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In vitro and *in silico* antioxidant and toxicological activities of *Achyrocline satureioides*

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ABSTRACT

Ethnopharmacological relevance: *Achyrocline satureioides* ("macela or marcela") is a medicinal plant, traditionally collected in "Good Friday" before sunrise. In traditional medicine, dried flowers of *A. satureioides* are used as anti-dyspeptic, antispasmodic and anti-inflammatory.
Aim of the study: To evaluate the phytochemical profile and to present an *in vitro* and *in silico* approach about toxicity and antioxidant potential of *A. satureioides* flowers extract and its major phytoconstituents.
Materials and methods: Plant were collected according to the popular tradition. Extract were obtained by infusion and analyzed from high-performance liquid chromatography. Toxicity was evaluated in *Artemia salina* and human lymphocytes. Extract antioxidant activity was determined with total antioxidant capacity, $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ scavenging, ferric reducing antioxidant power, deoxyribose degradation assay, and thiobarbituric acid reactive substances (TBA-RS) assay. TBA-RS inhibitions were evaluated in brain of rats for *A. satureioides* extract and its major phytoconstituents. Predictions of activity spectra for substances and *in silico* toxicity evaluation from major phytoconstituents were performed via computer simulation.
Results: Chromatographic data indicated isoquercitrin, quercetin and caffeic acid as main compounds in flowers extract. Toxicity tests demonstrated a very low toxic potential of *A. satureioides*. Extract exhibited antioxidant activities in low concentrations. Both extract and major phytochemicals standards showed protection against lipid peroxidation in brain of rats. Computer simulations pointed some biological activities in agreement with traditional use, as well as some experimental results found in this work. Moreover, *in silico* toxic predictions showed that the *A. satureioides* major compounds had low probability for toxic risk.
Conclusion: Our results indicate that *A. satureioides* infusion possesses low toxicological potential and an effective antioxidant activity. These findings confirm the traditional use of this plant in the folk medicine.

1. Introduction

Medicinal plants have been extensively used for centuries to treat several diseases. During the last decades, there has been interest in identifying metabolites from plants that can exert beneficial effects on human health. Among these metabolites, the antioxidants or free radical scavengers have received special attention for their pharmacological potential (Sen and Samanta, 2015).

Achyrocline satureioides (Asteraceae), popularly known as "macela or marcela", is one of the 25 Achyrocline spp. described in Brazilian territory. *A. satureioides is* a medium-sized aromatic annual herb, commonly found in tropical and subtropical America (Retta et al., 2012). In Brazilian southern, the medicinal herb *A. satureioides* is traditionally collected in "Good Friday". This collection is performed before sunrise and the naturally dried flowers are used along the year to treat several gastrointestinal disorders (Simoes et al., 1988).

A. satureioides is considered a promising medicinal and aromatic plant and is an official vegetable drug in the Brazilian Pharmacopoeia (Retta et al., 2012). In fact, previous *in vivo* and *in vitro* studies have confirmed the traditional use of *A. satureioides* as anti-inflammatory, hepatoprotective, antioxidant, immunomodulatory, antimicrobial,

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antitumoural and photoprotective (Arredondo et al., 2004; Cosentino et al., 2008; Kadarian et al., 2002; Morquio et al., 2005; Polydoro et al., 2004; Retta et al., 2012). Furthermore, *in vitro* examinations showed *A. satureioides* cytotoxicity at higher concentrations (Sabini et al., 2013).

Investigations about chemical composition found the flavonoids quercetin, 3-O-methylquercetin, and luteolin as the main compounds in *A. satureioides* inflorescences extracts (Carini et al., 2014). These isolated compounds have demonstrated *in vitro* some pharmacological activities, such as scavenging of reactive oxygen species (ROS) (Arredondo et al., 2004; Carini et al., 2014; Retta et al., 2012). Surely, this antioxidant property is very important considering that ROS and other reactive species have been implicated in the pathology of over 100 human diseases (Halliwell, 2001).

Considering the *A. satureioides* potential as a medicinal plant, this study aimed to identify phenolic content and to evaluate, *in silico* and *in vitro,* the antioxidant and toxicological potential of crude extract and its isolated major compounds.

2. Methods

2.1. Chemicals

Methanol, acetic acid, ascorbic acid, gallic acid, chlorogenic acid, caffeic acid, ellagic acid and ferrous sulfate were purchased from Merck (Darmstadt, Germany). Quercetin, quercitrin, isoquercitrin, rutin, luteolin, kaempferol, catechin, epicatechin, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (trolox); deoxyribose, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and histopaque® were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Plant material collection

Achyrocline satureioides (Lam.) DC. was collected in autumn, in "Good Friday", before sunrise. The collection area (Fig. 1) is located in Brazil-Uruguay-Argentina border (29°48'02.5"S 57°00'32.7"W), in the Brazilian Pampa Biome. Botanical identification of samples was confirmed and a voucher specimen (number 085/2016) was deposited at the Herbarium of the Federal University of Pampa. The plant name has been checked with "The Plant List" (www.theplantlist.org) (accessed 1 June 2016).

2.3. Extract obtainment

Flowers of the plant were submitted to dryness at ambient temperature (25–30 °C) for five days. In order to reproduce the traditional use, the flowers were submitted to extraction by infusion in hot water at 80 °C, for 15 min with a plant:solvent proportion of 1:100 (w/v).

2.4. Phytochemical analysis

2.4.1. Total phenolic and flavonoid content

Total phenolic and flavonoid content of *A. satureioides* extract was measured spectrophotometrically using the methods proposed by Nurmi et al. (1996) and Chang et al. (2002), respectively. A standard curve of gallic acid and quercetin was used to determine the polyphenols and flavonoids equivalents content.

2.4.2. High performance liquid chromatography with diode array detector (HPLC-DAD) analysis

HPLC-DAD analyses were performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20 A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

Reverse phase chromatographic analyses were carried out under gradient conditions, using C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles. The mobile phase was water containing 1% acetic acid (A) and methanol (B), and the composition gradient was: 4% (B) for 5 min; 25% (B) until 10 min; 40%, 50%, 60%, 70% and 80% (B) every 10 min (Barbosa-Filho et al., 2014). Extracts were analyzed at concentrations of 20 mg/mL. The flow rate was 0.7 mL/min and the injection volume was 40 μL. Sample and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and degassed by ultrasonic bath prior to use.

Stock solutions of standards references to *A. satureioides* extracts were prepared in the HPLC mobile phase at a concentration range of 0.020 – 0.350 mg/mL catechin, epicatechin, quercetin, isoquercitrin, quercitrin, luteolin, kaempferol and rutin, and 0.035 – 0.250 mg/mL for gallic, chlorogenic, caffeic and ellagic acids.

Quantification was carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid, 280 nm for catechin and epicatechin, 327 nm for ellagic acid, chlorogenic acid, caffeic acid and caffeic acid derivative, and 365 for quercetin, isoquercitrin, quercitrin, luteolin, rutin and kaempferol. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–500 nm). All chromatography operations were carried out at ambient temperature and in triplicate.

2.4.3. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Boligon et al. (2012). LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.5. Toxicity assays

2.5.1. Artemia salina toxicity bioassay

To estimate the *A. satureioides* infusion toxicity, we perform the *A. salina* toxicity bioassay (Meyer et al., 1982), with some modifications. Briefly, *A. salina* cysts were induced to hatch in aerating solution (water/sodium chloride 3%) in a conical flask at 18 °C for 24 h. The newly hatched nauplii were collected and transferred individually to a 96-well plate containing different *A. satureioides* concentrations or a control saline solution (blank). The plates were sealed and incubated. Incubation media was analyzed after 24 h in order to evaluate the number of dead nauplii. Assays were performed in triplicate and n = 30 nauplii were used in each assay.

The median lethal dose (LD_{50}) was the required concentration to kill 50% of nauplii. It was considered LD50 < 1000 μg/mL toxic, and ≥ 1000 μg/mL non-toxic.

2.5.2. Comet assay

Comet assay was performed in human lymphocytes according Singh et al. (1988). Briefly, samples of human blood (10 mL) were collected from healthy adult volunteers by venous puncture in heparinized tubes. After four hours of incubation with *A. satureioides* (concentrations showed in the figure) or hydrogen peroxide (positive control), lymphocytes were separated with histopaque®. Separated cells were suspended in agarose low melting point, and spread into a glass microscope slide. Dry slides were incubated in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH