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The anti-inflammatory effect of *Ilex paraguariensis* A. St. Hil (Mate) in a murine model of pleurisy

Ana Beatriz Gobbo Luz [a], Carlos Henrique Blum da Silva [b], Marcus Vinicius P.S. Nascimento [a], Bruno Matheus de Campos Facchin [a], Bruna Baratto [a], Tânia Silvia Fröde [a], Flávio Henrique Reginatto [b], Eduardo Monguilhott Dalmarco [a,c,*]

[a] Department of Clinical Analysis, Centre of Health Sciences, Federal University of Santa Catarina, 88040-900 Florianopolis, Brazil
[b] Department of Pharmaceutical Science, Centre of Health Sciences, Federal University of Santa Catarina, 88040-900 Florianopolis, Brazil
[c] Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University of Torino, 10124 Torino, Italy

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ABSTRACT

Ilex paraguariensis is a native plant from Southern America, where it is used as a beverage. In traditional medicine, it is used to treat many diseases including inflammation. However, we do not yet know precisely how this effect occurs. We therefore evaluated its anti-inflammatory effect in a murine model of pleurisy. The standardized CE, BF and ARF fractions, Caf, Rut and CGA were able to reduce leukocyte migration, exudate concentration, MPO and ADA activities and NOx levels. Moreover, *I. paraguariensis* also inhibited the release of Th1/Th17 pro-inflammatory cytokines, while increasing IL-10 production and improving the histological architecture of inflamed lungs. In addition, its major compounds decreased p65 NF-κB phosphorylation. Based on our results, we can conclude that *I. paraguariensis* exerts its anti-inflammatory action by attenuating the Th1/Th17 polarization in this model. This fact suggests that the use of this plant as a beverage can protect against Th1/Th17 inflammatory diseases.

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1. Introduction

Ilex paraguariensis A. St. Hil. (Aquifoliaceae), popularly known as yerba mate, is a native species from Southern Latin America, where it is used as a beverage and has great economic and social importance. In traditional medicine, it is used to treat arthritis, fatigue, obesity, and liver and intestinal affections [1]. The bioactive properties of *I. paraguariensis* are related to its phytochemical composition, especially methylxanthines [2], saponins and phenolic compounds [1]. In the last 15 years, several studies of *I. paraguariensis* have been performed, demonstrating interesting biological effects like anti-obesity [3], anti-diabetic [3], neuroprotective [4], antioxidant [5], antimicrobial [6], and anti-inflammatory [7] effects. However, as far as we know, there have been no studies to explore whether this anti-inflammatory effect is due to a possible effect on the cytokine environment involved in *in vivo* inflammatory process.

The non-resolving inflammatory process involves on a lack of immune system control and, in most cases, leads to chronic disease that require a long-term treatment and often result in loss of function of the affected organ. Notably, the disruption of normal balance of pro- and anti-inflammatory cytokines is a shared characteristic of these diseases *e.g.* neutrophilic asthma, rheumatoid arthritis, systemic erythematosus lupus, and many others [8,9]. In this context, the growing understanding about the immune modulation, Th1/Th2/Th17 balance and the intricate cell signaling related to these diseases had been leading researchers to take a different approach to deal with these conditions.

It is well known that adhesion to patient therapy in long-term treatments for non-self-resolving inflammatory process is very weak, which contributes to the high percentage of related disabilities and deaths [10]. For this reason, the discovery of natural source-derived compounds, specially from plants commonly used as food intake like *I. paraguariensis*, seems to have great potential in the treatment of these conditions [11,12].

In order to better clarify this point, in the present study, we focused on evaluating whether the anti-inflammatory property of this plant is related to its ability to change the cytokine environment. For this propose, *I. paraguariensis* standardized crude extract (CE), its related fractions – buthanolic (BF) and aqueous residue (ARF) fractions – and its major compounds caffeine (Caf), rutin (Rut) and chlorogenic acid (CGA) were tested in an *in vivo* model of pleurisy. We also investigated the effects on leukocyte migration and plasma exudation, myeloperoxidase (MPO)

* Corresponding author at: Department of Clinical Analysis, Centre of Health Sciences, Universidade Federal de Santa Catarina, Campus Universitário - Trindade, 88040-970 Florianópolis, SC, Brazil.
E-mail addresses: anaaabia@gmail.com (A.B.G. Luz), carloshblum@gmail.com (C.H.B. da Silva), mmmarcusster@gmail.com (M.V.P.S. Nascimento), brunomatheusfacchin@gmail.com (B.M. de Campos Facchin), bruhb90@gmail.com (B. Baratto), tania.frode@ufsc.br (T.S. Fröde), flavio.reginatto@ufsc.br (F.H. Reginatto), eduardo.dalmarco@ufsc.br, edalmarco@gmail.com (E.M. Dalmarco).

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and adenosine deaminase (ADA) activities, oxide nitric metabolite (NOx) concentration, pro- and anti-inflammatory cytokine levels, histological lung parameters, and finally, the action of its major compounds on phosphorylation of the p65 subunit of NF-κB (p-p65 NF-κB).

2. Materials and methods

2.1. Plant material

Ilex paraguariensis A. St. Hil. leaves were harvested in October/2012 in Erechim (27°38′03″ S and 52°16′26″ W), in the State of Rio Grande do Sul, Brazil. A voucher specimen was identified by Dr. Branca Maria Severo and deposited in the Herbarium of Universidade de Passo Fundo (RSPF 11074). The leaves were immediately frozen, lyophilized, crushed and stored at −20 °C until required for the preparation of extracts.

2.2. Extract preparation

The *I. paraguariensis* extracts were prepared by turboextraction in an Ultra-Turrax. Briefly, 200 g of leaves were extracted for five minutes, with 1000 mL of ethanol 20° GL (Gay Lussac) as the liquid extractor (1:5 m/v), yielding the hydroethanolic extract. After filtration, the ethanol was eliminated under reduced pressure, the volume was adjusted to 800 mL with distilled water, and the preparation was separated into two fractions. One fraction was evaporated under reduced pressure to dryness to obtain the crude extract (CE). The second fraction was then partitioned with *n*-BuOH, yielding the *n*-BuOH fraction (BF) and aqueous residual fraction (ARF). The *n*-BuOH of BF was evaporated under reduced pressure and all the samples, CE, BF and ARF, were dried by lyophilization.

2.3. Chromatographic separation

Chromatographic separation was performed using an Acquity-UPLC™ (Waters, MA, USA) system equipped with a quaternary pump, degasser and autosampler. Detection was carried out using a photodiode array detector (PDA). The column used was a Waters BEH C18 column, 1.7 μm, 50 × 2.1 mm at 40 °C. The method used a gradient combining solvent A (formic acid/water, pH 2.5) and solvent B (acetonitrile), programmed as follows: 0–5 min, linear change from A-B (97:3 v/v) to A-B (90:10 v/v); 5–6 min, isocratic A-B (90:10 v/v); 6–9 min, linear change to A-B (80:20 v/v) and 9–10 min, linear change to A-B (10:90 v/v). The flow rate was kept constant at 0.3 mL min^{-1} and the injection volume was 5 μL. The peaks were characterized by comparing the retention time, UV spectra and by co-injection of the sample with the reference standards. Quantification was performed by external calibration, using their corresponding standards. Caffeine was quantified at 280 nm, while the phenolic compounds chlorogenic acid and rutin were quantified at 320 nm. All the analyses were performed in triplicate, and the peak area measured. The standard solutions were analyzed in different ranges: 0.05–50 μg mL^{-1} for the caffeine (Sigma-Aldrich®); 0.05–100 μg mL^{-1} for the chlorogenic acid (Fluka®) and 0.25–50 μg mL^{-1} for the rutin (Sigma-Aldrich®). Quantification was performed using six to eight-point regression curves (caffeine, $r^2 = 1$; chlorogenic acid, $r^2 = 0.9999$; rutin, $r^2 = 0.9999$). The regression equations were "$y = 39.320x + 2074.9$" for caffeine, "$y = 46.696x - 1896$" for chlorogenic acid, and "$y = 15.618x + 178.92$" for rutin. The extracts were analyzed at a concentration of 500 μg mL^{-1}. The results were expressed as milligrams per gram of extract (mg compound/g^{-1} E).

2.4. LC–MS analysis

In addition to LC/PDA analysis, the identification was carried out by liquid chromatography (UPLC, Waters Acquity mode) coupled to a high-resolution mass spectrometer (Xevo G2 QTof model), equipped with an electrospray ionization source and controlled by MassLynx v.4.1 software for data acquisition. The mass spectrometer parameters were set as follows: ionization mode, electrospray negative ion; capillary voltage, 2.0 kV; source block temperature, 90 °C; desolvation temperature, 350 °C; nebulizer nitrogen flow rate, 30 L h^{-1}; desolvation nitrogen gas flow, 600 L h^{-1}; and cone voltage, 40 V. The spectra were recorded by scanning the mass range from m/z 100 to 1000 with scan time of 0.5 s.

2.5. LC-PDA validation procedure

The LC-PDA quantification method was validated according to the ICH guidelines (2005) to comply with the requirements for specificity, linearity, accuracy, precision (repeatability and intermediate precision), limit of quantification (LOQ) and limit of optical detection (LOD).

2.6. Animals

In this experimental protocol, we used female Swiss mice (18–22 g), housed under standardized and controlled conditions (20 ± 2 °C, 12 h light/dark periods) with free access to chow and water. All experiments were designed to minimize animal suffering and to use the minimum number of animals required to achieve a valid statistical evaluation. The experiments were performed according to the regulations of the Brazilian College of Animal Experimentation (COBEA) and are in accordance with the rules of the Committee for Ethics in Animal Research of the Federal University of Santa Catarina (CEUA - PP00965).

2.7. Experimental design of the murine model of pleurisy

The pleurisy was performed as previously reported [13]. Briefly, the experimental protocol was divided into two steps. First, to establish a dose–response curve, animals were randomly divided in different groups ($n = 6$) and challenged with Evans Blue dye solution (25 mg/kg) administered by the intravenous route (i.v). After 10 min, different groups were treated with different doses of CE (10–50 mg/kg), BF (0.1–10 mg/kg), ARF (0.1–10 mg/kg), Caf (0.1–5 mg/kg), Rut (0.01–1 mg/kg), CGA (0.01–1 mg/kg) or dexamethasone (0.5 mg/kg) (Dex) by the oral route (p.o.). Shortly after 0.5 h, pleurisy was induced by a single injection of 0.1 mL of sterile saline containing λ-carrageenan 1% (w/v) (Cg) administered by the intra-pleural route (i.pl). The animals were euthanized after 4 h with an overdose of pentobarbital (120 mg/kg) administered by the intraperitoneal route (i.p.), and the pleural cavity was exposed and washed with 1.0 mL of sterile phosphate buffered saline (PBS, pH 7.2) (Laborclin, Pinhais, Paraná, Brazil) containing heparin (20 IU/mL). The pleural fluid was used to measure the primary inflammatory parameters: total and differential leukocyte count and exudate concentration. In order to determine the time-course response of the plant material, other groups of animals were pre-treated with the lowest effective dose of CE obtained above (25 mg/kg) at 0.5, 1 and 2 h before pleurisy induction.

In another set of experiments, previously selected doses were used to perform the MPO, ADA and NOx assays and quantification of pro- and anti-inflammatory cytokines in the pleural fluid. Also, p65 NF-κB phosphorylation and histological changes were performed on lung tissue samples. For these experiments, the animals were orally treated with CE (25 mg/kg), BF (1 mg/kg), ARF (1 mg/kg), Caf (5 mg/kg), Rut (1 mg/kg) or CGA (0.1 mg/kg), followed by induction of pleurisy by Cg 1% (i.pl.). In parallel with all the experiments, a group of animals was challenged with 0.1 mL of Cg 1% (i.pl.) only, and was named as a positive control group, while another group received 0.1 mL of saline (0.9% NaCl) (i.pl.), and was considered a negative control group.

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