid chromatography chromatograms of (a) standards and (b) alkaloids from *Hypecoum leptocarpum*, measured at 285 nm. The mobile phase was acetonitrile and 0.2% aqueous formic acid with a gradient of 5%–20% acetonitrile from 0 to 30 min and 20%–90% acetonitrile from 30 to 54 min. Peaks: (1) protopine; (2) corydamine

Table 1: Alkaloids characterized in total alkaloid fraction from *Hypecoum leptocarpum* using liquid chromatography/quadrupole-time-of-flight mass spectrometry

Number	Retention time (min)	Experimental *m/z* ([M+H]$^+$)	Molecular formula
1	10.98	354.1343	$C_{20}H_{19}NO_5$
2	11.47	370.1662	$C_{21}H_{23}NO_5$
3	13.98	368.1147	$C_{20}H_{17}NO_6$
4	14.89	384.1463	$C_{21}H_{21}NO_6$
5	15.62	351.1346	$C_{20}H_{18}N_2O_4$
6	22.09	370.1319	$C_{20}H_{19}NO_6$
7	28.52	206.0823	$C_{11}H_{11}NO_3$

shown in Table 1, which provides retention times, experimental m/z, and most probable molecular formulae. Using information from the high-resolution mass spectra and literature data,[26,39,40] together with data for the two compounds isolated in the present study, five additional compounds were characterized, with putative structures shown in Figure 1b. The seven compounds are all isoquinoline alkaloids and have been previously described in *H. leptocarpum*. In the diode-array detector (DAD)-HPLC analysis of protopine and corydamine in AHL, the chromatographic peaks were identified by comparing the retention times and UV spectra with those of standards [Figure 2]. AHL contained 13.3% protopine and 1.5% corydamine.

Effects of alkaloids from *Hypecoum leptocarpum* and protopine on cell viability

To ensure that inhibition of cytokine secretion by AHL and protopine was not caused simply by a cytotoxic effect on the cells, an MTT assay was performed before deciding the maximum dose for cytokine measurements. AHL and protopine were somewhat toxic to the cells at concentrations >25 μg/mL [Figure 3a]. The maximum dose of AHL was thus set at 25 μg/mL for additional experiments. Following a report by Bae *et al.* in 2012[41] describing the anti-inflammatory activity of protopine, both AHL and protopine (positive control) were used in subsequent tests at concentrations of 1.56, 3.13, 6.25, 12.50, and 25 μg/mL.

Inhibitory effects of alkaloids from *Hypecoum leptocarpum* on nitric oxide, interleukin-1 β, interleukin-6, and tumor necrosis factor-α production

Concentrations of NO, IL-1 β, IL-6, and TNF-α were measured to evaluate the effect of AHL and protopine on cytokine production in LPS-induced RAW 264.7 cells. Both AHL and protopine inhibited cytokine production in an almost dose-dependent manner, with nearly complete inhibition at a concentration of 25 μg/mL [Figure 3b-e]. Maximum inhibitory effects and IC_{50} values of AHL and protopine for the production of NO, IL-1 β, IL-6, and TNF-α are listed in Table 2.

Effects of alkaloids from *Hypecoum leptocarpum* and protopine on mRNA expression of inducible nitric oxide synthase, interleukin-1 β, interleukin-6, and tumor necrosis factor-α

To confirm the ability of AHL and protopine to suppress cytokine production and better understand the inhibitory mechanism of the pro-inflammatory mediators, expression levels of mRNA for iNOS, IL-1 β, IL-6, and TNF-α in LPS-induced RAW264.7 cells were evaluated by reverse transcription-PCR. AHL and protopine downregulated mRNA levels of the tested cytokines to different degrees [Figure 4]. In agreement with the ELISA results, all doses of AHL markedly decreased the expression levels of mRNA for iNOS, IL-1 β, and IL-6. Expression of iNOS mRNA was reduced to the control level in the high dose (25 μg/mL) AHL group while expression of IL-6 mRNA was downregulated to below the normal levels at all the three concentrations of AHL. All doses of AHL downregulated mRNA expression of iNOS and IL-1 β to a greater extent than protopine and high doses of AHL and protopine decreased gene expression of IL-6 and TNF-α to a similar extent.

DISCUSSION

Nonsteroidal and steroidal anti-inflammatory drugs are currently the most widely used anti-inflammatory agents. Although they usually produce a beneficial effect, the long-term use of these drugs is limited by side effects.[42] There is thus a clinical need for new and safe therapies to treat inflammation. Phytochemicals are a potentially rich source of inhibitors of pro-inflammatory cytokines, which have potential as alternative therapies for inflammation. Extracts derived from medicinal plants may contain combinations of chemical components with different beneficial effects, and in some cases, the different components may act synergistically.[43,44]

In the present study, the total alkaloid fraction and protopine isolated from *H. leptocarpum* were shown to inhibit the production of NO, IL-1 β, IL-6, and TNF-α in LPS-induced RAW264.7 cells. AHL more effectively reduced the production of IL-1 β (IC_{50} value, 0.12 ± 0.28 μg/mL) whereas protopine more effectively reduced the production of NO, IL-6, and TNF-α (IC_{50} value, 6.10 ± 0.86, 0.05 ± 0.11, and 0.28 ± 0.19 μg/mL, respectively). Using carrageenan-induced edema and the formalin test, we have previously shown that AHL has anti-inflammatory and analgesic effects *in vivo*.[35] The present study also suggested that total alkaloids might be responsible for the anti-inflammatory activity of *H. leptocarpum*.

Gene expression levels were measured to investigate the effect of AHL and protopine on the production of major macrophage-derived inflammatory cytokines and to better understand the inhibitory mechanism of the pro-inflammatory mediators. Both AHL and protopine downregulated mRNA expression of the pro-inflammatory mediators iNOS, IL-1 β, IL-6, and TNF-α, further demonstrating that alkaloids are

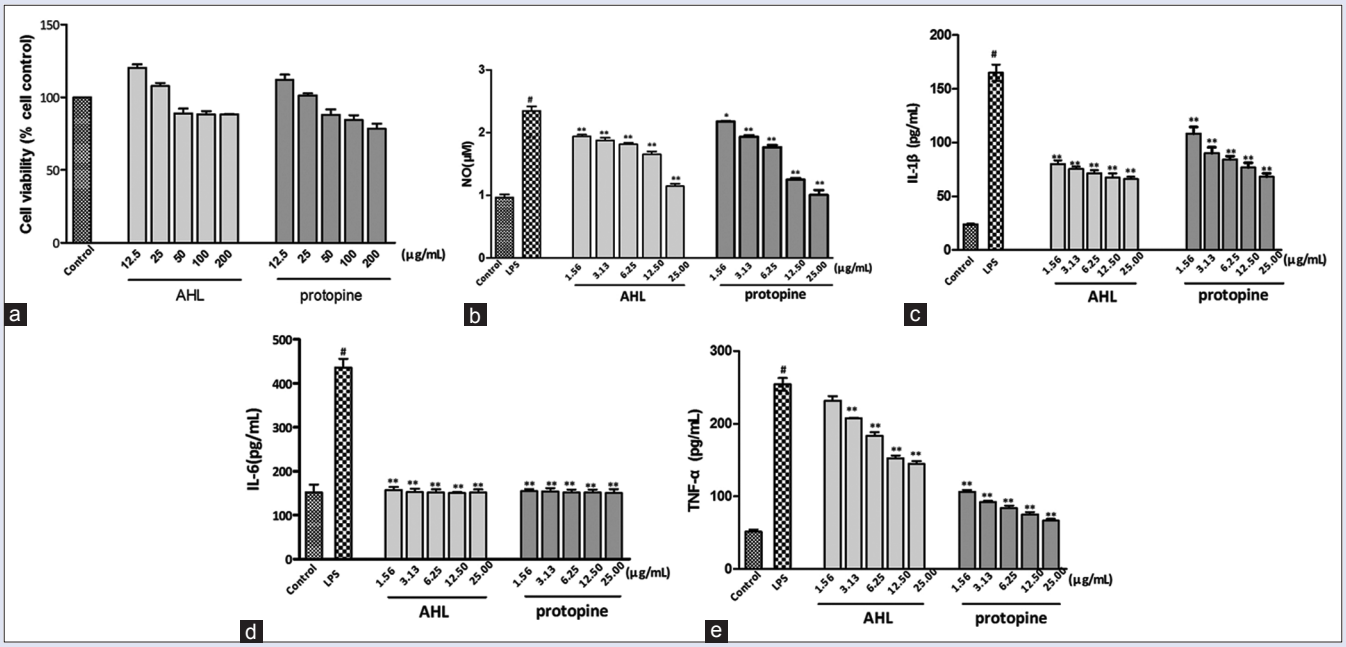

Figure3: Alkaloids from *Hypecoum leptocarpum* and protopine from *Hypecoum leptocarpum* inhibited inflammatory cytokine release in lipopolysaccharide-induced RAW264.7 cells. (a) Viability of RAW264.7 cells, determined by 3-(4, 5-dimethylthylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide assay, following treatment with alkaloids from *Hypecoum leptocarpum* or protopine (12.5, 25, 50, 100, or 200 μg/mL) for 20 h. (b-e) Macrophages were treated with lipopolysaccharide (1 μg/mL) and alkaloids from *Hypecoum leptocarpum* or protopine (1.56, 3.13, 6.25, 12.50, or 25 μg/mL) for 24 h. The culture supernatants were collected and nitric oxide (b), interleukin-β (c), interleukin-6 (d), and tumor necrosis factor-α (e) were measured. Data represent the mean ± standard deviation from three separate experiments. $^{\#}P < 0.01$, significant compared with vehicle-treated control; $^{*}P < 0.05$, $^{**}P < 0.01$ significant compared with lipopolysaccharide alone

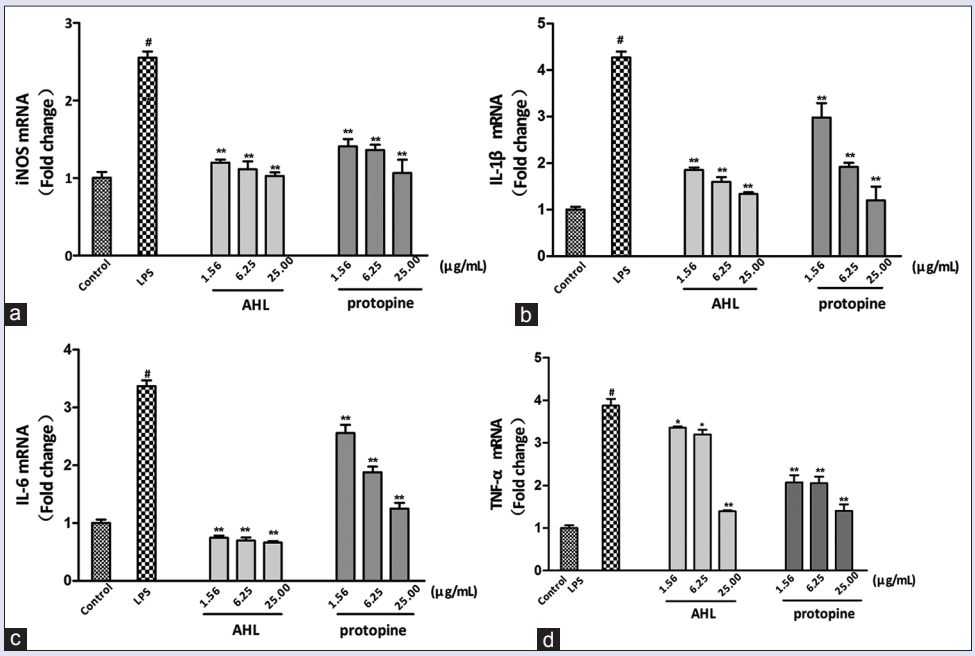

Figure 4: Alkaloids from *Hypecoum leptocarpum* and protopine from *Hypecoum leptocarpum* inhibited mRNA expression of inflammatory cytokines in lipopolysaccharide-induced RAW264.7 cells. Cells were treated with lipopolysaccharide (1 μg/mL) and different concentrations (1.56, 6.25, or 25 μg/mL) of alkaloids from *Hypecoum leptocarpum* or protopine for 24 h. mRNA levels of inducible nitric oxide synthase (a), interleukin-β (b), interleukin-6 (c), and tumor necrosis factor-α (d) were determined by reverse transcription-polymerase chain reaction. Data represent the mean ± standard deviation from three separate experiments. $^{\#}P < 0.01$, significant compared with vehicle-treated control; $^{*}P < 0.05$, $^{**}P < 0.01$ significant compared with lipopolysaccharide alone

the crucial anti-inflammatory components of *H. leptocarpum*. Chemical characterization of AHL by LC/Q-TOF MS and DAD-HPLC revealed the presence of a mixture of alkaloids containing 13.3% protopine and 1.5% corydamine. The study also confirmed the anti-inflammatory

Table 2: Inhibition by alkaloids from *Hypecoum leptocarpum* and protopine of production of nitric oxide, interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha in lipopolysaccharide-induced RAW264.7 cells

	Components	Cytokines			
		NO	IL-1β	IL-6	TNF-α
Inhibition ratio (%)[a]	AHL	86.8±2.2	70.1±1.5	100.1±2.5	50.8±3.6
	Protopine	97.1±3.4	68.5±2.1	100.3±2.9	92.0±2.1
IC_{50} (μg/mL)	AHL	7.47±2.81	0.12±0.28	0.56±0.37	18.95±5.23
	Protopine	6.10±0.86	3.20±0.92	0.05±0.11	0.28±0.19

[a]Inhibition ratio by AHL or protopine at a concentration of 25 μg/mL. AHL: Alkaloids from *Hypecoum leptocarpum*; NO: Nitric oxide; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; TNF-α: tumor necrosis factor-alpha

effect of total alkaloids in *H. leptocarpum* and further indicated that protopine is one of the main anti-inflammatory substances in the herb.

The *in vitro* experiments performed in this study measured the expression levels of mRNA for four inflammatory cytokines NO (iNOS was measured), IL-1 β, IL-6, TNF-α, which are involved in various inflammatory signaling pathways. Nuclear factor-kappa B (NF-κB) signaling is the most critical downstream pathway during inflammation mediated by LPS.[45] Activation of this pathway leads to the release of inflammatory mediators, including IL-1 β, IL-6, and TNF-α, and synthetases, such as iNOS. Overexpression of these factors is involved in many inflammation-related diseases.[46] Suppression of these cytokines by AHL could thus be mediated by the inhibition of NF-κB activation.

Janus kinase-signal transducers and activators of transcription (JAK-STAT) is another signaling pathway involved in LPS-stimulated inflammation.[47] JAK autophosphorylation induces a conformational change within the protein, which enables it to transduce the intracellular signal by further phosphorylating and activating transcription factors, STATs.[48-50] The STAT protein family comprises seven members, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, which regulate growth, survival, and differentiation in cells.[51] STAT1 and STAT3 have been implicated as crucial transcription factors in both immune and inflammatory pathways.[52,53] An STAT3-specific inhibitor has also been shown to block LPS-mediated STAT3 tyrosine phosphorylation, leading to inhibition of IL-1 β and IL-6 production, in an LPS-induced inflammation model.[54] This suggests that AHL may exert its anti-inflammatory effects by blocking LPS-regulated tyrosine phosphorylation of STAT3 in the JAK/STAT pathway. IL-1 β regulates the production of IL-6, a pleiotropic inflammatory cytokine that plays an important role in pathophysiological conditions, such as acute and chronic fibrotic lung disease.[55] It has also been reported that the cytokines such as IL-6 and TNF-α cause inflammatory cascade injury and stimulate neutrophil transfer into lung tissues.[56,57] In this context, *H. leptocarpum* is widely used in traditional Tibetan medicine to treat pneumonia and it is possible that the beneficial effects may be associated with the suppression of IL-1 β, IL-6, and TNF-α. A study by Liu *et al.* also provided new *in vitro* evidence for the inhibitory effect of *H. leptocarpum* on pulmonary inflammation.[21]

CONCLUSION

We have shown that AHL downregulated mRNA expression of iNOS, IL-1 β, IL-6, and TNF-α and significantly suppressed the production of NO, IL-1 β, IL-6, and TNF-α in LPS-induced RAW 264.7 cells. These findings provide convincing evidence that AHL, which contains 13.3% protopine and 1.5% corydamine, could act as a potential therapeutic and complementary agent in the treatment of inflammatory diseases. However, further studies should be carried out to identify more active constituents and explore efficiency in other experimental animal models.

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Conflicts of interest

There are no conflicts of interest.

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