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

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\textbf{ABSTRACT}

\textit{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.

\textit{Materials and methods:} The chemical profile of TSL-IHL was analyzed by HPLC-UV. Its \textit{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 \textit{in vivo} anti-inflammatory effects were evaluated in adjuvant- and collagen-induced arthritis rat models.

\textit{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-\(\alpha\)-induced activation of NF-\(\kappa\)B and MAPK pathways in b End3 cells, suppress the expressions of MMP-3, MCP-1, and IL-1 in TNF-\(\alpha\)-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 0.6–24 \(\mu\)g/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.

\textit{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-\(\alpha\), 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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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 degraded 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 (Thalhammer 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; Pannovic 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 medicinal plants are also used for the treatment of rheumatic diseases in traditional Western medicine (Jarić et al., 2015; Hambetamiam, 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, tuberculous enterorrhrea, 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.

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 polyostere base 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 and the eluate 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 C18 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
was 1.0 mL/min.

2.4. Identification and quantification of chemical constituents in TSL-IHL

To identify the constituents in TSL-IHL, the chromatograms were recorded at 205 nm, and the identification of the peaks was conducted by comparing their retention times and diode-array spectra in a real-time analysis, with the corresponding data obtained by analyzing standard compounds. After identifying AL and IAL as the main constituents of TSL-IHL, their quantification was conducted with the external standard HPLC method. Calibration curves were obtained using five concentration levels and fitted to linear equations. The method was validated in terms of linearity, limits of detection, limits of quantification, precision, accuracy, and stability. The above parameters were presented in Table 1.

| Table 1 |
| The linearity, limits of detection, limits of quantification, precision, accuracy, and stability of AL and IAL. |
| Equation |
| AL | IAL |
| Range of linearity (μg) | 0.474–4.74 | 0.484–4.84 |
| Determination coefficient (r²) | 1 | 1 |
| Limits of detection (LOD) (ng) | 1.254 | 1.254 |
| Limits of quantification (LOQ) (ng) | 4.18 | 4.18 |
| Precision (R.S.D, %) | 0.440 | 0.470 |
| Accuracy (R.S.D, %) | 0.686 | 0.542 |
| Stability (R.S.D, %) | 0.982 | 1.026 |

2.5. Cell cultures

bEnd.3 mouse endothelial cells and RAW264.7 cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, BRL) supplemented with 10% fetal bovine serum (GIBCO, BRL) at 37 °C in a humidified atmosphere with 5% CO₂. Synovial fibroblasts were isolated from synovial tissue specimens obtained from RA patients who have experienced total joint replacement surgery, as previously described (Zimmermann et al., 2001). The study was approved by the Second Military Medical University Institutional Review Board. Synovial fibroblasts were grown in DMEM supplement with 10% FBS and 1% synoviocyte growth supplement (Scientcell, Carlsbad, CA).

2.6. Western blotting

b.End.3 cells were cultured in 6-well plates at 2×10⁵ cells/well for 20 h, then treated with or without AL, IAL, and TSL-IHL (0.6, 1.2, 2.4 μg/mL) for 2 h, followed by stimulation with 20 ng/mL TNF-α or LPS for 6 h. Total RNA of Synovial fibroblasts and RAW 264.7 cells were extracted using RNA Miniprep kit (BioMiga) according to the manufacturer’s instruction. Then 1 μg of total RNA was reverse transcribed using PrimeScript RT reagent Kit (Takara, Dalian, China). The primers used were designed and synthesized by Takara, the sequences of the primers are indicated in Table 2. PCR amplification was performed on an iCycler (Bio-Rad Laboratories).

2.8. Quantitative real-time PCR (qRT-PCR)

Human synovial fibroblasts or RAW264.7 cells were cultured in 6-well plates at 2×10⁵ cells/well for 36 h, then treated with 0.6, 1.2, 2.4 μg/mL of TSL-IHL, AL, or IAL for 2 h, followed by stimulation with 20 ng/mL TNF-α or LPS for 6 h. Total RNA of Synovial fibroblasts and RAW 264.7 cells was extracted by using RNA Miniprep kit (BioMiga) according to the manufacturer’s instruction. Then 1 μg of total RNA was reverse transcribed using PrimeScript RT reagent Kit (Takara, Shanghai, China). The primers used were designed and synthesized by Takara, the sequences of the primers are indicated in Table 2. PCR amplification was performed on an iCycler (Bio-Rad Laboratories).

2.9. Animals

Male Wistar rats (160–180 g) were purchased from Slacom Experimental Animal Company (Shanghai, China). Rats were housed in standard laboratory conditions with ambient temperature (22 ± 1 °C) and relative humidity (55 ± 5%) in a 12 h light/dark cycle. They were fed with sterilized pelleted diet and had free access to water, and acclimatized for at least one week prior to experiment. All animal experiments were approved by the Administrative Committee of Experimental Animal Care.

2.10. Induction of arthritis and TSL-IHL treatment

2.10.1. Adjutant-induced arthritis (AA) model

AA was induced in rats as previously described (Kim et al., 2004). Briefly, arthritis was induced by an intradermal injection of 0.1 mL of CFA into the bases of left hind paw. The day of CFA injection was designated as day 0. To investigate the preventive and therapeutic effects of TSL-IHL, it was given following the prophylactic and therapeutic protocols, respectively. For the prophylactic protocol, 3 groups (10 rat/group) were orally administered with TSL-IHL (12.5, 25, 50 mg/kg) dissolved in 0.3% sodium carboxymethylcellulose once a day for 14 days starting from the day 0, and the group treated with MTX (1 mg/kg, po, once every two days, starting from the day 0) was used as positive control; for therapeutic protocol, 3 groups (10 rat/group) were orally administered with TSL-IHL (25, 50, 100 mg/kg)
daily starting from day 10 after CFA immunization, and a MTX treatment group (1 mg/kg, po, once every two day, starting from day 10 of CFA immunization) was used as positive control. The rats in normal control and AA model groups were given an equal volume of 0.3% sodium carboxymethylcellulose at the same time of TSL-IHL administration.

2.10.2. Collagen-induced arthritis (CIA) model

CIA were induced in rats as previously described (Bashi et al., 2016). Bovine type II collagen (CII) was dissolved in 0.1 M acetic acid at a concentration of 13.33 mg/mL, emulsified with an equal volume of CFA containing Mycobacterium tuberculosis H37 RA and stored in ice before use. CIA induction was carried out by injecting 200 μL of the emulsion into the base of the tail. Rats were immunized a second time one week later using the same method. The day of second immunization was defined as day 0. For the prophylactic protocol, 3 groups (10 rat/group) were orally administered with TSL-IHL (12.5, 25, 50 mg/kg) daily starting from the day 0. One group treated with MTX (1 mg/kg, po, once every two day, starting from the day 0) was used as positive control. For therapeutic protocol, 3 groups (10 rat/group) were orally administered with TSL-IHL (12.5, 25, 50 mg/kg) daily starting from day 15. A MTX treatment group (1 mg/kg, po, once every two day, starting from day 15 after second immunization) was used as positive control. The rats in normal control and CIA model groups were given an equal volume of 0.3% sodium carboxymethylcellulose at the same time of TSL-IHL administration.

2.11. Assessment of arthritis severity

To evaluate the severity of arthritis, the volume of left and right hind paw was measured with a volume meter (YLS-7B, Ji Nan, China). The arthritis index was graded on a scale of 0–4 as previously described (Chen et al., 2014): grade 0, no swelling and focal redness; grade 1, swelling of finger joints; grade 2, mild swelling of ankle or wrist joints; grade 3, swelling of the entire paws; and grade 4, swelling of entire paw, including ankle. The cumulative score for four paws of each rat was used as the clinical score with a maximum value of 16.

2.12. Statistical analysis

All data are presented as the mean ± SEM, and were evaluated with one-way ANOVA following by Dunnett’s multiple comparisons test between different groups. Statistical significance was determined as P < 0.05.

3. Result

3.1. AL and IAL are main constituents of TSL-IHL

Chemical constituent analysis showed that AL and IAL represent main constituents of TSL-IHL prepared by us. Fig. 2 depicts the fingerprint of the constituents and their UV spectra. The contents of AL and IAL in TSL-IHL were quantified by the validated HPLC method. The contents of AL and IAL account for 35.29 ± 0.63 and 54.70 ± 0.42 (%) of the extract, respectively (Data obtained from 6 determinations).

3.2. TSL-IHL exhibits inhibitory effect on TNF-α induced activation of NF-κB and MAPK pathways, which is comparable to those of AL and IAL

The anti-inflammatory potentials of AL and IAL have been indicated by previous findings that both of them could effectively block the NF-κB and MAPK pathways. Since TSL-IHL contains mainly AL and IAL, it should also have anti-inflammatory potential. To provide experimental evidence, we tested its effect on the activation of NF-κB and MAPK pathways using western blotting assay, and the effects of AL and IAL were simultaneously studied for comparison. The results revealed that treatment of AL, IAL, and TSL-IHL suppressed TNF-α-induced IκB phosphorylation and degradation, as well as the phosphorylations of NF-κB p65 subunit, p52, and JNK in b. End cells, in a dose-dependent manner in the range of 0.6–2.4 μg/mL (Fig. 3), indicating that AL, IAL, and TSL-IHL could all dose-dependently inhibit TNF-α-induced activation of NF-κB and MAPK pathways. It should be noted that the inhibitory effects of TSL-IHL, AL and IAL were comparable.

3.3. TSL-IHL, AL, and IAL, could all similarly suppress the expression of inflammatory genes in activated synovial fibroblasts and RAW 264.7 cells

Synovial fibroblasts and macrophages are two major cells that produce inflammatory mediators in RA (Bartok and Firestein, 2010; Soler Palacios et al., 2015; Mulherin et al., 1996). To further investigate the anti-inflammatory potential of TSL-IHL we examined its effect on the expression of inflammatory genes in activated synovial fibroblasts and RAW 264.7 cells. The results indicated that TSL-IHL (0.6, 1.2, 2.4 μg/mL) dose-dependently suppressed expressions of inflammatory genes, including IL-1, MCP-1, and MMP-3 in TNF-α-stimulated synovial fibroblasts (Fig. 4A), as well as IL-1, IL-6, TNF, and RAW 264.7 cells (Fig. 4B). In these cellular experiments, AL and IAL also showed similar inhibitory activities on these pro-inflammatory gene expressions. CCK8 assay showed that TSL-IHL, AL and IAL are not cytotoxic to these cells within the same dose range of 0.6–2.4 μg/mL (Data not shown), excluding the possibility that the observed inhibition on inflammatory gene expressions by them arisen from cytotoxicity.

3.4. TSL-IHL ameliorate arthritis severity of AA in rats

To evaluate the in vivo anti-inflammatory activity of TSL-IHL, we tested its anti-arthritic effect in AA rat model. Inflammatory polyarthritis was induced in rats by a single subcutaneous injection of CFA into their footpad, as indicated by marked and steady increase in arthritis index and hind paw volume from day 12 after adjuvant injection in all immunized rats, compared with normal control group (Fig. 5). To test the anti-arthritic effect of TSL-IHL, different doses of TSL-IHL were orally given once a day starting from day 0 (prophylactic protocol) or day 10 (therapeutic protocol) after CFA injection. Both prophylactic and therapeutic treatment with TSL-IHL resulted in significant and dose-dependent decreases in the arthritis index score and hind paw swelling rate, compared to the AA model control group (Figs. 5A and 5B), indicating that TSL-IHL has a substantial in vivo anti-inflammatory activity. As a positive drug control, MTX (1 mg/kg) also significantly diminished the arthritis index and hind paw volume swelling induced by CFA.

3.5. TSL-IHL ameliorate arthritis severity of CIA in rats

In order to further evaluate the potential of TSL-IHL in treatment of RA, we also tested its anti-arthritic effect in CIA rat model. As shown in Fig. 6, significant increasement of clinical scores and hind paw volume were observed after immunized with CII twice, compared with the normal control rats. Oral treatment with TSL-IHL (12.5, 25, 50 mg/kg) or MTX (1 mg/kg) daily starting from day 0 (preventive protocol) or day 15 (therapeutic protocol) of second immunization significantly decreased the arthritic score and paw swelling in a dose-dependent manner (Figs. 6A and 6B), indicating that TSL-IHL treatment reduces the arthritic inflammatory response induced by CII in rats. No deaths or any other obvious adverse effects were detected in the TSL-IHL-treated rats during the study, indicating that TSL-IHL is safe in rats at its effective anti-inflammatory dose.
It has been well-established that a range of inflammatory diseases are commonly aggravated by the uncontrolled expression of a broad spectrum of different pro-inflammatory mediators, such as cytokines and chemokines, and various immune response regulators. Their production and activity are, in turn, tightly controlled by different signaling systems including NF-κB, MAPK, JAK/STAT, etc. Therefore the novel small molecule regulators of NF-κB and MAPK activation within the dose range of 0.6–2.4 μg/mL, indicating that TSL-IHL may have therapeutic potential for RA.

The components in a plant extract could often act synergistically on the same signaling pathway or simultaneously affect multiple pharmacological targets, thus may provide clinical efficacy beyond the reach of single compound-based drugs (Schmidt et al., 2007). However, it is also possible that extract components just as well act antagonistically to each other. The chemical analysis shows that TSL-IHL could inhibit the expression of MMP-3, IL-1 and MCP-1 in TNF-α-activated synovial fibroblasts, as well as the expression of IL-1, IL-6 and iNOS in LPS-activated RAW264.7 macrophage cells, in a dose-dependent manner in the dose range of 0.6–2.4 μg/mL, indicating that TSL-IHL may have therapeutic potential for RA.

In the present study, we firstly confirmed the inhibitory effect of TSL-IHL on the activation NF-κB and MAPK pathways with western blotting assay, then, in order to evaluate its potential in RA treatment, we chose synovial fibroblasts and RAW264.7 macrophage cells as cellular models to further test its effect on the expression of pro-inflammatory mediators. Synovial fibroblasts and macrophages are deeply implicated in RA disease process. The main pathogenesis character of RA is synovial hyperplasia, which is ascribed to the increased number and cellular activation of synovial fibroblasts and macrophages. Activated RA synovial fibroblasts are directly involved in cartilage destruction by producing cytokines that perpetuate inflammation and proteases that contribute to cartilage destruction (Noss and Brenner, 2008). MMP-3 is the most important protease involved in cartilage degradation, because it could not only directly degrade cartilage and bone but also activate other MMPs and degrade multiple proteins, such as cartilage link protein, fibronectin, and collagen types, thereby damaging the articular cartilage and bone of the RA patients (Sun et al., 2014; Ma et al., 2015). IL-1 and MCP-1 which can be detected in synovial cells as well as in synovial fluid could also exert local effects on cartilage and bone matrix metabolism and enhance joint destruction indirectly (Szekanecz et al., 1998; Klimiuk et al., 2005). Macrophages also play a pivotal role in promoting inflammation and joint destruction in RA. Increased numbers of macrophages in the synovial tissue produce high levels of cytokines such as IL-1, IL-6, iNOS, which contribute to the bone destruction. Our experiments demonstrated that TSL-IHL could inhibit the expression of MMP-3, IL-1 and MCP-1 in TNF-α-activated synovial fibroblasts, as well as the expression of IL-1, IL-6 and iNOS in LPS-activated RAW264.7 macrophage cells, in a dose-dependent manner in the dose range of 0.6–2.4 μg/mL, indicating that TSL-IHL may have therapeutic potential for RA.

Fig. 2. HPLC fingerprint and UV spectra of TSL-IHL. AL and IAL were identified by comparing with the retention times and UV spectra of standard compounds.

Fig. 3. TSL-IHL, AL, and IAL inhibit TNF-α-induced activation of NF-κB and MAPK pathways. h-End3 cells were treated with TSL-IHL, AL, or IAL (0.6, 1.2, 2.4 μg/mL) for 2 h and stimulated with 20 ng/mL TNF-α for 20 min, and then the levels of IkBα, p65, p38 and JNK and their phosphorylated levels were detected by immunoblotting. The result is representative of three experiments.

**4. Discussion**

The components in a plant extract could often act synergistically on the same signaling pathway or simultaneously affect multiple pharmacological targets, thus may provide clinical efficacy beyond the reach of single compound-based drugs (Schmidt et al., 2007). However, it is also possible that extract components just as well act antagonistically to each other. The chemical analysis shows that TSL-IHL mainly consists of AL and IAL, both of which have been previously found to be able to inhibit NF-κB activation. In order to investigate whether AL and IAL could act synergistically in TSL-IHL, we compared their pharmacological activities with that of TSL-IHL in our in vitro study. No remarkable difference was detected among their inhibitory effects on NF-κB and MAPK activation within the dose range of 0.6–2.4 μg/mL in our western blotting assay (Fig. 3). In the quantitative real-time PCR assays, AL, IAL, and TSL-IHL also displayed similar dose-dependent...
inhibitory effects on the inflammatory gene expressions in the dose range of 0.6–2.4 μg/mL. However, it should be noted that, at the high dose of 2.4 μg/mL, TSL-IHL seemed more effective than AL and IAL in suppressing the expressions of most genes examined (Fig. 4). Even though this result is not enough to support the conclusion that AL and IAL act synergistically, it at least rules out the possibility that they act antagonistically to each other in TSL-IHL, or rather, it can be concluded that the purified AI or IAL is not superior to TSL-IHL in inhibiting the activation of NF-κB and MAPK pathways and suppressing the expressions of inflammatory genes.

Even though AL and IAL are abundant in *I. helenium*, the isolation and purification of each isomer from the plant is still difficult and complicated with low yields and high cost, comparing with the preparation of TSL-IHL. Their organic synthesis is not economic too.

**Fig. 4.** TSL-IHL, AL, and IAL inhibit inflammatory gene expression. (A) Human synovial fibroblasts or (B) RAW264.7 cells were treated with TSL-IHL, AL, or IAL (0.6, 1.2, 2.4 μg/mL) for 2 h, and stimulated with 20 ng/mL TNF-α or LPS for 6 h, then the mRNA levels of indicated genes were detected by qRT-PCR. The results are represented as means ± SEM obtained from three independent experiments. Statistic difference is indicated as # p < 0.05 vs. medium control and * p < 0.05 vs. TNF-α or LPS alone control.

**Fig. 5.** TSL-IHL ameliorates AA in rats. Arthritis was induced in rats by a single subcutaneous injection of CFA into their footpad. The day of CFA injection was designated day 0. TSL-IHL was administered po at 12.5, 25, 50 mg/kg daily starting from (A) day 0 (preventive protocol) or (B) at 25, 50, 100 mg/kg daily starting from day 10 (therapeutic protocol) of CFA injection. MTX (1 mg/kg, po, once every two day starting from day 0 or day 10) was used as positive control. The arthritis index was evaluated and the volume of left and right hind paw was measured in control and TSL-IHL-treated rats every 3 days (n=10). The results are represented as means ± SEM of 10 rats per group. Statistic difference is indicated as # p < 0.05 vs normal control and * p < 0.05 vs CIA control.
Here the in vitro data indicated that TSL-IHL has anti-inflammation pharmacological activity which is not inferior to purified AL and IAL. So we think it is more suitable to be developed in to a therapeutic material. To find out whether it is also active in vivo, we further tested its anti-inflammation effects in AA and CIA rat models. The results demonstrated that oral administration of TSL-IHL at the dose level of 12.5–50 mg/kg in either developing or developed phases of arthritis could dose-dependently reduce the arthritic severity and paw swelling induced by adjuvant or collagen in rats. These in vivo data further validated the preventative and therapeutic potential of TSL-IHL for RA. It should be especially noted that TSL-IHL is well tolerated by rats after multiple doses. In the animal experiments, we observed no deaths or any other serious adverse effects in the TSL-IHL-treated rats, indicating that TSL-IHL is safe in rat at a dose capable of exerting an in vivo anti-inflammatory effect sufficient to bring about amelioration of arthritis in these animals. Of course, further investigations of the safety and efficacy of TSL-IHL for RA will be needed before it can be used in clinical trials in humans.

5. Conclusion

The present study provided experimental evidences for the therapeutic potential of TSL-IHL, a SL-enriched extract of I. helenium with AL and IAL as the main constituents, in the treatment of RA. Our results demonstrated that TSL-IHL could effectively inhibit the activation of NF-κB and MAPK pathways and suppress the inflammatory gene expressions in activated synovial fibroblasts and RAW 264.7 macrophages. More importantly, our experiments showed that oral treatment with TSL-IHL alleviated the paw swelling and arthritic severity in both AA and CIA rat arthritis models. These findings suggested that TSL-IHL may have applications in prevention and treatment of RA.

Declaration of interest

The authors report that they have no conflicts of interest.

Author contributions

Z.W.D. and H.Z.L. designed the experiments, analyzed the experimental data and wrote the paper. G.S. conducted the in vitro experiments. W.Q. collected the plant material and prepared total sesquiterpene lactones from Inula helenium (TSL-IHL). T.X.H. identified and quantified the chemical constituents of TSL-IHL. L.H.L., S.Y.H., X.X.K. and W.G.Z. conducted the in vivo experiments.

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