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The sedative activity of flavonoids from *Passiflora quadrangularis* is mediated through the GABAergic pathway

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ABSTRACT

The aim of this study was to investigate the sedative activity of the aqueous leaf extract of *Passiflora quadrangularis*, a species that is widely cultivated and consumed in South America, and to identify its main constituents and elucidate the involvement of the GABAergic pathway in its mechanism of action. The bioguided fractionation of the crude extract showed a positive relationship between the sedative activity of the extract and its flavonoids. The methods employed to identify and isolate its main flavonoids resulted in the identification of vitexin-2''-*O*-xyloside, vitexin-2''-*O*-glucoside, orientin-2''-*O*-xyloside and orientin-2''-*O*-glucoside. Vitexin-2''-*O*-xyloside, the major flavonoid of the extract, showed sedative activity after oral administration in mice.

1. Introduction

The genus *Passiflora* is the largest and most important genus of the Passifloraceae family, comprising about 500 species. *Passiflora quadrangularis* L. is a species cultivated throughout tropical America for its edible fruits, at elevations below 2500 m [1]. As occurs with other *Passiflora* species [2], the leaves of *P. quadragularis* are used in traditional medicine as a sedative and mild tranquilizer [3,4]. Concerning the chemical composition of extracts obtained from its leaves, reports show the presence of flavonoids [5,6] and saponins [7–9]. Neuropharmacological data reported that a hydroethanolic extract, but not the aqueous extract from leaves of the species, presented anxiolytic activity in rats tested in the elevated plus maze (500 mg/kg), open field (100, 250 and 500 mg/kg) and hole-board tests (250 mg/kg) after oral administration [10]. Regarding sedative activity, Gazola and coworkers [11] showed that oral administration of aqueous extract of pericarp (100 and 300 mg/kg) extended sleep duration in the ethyl ether-induced hypnosis test in mice. Also, apigenin (0.6 mg/kg), the main flavonoid of the extract, induced a similar sedative response at a dose equivalent to that found in the extract. Furthermore, the authors suppose that the sedative activity of apigenin may be due to an enhancement of the GABAergic system [11]. Thus, the aim of the present study was to investigate the sedative activity of the aqueous leaf extract of *P. quadrangularis*, and of its main constituents, and the role of the GABAergic pathway in its mechanism of action.

2. Material and methods

2.1. Plant material and extraction

Leaves of *Passiflora quadrangularis* L., Passifloraceae, collected in Colombia [Neiva, Huila (2°59′55″, −75°18′16″) in July 2011, and identified by Prof. Luis Carlos Jimenez (Instituto Nacional de Ciencias, Universidad Nacional de Colombia), with a voucher specimen deposited in the Herbarium of the Universidad Nacional de Colombia (COL 572634), were air-dried at temperatures below 40 °C, powdered, and extracted by infusion with hot water (90 °C - plant: solvent, 1:10, w/v) for 10 min. Thereafter, the aqueous extract was filtered and freeze-dried, yielding the aqueous extract of leaves from *P. quadrangularis*.

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2.2. Fractionation of aqueous extract of P. quadrangularis

For the fractionation, 1 g of the aqueous extract of leaves from *P. quadragularis* was dissolved in distilled water (1: 20, w/v) and fractionated using the ion exchange resin Amberlite IR120 (40 g). Previously, Amberlite IR120 was added to a glass column, activated with 150 mL of hydrochloric acid solution (HCl 1N) and washed with distilled water until neutralization. After elution of the extract, the column was washed with 300 mL of distilled water, which was added to residual extract solution to yield the amino acid free extract solution. Next, 500 mL of sodium hydroxide solution (NaOH 3N) was passed through the column, giving an ammonia solution containing the amino acids from the extract. The amino acid free extract solution and the ammonia solution containing the amino acids were dried under reduced pressure (temperature below 40 °C) yielding the amino acid free fraction and the amino acid fraction (AAF).

The amino acid free fraction (150 mg) was solubilized in methanol. This solution was applied to a Sephadex LH-20 (40 g) column and eluted with methanol. Fractions were subjected to thin layer chromatography (TLC) [silica gel $60F_{254}$ plates - ethyl acetate: acetone: acetic acid: water (8: 2: 1: 1, v/v/v/v) - natural reagent/365 nm]. In the first 38 fractions, no presence of compounds with fluorescence or fluorescence quenching was observed, but when the fractions were sprayed with sulfuric acid/anisaldehyde and heated, the presence of saponins was indicated; thereafter the presence of compounds with fluorescence quenching and fluorescence was observed in the last 33 fractions (40–73), characterizing the presence of flavonoids. Fractions 1–38 and 40–73 were grouped, dried under reduced pressure (temperatures below 40 °C) and resulted in a saponin (SF) and a flavonoid fraction (FF), respectively.

2.3. Isolation and characterization of the main flavonoids in aqueous leaf extract of P. quadrangularis

To isolate main flavonoids of the aqueous extract of *P. quadrangularis*, the aqueous extract was subjected to gradient preparative High-Speed Counter-Current Chromatography (HSCCC) using a P. C. Inc. apparatus. The proportions of the solvent system ethyl acetate: butanol: water (1: X: 1) were adjusted according to the flavonoid partition coefficients observed by TLC in a four-step gradient (A–D; X = 0.2, 0.4, 0.6 and 0.8, respectively). The lower aqueous phase of system A was employed as stationary phase, while the organic phases of the systems A–D were used as mobile phase in tail-to-head mode, at a flow rate of 3 mL/min and 800 rpm. Next, 1 g of the aqueous leaf extract of *P. quadrangularis* was dissolved in 6 mL of both phases (1: 1 proportion) of system A and injected in a 400 mL coil, previously equilibrated with stationary and mobile phase of system A (V_S = 250 mL, S_F = 62,5%). A hundred and forty three fractions of 6 mL each were collected. Fractions containing the four main flavonoids from the extract were grouped into four types, according to their TLC profiles (Fraction I: 93.2 mg, Fraction II: 15.0 mg, Fraction III: 81.0 mg, Fraction IV 22.8 mg). These fractions were then purified in Sephadex LH-20 columns, using methanol as eluent, giving the purified compounds **1** (31.0 mg), **3** (8.4 mg), **2** (16.8 mg) and **4** (6.8 mg).

Solutions of the extract (3 mg/mL) and isolated compounds (0.5 mg/mL) were analysed based on the techniques previously reported by Costa et al. [5] and are briefly presented below. All samples were solubilized in methanol:water (5: 5, v/v) and filtered in a 0.45 μm membrane.

2.3.1. HPLC-DAD analysis

In the High Performance Liquid Chromatography (HPLC) analysis, 20 μL of the samples were injected into the HPLC (PerkinElmer Series 200) equipped with Diode Array Detection (DAD), quaternary pump, on-line degasser and auto-sampler. The data were processed using the TotalChrom Workstation software. The UV spectra were monitored over a range of 400–190 nm. A Vertical VertSep C_{18} column (250 × 4.6 mm i.d.; 5 mm) was used, with a gradient system of acetonitrile (solvent A) and formic acid 0.5% (solvent B), in a single step: 15–35% A (0–15 min). The flow rate was kept constant at 1.2 mL/min and the chromatograms were recorded at 340 nm.

2.3.2. LC–MS analysis

The analysis by Liquid Chromatography-Mass Spectrometry (LC–MS) was carried out using a Shimadzu LC-10A coupled to a selective mass detector (LCMS-2010EV). Electrospray ion source (ESI)-MS spectra were acquired in negative ion mode, and the interface and MS detector parameters were as follows: detector voltage, 1.5 kV; CDL voltage, 150 V, CDL temperature, 250 °C; heat block temperature, 250 °C; QarrayRF voltage, 150 V and nitrogen as nebulizing gas, at a flow rate of 1 L/min. The chromatographic conditions were the same as those used in the HPLC-DAD analysis.

2.4. Animals

Male adult Swiss mice (3–4 months), weighing 35–50 g, were used for behavioural experiments. Animals were maintained in a 12-h light-dark cycle (lights on at 7:00 a.m.) at constant room temperature (23 ± 2 °C). Mice were housed in groups of 20 per plastic cage (30 × 37 × 16 cm) with food and water *ad libitum*, except during the experiments. All animals were allowed to adapt to the laboratory conditions for at least one week prior to the behavioural assessments of the study. On the day of the experiment, animals were housed in the experimental room at least 1 h prior to the start of the testing procedures. Each animal was used only once. Experiments were conducted in accordance with national and international standards of animal welfare (Brazilian Law #11,794, 10/08/2008; NIH publication #85-23, revised in 1996) and approved by the local Committee for Animal Care in Research (#23080.044085/2009-37/CEUA/UFSC).

2.4.1. Drugs and Treatments

Mice were orally treated (p.o.) through an intra-gastric cannula and received a constant volume of 0.1 mL/10 g per animal weight in all treatments. Animals of the test group received the compounds dissolved in distilled water and the control group received only distilled water. In the ethyl ether-induced hypnosis test, diazepam (DZP; Dienpax, Sanofi-Winthrop Lab.) was administered (p.o.) to the mice of the positive control group, at a dose of 1 mg/kg [11,12]. The extract was tested at doses of 30, 60, 100, 300, and 600 mg/kg; AAF, SF and FF at a dose of 6 mg/kg and vitexin-2''-*O*-xyloside (V2OX) at doses of 1, 3 and 6 mg/kg.

To assess the involvement of the GABAergic system, animals were pretreated (i.p.) with physiological saline solution (Quimibrás Indústrias Químicas S.A.) or flumazenil (FMZ; Flumazen, União Química Lab. −1 mg/kg) [20], fifteen minutes prior to the oral treatments with the test compound V2OX (1 mg/kg), the reference drug DZP (1 mg/kg), or distilled water (control group).

2.4.2. Ethyl ether-induced hypnosis test

Animals were treated (p.o.) with tested compounds, DZP or distilled water. One hour after their treatment (30 min for DZP) mice were individually placed in an ethyl ether-saturated glass cage (6 mL, 13 min of saturation, 30 cm × 20 cm glass cage), where they remained for up to 1 min after the loss of the righting reflex. The hypnosis time was measured by the loss of righting reflex, and the recovery of this reflex was considered to be the endpoint of the hypnosis. A stopwatch was used to record the duration of hypnosis in seconds(s) [11].

2.5. Statistical analysis

the results of the sedative experiments are expressed as means ± S.E.M. The data from the experiments without pretreatment