Industrial Crops & Products 125 (2018) 50–58

Contents lists available at ScienceDirect

Industrial Crops & Products

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

Triterpenoid saponins and flavonoids from licorice residues with anti-inflammatory activity

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ARTICLE INFO

Keywords:
Licorice residues
Triterpenoid saponins
Flavonoids
Anti-inflammatory

ABSTRACT

Industrial processing of licorice (*Glycyrrhiza glabra* L.) leads to a considerable quantity of residues which are normally discarded randomly or treated by landfill. Therefore, the aim of this study was to develop licorice residues as a source of bioactive compounds with potentially applications. Chemical investigation of licorice residues led to the isolation of four new triterpenoid saponins (**1–3**, **12**), along with ten known saponins (**4–11**, **13–14**) and five flavonoids (**15–19**). The structures were established by comprehensive spectroscopic analyses. All the isolated compounds were tested for their inhibitory effects on nitric oxide (NO) production induced by lipopolysaccharide (LPS) in RAW 264.7 cells. Among the results, compound **18** displayed the most potent NO inhibitory effect (IC_{50} 9.89 μM) compared with the positive control drug minocycline (IC_{50} 33.20 μM). Further studies showed that the levels of pro-inflammatory cytokines interleukin (IL)-1β and interleukin (IL)-6 and inducible enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were markedly down-regulated by compound **18**. In addition, the Western blotting assay showed that compound **18** might reduce the inflammatory effect by down-regulating autophagy level. Overall, this study suggest that licorice residues are a promising waste and a valuable source of bioactive compounds for the pharmaceutical industry.

1. Introduction

Inflammation is the normal, protective, and temporary response of the innate immune system to cope with infections and injury (Nguyen et al., 2015). In recent years, inflammatory responses have caused a great number of diseases with a high incidence among the general population, such as lung disease (Wang et al., 2016) and hepatitis (Jiang et al., 2017). Inflammation involves a complex series of reactions regulated by a cascade of pro-inflammatory cytokines, such as interleukin (IL)-1β, interleukin (IL)-6, inducible enzymes including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and nitric oxide (NO) produced by active macrophages (Patel et al., 2015). Hence, the inhibition of pro-inflammatory cytokines, inducible enzymes and NO will be an important strategy for the treatment of inflammation. In addition, it is commonly accepted that autophagy could promote inflammatory responses (Liu et al., 2017), suggesting that autophagy is a possible mechanism contributing to inflammation.

Licorice, the roots and rhizomes of *Glycyrrhiza* species, is an important industrial crop and cultivated around the world (Han et al., 2018a). Licorice also has been increasingly used as a healthy additive formulated into a variety of commercial products, including drugs, foods, drinks and cosmetics, which are marketed in Asia as well as in many other countries around the world (Wang et al., 2013). With the rapid development of the industry, more than 200 million tons of licorice were consumed in industrial products per year (Guo et al., 2015) and industrial processing of licorice products leads to a considerable quantity of residues (Xin et al., 2018). Licorice residue, as industrial waste, is often normally discarded randomly or directly burnt in the field, leading to serious environmental problems (Han et al., 2018b). Therefore, it is of great importance to transform these residues into a source of valuable products, not only for minimizing the environmental pollution, but also for generating a significant profit.

In recent years, exploitation of industry residues as a source of bioactive products, such as triterpenoid saponins (Ralla et al., 2018),

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https://doi.org/10.1016/j.indcrop.2018.08.075
Received 16 May 2018; Received in revised form 28 August 2018; Accepted 29 August 2018

flavonoids (Wong et al., 2015) and lignans (Huang et al., 2015), has become a useful way to increase the economic value of residues and to be of significant environmental benefit. The flavonoids isolated from licorice residues have been applied as chemo-preventive agents (Li et al., 2015), which is a good case for developing and utilizing licorice residues. Therefore, a phytochemical investigation of licorice residues has been carried out to yield fourteen triterpenoid saponins (**1–14**) including four new triterpenoid saponins (**1–3**, **12**) and five flavonoids (**15–19**). All the compounds isolated were screened for the inhibitory activities on NO production induced by lipopolysaccharides (LPS) in RAW 264.7 cells and the results revealed that compound **18** markedly suppressed the NO production. The variations in pro-inflammatory cytokines and inducible enzyme levels, including IL-1β, IL-6, iNOS and COX-2, were investigated. Moreover, autophagy-related protein was also identified.

2. Materials and methods

2.1. Experimental instruments

The UV spectra were obtained on Shimadzu UV-1700 spectrometer (Shimadzu, Tokyo, Japan). Optical rotations were measured on a JASCO DIP-370 digital polarimeter (Jasco Co., Sapporo, Japan). The IR spectra were provided by Bruker IFS-55 spectrometer in KBr pellets (Bruker Co., Karlsruhe, Germany). The 1D and 2D NMR spectra were recorded on a Bruker ARX-400 NMR spectrometer (Bruker Corporation, Bremen, Germany) using trimethylchlorosilane (TMS) as the internal standard, with chemical shifts recorded as δ values. HR-ESI-MS data were collected on a Bruker Micro Q-TOF spectrometer (Bruker Daltonics, Billerica, USA). A Waters 1525–2489 HPLC (Waters Corporation, Massachusetts, USA) was used for isolation and purification with a preparative HPLC C18 column (5 μm, 250 × 10 mm, Shimadzu, Tokyo, Japan). The absorbances in bioassays were measured and recorded on a Thermo Varioskan Flash Multimode (Thermo Scientific Co. Ltd, Massachusetts, USA).

2.2. Chemicals and reagents

D101 macroporous resin (Cangzhou Baoen Chemistry Ltd, Hebei, P. R China). MCI gel (75–150 μm; Mitsubishi Chemical Industries Ltd. Tokyo, Japan). ODS silica gel (60–80 μm, Thermo scientific Co. Ltd., USA). The fractions were monitored by TLC (Qingdao Marine Chemical Factory, Qingdao, P. R. China). All solvents for extraction and chromatography were commercially purchased and routinely distilled prior to use. MTT, LPS, minocycline and the antibody specific to LC3 were purchased from Sigma Chemical Co. Ltd. (St. Louis, Missouri, USA). Dulbecco's modified Eagle's medium (DMEM), PMSF and RIPA lysis buffer were obtained from Beyotime Institute of Biotechnology (Haimen, China). Fetal bovine serum (FBS) and Griess reagent were purchased from Gibco Company (USA). Antibodies specific to COX-2, iNOS, β-actin and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). The Mouse IL-6 and IL-1β ELISA kits were purchased from Dakewe Biotech Co. LTD. (Shenzhen, China). ECL was obtained from Thermo Scientific (Rockford, Illinois, USA). Millipore Immobilon-P transfer Membranes were acquired from Millipore (Billerica, Massachusetts, USA).

2.3. Plant materials

The licorice (*Glycyrrhiza glabra* L.) residues were supplied by a drug manufacturing company from Liaoning Province, P. R. China in May 2014. A voucher specimen (No. 20140527) has been deposited in the Nature Products Laboratory, Shenyang Pharmaceutical University, Liaoning, P. R. China.

2.4. Extraction and isolation

The dry licorice residues (40 kg) were extracted using 80% ethanol (200 L × 2 h) at 80 °C for three times. The combined ethanol extracts were filtered and concentrated under reduced pressure using a rotary evaporator. Then, the extracts obtained were suspended in H_2O and partitioned successively with petroleum ether, ethyl acetate and *n*-BuOH. The *n*-BuOH extracts were subjected to macroporous resin eluted with H_2O, which was gradually enriched with ethanol to afford 6 fractions (A–F). Fraction B was then chromatographed over MCI gel column eluted with increasing gradient of ethanol to afford six subfractions (B1-B6). Subfractions B3 and B4 were further purified *via* ODS column eluted with MeCN/H_2O/TFA (from 20:80:0.05 to 40:60:0.05) to obtain B3-1 to B3-4 and B4-1 to B4-3. Subfraction B3-1 was subjected to semipreparative HPLC eluted with MeOH/H_2O (45:55, 3 ml/min, UV 254 nm) to yield compound **1** (54.2 mg, t_R 16.2 min), **2** (33.1 mg, t_R 17.1 min). Subfraction B3-2 was separated by the same method to afford compounds **3** (19.9 mg, t_R 20.1 min), **4** (24.0 mg, t_R 20.9 min). Likewise, compounds **5** (31.5 mg, t_R 22.8 min), **7** (12.3 mg, t_R 23.5 min) were acquired from B3-4. Compounds **6** (20.7 mg, t_R 25.1 min), **9** (14.3 mg, t_R 26.8 min), **10** (45.7 mg, t_R 27.7 min) were isolated from B4-1. Compounds **13** (33.1 mg, 27.9 min), **14** (24.3 mg, 28.3 min) were acquired from B4-2. Further separation of fraction B4-3 led to the isolation of compounds **8** (39.8 mg, t_R 28 min), **11** (28.3 mg, t_R 28.9 min), **12** (40.7 mg, t_R 31.2 min). Besides, the ethyl acetate extracts were subjected to macroporous resin eluted with ethanol/H_2O with a gradient from 10% to 90% to obtain fraction G to L. Fraction K was subjected to MCI gel column to yield fraction K1-K7. Then, further separation of K5 resulted in the isolation of K5-1 to K5-5. Compounds **18** (50.1 mg, t_R 28.4 min) and **19** (10.4 mg, t_R 31.2 min) were obtained from K5-1 using semipreparative HPLC eluted with MeCN/H_2O (45:55, 3 ml/min, UV 254 nm). Meanwhile, compound **15** (37.6 mg, t_R 25.1 min) was acquired from K5-3. Besides, further isolation of fraction K5-4 produced compounds **16** (40.3 mg, t_R 19.1 min), **17** (31.8 mg, t_R 23.2 min).

Licorice saponin M1 (**1**): white amorphous powder (MeOH); $[\alpha]_D^{20}$ + 95.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (logε): 250 nm (3.39); IR (KBr) ν_{max} cm^{-1}: 3428, 2920, 1727, 1643, 1454, 1385, 1261, 1213, 1163, 1086, 879, 618 cm^{-1}; The ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 873.4257 $[M + Na]^+$ (calcd for $C_{44}H_{66}O_{16}Na$, 873.4243).

Licorice saponin M2 (**2**): white amorphous powder (MeOH); $[\alpha]_D^{20}$ + 87.8 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (logε): 250 nm (3.41); IR (KBr) ν_{max} cm^{-1}: 3435, 2920, 1727, 1644, 1462, 1384, 1262, 1211, 1119, 1048, 879, 618 cm^{-1}; The ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 873.4207 $[M + Na]^+$ (calcd for $C_{44}H_{66}O_{16}Na$, 873.4243).

Licorice saponin M3 (**3**): white amorphous powder (MeOH); $[\alpha]_D^{20}$ + 73.4 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (logε): 249 nm (3.43); IR (KBr) ν_{max} cm^{-1}: 3431, 2923, 1634, 1384, 1048, 537 cm^{-1}; The ^1H and ^{13}C NMR data, see Table 1; HRESIMS at *m/z* 875.4036 $[M + Na]^+$ (calcd for $C_{43}H_{64}O_{17}Na$, 875.4036).

Licorice saponin M4 (**12**): white amorphous powder (MeOH); $[\alpha]_D^{20}$ − 28.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (logε): 241 nm (3.35), 250 nm (3.58), 259 nm (3.30); IR (KBr) v_{max} cm^{-1} 3426, 2922, 1734, 1648, 1383, 1047 cm^{-1}; The ^1H and ^{13}C NMR data, see Table 1; HRESIMS at *m/z* 843.4178 $[M + Na]^+$ (calcd for $C_{43}H_{64}O_{17}Na$, 843.4245).

2.5. Cell culture and cell viability assay

RAW 264.7 cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (10 μg/mL) in a humidified atmosphere of 5% CO_2 at 37 °C. Besides, the compounds isolated were screened for their cytotoxicity *via* MTT method (Chiu et al., 2016). Briefly, RAW 264.7 macrophage cells