Food and Chemical Toxicology 47 (2009) 2142–2149

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Boerhaavia diffusa: Metabolite profiling of a medicinal plant from Nyctaginaceae

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ARTICLE INFO

Article history:
Received 6 May 2009
Accepted 28 May 2009

Keywords:
Boerhaavia diffusa
Metabolite profiling
Phenolics
Organic acids
Acetylcholinesterase inhibition
Antioxidant activity

ABSTRACT

Boerhaavia diffusa is a plant which is extensively used in folk medicine. However, when it comes to its phytochemical characterization, little attention has been given to secondary metabolites other than rotenoids and alkaloids. A metabolite profiling and biological study was undertaken in this species' leaves and roots and substantial differences were found between the two parts of the plant. The volatile composition was analysed for the first time using HS–SPME–GC–MS and several compounds, including terpenes, phenylpropanoids, indol compounds, norisoprenoids, among others, were identified. Organic acid analysis was also performed, allowing their characterization in this species for the first time, and oxalic, ketoglutaric, pyruvic, quinic and fumaric acids were identified. Quantitative differences between the two vegetal materials were found. Additionally, several flavonoids and one phenolic acid were also confirmed. Concerning the biological potential, the aqueous extract of each plant part was tested against DPPH radical, one reactive oxygen species ($O_2^{\cdot-}$) and one reactive nitrogen species ($^{\cdot}NO$). Moreover, activity against acetylcholinesterase, an enzyme with a well-known role in several physio-pathological processes, was assayed. When possible, the relation between the chemistry and activity displayed was established. Leaves revealed stronger antioxidant activity than roots, and acetylcholinesterase inhibition was not found in neither plant part.

1. Introduction

Boerhaavia diffusa (Nyctaginaceae) is one of the most famous medicinal plants in India, where it is widely used in folk medicine. This plant's medicinal properties are recognized worldwide, with its leaves and roots being used in several conditions, such as asthma, urinary disorders, rheumatism and pain states (Awasthi and Verma, 2006; Hiruma-Lima et al., 2000), among many others. Most of the times, its consumption takes place as decocts or infusions.

Verma et al. (1979) showed the antiviral activity of this species against phytopathogenic viruses, in various host–virus combinations, both *in vitro* and *in vivo*. Later, the antiviral agent was identified to be an extremely thermostable basic glycoprotein.

More recently, extracts of *B. diffusa* have equally proved to display anti-proliferative activity against a variety of established tumour cell lines (Mehrotra et al., 2002). Posterior studies showed that methanolic extracts of this species were effective in reducing metastases formation in some melanoma cells (Leyon et al., 2005).

Metabolite profiling is a promising approach that differentiates genotypes based on metabolite levels that may or may not produce visible phenotypes (Fiehn, 2002). Furthermore, different parts of a plant can be distinguished by taking into account their specific metabolites.

Most volatile compounds from living organisms are produced through primary and secondary metabolic pathways, presenting low molecular weight and a lipophilic behaviour (Goff and Klee, 2006). Non-conjugated compounds can freely cross membranes and be released in the atmosphere when there are no barriers for their diffusion.

Within the several volatile compounds produced by a species, only a small group constitutes the so-called flavor fingerprint, which helps animals recognizing these species (Goff and Klee, 2006). The most important class of volatiles is derived from the isoprene pathway. In plants, mevalonate and/or deoxyxylulose are the two possible pathways that lead to terpenes' precursors, isopentenyl pyrophosphate and dimethylallyl pyrophosphate. These are the precursors of monoterpenes and iridoids (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), among others (Bruneton, 2001). Within these classes, monoterpenes and sesquiterpenes are the most volatile ones.

The study of a species' volatiles compounds can be valuable, as these compounds are described to have several biological functions, such as antioxidant, antiseptic or anti-atherosclerotic (Edris, 2007).

The aim of this work was to provide the first analysis of volatile compounds of a widely used medicinal plant, *B. diffusa,* using a HS–SPME–GC–MS technique directly into the headspace of the

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doi:10.1016/j.fct.2009.05.033

aqueous extract of the leaves and roots. In addition to phenolics (determined by HPLC–DAD), the organic acids (HPLC–UV) profile and *in vitro* antioxidant and anti-acetylcholinesterase activities are described for the first time, providing further knowledge on this species' chemistry and biological potential.

2. Materials and methods

2.1. Standards and reagents

Reference compounds were purchased from various suppliers: octanal, (*E*)-2-octenal, (*E*)-2-nonenal, hexadecanoic acid methylester, geranylacetone, β-cyclocitral, α-pinene, β-pinene, linalool, limonene, eugenol, (*E*)-2-decen-1-ol; (*Z*)-2-hexenol, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, 6-methyl-5-hepten-2-one, methyldihydrojasmonate, 5-*O*-caffeoylquinic acid, oxalic, citric, malic, shikimic and fumaric acids, dabsyl chloride reagent, sodium hydrogen carbonate, dimethylformamide, triethylamine, sodium dihydrogenphosfate, acetylcholinesterase, acetylthiocholine iodide (ATCI), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), 2,2-diphenyl-1-picrylhydrazyl (DPPH·), β-nicotinamide adenine dinucleotide reduced form (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT) and sulfanilamide were from Sigma–Aldrich (St. Louis, MO, USA); (*E*)-2-nonenal; hexanal, (*E*)-2-hexanal, phenylacetaldehyde, β-ionone, dimethyl disulfide; dimethyl trisulfide and *cis*-3-hexenyl acetate were obtained from SAFC (Steinheim, Germany); pyruvic and *cis*-aconitic acids, eucalyptol, *o*-cymene, rutin and kaempferol-3-*O*-rutinoside were from Extrasynthese (Genay, France); acetic acid, hexyl ester and menthol were obtained from Fluka (Buchs, Switzerland); sodium nitroprussiate dihydrate and allylisothiocyanate were from Riedel de Haën (Seelze, Germany).

Methanol, sulphuric and formic acids were from Merck (Darmstadt, Germany). Water was treated in a Milli-Q (Millipore, Bedford, Massachusetts) water purification system.

2.2. Samples

Leaves and roots of *B. diffusa* were collected in Luanda (Angola) in 2002. The plant was identified at LESRA (Universidade Agostinho Neto, Luanda, Angola) and a voucher specimen was deposited at the Laboratory of Pharmacognosy, Faculty of Pharmacy, Porto University, Porto, Portugal (BdPK0702).

Samples were stored at room temperature, in a dessicator in the dark until required for analysis.

2.3. Sample preparation

An aqueous extract was used for the phytochemical characterization and in the biological assays: About 1.5 g of each *B. diffusa* dried material (leaves and roots) were boiled for 20 min in 300 mL of water and filtered over a Büchner funnel. The resulting extracts were then frozen and lyophilized. The lyophilized extracts were kept in a dessicator in the dark.

2.4. Phenolics analysis by HPLC–DAD

Twenty microlitres of the several lyophilized plant parts were redissolved in water (100 mg/ml for roots and 50 mg/ml for leaves) and analysed using an HPLC unit (Gilson) and a Spherisorb ODS2 column (4.6 × 250 mm, 5 μm particle size). Solvents used were methanol (A) and 5% formic acid (B). Elution was performed with a flow of 0.9 mL/min and gradient was as follows: 95% B at 0 min, 85% B at 3 min, 75% B at 13 min, 70% B at 25 min, 65% B at 35 min, 55% B at 39 min, 50% B at 44 min. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 330 nm. The data were processed on a Unipoint Software system (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Quantification of phenolic compounds was achieved by comparison of their absorbance with that of external standards commercially available: caffeoyltartaric acid was quantified as 5-*O*-caffeoylquinic acid and quercetin derivatives were quantified as rutin, while kaempferol and eupalitin derivatives were quantified as kaempferol-3-*O*-rutinoside.

2.5. Organic acids analysis by HPLC–UV

For organic acids determination, the sample was redissolved in 0.01 N sulphuric acid (100 mg/mL) prior to analysis by HPLC–UV.

The separation of organic acids was carried out as previously reported (Pereira et al., 2009) in a system consisting of an analytical HPLC–UV unit (Gilson) with an ion exclusion column, Nucleogel Ion 300 OA (300 × 7.7 mm) in conjunction with a column heating device set at 30 °C. Elution was performed in isocratic mode with sulphuric acid 0.01 N, under a flow rate of 0.2 mL/min. The detection was achieved with an UV detector set at 214 nm. Identification was performed by comparison of the retention times with those of authentic standards.

Each organic acid was quantified by the absorbance recorded in the chromatograms relative to that of external authentic standards. The peaks in the chromatograms were integrated using a default baseline construction technique.

2.6. Extraction methodologies – SPME technique and SPME fibres

Several commercial fibres can be used to extract volatiles. According to bibliography, recommendations of supplier (Supelco, Bellefonte, PA, USA) and to our own knowledge (Guedes de Pinho et al., 2008) three of them are the most adaptable to the intended compounds and to the matrix under study. The fibres used were coated with different stationary phases and various film thicknesses: black – Carboxen TM/polydimethylsiloxane (CAR/PDMS), 75 μm; orange – Carbowax/Divinylbenzene (CW/DVB), 65 μm; blue – Divinylbenzene/PDMS (DVB/PDMS), 50/30 μm. They were conditioned by inserting them into the GC injector; temperature and time were used according to the procedure recommendation of Supelco: 300 °C for 1 h, 220 °C for 30 min, and 250 °C for 30 min, respectively.

2.7. Qualitative and Semi-quantitative SPME analysis

2.7.1. Headspace solid phase microextraction (SPME) (lyophilized aqueous extract)

Approximately 0.2 g of lyophilized aqueous extract was dissolved in 5 mL of a 5% ethanol solution in a 15 mL vial, and 0.5 g of anhydrous sodium sulphate was added to help the release of analytes from the matrix. It was then sealed with a polypropylene hole cap and PTFE/silicone septa (Supelco, Bellefonte, PA, USA). The mixture was magnetically stirred at 760 rpm, 55 °C, for 5 min. The fibre was then exposed to the headspace for 20 min, with agitation (800 rpm). Afterwards, the fibre was pulled into the needle sheath and the SPME device was removed from the vial and inserted into the injection port of the GC system for thermal desorption. After 1 min the fibre was removed and conditioned in another GC injection port for 15 min at 250 °C.

2.7.2. Gas chromatography–mass spectrometry analysis of volatile compounds

HS-SPME analysis was performed using a Varian CP-3800 gas chromatograph (USA) equipped with a VARIAN Saturn 4000 mass selective detector (USA) and a Saturn GC/MS workstation software version 6.8. The column used for samples analysis was VF-5 ms (30 m × 0.25 mm × 0.25 μm) from VARIAN. Stabilwax-DA fused silica column (60 m × 0.25 mm, 0.25 μm) (Restek, USA) was used in order to check the identity of some compounds found in the first column. The injector port was heated to 220 °C. The carrier gas was Helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. The oven temperature was set at 40 °C for 1 min, then increasing 2 °C/min to 220 °C and held for 30 min. All mass spectra were acquired in electron impact (EI) mode. Ionization was maintained off during the first 2 min, to avoid solvent overloading. The Ion Trap detector was set as follows: the transfer line, manifold and trap temperatures were, respectively, 280, 50 and 180 °C. The mass ranged from 40 to 350 *m/z*, with a scan rate of 6 scan/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionization time was 25,000 μs, with an ionization storage level of 35 *m/z* and the analysis was performed in Full Scan mode.

Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic compounds, when available, run under the same conditions, and by comparison of the retention indices (as Kovats indices) with the literature data. The comparison of MS fragmentation pattern with those of pure compounds and mass spectrum database search was performed using the National Institute of Standards and Technology (NIST) MS 05 spectral data base. Confirmation was also conducted using the laboratory built MS spectral database, collected from chromatographic runs of pure compounds performed with the same equipment and conditions. For quantitative evaluation, diagnostic mass fragments and target ion of the compounds (Table 1) were selected from full scan mode.

2.8. Evaluation of antioxidant activity

2.8.1. DPPH· scavenging activity

The disappearance of DPPH· was monitored spectrophotometrically at 515 nm on a Multiskan Ascent plate reader (Thermo, Electron Corporation), following a described procedure (Pereira et al., 2009). For each extract, a dilution series (five different concentrations) was prepared in a 96-well plate. The reaction mixtures in the

Table 1
Phenolics quantification of *B. diffusa* leaves and roots (mg/kg).[a]

Compound	Leaves	Roots
Quercetin 3-*O*-robinobioside	6726.26 ± 26.30	484.39 ± 33.19
Eupalitin 3-*O*-galactosyl(1 → 2)-glucoside	1066.07 ± 7.87	61.38 ± 3.23
Kaempferol 3-*O*-robinobioside	450.19 ± 19.17	–
Eupalitin 3-*O*-galactoside	2198.73 ± 126.49	–
Quercetin	nq	–
Caffeoyltartaric acid	–	503.32 ± 6.77
Total	10441.25	1049.09

[a] Results are expressed as means ± standard deviations of three determinations.