Journal of Ethnopharmacology 196 (2017) 39–46

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Total sesquiterpene lactones prepared from *Inula helenium* L. has potentials in prevention and therapy of rheumatoid arthritis

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ARTICLE INFO

Keywords:
Inula helenium L.
Total sesquiterpene lactones
Anti-inflammatory
Rheumatoid arthritis

ABSTRACT

Backgrounds: *Inula helenium* L. is an herb with anti-inflammatory properties. Sesquiterpene lactones (SLs), mainly alantolactone (AL) and isoalantolactone (IAL), are considered as its active ingredients. However, the anti-inflammatory effects of SL-containing extracts of *I. helenium* have not been explored. Here we prepared total SLs from *I. helenium* (TSL-IHL), analyzed its chemical constituents, and performed cellular and animal studies to evaluate its anti-inflammatory activities.

Materials and methods: The chemical profile of TSL-IHL was analyzed by HPLC-UV. Its *in vitro* effects on the activation of signaling pathways and expression of inflammatory genes were examined by western blotting and quantitative real-time PCR, respectively, and compared with those of AL and IAL. Its *in vivo* anti-inflammatory effects were evaluated in adjuvant- and collagen-induced arthritis rat models.

Results: Chemical analysis showed that AL and IAL represent major constituents of TSL-IHL. TSL-IHL, as well as AL and IAL, could inhibit TNF-α-induced activation of NF-κB and MAPK pathways in b. End3 cells, suppress the expressions of MMP-3, MCP-1, and IL-1 in TNF-α-stimulated synovial fibroblasts, and IL-1, IL-6, and iNOS in LPS-activated RAW 264.7 cells in a dose-dependent manner in the range of 0.6–2.4 μg/mL. Oral administration of TSL-IHL at 12.5–50 mg/kg could dose-dependently alleviate the arthritic severity and paw swelling in either developing or developed phases of arthritis of rats induced by adjuvant or collagen

Conclusions: These results indicated potentials of TSL-IHL in prevention and therapy of rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterized by synovial hyperplasia, inflammatory cells infiltration, cartilage and bone destruction. It affects about 1% population in the world and associated with severe morbidity, functional impairment, permanent disability and increased mortality. It's reported that the pathologic processes of RA are mediated by a number of pro-inflammatory cytokines, chemokines, and matrix metalloproteinases, whose overexpression in synovial fluid results in chronic and persistent inflammation (Okamoto et al., 2008; Montecucco and Mach, 2009; Hopkins and Meager, 1988). RA still remains a formidable clinical challenge until now. Since the mid-1980s, methotrexate (MTX) has been the standard disease-modifying anti-rheumatic drug (DMARD) ascribing to its satisfactory efficacy and affordability (Welles et al., 1985). However, a significant proportion of patients become refractory to MTX therapy, probably due to the onset of drug resistance (Morgan et al., 2003). In recent years, biologics, typically antibodies or decoy receptors designed to inhibit pathogenic cytokines such as TNF-α, IL-1 and IL-6, have achieved major advances in RA management. Unfortunately, these protein-based agents require parenteral administration and may leave patients at increased risk of serious infection and cancer. However, the success of biologics has identified the therapeutic potential in targeting unbalanced cytokine networks. Therefore, the attention has been focused on orally bioavailable small-molecule inhibitors of key signaling elements in pathogenic cytokine production

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http://dx.doi.org/10.1016/j.jep.2016.12.020
Received 13 August 2016; Received in revised form 25 November 2016; Accepted 14 December 2016
Available online 15 December 2016

and downstream receptor cascades. NF-κB and Mitogen-activated protein kinases (MAPKs) were considered as candidate druggable targets because they are not only key regulators of pro-inflammatory cytokine production but also play important roles in the downstream signaling cascades of cytokine receptors (Alghasham and Rasheed, 2014).

NF-κB is family of transcription factors which play a critical role in mediating a variety of essential cellular process including immune and inflammatory responses. In un-stimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form associated with inhibitors of NF-κB (IκB). In response to stimuli such as cytokines and microbial products, IκB is rapidly phosphorylated by IκB kinase. Phosphorylated IκB is subsequently ubiquitinated and degradated by 26 s proteasome, liberating NF-κB to translocate to the nucleus to regulate gene expression (Pomerantz and Baltimore, 2002). NF-κB has been considered as one of the master regulators of inflammatory cytokine produced in RA (Tak and Firestein, 2001; Han et al., 1998). Researches demonstrated that NF-κB is activated in the inflamed synovium and rheumatoid cartilage, and inhibition of NF-κB can attenuate synovial and joint inflammation in animal models of arthritis (Tak et al., 2001; Tas et al., 2006; Wang et al., 2011), suggesting that NF-κB may be a promising target in developing effective therapeutics for RA.

MAPK also play a critical role in the pathogenesis of RA. There are three classes of MAPKs: the extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK). The three MAPK families expressed in their active phosphorylated forms have been described in RA synovium (Thalhamer et al., 2008). Activated p38 is overexpressed in the synovial microvasculature and synovial lining layer, JNK is a key MAP kinase involved in the induction of metalloproteinase genes and mononuclear infiltrates in RA synoviocytes (Ralph and Morand, 2008; Paunovic and Harnett, 2013). A wealth of pre-clinical data have demonstrated the efficacies of small-molecule inhibitors specifically targeting the MAPK pathways in protecting against RA in a wide range of animal models. Many of these inhibitors have gone on to phases I and II clinical trials as mono- and co-therapies (with MTX).

Medicinal plant-derived compounds are wealthy sources of anti-inflammatory agents. Sesquiterpene lactones (SLs) constituted a large group of secondary plant metabolites mostly found in the Asteraceae family (Merfort, 2011). These natural compounds represent active principles of many herbs used in traditional medicine as anti-inflammatory remedies. SL-enriched plant extracts are frequently used in traditional medicine for the treatment of infections and inflammation (Heinrich et al., 1998). Alcoholic preparations of some SL-rich Asteraceae plants are also used for the treatment of rheumatic diseases in traditional Western medicine (Jarić et al., 2015; Habtemariam, 1998). In recent years, the anti-inflammatory properties of SLs have attracted a great deal of interest, and many plant-derived SLs were found to exhibit potent anti-inflammatory activities (Zhou et al., 2008; Lyss et al., 1998). *Inula helenium* L. belongs to Asteraceae family, and its dried roots referring to as “Tumuxiang”, is commonly used as traditional Chinese medicine for treatment of inflammatory diseases such as enterogastritis, tuberculotic enterorrhea, and bronchitis (Editorial board of Flora of China, 1979; Chinese Pharmacopoeia Commission, 2015). Modern scientific research indicated that main active ingredients of Tumuxiang are SLs, mainly alantolactone (AL) and isoalantolactone (IAL) (Fig. 1). This pair of structural isomers has been demonstrated to have numerous biological activities such as anti-inflammatory, anti-bacterial, and anti-tumor activities, *etc* (Rasul et al., 2013). With regard to the anti-inflammatory activity, a previous research has demonstrated that AL suppressed inducible nitric oxide (iNOS) and cyclooxygenase-2 expression by inhibiting NF-κB and MAPK pathways in LPS-activated RAW 264.7 cells (Chun et al., 2012). In addition, AL and IAL were found to inhibit NF-κB signaling pathway in some tumor cell lines (Di et al., 2014; Wu et al., 2013; Wei et al., 2013). However, no studies have explored the anti-inflammatory effects of well-characterized SL-containing extracts of *I. helenium*. In present study, we prepared total sesquiterpene lactones from *I. helenium* (TSL-IHL), analyzed its chemical constituents, and performed studies to evaluate its anti-inflammatory activities both *in vitro* and *in vivo*.

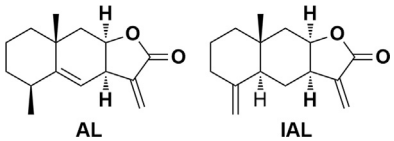

Fig. 1. Chemical structures of AL and IAL.

2. Materials and Methods

2.1. Reagent

AL (95% purity) and IAL (95% purity) were purchased from National Institutes for Food and Drug Control (Beijing, China). HPLC-grade acetonitrile was obtained from J.T. Baker. Analytical grade ethanol and petroleum ether (60–90 °C) were purchased from SinoPharm. Diaion®. HP20, a polystyrene based synthetic macroporous resin was obtained from Mitsubishi Chemical Corp. (Japan). Recombinant human TNF-α was purchased from Peprotech (Rocky Hill, NJ). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO). MTX was obtained from Pude Pharmaceutical Co., Ltd. (Shanxi, China). Bovine type II collagen (CII) was obtained from Collagen Research Center (Tokyo, Japan). Complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* strain H37Rv was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Plant material and preparation of TSL-IHL

The roots of *Inula helenium* L. (Asteraceae) were obtained from Anguo county, Hebei province, China, in September 2013. The identification and authentication of the plant material was carried out by Prof. Bao-Kang Huang from the department of pharmacognosy of Second Military Medical University (Shanghai, China). A voucher specimen (No. 2013.09.22) is deposited in the herbarium of School of Pharmacy, Second Military Medical University.

The TSL-IHL was prepared as follows: Air-dried roots of *I. helenium* (5 kg) were powdered and extracted with 80% ethanol (80 L) two times (2 h) under condition of reflux. The solvent was removed under low pressure to afford a crude extract, which was then suspended in water and extracted with petroleum ether (3×10 L), affording 313.3 g of extract. The petroleum ether extract was suspended with 5 L of 85% aqueous ethanol and filtered to obtain the solution. The solution was loaded onto a 1:10 (diameter to height ratio) HP20 packed column (Bed volume=3 L) at the flow rate of 3 L/h. The outlet solvent was collected. The column was then eluted with 24 L of 85% aqueous ethanol at the flow rate of 6 L/h. The outlet and the eluate were combined and concentrated to the volume of 0.8 L. Then, the concentrated solution was mixed with 0.4 L petroleum ether to crystallize at 4 °C for 24 h. Finally, the crystals were filtered and dried to obtain 89.0 g of TSL-IHL.

2.3. HPLC-UV

The HPLC system consisted of an HP1100 quaternary pump (G1311A), an autosampler (G1313A), an HP1100 VWD detector (G1321B) and an HP1100 DAD detector (G1315B). The separation was performed on an Agilent Zorbax SB C_{18} column (4.6×250 mm, 5 μm) at 30 °C. Chromatographic analysis was performed using a gradient elution consisting of acetonitrile and water. The flow rate