Journal of Ethnopharmacology 189 (2016) 1–9

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Iridoid glycosides from the flowers of *Gentiana macrophylla* Pall. ameliorate collagen-induced arthritis in rats

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ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form
10 May 2016
Accepted 11 May 2016
Available online 12 May 2016

Keywords:
Gentiana macrophylla Pall.
Iridoid glycosides
Rheumatoid arthritis
Collagen-induced arthritis
Gentiopicroside

ABSTRACT

Background: The flowers of *Gentiana macrophylla* have been usually applied to cure the joint inflammation and rheumatoid arthritis in Traditional Chinese Medicine.
Hypothesis/purpose: This work aimed to investigate the anti-rheumatoid arthritic effect and possible mechanism of iridoid glycosides from *G. macrophylla* (GMI) using an animal model of collagen-induced rheumatoid arthritis (CIA) in rats.
Study design: All rats were randomly divided into five groups: normal control, CIA, dexamethasone, 15 mg/kg and 30 mg/kg GMI.
Methods: CIA was induced (day 0) in male Sprague-Dawley rats by intradermal injection of complete Bovine CII at the base of the tail. Dexamethasone was chosen as the positive drug. The administration of different drugs started from day 1 and continued for 28 days. Paw swelling, arthritis score and histopathological changes were examined to assess the severity of arthritis. In addition, the serum levels of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expressions in joint synovial tissues were detected.
Results: GMI reduced paw edema, arthritis scores and the index of spleen and thymus from day 7 to 21 after CIA compared with those in the CIA group. Our data also demonstrated that GMI inhibited pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, regulated the expression of iNOS and COX-2 compared with those in the CIA group. We also obtained four major components from GMI, identified as loganic acid, swertamarin, gentiopicroside and sweroside, and the contents of them were also calculated respectively.
Conclusion: Taken together, our results shed light on the therapeutic efficacy of GMI in rats rheumatoid arthritis model by reducing the levels of IL-1β, IL-6 and TNF-α in serum as well as down-regulating the levels of iNOS and COX-2. Therefore, GMI may be an effective therapy for the treatment of rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is one of the most chronic destructive diseases to human health, which is characterized by chronic inflammatory cell infiltration and proliferation of synovial tissue, accompanied by destruction of cartilage and bone (Parolini et al., 2014). The treatment goals for this disease are long-term relief of pain, prevention of joint inflammation and suppression of pannus formation and morphological changes (Li et al., 2012). According to the guidelines of the American College of Rheumatology, disease-modifying anti-rheumatic drugs (DMARDs), anti-inflammatory medications, such as non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular and oral corticosteroids are commonly used clinical drugs for the treatment of RA. Nevertheless, administration of these drugs is associated with severe adverse

Abbreviations: RA, rheumatoid arthritis; DMARDs, disease-modifying anti-rheumatic drugs; NSAIDs, non-steroidal anti-inflammatory drugs; GMI, iridoid glycosides from *Gentiana macrophylla* Pall.; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; NO, Nitric oxide; NOS, nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; PGE2, prostaglandin E2; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; CIA, collagen-induced rheumatoid arthritis; DAD, photodiode array detector; LOD, limit of detection; LOQ, limit of quantification; SD, Sprague-Dawley; DMEM, Dulbecco's Modified Eagle's Medium; DEX, dexamethasone; HE, hematoxylin and eosin; ELISA, enzyme-linked immunosorbent assay; RSD, relative standard deviations

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http://dx.doi.org/10.1016/j.jep.2016.05.027
0378-8741/

effects, such as gastrointestinal injury, cardiovascular risk and renal irritations (Doan and Massarotti, 2005; Saag et al., 2008; Singh et al., 2012). Fortunately, natural products are expected to heal RA with their advantages of higher efficacy and fewer side effects and lower toxicity (Wang et al., 2012).

Gentiana macrophylla Pall. (Gentianaceae) is the perennial herb of Radix Gentianae Macrophyllae. It has long history for the treatment of various diseases including inflammation, rheumatoid arthritis, osteoarthritis and nociceptive pain in China, Japan and South Korea (Commission, 1999; Guo et al., 2014). The flowers of this plant named Hare Jilezhe have been traditionally used in Mongolian medicine. They contain several active components such as iridoid glycosides, flavonoids and lignans (Commission, 1999). Our previous studies have demonstrated anti-inflammatory and analgesic effects of *G. macrophylla* in several animal models (Jia et al., 2012). Further we also found that the iridoid glycosides from *G. macrophylla* (GMI) might be one of the main biological active compounds and observed pharmacological effects (Jia et al., 2012). However, no systematic study on chemical composition and potential therapeutic action of GMI in RA has been reported. In addition, the molecular and cellular mechanisms underlying the modulation of the inflammatory response of GMI still remain obscure.

Inflammatory mediators can induce tissue damage and inflammatory response through up-regulation of several reactive genes, including cyclooxygenase-2 (COX-2), inducible nitric oxide (iNOS), interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Nitric oxide (NO) is an important messenger that has considerable functions in vascular regulation, autoimmunity defense, neuronal signal transduction and so on (Paige and Jaffrey, 2007). NO is created via the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) in the presence of molecular oxygen and NADPH. Especially, iNOS, a ubiquitous mediator of a wide range of inflammatory condition, becomes active following infections and then abundant of NO are generated (Breitbach et al., 2006). COX-2 also plays important role in immune modulation and pathophysiological process (Rahman et al., 2006). Abundant of prostaglandin E2 (PGE2) derived from COX-2 following induction by pro-inflammatory mediators, such as bacterial lipopolysaccharide (LPS), TNF-α and IL-6, are implicated in the pathogenesis of RA and inflammation (Geng et al., 1993). These findings inspired us to infer that GMI ameliorate RA through these important targets.

In order to verify our speculation, the present study was designed to investigate the anti-rheumatoid arthritis property of GMI and the potential mechanisms using an animal model of collagen-induced rheumatoid arthritis (CIA) in rats. This model has been widely used to study disease mechanisms and potential therapies for RA. Indeed, CIA model has many morphological features similar to those of human RA including patterns of synovitis, pannus formation, and erosion of articular cartilage and bone. Moreover, CIA shares with RA many of the cytokines and biological factors in the synovium and cartilage (Wang et al., 2014).

2. Materials and methods

2.1. Plant materials and reagents

The flower samples of *G. macrophylla* were collected over four months of 2013 (June, July, August and September) in Longxian County, Shaanxi Province, China. The plant materials were identified by Professor Zhenghai Hu (Northwest University, Xi'an, 710,069, China). Sample was deposited in Biomedicine Key Laboratory of Shaanxi Province, Northwest University. Four standard references, namely, loganic acid (IG1, > 98%, CAS: 22255-40-9, $[\alpha]^{26}D^{-86.2}$ (c, 0.5 in H_2O)); swertamarin (IG3, > 98%, CAS: 17388-39-5, $[\alpha]^{20}D^{-127}$ (c, 1 in 96% ethanol)); gentiopicroside (IG4, > 98%, CAS: 20831-76-9, $[\alpha]D^{-196.3}$ (H_2O)) and sweroside (IG5, > 98%, CAS: 14215-86-2, $[\alpha]^{26}D^{-236}$ (H_2O)) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ELISA kits of TNF-α, IL-1, and IL-6 were purchased from R&D systems, (Minneapolis, MN, USA). Anti-rabbit inducible NO synthase (iNOS) was obtained from Assay designs (Ann Arbor, MI, USA). Anti-goat COX-2 and β-actin antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the other chemicals and biochemical used were of the highest grade available.

2.2. Preparation of herbal extracts

The iridoid glycosides extract of *G. macrophylla* was prepared as previously described (Chen et al., 2009). Briefly, 300 g powder of flowers of *G. macrophylla* was extracted with 75% ethanol by means of percolation at room temperature (25 °C). The operations were repeated until the extract was near to colorless. After removal of the solvent in vacuo, the residue was suspended in water. This suspension was then fractionated with petroleum ether, $CHCl_3$, EtOAc and *n*-BuOH, successively. The *n*-BuOH fraction was subjected to column chromatography (silica gel (1000 g) 9×100 cm column; $CHCl_3$/MeOH/H_2O gradient (10:3:1–5:3:1)) to yield iridoid glycosides fraction. Iridoid glycosides extract of *G. macrophylla* (GMI) was obtained after they were vacuum dried at 60 °C.

2.3. Qualitative and quantitative analysis of GMI

A Beckman 125 HPLC instrument (Beckman, USA), equipped with a Hamilton autosampler and a 168 photodiode array detector (DAD) was used. The UV spectra were recorded between 190 and 490 nm for peak characterization, and the detection wavelength was set at 254 nm. A Waters SunFire C18 (4.6 mm × 250 mm, 5 μm) was used with the column temperature held at 25 °C. Elution was carried out at a flow rate of 0.8 ml/min with mobile phases consisting of 1‰ acetic acid (A) and acetonitrile (B). The gradient elution was as follows: 0–20 min, 95–90% (B); 21–40 min, 90–87.5% (B); 41–60 min, 87.5–80% (B); 61–90 min, 80% (B); 91–100 min, 80–95% (B). The mobile phase was filtered through a Millipore 0.45 μm filter and degassed prior to use. The peaks were detected at 254 nm and loganic acid, swertamarin, gentiopicroside and sweroside were detected by comparing with the chemical marks, which were identified with MS, ^{1}H NMR, and ^{13}C NMR.

2.4. Method validation

Analytical method's linearity, limit of detection and quantification (LOD and LOQ) and inter-day/intra-day precision were validated following the ICH guidelines (ICH 1996). Recovery was used to evaluate the accuracy of the method.

2.5. Animals

All experiments were performed in adherence with the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care (approval number: XJYYLL-2014302). The male Sprague-Dawley (SD) rats (weight 180–220 g) were obtained from the animal research center at the Fourth Military Medical University. Animals were kept in polyethylene boxes under controlled temperature (22 ± 2) °C, humidity (55 ± 5)% and circadian cycle, with free access to standard food and water. All experiments were performed by the same researcher in a quiet test room close to the storage room.