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Calea uniflora Less. attenuates the inflammatory response to carrageenan-induced pleurisy in mice

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ABSTRACT

Calea uniflora Less. (family Asteraceae), also named "arnica" and "erva-de-lagarto", is a native plant to the South and Southeast of Brazil. This species was used to treat rheumatism, respiratory diseases, and digestive problems in Brazilian folk medicine. *In vitro* studies have shown the important biological effects of *C. uniflora*. However no studies have focused on the mechanism of action of anti-inflammatory activity of *C. uniflora*. The aim of this study was to evaluate the anti-inflammatory effects of the crude extract, its fractions, and isolated compounds obtained from of *C. uniflora*, using mouse model of carrageenan-induced inflammation. The following inflammatory parameters: leukocyte influx, degree of exudation, myeloperoxidase (MPO) and adenosine deaminase (ADA) activities, nitric oxide metabolites (NOx), proinflammatory cytokines and phosphorylation of the p65 subunit of NF-κB (p-p65 NF-κB), and p38 mitogen-activated protein kinase (p-p38 MAPK) levels were determined. The crude extract of *C. uniflora*, its fractions and its isolated compounds reduced the leukocyte influx, degree of exudation, MPO and ADA activities, NOx, TNF-α, IFN-γ, MCP-1 and IL-6 levels ($p < 0.05$). The isolated compounds reduced p-p65 NF-κB and p-p38 MAPK levels ($p < 0.01$). This study demonstrated that *C. uniflora* exhibits a significant anti-inflammatory activity *via* inhibition of the leukocyte influx and degree of exudation. These effects were associated with a decrease in the levels of several proinflammatory mediators. The mechanism of the anti-inflammatory action of *C. uniflora* may be, at least in part, *via* the inhibition of p65 NF-κB and p38 MAPK activation by the isolated compounds.

1. Introduction

Inflammation occurs because of an imbalance of the cellular and tissue homeostasis; it is an essential defense mechanism against pathogens and for the repair of damaged tissues [1,2]. It is noticed that inflammation is a key component in the pathogenesis of various multifactorial diseases, such as rheumatoid arthritis, asthma, chronic inflammatory bowel disease, type 2 diabetes, neurodegenerative diseases and cancer [3]. Although several anti-inflammatory agents are approved by different pharmaceutical regulatory agencies, the therapy is often ineffective. Therefore, the scientific community has been interested in the clinical trials of the natural products to provide safe and effective treatments to patients with inflammatory disorders [2,4].

It is important to mention that the 2015 Nobel Prize in Medicine was awarded to three scientists for their discovery of drugs based on natural products [5]. Natural products have a significant potential to expand the field of disease prevention and treatment. The development of natural products-based drugs has become popular, in part, owing to their

Abbreviations: ADA, adenosine deaminase; AH-BU, α-hydroxy-butein; Aq, Aqueous fraction; *C. uniflora*, *Calea uniflora* Less.; CBA, Cytometric Bead Array; CC, column chromatography; CE, crude extract; Cg, carrageenan; CONCEA, National Council of Animal Experimentation Control; COSY, correlation spectroscopy; COX-2, cyclooxygenase; DCM, Dichloromethane fraction; Dex, dexamethasone; ELISA, enzyme-linked immunosorbent assay; EtOAc, ethyl acetate fraction; Hex, hexane fraction; HMBC, Heteronuclear Multiple Bond Correlation; HSQC, Heteronuclear Single Quantum Correlation; IFN-γ, interferon gamma; IL-6, interleukin 6; Indo, indomethacin; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; i.pl., intrapleural; i.v., Intravenous; MAPK, Mitogen-activated protein kinases; MCP-1, Monocyte chemoattractant protein-1; MPO, myeloperoxidase; NF-κB, Nuclear factor kappa B; NMR, Nuclear magnetic resonance; NOx, nitric oxide metabolites; NO_2^-, nitrite; NO_3^-, nitrate; NO, noreugenin; OR + BU, Orobol + Butein; p38 MAPK, p38 mitogen-activated protein kinase; p-p38 MAPK, Phosphorylated p38 mitogen-activated protein kinase; p-NF-κB p65, Phosphorylation of p65 subunit of NF-κB; TMS, tetramethylsilane; TLC, thin-layer chromatography; TNF-α, tumor necrosis factor alpha; TPA, 12-O-tetradecanoylphorbol-13-acetate; VLC, vacuum liquid chromatography (VLC).

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structural diversity. Therefore, natural products remain important source for drug discovery and development [6,7].

Calea is a genus of the Asteraceae family, with almost 110 identified species. It is widely distributed in the tropical and subtropical regions of the world [8].

In vivo and *in vitro* studies have revealed several pharmacological properties of various species in the Calea genus, for example, *Calea zacatechichi* Schltdl. has demonstrated hypoglycemic [9], antiparasitic [10,11], antinociceptive, and/or anti-inflammatory effects [12,13]. Gomes and Gil also observed that *Calea prunifolia* Hunth. displayed an anti-inflammatory effect using a model of inflammation induced by topical application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the ear of mice [14]. Antihypertensive and vasodilator effects have also been reported for *Calea glomerata* Klatt. in spontaneously hypertensive rat (SHR) and in isolated rat aortic rings [15]. These effects are mainly mediated by the chemical constituents of these herbs, such as chromanones [8], chromenes [16], sesquiterpene lactones [17], phenolic compounds [18], and essential oils [19].

Calea uniflora Less. is a perennial plant, mainly found in the southern region of Brazil and in Uruguay [20]. This plant is also known as "arnica" or "erva-de-lagarto" [18,21].

Few studies focused on the pharmacological properties of *C. uniflora*. They are mainly *in vitro* studies that evaluated its antiparasitic activity using cultures of the trypomastigote forms of *Trypanosoma cruzi* [22, 23] and the promastigote forms of *Leishmania major* [8], as well as its antifungal effect against pathogenic *Candida* spp. and dermatophytes using a minimal inhibitory concentration [23], and its genotoxic effects through DNA damage in rat brain tissues assessed by the comet assay [20]. However, there are no *in vivo* studies in the literature that reported the anti-inflammatory properties of *C. uniflora.*

In this context, the present study was designed to elucidate the mechanism of the anti-inflammatory action of *C. uniflora* using a murine model of carrageenan (Cg)-induced pleurisy. We evaluated the effect of the crude extract (CE) of *C. uniflora*, its derived fractions and isolated compounds on several pro-inflammatory markers, including leukocyte migration, degree of exudation, myeloperoxidase (MPO) and adenosine deaminase (ADA) activities, nitric oxide metabolites (NOx), and proinflammatory cytokines (TNF-α, IFN-γ, MCP-1 e IL-6) levels. Furthermore, we evaluated the effect of the isolated compounds on the levels of nuclear factor-kappa B (NF-κB) and p38 mitogen-activated protein kinase (p38 MAPK).

2. Materials and methods

2.1. Plant material

The leaves of *Calea uniflora* Less. were collected from Imbituba, Santa Catarina, Brazil, on October 2012. The plant material was authenticated by Dr. John F. Pruski (New York Botanical Garden) and a voucher specimen (MO-2383317) was documented at the Missouri Botanical Garden Herbarium (MO), St. Louis, Missouri, USA.

2.2. Extraction and isolation of the major chemical constituents from the active plant fractions

2.2.1. General information

1D and 2D NMR experiments were carried out using Bruker AVANCE 400 MHz and/or Ascend 600 MHz spectrometers, operating at 400 MHz and 600 for ^{1}H NMR and at 100 and 150 MHz for ^{13}C NMR. Acetone-d_6 and methanol-d_4 were used as solvents, and tetramethylsilane (TMS) was employed as a chemical shift reference (0.00 ppm). Chemical shifts (δ) were given in parts per million (ppm) and coupling constants (J) were expressed in hertz (Hz). The analytical thin-layer chromatography (TLC) system was acquired from Silicycle and silica gel 60 F_{254} (0.04–0.63 μm, 240–400 mesh) used for vacuum liquid chromatography (VLC) and column chromatography (CC) was obtained from Vetec (Rio de Janeiro, RJ, Brazil).

2.2.2. Extraction and isolation

C. uniflora was extracted, fractionated, and purified exactly as previously described by Lima and co-workers (2015) [18]. The fresh leaves of *C. uniflora* (2.3 kg) were crushed into small fragments and extracted by maceration with 92% ethanol (EtOH) three times for 15 days at room temperature (approximately 25 °C). The leaves were pooled and evaporated to dryness under reduced pressure using a rotary evaporator at a temperature below 45 °C to provide 104.0 g of the crude extract. The crude extract was then dissolved in H_2O and extracted sequentially in three different solvents: *n*-hexane (Hex), dichloromethane (CH_2Cl_2), and ethyl acetate (EtOAc). The resulting fractions were concentrated to dryness in a rotary evaporator to provide hexane (Hex) (26.8 g), dichloromethane (DCM) (6.2 g), ethyl acetate (EtOAc) (3.2 g), and aqueous (Aq) (67.8 g) fractions. A previous study conducted in our laboratory, which has not been published yet, have demonstrated that the DCM and EtOAc fractions exhibited a more pronounced anti-inflammatory activity than that of the Hex and Aq fractions, therefore, these fractions were selected for further fractionation and purification. The DCM fraction (3.2 g) was submitted to VLC on silica gel and eluted with solvents of increasing polarity (hexane, CH_2Cl_2, EtOAc and methanol (MeOH)) resulting in the production of twelve subfractions, namely, A-M. The F subfraction (661.0 mg) was purified by CC on silica gel and eluted with a gradient of hexane, EtOAc and MeOH. The obtained eluates were pooled for TLC analysis to furnish 17 fractions. The F114-144 fraction (hex:EtOAc, 80:20) yielded 76.3 mg of compound **1**. In addition, the EtOAc fraction (6.2 g) was subjected to VLC on silica gel employing mixtures of hexane, EtOAc, acetone and MeOH as a mobile phase. Eight subfractions, namely, A-H, were obtained. Approximately 2.5 g of the D subfraction was purified by CC using CH_2Cl_2 with an increasing gradient of MeOH up to 100% as a mobile phase to give 151 fractions. F40–43 and F55–67 fractions were combined to render 61.9 mg of a 1:1 mixture of the compounds **2** and **3**, and the compound **4** (122.7 mg), respectively. The chemical identity of the isolated compounds was established by analyzing their spectral data (^{1}H NMR, correlation spectroscopy (COSY), Heteronuclear Single Quantum Correlation (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC)) as previously reported by Lima and collaborators (2015) [18].

2.3. Animals

Female Swiss mice (18–22 g, aged 1 month) were used in the experiments. The animals were housed under standardized conditions (room temperature of 22 ± 2 °C, under a 12-h light/12-h dark cycles and 50–60% humidity) with free access to standard mouse diet and water *ad libitum*. The number of animals used was the minimum necessary to demonstrate consistent effects of the treatment. This study was approved by the Committee for Ethics in Animal Research of the Federal University of Santa Catarina (License No. – PP00757), and the experiments were performed in accordance with the norms of the National Council of Animal Experimentation Control (CONCEA).

2.4. Carrageenan-induced pleurisy

Pleurisy was experimentally induced by a single intrapleural (i.pl.) injection of 0.1 mL of Cg (1%), in accordance with the methodology described by Saleh and co-workers [24]. After 4 h, animals were euthanized with an overdose of pentobarbital (120 mg/kg) administered intraperitoneally (i.p.). The thorax was opened, and the pleural cavity was washed with 1.0 mL sterile phosphate-buffered saline (PBS, pH 7.6, composition: NaCl (130 mmol), Na_2HPO_4 (5 mmol), KH_2PO_4 (1 mmol)) in distilled water containing heparin (20 IU/mL). The leaking fluid obtained from the pleural cavity was collected for further determination of inflammatory cell migration, degree of exudation, MPO, and