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Comprehensive identification of active triterpenoid metabolites in frankincense using a coupling strategy

Yuanyan Liu[a,*,1], Zhenli Liu[b,*,3], Cheng Lu[c], Jianbei Li[d], Zhangchi Ning[a,b], Zhiqian Song[b], Chun Wang[b], Zhiyong Du[b], Xinmiao Lu[a], Siyu Zhao[a], Aiping Lu[c,e,*,2]

[a] School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, China
[b] Institution of Basic Theory, China Academy of Chinese Medical Sciences, Beijing, China
[c] Institute of Basic Research in Clinical Medicine, China Academy of Chinese Medical Sciences, Beijing, China
[d] Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China
[e] Institute for Advancing Translational Medicine in Bone & Joint Diseases, School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China

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ABSTRACT

Frankincense resins are extensively used as natural remedies in regions ranging from North Africa to China. Triterpenoid metabolites from frankincense exhibit notable anti-inflammatory and anti-tumor properties. In the present paper, without the use of an isolation process, the fragmentation rules and NMR spectral characteristics of triterpenoid metabolites in frankincense are summarized through a coupling method using high performance liquid chromatography-diode array detection/electrospray ionization tandem mass spectrometry (HPLC-DAD/ESI-MS^n) combined with HPLC-nuclear magnetic resonance (NMR) experiments. Based on this groundwork, a coupling strategy for the comprehensive metabolic profiling of active triterpenoid metabolites from enriched fractions of frankincense was developed. The proposed strategy may serve as a method for the holistic screening of bioactive metabolites in complex TCM samples.

1. Introduction

Frankincense resins are obtained from trees of the *Boswellia* genus, which are widely distributed in the tropical regions of India, Arabia and the northeastern coast of Africa [1]. Frankincense resins contain different types of secondary metabolites, such as essential oils, sesquiterpenoids, diterpenoids and triterpenoids [2]. Among these, the most abundant compounds in frankincense resins are reportedly triterpenoids [3]. The *Boswellia* genus is an important source of biologically active triterpenoids. Plants accumulate an overwhelming variety of secondary metabolites that play important roles in defense and the interaction of the plant with its environment. *Boswellia* plants in tropical regions experience non-extreme ambient temperatures and moisture fluctuations. Meanwhile, to defend against attack by pathogens and insects, plants in tropical regions usually produce pharmacologically active metabolites, which help the plant adjust to the surroundings. Triterpenoid metabolites in frankincense resin often play a role in the interaction of a cell with its environment and have exhibited extensive therapeutic effects on allergic asthma, inflammatory bowel diseases, rheumatoid arthritis, osteoarthritis, brain tumors and edema [4,5].

Moreover, frankincense has enormous socioeconomic importance. Historically, it has been used in religious and cultural ceremonies and as an ingredient in traditional medicines, whereas currently, much attention has been focused on the medicinal properties of frankincense [4,6], as well as on the role in the field of archeological sciences [7]. China is the largest market for frankincense in the world, mainly due to the use of this material in

Abbreviations: HPLC-DAD/ESI-MS^n, High performance liquid chromatography-diode array detection/electrospray ionization tandem mass spectrometry; NMR, Nuclear magnetic resonance; GC, Gas chromatography; RT, Retention time; TOF, Time-of-flight; PFG, Pulsed field gradient; MW, Molecular weight; BAs, Boswellic acids; K, Keto substitution; A, Acetyl substitution.

* Corresponding author at: School of Chinese Medicine, Hong Kong Baptist University, Hong Kong SAR, China. Tel.: +852-34112456; fax: +852-34112461.
E-mail addresses: yyliu_1980@163.com (Y. Liu), zhenli_liu@sina.com (Z. Liu), lap64067611@126.com (A. Lu).
[1] These authors contributed equally to this work.
[2] Tel: +86 10 84738657; fax: +86 10 84738611.
[3] Tel: +86 10 64067611 2503; fax: +86 10 64013896.

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traditional Chinese medicine. It is primarily applied for the treatment of blood stagnation and inflammatory diseases, as well as for the relief of swelling and pain [8,9]. As summarized in our latest review [10], triterpenoid metabolites in frankincense are widely used as anti-inflammatory agents. Many studies have demonstrated the potent anti-tumor activities of triterpenoids obtained from frankincense [3,11,12]. To further elucidate the mechanism of their pharmacodynamic effect and to ensure their safety and effectiveness in clinical use, it is essential to develop fast and reliable methods for the chemical screening of triterpenoid metabolites in frankincense.

Within metabolomics, metabolic profiling can be defined as an analytical method for comprehensively depicting a number of metabolites from biological samples and is potentially restricted to a certain range of compounds or even to screening a predefined number of members of a compound class [13,14]. Metabolic profiling of herbal extracts is essential for the standardization of medicinal plants and the establishment of the scientific basis of their pharmacological action [15]. Metabolomics platforms using gas chromatography-mass spectrometry (GC-MS), LC-MS or nuclear magnetic resonance (NMR) method have been reported to be effective tools for the quality control of medicinal plants and their products [16]. LC-MS^n is one of the most powerful analytical tools for the identification and discrimination of metabolic profiling in plant extracts. NMR spectroscopy is immensely important for structural elucidation, particularly of complex molecules with (multiple) chiral center(s), but its limited sensitivity has placed constraints on its online use with LC [17]. In the present paper, LC-MS^n and NMR techniques are integrated for metabolite identification with complementary structure information. To date, a metabolic approach to the metabolic profiling of active triterpenoids in frankincense using HPLC-DAD/MS^n combined with NMR has not been described.

To avoid complicated isolation procedures followed by chemical manipulations and spectroscopic analysis, in the present paper, a coupling characteristic strategy for the metabolic profiling of frankincense was established. First, the methanol extract of frankincense was screened using HPLC-DAD/ESI-MS^n combined with HPLC-NMR. Without having undergone an isolation process, representative triterpenoid metabolites were identified or tentatively characterized. Among them, 11 boswellic acids metabolites were unambiguously identified as 'references'. Boswellic acids are the main bioactive metabolites in resin, however they are hardly found in the hydro distilled frankincense essential oil [18]. Their MS fragmentation behaviors and NMR spectral characteristics were systematically compared and summarized. Second, to decrease the interference from the major constituents and simultaneously increase the detection sensitivity, the methanol extract was divided into 4 subfractions prior to LC-MS so that minor and trace metabolites could be enriched and thus detected. Finally, HPLC-DAD/ESI-MS^n and HPLC-NMR experiments were integrated to depict metabolic profiling of fractions from frankincense based on the summarized fragmentation rules and NMR spectral characteristics. These studies can assist in effective analyses of active triterpenoid metabolites in frankincense and provide a strategy for the holistic screening of bioactive metabolites in complex TCM samples.

2. Materials and methods

2.1. Materials

Frankincense resins were purchased from the Beijing Tongrentang Medicine Corporation Ltd. (Beijing, China). HPLC-grade acetonitrile and methanol were from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was redistilled.

2.2. Sample preparation

Accurately weighed frankincense resin powder (1.0 g) was placed in a conical flask. The material was sonicated twice with 15 mL of pure MeOH for 30 min. The supernatant solution was combined, filtered and evaporated to dryness under vacuum. The residue (282 mg) was separated into 4 subfractions according to retention time (RT) on preparative HPLC. The methanol extract of frankincense 50 mg mL^{-1} was injected into an Agilent Zorbax SB-C_{18} (21.2 mm × 250 mm, 7 μm) column twice 100 μL each time. Eluents of the target filtered fractions from the column were collected in a flask. The four collected eluents were removed and dried under a stream of nitrogen before lyophilization to yield four enriched subfractions (Fr.1, 1.78 mg; Fr.2, 1.56 mg; Fr.3, 1.10 mg and Fr.4, 2.34 mg). The enriched subfractions were dissolved in MeCN, filtered through a 0.45 μm microporous membrane and stored at 4 °C until analysis.

2.3. HPLC-DAD/ESI-MS^n analyses

For the online HPLC-DAD/ESI-MS^n analyses, an Agilent 1100 Series liquid chromatography system, which was coupled to an ion trap mass spectrometer was utilized. Positive and negative ion ESI-MSn experiments were performed on an Agilent 1100 Series LC/MSD Trap/SL mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The ESI conditions were as follows: HV capillary voltage 3.5 kV (positive and negative); drying temperature 325 °C (positive), 350 °C (negative); drying gas (N_2) 6.0 L min^{-1} (positive), 9.0 L min^{-1} (negative); nebulizer gas (N_2) 15 psi (positive and negative); capillary exit voltage 106.8 V (positive), −121 V (negative) and injection rate 5 μL min^{-1} (positive and negative). The smart fragmentation function was on (SmartFrag Ampl was 30–200%). The concentration of each sample was 1 mg mL^{-1}.

HPLC separation was carried out on an Agilent XDB-C_{18} column (2.1 mm × 150 mm, 5 μm) (0.2 mL min^{-1}) using a mobile phase of acetonitrile and water, each containing 0.01% acetic acid. The gradient program (MeCN/H_2O, v/v) was 40:60 (t = 0 min), 50:50 (t = 10 min), 70:30 (t = 15 min), 85:15 (t = 25 min), 90:10 (t = 40 min), 95:5 (t = 50 min). The injection volume of each sample was 10 μL. Each sample was prepared in triplicate.

2.4. HPLC-HRMS analyses

High-resolution mass spectra were measured on an Agilent 6520 Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA). The negative ion ESI conditions were as follows: gas temperature, 300 °C, drying gas, 8 L min^{-1} and nebulizer, 20 psi. The TOF conditions were as follows: fragmentor, 200 V; skimmer, 60 V and OCTRFV, 200 V. HPLC separation was performed on an Agilent XDB-C_{18} column (4.6 mm × 150 mm, 5 μm). The concentration of each sample was 1 mg mL^{-1}, and the injection volume was 10 μL.

2.5. HPLC-NMR analyses

HPLC-NMR data were acquired using a Varian INOVA-500 spectrometer equipped with a ^1H {^{13}C} pulsed field gradient (PFG) HPLC-NMR flow probe with a 60 mL flow cell. ^1H NMR and HMBC spectra were obtained in stop-flow mode at 500.13 MHz. Varian WET solvent suppression and related sequences were used to suppress the acetonitrile, its ^{13}C satellites, and residual water peaks. Free induction decays were collected with 16 K data points, a spectral width of 8000 Hz, a 1.0 s acquisition time, and a 1 s pulse delay. A total of 256 transients were acquired to obtain the ^1H NMR data.

The HPLC system consisted of a Varian Prostar 230 solvent delivery system and Varain Prostar 330 photodiode array detector. The HPLC method was the same as that used in HPLC-MS experiments except that the mobile phase was acetonitrile and deuterated water.