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The use of chemometrics to study multifunctional indole alkaloids from *Psychotria nemorosa* (*Palicourea comb. nov.*). Part II: Indication of peaks related to the inhibition of butyrylcholinesterase and monoamine oxidase-A

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ABSTRACT

Psychotria nemorosa is chemically characterized by indole alkaloids and displays significant inhibitory activity on butyrylcholinesterase (BChE) and monoamine oxidase-A (MAO-A), both enzymes related to neurodegenerative disorders. In the present study, 43 samples of *P. nemorosa* leaves were extracted and fractionated in accordance to previously optimized methods (see Part I). These fractions were analyzed by means of UPLC-DAD and assayed for their BChE and MAO-A inhibitory potencies. The chromatographic fingerprint data was first aligned using correlation optimized warping and Principal Component Analysis to explore the data structure was performed. Multivariate calibration techniques, namely Partial Least Squares (PLS1), PLS2 and Orthogonal Projections to Latent Structure (O-PLS1), were evaluated for modelling the activities as a function of the fingerprints. Since the best results were obtained with O-PLS1 model (RMSECV = 9.3 and 3.3 for BChE and MAO-A, respectively), the regression coefficients of the model were analyzed and plotted relative to the original fingerprints. Four peaks were indicated as multifunctional compounds, with the capacity to impair both BChE and MAO-A activities. In order to confirm these results, a semi-prep HPLC technique was used and a fraction containing the four peaks was purified and evaluated *in vitro*. It was observed that the fraction exhibited an IC_{50} of 2.12 μg mL^{-1} for BChE and 1.07 μg mL^{-1} for MAO-A. These results reinforce the prediction obtained by O-PLS1 modelling.

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1. Introduction

Plants are known as impressive chemical factories and have been playing an important role in drug discovery. Nevertheless, pharmaceutical companies have reduced the economic input in this research field, mainly because of difficulties related to the re-isolation of known metabolites and the lack of reliable tools for the indication of active compounds [1]. However, several new strategies and technologies are nowadays available and have been successfully applied for the identification of leads in natural products research [1–5]. One of these strategies is the metabolic profiling approach. It correlates the chemical profile and the biological activity of extracts or fractions, guiding the isolation and early identification of the targeted secondary metabolites [2,6–10].

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Different plants have been studied by this metabolic profiling strategy, focusing on a wide variety of secondary metabolites, such as terpenoids [11], flavonoids [2], and alkaloids [12]. Alkaloids make up around 20% of the natural substances described so far, and have a structural diversity that is comparable to terpenoids. These nitrogenous compounds usually are pharmacologically active, being the main group of secondary metabolites of interest to researchers and the pharmaceutical industry. These metabolites are predominantly found in angiosperms, mainly in Apocynaceae, Solanaceae, Papaveraceae, Loganiaceae, and Rubiaceae [13,14].

Alkaloids are widely known for their potential to treat central nervous system (CNS) related diseases. Several bioactive alkaloid-like structures are reported in the literature, however probably the best known examples are the indole alkaloids derivatives, as rivastigmine and galantamine, used for the treatment of Alzheimer's disease [15]. In addition, some of these compounds have been investigated because of their multifunctional activities, also related to other targets in neurodegenerative processes, such as the inhibition of monoamine oxidases (MAO) [16].

The plants belonging to the genus *Psychotria* L. (Rubiaceae) are widely used because of the different effects they can promote in the CNS. Amazon Indian tribes use these plants for the preparation of Ayahuasca, a hallucinogenic beverage for medicinal, ritual and recreational purposes [17]. In the traditional medicine of Middle America, *Psychotria* species are used for the treatment of dementia related effects [18]. In fact, our research group has also demonstrated the modulatory action of *Psychotria* alkaloid fractions and isolated compounds on enzymes related to neurodegenerative disorders, such as acetylcholinesterase (AChE), butyrylcholinesterase (BChE), MAO-A, sirtuins, and catechol-*O*-methyltransferase (COMT) [16,19–22]. It is worthwhile to mention that several *Psychotria* subg. *Heteropsychotria* species have been transferred to the *Palicourea* genus, based on taxonomic and chemical aspects [23,24]. Taking this into account, *Palicourea comb. nov.* was added to the original *Psychotria* subg. *Heteropsychotria* names, in accordance with this trend [23,24].

Recently, we demonstrated that the alkaloid fraction obtained from *Psychotria nemorosa* Gardner (*Palicourea comb. nov.*), was able to significantly inhibit MAO-A and BChE activities [25]. In addition, the species exhibited a high chemical diversity. In order to access the alkaloid metabolite profile, optimized extraction and fractionation methods were developed in the first part of this study [26]. In the actual paper, this optimized extraction procedure was applied to several samples of *P. nemorosa* and the alkaloid fractions were analyzed by means of UPLC-DAD. All fractions were evaluated *in vitro* for their MAO-A and BChE inhibitory activities and these results were modelled by different multivariate calibration techniques as a function of their chromatographic fingerprints, aiming the indication of peaks potentially responsible for the pharmacological activities.

2. Material and methods

2.1. Chemicals

Kynuramine dihydrobromide, clorgyline hydrochloride, pargyline hydrochloride, tacrine, galanthamine, 5,5′-dithiobis-(2-nitrobenzoic acid), acetylthiocholine iodide, electric eel acetylcholinesterase, *S*-butyrylthiocholine iodide, horse serum butyrylcholinesterase, and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human MAO-A and MAO-B SupersomesTM were acquired from BD Gentest (Woburn, MA, USA). UPLC–MS grade solvents were acquired from Actu-All Chemicals (Oss, The Netherlands). All remaining solvents were acquired from Tedia Company (Fairfield, CA, USA).

2.2. Plant material

The leaves of *P. nemorosa* were collected from forty three individuals distributed in five different places of Blumenau/SC, Brazil (Fig. 1). Vouchers of each collection were deposited in the Dr. Roberto Miguel Klein Herbarium under the numbers FURB 43759, 43756, 43758, 43755, 43580. The identification of the species was performed by the botanic André L Gasper (FURB/Brazil). Access authorization was given by CNPq/Brazil under the number 010772/2014-6. The vegetal material was dried in an air oven at 40 °C for 48 h (Lawes, Brazil), ground using an analytical mill (IKA, Königswinter, Germany), and sieved (≤180 μm) using a mechanical shaker Retac 3D (RETSCH, Haan, Germany).

2.3. Extraction and fractionation procedures

The optimized extraction and fractionation methods were previously described [26]. Briefly, vegetal material was submitted to ultrasound assisted extraction using an ultrasonic bath (132 W; 40 kHz) during 65 min at 45 °C. Methanol was used as extraction solvent at a 1:50 (m/v) drug:solvent ratio. Vegetal material particle size was ≤ 180 μm. The extracts were filtered and then evaporated at 40 °C using a rotary evaporator with a vacuum pump (V-710, Büchi, Flawil, Switzerland). Before fractionation experiments, the samples were kept for two weeks under vacuum in a desiccator containing activated silica gel beads.

For fractionation, a solid phase extraction method was developed applying a Box-Behnken design [26]. Briefly, normal-phase silica cartriges (Supelclean LC-Si, Supelco, PA, USA) were equilibrated with 10 mL of HCl 1 M. Before dryness, the samples (150 mg mL^{-1} in HCl 1 M) were loaded and the cartridges dried. Then, the cartridges were washed using 10 mL of dichloromethane. After dryness, the samples were eluted with 30 mL of 5% NH_4OH in dichloromethane/acetonitrile (6:4, v/v) (gravity flow). The resulting organic extracts were concentrated to dryness by a tube evaporator at 40 °C, resulting in the fractions enriched in alkaloids. These samples were kept for one week under vacuum in a desiccator containing activated silica gel beads.

2.4. Fingerprint development

A system composed by an ACQUITY I-class UPLC® from Waters (Milford, MA, USA) was used. The separation was performed on a 50 mm × 2.1 mm i.d., 1.7 μm, Acquity BEH C_{18} UPLC column (Waters) at 40 °C and a flow rate of 0.3 mL min^{-1} with a mobile phase consisting of water (formic acid 0.1%) (A) and methanol (B) in the following gradient: 0 min (99 (A): 1 (B), v/v), 1 min (94:6), 4 min (94:6), 24 min (54:46), 25 min (54:46), 26 min (48:52), 28 min (48:52), 29 min (0:100), 33 min (0:100), 35 min (99:1), 40 min (99:1). A 2 μL aliquot of the samples was injected twice and the detection was performed at 280 nm. The solutions were freshly prepared in methanol before each experiment and filtered through a 0.22 μm cellulose regenerated membrane filter. Data was processed using Waters MassLynx software.

2.5. Enzymatic assays

2.5.1. Cholinesterases inhibitory assays

First, all fractions were assayed both for AChE and BChE at 100 μg mL^{-1}. For the AChE inhibitory assay, wells were filled with 158 μL Ellman's reagent (0.15 mM 5,5′-dithiobis-(2-nitrobenzoic acid) in 0.1 M phosphate buffer pH 7.4), 20 μL acetylthiocholine iodide solution (0.33 mM), and 2 μL test compound solution in DMSO. The addition of 20 μL electric eel AChE solution (1 U.I. mL^{-1} in 0.1 M phosphate buffer pH 7.4, containing human serum albumin at 1 mg mL^{-1}) started the reaction and the absorbance at 412 nm

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