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Original article

The anti-inflammatory effects of N-methyl-(2S,4R)-*trans*-4-hydroxy-L-proline from *Syderoxylon obtusifolium* are related to its inhibition of TNF-alpha and inflammatory enzymes

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ABSTRACT

Background: *Sideroxylon obtusifolium* (Roem. & Schult.) T.D. Penn., Sapotaceae family, is a medicinal species native to the Brazilian Northeastern region. The plant is popularly used as an anti-inflammatory and hypoglycemic.
Purpose: To evaluate the anti-inflammatory properties of the N-methyl-(2S,4R)-*trans*-4-hydroxy-L-proline (NMP) from *S. obtusifolium* leaves in models of inflammation and to clarify its action mechanisms.
Methods: Male Swiss mice were distributed intocontrols and groups treated with NMP (25, 50 and 100 mg/kg, p.o.), indomethacin or morphine (reference drugs). The animals were subjected to the formalin, carrageenan-induced edema and peritonitis tests. Furthermore, peritoneal lavage and slices from edematous paws were used for histological and immunohistochemical (iNOS, TNF-alpha, COX-2 and NF-kB) assays.
Results: Decreases in licking time, in the 1st and mainly in the 2nd phases of the formalin test, were shown after NMP treatments. In addition, decreases (around 50%) in paw edema were noticed at the 3rd h. The HE staining of paw slices demonstrated a complete reversion of the increased PMN cell number after NMP treatment. Similarly, decreases higher than 70% were also demonstrated in PMN cells, in the peritoneal fluid. Furthermore, NMP significantly decreased iNOS, TNF-alpha, COX-2 and NF-kB immunoreactivities.
Conclusions: We showed that *S. obtusifolium* presents a potent anti-inflammatory activity, due to the presence of the N-methyl-(2S,4R)-*trans*-4-hydroxy-L-proline(NMP) in the plant extract. This action is related to the inhibition by NMP of TNF-alpha and inflammatory enzymes.

Introduction

The species *Sideroxylon obtusifolium* (Roem. & Schult.) T.D. Penn. belongs to the Sapotaceae family and is a tree native to Central and South America. In Brazil, it is found in the "caatinga" (xeric shrubland and thorn forest), an eco region characteristic of the Brazilian Northeastern region. *S. obtusifolium* is popularly used for its antinociceptive, anti-inflammatory and hypoglycemic properties, among others (Araujo-Neto et al., 2010). For this purpose, several parts of the tree, as the inner bark and leaves, are used.

Chemical and pharmacological studies on *S. obtusifolium* are few and, until some years ago, only common pentacyclic triterpenoids had been reported. Lately (Passos-Oliveira et al. 2012), saponins and flavonoids were shown to be the main constituents of the leaves of *S. obtusifolium*. Thus, four saponins and ten flavonol glycosides were isolated, by those authors, from the butanol-soluble fraction of an ethanolic extract. The compounds include a new

Abbreviations: NMP, N-methyl-(2S,4R)-trans-4-hydroxy-L-proline; MPO, myeloperoxidase; PMA, phorbol myristate acetate; COX-2, cyclooxygenase 2; INDO, indomethacin; DEXA, dexamethasone; PBS, phosphate buffered saline; HE, hematoxylin-eosin; TNF-alpha, tumor necrosis factor alpha; iNOS, inducible isoform of nitric oxide synthase; PMN, polymorphonuclear cells; NO, nitric oxide.

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triterpene glycoside, as well as new flavonol glycosides. In addition, catechin and a glycerogalactolipid were obtained from the ethyl acetate-soluble fraction.

In the present work, N-methyl-(2S,4R)-*trans*-4-hydroxy-L-proline (NMP) was isolated from the methanolic extract of *S. obtusifolium* leaves, as its main chemical component. This compound was evaluated in experimental models of nociception and inflammation, as well as *in vitro* assays (measurement of myeloperoxidase, MPO, activity in human neutrophils). In addition, the inflamed mouse paw was processed for histological and immunohistochemical studies, in order to clarify the possible mechanism of action involved with the observed effects.

Materials and methods

Drugs and reagents

Lambda carrageenan, phorbol myristate acetate (PMA) and indomethacin were purchased from Sigma-Aldrich (Mo, USA). The antibodies: COX-2 (M-19), sc-1747, goat polyclonal; TNF (52B83), sc-52746, mouse monoclonal; NOS-2 (H-174), sc-8310, rabbit polyclonal; NF-kB p50 (NLS), sc-114, rabbit polyclonal were from Santa Cruz Biotechnology Inc. (CA, USA). All other drugs were of analytical grade.

Plant material

Leaves from the species *S. obtusifolium* were collected at the municipality of Mauriti, Ceará State, in August 2014. A voucher specimen (number 10,648) representing the field collection has been stored at the "Herbário Caririense Dárdano de Andrade Lima", Regional University of Cariri (URCA), Ceará, Brazil, after identification by Dr. Maria Arlene Pessoa da Silva. One hundred grams of ground dry leaves were conditioned into a cotton fabric bag and then boiled, for 15 min, with 500 ml distilled water. The process was repeated once more. Both water soluble materials were pooled together and lyophilized to yield 34.86 g of a light brown residue, designated SOL-dec (decoction from *S. obtusifolium* leaves). Then, 10.0 g SOL-dec were poured into a Whatman® cellulose extraction timble and, then, extracted with methanol on a glass sohxlet apparatus. The methanol solution was rotoevaporated under low pressure to yield 6.76 g of a yellowish amorphous powder, designated SOL-decM. One gram aliquots (6×) of this SOL-decM, diluted into 3.5 ml distilled water, were chromatographed on a Phenomenex solid-phase-extraction (SPE),Strata® C-18 reverse phase giga tube (20 g/60 ml), previously conditioned with MeOH and equilibrated with distilled water. Fractions (20 ml) of this chromatographed material were collected, initially using water as eluent, followed by a gradient mixture of H_2O/MeOH (varying from 10 to 50%, and then to MeOH) and finally washed with a THF/MeOH 1:1 solution. After TLC analysis of all fractions, the H_2O fractions were pooled together and lyophilized to afford 1.7 g of compound **1**.

Structure determination

Compound **1**, a dark brown resin, $[\alpha]^{21}{}_D = -31°$ (c. 0.1, H_2O), showed on its positive ion mode HR-ESI mass spectrum the protonated molecule ion peak at 246.0872 ($C_6H_{11}NO_3$,), 168.0655 (Na^+ adduct) and 184.0586 (K^+ adduct). Its IR, 1H and 13C NMR data were compatible with the structure of a 4-hydroxy-proline derivative (Fig. 1). The suggested structure was confirmed by analyses of the COSY and HMBC NMR spectra. The relative stereochemistry of all stereogenic centers was suggested based on the observed *J* values for the scalar coupling splitting pattern and by the NOESY spectrum analysis. The final structure, including the absolute stereochemistry, was accomplished from the negative specific rotation of compound **1** (see supplementary material), and by comparison to data available from the literature (Winkler, 2006). Thus, compound **1** was characterized as the N-methyl-(2S,4R)-*trans*-4-hydroxy-L-proline (Fig. 1), otherwise designated hydroxylhygrinic acid or (2R,4S)-4-hydroxy-1-methyl-2-pyrrolidine carboxylic acid (Krebs and Ramiarantsoa, 1996; Dekebo et al., 2007).

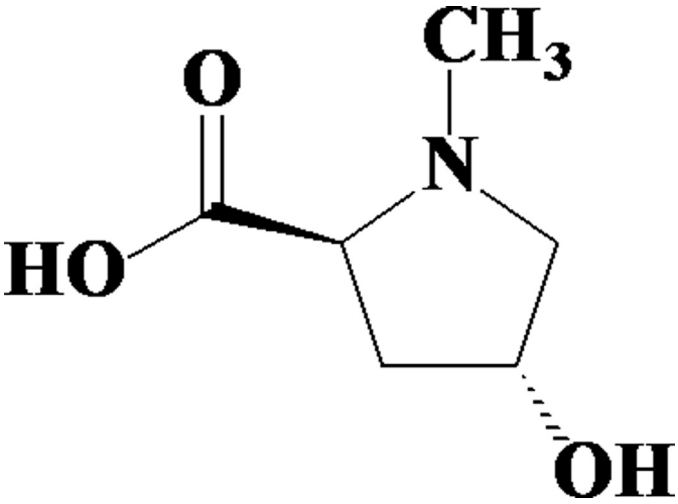

Fig. 1. Chemical Structure of N-methyl-(2S,4R)-*trans*-4-hydroxy-L-proline (NMP) isolated from the leaves of *Sideroxylon obtusifolium*.

Animals

Male Swiss mice (25 – 30 g) were provided by the Animal House of the Federal University of Ceará (UFC), Brazil. The animals were housed into plastic cages with sawdust as beddings and kept in a room with controlled temperature (25 ± 2 °C), under a 12 h light/12 h dark cycle and food and water supplied *ad libitum*. The experiments were carried out according to the Guide for the Care and Use of Laboratory Animals, of the U.S. Department of Health and Human Services (USA, 2011). The project was previously approved by the Animal's Ethics Committee, of the Faculty of Medicine of the Federal University of Ceará. In all tests, the drug was dissolved in distilled water prior to use.

Formalin test in mice

Twenty microliters 2% formalin were administered s.c. to the mouse's right hind paw, and the licking time was recorded from 0 to 5 min (phase 1, neurogenic) and from 20 to 25 min (phase 2, inflammatory) after the formalin injection. The animals (6 to 17 per group) were treated with distilled water (Control, 0.1 ml/100 g, i.p.), morphine (MOR, 5 mg/kg, i.p.) or NMP(25, 50 and 100 mg/kg, p.o.). Morphine was used as reference. The treatments were performed 30 min before the formalin injection.

Carrageenan-induced mouse paw edema

In this test, the mice were randomly chosen, divided into the following groups (ranging from 6 to 8 animals): Control (administered with distilled water, 0.1 ml/100 g) and groups administered with NMP (25, 50 and 100 mg/kg, p.o.). Another group was injected with the reference drug, indomethacin (INDO, 20 mg/kg, i.p.). Sixty minutes later, the edema was induced by the injection of 40 µl 1% carrageenan solution into the animal's right hind paw. Measurements of the paw volume were done by means of a plethysmometer (Ugo Basile, Italy), immediately prior to the carrageenan injection and 1, 2, 3, 4 and 24 h after. The paw edema volume (ml)