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Mass spectrometric dereplication of nitrogen-containing constituents of black cohosh (*Cimicifuga racemosa* L.)

Dejan Nikolić *, Tanja Gödecke, Shao-Nong Chen, Jerry White, David C. Lankin, Guido F. Pauli, Richard B. van Breemen

Department of Medicinal Chemistry and Pharmacognosy, UIC/NIH Center for Botanical Dietary Supplements Research, University of Illinois College of Pharmacy, 833 S. Wood Street, Chicago, IL 60612-7231, United States

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ABSTRACT

Black cohosh preparations are popular dietary supplements among women seeking alternative treatments for menopausal complaints. For decades, triterpene glycosides and phenolic acids have dominated the phytochemical and biomedical research on this plant. In this study, we provide evidence that black cohosh contains an unexpected and highly diverse group of secondary nitrogenous metabolites previously unknown to exist in this plant. Using a dereplication approach that combines accurate mass measurements, database searches and general knowledge of biosynthetic pathways of natural products, we identified or tentatively identified 73 nitrogen-containing metabolites, many of which are new natural products. The identified compounds belong to several structural groups including alkaloids, amides or esters of hydroxycinnamic acids and betains. Among the alkaloids, several classes such as guanidino alkaloids, isoquinolines and β-carbolines were identified. Fragmentation patterns for major compound classes are discussed, which provides a framework for the discovery of these compounds from other sources. Identification of alkaloids as a well-known group of bioactive natural products represents an important advance in better understanding of the pharmacological profile of black cohosh.

1. Introduction

The roots/rhizomes of black cohosh (*Cimicifuga racemosa* (L.) Nutt., syn. *Actaea racemosa* L.) have traditionally been used by Native Americans for treating a variety of medical conditions such as colds, rheumatism as well as for alleviating menopausal symptoms such as hot flashes [1]. Because of the risks associated with hormone replacement therapy, black cohosh preparations have become popular dietary supplements among women seeking alternative treatments for menopausal complaints [2]. Extensive preclinical and clinical investigations have provided conflicting evidence regarding the efficacy of black cohosh [3]. Early studies suggested that black cohosh extracts were effective in reducing the frequency and intensity of hot flashes among perimenopausal and postmenopausal women [4–7], while several recent trials including double-blind placebo-controlled studies demonstrated no vasomotor symptom benefits [8–11].

In terms of the chemical composition of black cohosh, triterpene glycosides and phenolic acids represent the major constituents of black cohosh extracts and interest in them has dominated the phytochemical and biomedical research on this plant for decades [12]. Abundant triterpenes such as actein and 23-*epi*-26-deoxyactein are often used as markers for the standardization of black cohosh preparations. The major phenolic constituents are hydroxycinnamic acids (caffeic, ferulic and isoferulic acid) and their condensation products with glycoloyl phenylpropanoids, commonly known as cimicifugic acids [13]. A third group of black cohosh constituents that has

* Corresponding author at: Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, 833 S. Wood St., Chicago, IL 60612-7231, United States. Tel.: +1 312 413 5867; fax: +1 312 996 7107.
E-mail address: dnikol1@uic.edu (D. Nikolić).

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received far less attention is the alkaloids. We recently described the isolation and identification of several guanidine alkaloids from black cohosh including cimipronidine, cyclocimipronidine and dopargine as well as salsolinol, a member of the tetrahydroisoquinoline (THIQ) group of alkaloids [14,15]. Apart from these compounds, little is known about the presence of nitrogen-containing compounds in black cohosh, which prompted us to explore further this part of the black cohosh secondary metabolome. In this study, we carried out a detailed mass spectrometric investigation of the nitrogen-containing metabolome of a 75% ethanolic extract of black cohosh roots/rhizomes. The results revealed that black cohosh contains an unexpected and remarkably diverse group of nitrogenous metabolites previously unknown to exist in this plant. These results may provide important insights into the future investigation and understanding of the biological activities of this popular botanical dietary supplement.

2. Experimental

2.1. Chemicals

All organic solvents were HPLC-grade or better and were purchased from Fisher Scientific (Fair Lawn, NY). All chemicals used for synthesis were purchased from Sigma-Aldrich (St. Louis, MO). Authentic standards for compound identification were either commercially available, synthesized in-house or previously isolated from other plants. All of the commercial standards were purchased from Sigma-Aldrich except allocryptopine and protopine which were purchased from MP Biosciences (San Diego, CA) and ChemDiv (San Diego, CA), respectively. Magnoflorine, menisperine, magnocurarine, reticuline, laurotetatine, and laurolitsine were kind gifts from Drs. Jan Glinski (Planta Analytica), Yimin Zhao (Beijing Institute of Pharmacology) and Shoei-Sheng Lee (National Taiwan University).

2.1.1. Synthesis of ferulic and isoferulic acid amides

Small-scale synthesis of ferulic and isoferulic acid amides was carried out using routine synthetic coupling reactions utilizing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) as the activating agent.

2.1.2. Synthesis of feruloyl and isoferuloyl choline

Feruloyl (**47**) and isoferuloyl choline (**48**) were synthesized according to the protocol of Boettcher et al. [16].

2.1.3. Synthesis of N-formyl arginine

N-formyl arginine (**25**) was prepared by treating arginine with formic acid at elevated temperature according to the method of Karapetyan et al. [17].

2.1.4. Pictet Spengler adducts

2(*N*)-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (**58**) and cimitrypazepine (**59**) were synthesized by condensation of N_{ω}-methylserotonin and formaldehyde according to the method of Somei et al. [18]. (3S)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**46**) was synthesized by condensation of tryptophan and formaldehyde under acidic conditions as described by Brossi et al. [19]. *N*(2)-methyl-6-hydroxy-3,4-dihydro-β-carboline (**53**) was prepared by treating N_{ω}-methylserotonin with glyoxylic acid under alkaline conditions according to the protocol of Yamano et al. [20].

Cimitrypazepine (**59**) ^{1}H-NMR (CD_3OD) δ: 2.75 (3H, s), 3.16 (2H, m), 3.31 (2H, m), 4.34 (2H, s), 7.02 (1H, brs), 6.68 (1H, d, $J = 8.6$ Hz), 7.1 (1H, d, $J = 8.6$ Hz).

N(2)-methyl-6-hydroxy-3,4-dihydro-β-carboline (**53**) ^{1}H-NMR (CD_3OD) δ: 7.42 (*d*, $J = 9.2$ Hz, 1H), 6.94 (brs, 1H), 6.33 (d, $J = 9.2$ Hz, 1H), 3.78 (*m*, 2H), 3.63 (*s*, 3H), 3.11 (*m*, 2H).

2.2. Plant material

The raw plant material and the corresponding 75% ethanolic extract used in this study were identical to the materials used in our recent Phase IIb clinical trial and were described previously [11,21–23]. Briefly, the plant material was acquired from Naturex (previously Pure World, South Hackensack, NJ) and botanically authenticated using PCR and microscopy [24]. Milled roots/rhizomes were extracted with 75% ethanol by large-scale percolation, vacuum-dried at 45 °C and milled though a 60-mesh screen to yield a powdered extract.

2.3. Fractionation

The 75% ethanolic extract of black cohosh roots/rhizome was partitioned between water and ethyl acetate. The water partition was further fractionated on a column filled with Amberlite XAD-2 resin to yield water and methanol-soluble fractions. The methanol fraction was then subjected to pH-zone refinement fast centrifugal partition chromatography (FCPC) using water/butanol/ethyl acetate (5:4:1) as the solvent system. This approach yielded six chemically distinct fractions labeled FCPC 1–6. More detailed description of the fractionation procedure has been published elsewhere [13,21].

2.4. Dereplication

We followed a standard dereplication approach used in mass spectrometry-based metabolomics studies beginning with the determination of elemental composition by accurate mass measurement, followed by the acquisition of product ion tandem mass spectra. Since product ion mass spectra were acquired using accurate mass measurement, the elemental composition of the fragment ions could also be determined. The validity of the molecular formula obtained from accurate mass measurements was established using additional criteria such as isotope pattern, elemental composition of fragment ions as well as general plausibility of the formula based on general knowledge of natural product chemistry. The elemental composition was then searched in SciFinder and Beilstein CrossFire Commander databases of natural products as well as in the MassBank (www.massbank.jp) database of tandem mass spectra [25]. If a match was obtained in the MassBank database, final confirmation of compound identity was obtained by comparing the retention time and fragmentation pattern with those of authentic standards. This was a necessary precaution due to well-known differences in appearance of product ion spectra obtained using different instruments [26]. For compounds for which there were no spectra in the MassBank database, the hits obtained in the SciFinder or Beilstein databases provided clues as to possible structure. Based on the interpretation of product