Original article

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

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ABSTRACT

Background: Syderoxyylon 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 into controls 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.

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Introduction

The species Syderoxyylon 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 antiinociceptive, 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
triterpene glycoside, as well as new flavonol glycosides. In addition, catechin and a glycerolgalactolipid 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 noiception and inflammation, as well as in *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 (S2883), sc-52746, mouse monoclonal; NOS-2 (H-174), sc-8310, rabbit polyclonal; NF-κB 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 Carriense Dârdo 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 timber and, then, extracted with methanol on a glass soxhlet apparatus. The methanol solution was rotaryevaporated under low pressure to yield 6.76 g of a yellowish amorphus 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 H₂O/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 H₂O fractions were pooled together and lyophilized to afford 1.7 g of compound 1.

**Structure determination**

Compound 1, a dark brown resin, [α]D²¹ = −31° (c. 0.1, H₂O), showed on its positive ion mode HR-ESI mass spectrum the protonated molecular ion peak at 246.0872 ([C₇H₁₂NO₃]⁺, 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 hydroxyhygrinonic acid or (2R,4S)-4-hydroxy-1-methyl-2-pyrrolidine carboxylic acid (Krebs and Kamiarantsoa, 1996; Dekebo et al., 2007).

**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 bedding 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 dissolvd 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 (μl)
was determined by the difference between the final and initial volumes.

In vivo carrageenan-induced neutrophil migration into mice peritoneal cavities

Groups of 6 animals were treated with NMP (100 mg/kg, p.o.), dexamethasone (DEXA, 4 mg/kg, i.p.) or distilled water (Control), 60 min before the induction of inflammation by means of 1% carrageenan (250 μl). All drugs were administered at a volume of 10 ml/kg. Then, the animals were returned to their cages and left with free access to water. After 4 h, the animals were euthanized and 3 ml PBS containing heparin (5 IU/ml) were interjected into their peritoneal cavities. Then, the peritoneal fluid was collected with a Pasteur pipette, through abdominal laparoscopy. A sample of the peritoneal lavage fluid was diluted (1:20) in Turk liquid, for quantification of cell numbers, using a Neubauer chamber. The cells were stained by a conventional fast pigment (panoptic-stained blood smears) and the results were expressed in number of cells/mm³.

Myeloperoxidase (MPO) release from human neutrophils

Polymorphonuclear cells, predominantly neutrophils (80–90%) presenting a 90% viability were obtained from the Hematology and Hemotherapy Center of the Ceará State (HEMOCE, Fortaleza, Brazil) and isolated according to a previously described method (Lucisano and Mantovani, 1984). The blood was centrifuged, the plasma discarded and the serum washed several times with saline, using a 2.5% gelatin solution as a separation gradient of blood cells. Then, a suspension of neutrophils (5 × 10⁸ cells/ml) was incubated for 15 min with HBSS (Hanks’ balanced salt solution), distilled water (vehicle, control), NMP (10–100 μg/ml) or indomethacin (INDO, as reference), at 37 °C. Additionally, except for the HBSS, all other assay tubes received phorbol 12-myristate 13-acetate (PMA, 0.1 μM), for 15 min at 37 °C. This was followed by centrifugation (800 g, 4 °C), then PBS (100 μl), phosphate buffer (50 μl) and H₂O₂ (0.012%) were added to the supernatant (50 μl). After 5 min at 37 °C, 20 μl tetramethylbenzidine (TMB, 1.5 mM) were added and the reaction stopped by the addition of 30 μl sodium acetate (1.5 M, pH 3.0). The absorbance was determined at 620 nm.

Histological analyses of HE stained sections from paws with carrageenan-induced edema in NMP-treated mice

Sections (10 μm) from mice paws submitted to carrageenan-induced edema were used for HE staining. The edematous paw was cut, sagittally dissected and fixed in buffered formalin for 24 h, followed by dehydration in a 70% ethanol solution for paraffin embedding and sectioning. The slices were analyzed by optic microscopy for images capture and cell counting.

Immunohistochemistry analyses for TNF-alpha, iNOS, COX-2 and NF-kB

For immunohistochemistry assays, the streptavidin-biotin-peroxidase method was used (Hsu and Raine, 1981). Three mice of each group were treated either with distilled water or NMP (100 mg/kg, p.o.) or indomethacin (INDO, 20 mg/kg) and, 60 min later, with an intraplantar injection of carrageenan (protocol previously described). When necessary, a 4th untreated group was included (normal control). Three hours later, the animals were sacrificed and 5 mm plantar region sections of the carrageenan-injected hind paw were immersed in 10% buffered formol for 48 h, followed by immersion into a 70% alcohol solution. The sections were then deparaffinized, hydrated in xylol and ethanol, and immersed in 0.1 M citrate buffer (pH 6) under 18 min microwave heating, for antigen recovery. After cooling at room temperature for 20 min, the sections were washed with a phosphate-buffered solution, followed by a 15 min blockade of endogenous peroxidase, with a 3% H₂O₂ solution. The sections were incubated overnight (4 °C) with the primary antibodies (anti-iNOS, anti-COX-2, anti-TNF-alpha and anti-NF-kB) diluted in PBS, according the manufacturers’ instructions. On the next day, the sections were washed in PBS and incubated for 30 min with the secondary biotinilated rabbit antibody (anti-lgG), also diluted in PBS (1:200 dilution). After washing in PBS, the sections were incubated for 30 min with the conjugated streptavidin peroxidase complex (ABC Vectastain® complex, Vec-
Fig. 3. N-methyl-(25,4R)-trans-4-hydroxy-L-proline (NMP) isolated from the methanolic extract of S. obtusifolium leaves decreases the carrageenan-induced paw edema at the 3rd h, similarly to indomethacin (INDO, 20 mg/kg) used as reference. a. vs. control, \( q = 6.682^{\ast \ast \ast} \); b. vs. control, \( q = 7.836^{\ast \ast \ast} \); c. vs. control, \( q = 11.29^{\ast \ast \ast} \); d. vs. Control, \( q = 10.54^{\ast \ast \ast} \) (ANOVA and Tukey as the post hoc test).

Fig. 4. N-methyl-(25,4R)-trans-4-hydroxy-L-proline (NMP) isolated from the methanolic extract of S. obtusifolium leaves decreases the carrageenan-induced PMN migration to the mouse peritoneal cavity. a. vs. control, \( q = 17.44^{\ast \ast \ast} \); b. vs. control, \( q = 16.37^{\ast \ast \ast} \). The control was injected with carrageenan (Cg) only, and dexamethasone (Dexa) was used as reference. For statistical analyses, ANOVA and Tukey as the post hoc test were used.

Fig. 5. N-methyl-(25,4R)-trans-4-hydroxy-L-proline (NMP) isolated from the methanolic extract of S. obtusifolium leaves decreases the myeloperoxidase release from human neutrophils. HBSS=Hank’s balanced salt solution; vehicle=distilled water; indomethacin (Indo) was used as reference. a. vs. HBSS, \( q = 12.57^{\ast \ast \ast} \); b. vs. HBSS, \( q = 12.72^{\ast \ast \ast} \); c. vs. HBSS, \( q = 7.786^{\ast \ast \ast} \); d. vs. HBSS, \( q = 5.701^{\ast \ast \ast} \); e. vs. vehicle, \( q = 5.138^{\ast \ast \ast} \); f. vs. vehicle, \( q = 6.087^{\ast \ast \ast} \); g. vs. vehicle, \( q = 9.959^{\ast \ast \ast} \); h. vs. Indo, \( q = 13.23^{\ast \ast \ast} \) (ANOVA and Tukey as the post hoc test).

tor Laboratories, Burlingame, CA, USA). After another washing with PBS, the sections were stained with 3,3’-diaminobenzidine-peroxide (DAB) cromophore, counter-stained with Mayer hematoxylin, dehydrated and mounted in microscope slides for analyses, and the data were semiquantified (as relative optic density) with the Image J (NIH, USA) software.

Statistical analysis

All results are presented as mean ± S.E.M. One-way ANOVA and Tukey as a post hoc test were used for comparing the results among treatments. The significance level was set at \( p < 0.05 \).

Results

Effects of NMP on the formalin test in mice

This is a tonic model of continuous pain, particularly useful for the evaluation of inflammatory, neurogenic and central mechanisms of nociception. The formalin-induced nociceptive behavior shows an early phase, starting immediately after the formalin injection and lasting for 5 min, and a late phase, lasting for 20 to 40 min and starting 15 to 20 min after the formalin injection. The 1st phase (Fig. 2A) is due to a direct chemical stimulation of nociceptors, and the 2nd phase involves peripheral inflammatory processes (Haley et al., 1989; Meunier et al., 1998). NMP (25, 50 and 100 mg/kg) reduced the licking time (s) in the 1st phase of the test by 35, 42 and 52%, respectively. MOR (5 mg/kg) decreased this nociceptive behavior by 65%. A similar but more intense effect was observed in the 2nd phase (Fig. 2B) of the formalin test, where NMP at the same doses decreased by 30, 61 and 78% the licking time, as related to controls. MOR showed an 83% reduction of this nociceptive behavior. Thus, our results indicated that NMP is effective in both neurogenic and inflammatory phases of the formalin test (Fig. 2A and B).
Effects of NMP on the carrageenan-induced paw edema in mice

This test is widely used to test new anti-inflammatory drugs, as well as to study the mechanisms involved in inflammation. Carrageenan caused a progressive increase in the paw edema, presenting a peak between 3 and 4 h and decreasing towards the basal level afterwards. In the present work, edema measurements were performed 3 h after the carrageenan injection. We showed that NMP, at the doses of 25, 50 and 100 mg/kg, reduced by 30, 37 and 51% the edema volume, respectively, in relation to controls. The reduction was of 48% after INDO (20 mg/kg) injection (Fig. 3).

Effects of NMP on the carrageenan-induced peritonitis in mice

The intraperitoneal administration of carrageenan produces a sustained increase in post-capillary venule permeability, thereby leading to increased infiltration of PMN cells, particularly neutrophils. NMP at the dose of 100 mg/kg reduced by 77% the PMN cells (predominantly neutrophils) infiltration, as related to controls, while the reduction was of 72% after DEXA (4 mg/kg) injection (Fig. 4).

In vitro effect of NMP on myeloperoxidase (MPO) activity in human neutrophils

MPO is an enzyme largely expressed in neutrophils. It has been demonstrated to be involved in cellular homeostasis and plays an important role in the initiation and progression of acute, as well as chronic inflammatory diseases (van et al., 2009). NMP, at concentrations of 25, 50 and 100 μg/ml, decreased by 24, 31 and 40%, respectively, the absorbance measurements, in relation to controls (Fig. 5).

Effect of NMP on PMN cell migration (HE staining) in mice edematous paws from carrageenan-induced edema

The objective was to detect PMN cell migrations to the inflammation site and its possible inhibition after NMP treatment. The HE staining of edematous paw sections revealed a significant 2-fold increase in the average number of PMN cells, 3 h after the carrageenan injection, as related to normal controls (carrageenan-untreated animals). On the other hand, these values were similar to those of normal controls, in groups pretreated with NMP100 and INDO20 (Fig. 6).
Effects of NMP on iNOS, COX-2, TNF-alpha and NF-kB immunohistochemical assays in edematous mice paws from carrageenan-induced edema

The objectives were to demonstrate, in mice paws from carrageenan-induced edema, attenuation or reversion of the inflammatory changes after NMP treatments. For that, immunohistochemistry assays for iNOS and COX-2 (inflammation-related enzymes), TNF-alpha (a pro-inflammatory cytokine) and the nuclear factor NF-kB (a transcription factor) were performed. NF-κB has long been considered a prototypical proinflammatory signaling pathway, largely based on its activation by proinflammatory cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor-alpha (TNF-alpha) (Lawrence, 2009). The assays performed with the carrageenan group (Cg) revealed an almost 17-fold increase in iNOS immunoreactivity, as related to the normal control. Lower increases (4-fold) were observed after treatments with NMP100 (Cg+NMP100), and also INDO20 (6-fold), used as a reference drug (Fig. 7). A 7-fold increase in immunoreactivity for TNF-alpha was seen in the Cg group, as related to normal controls. However, increases of the order of 2-fold, approximately, were observed in the Cg groups after the same treatments with NMP100 and INDO20 (Fig. 8). A 2.7-fold increase was demonstrated for COX-2 immunoreactivity in the Cg-induced edematous paw, as compared to the normal paw, while only around 1.8-fold was observed for paws after both NMP100 and INDO 20 treatments (Fig. 9). A similar increase of 2.8-fold in the NF-kB immunostaining was demonstrated in the Cg group, as related to normal controls. Immunostaining values in the Cg+NMP100 group were similar to those of controls, while a 1.5-fold increase was seen in the Cg+INDO20 group (Fig. 10).

Discussion

N-methyl-(25,4R)-trans-4-hydroxy-L-proline (NMP) isolated from S. obtusifolium leaves caused significant and dose-dependent decreases in the 1st (neurogenic) and mainly in the 2nd phase (inflammatory) of the formalin test. Acute nociceptive, inflammatory and neuropathic pains depend, in some degree, on the peripheral activation of primary sensory afferent neurons. These neurons can be activated by a range of inflammatory mediators, such as prostanooids, bradykinin, ATP, histamine, and serotonin (Sawynok, 2003). Furthermore, the inhibition of their actions represents a strategy for the development of new analgesic and anti-inflammatory drugs.

NMP also decreased the carrageenan-induced paw edema in mice. Several mediators, as histamine, serotonin, bradykinin, prostaglandins, oxygen and nitrogen reactive species are known to be involved in the increased vascular permeability observed in inflammatory processes. Nonsteroidal anti-inflammatory drugs have been shown to be active in this model, as indomethacin used in the present work as a reference drug. In addition, the signs
of inflammation, such as edema, hyperalgesia, and erythema develop immediately after the intraplantar injection of carrageenan, resulting from the action of the above proinflammatory mediators that can be generated at the site of the insult and by infiltrating cells. Rapid migration and degranulation of neutrophils provide high MPO contents, at the inflammation sites.

Others (Araujo-Neto et al., 2010) reported that the oral treatment with the ethanol extract from S. obtusifolium elicited an inhibitory activity on acetic acid writhings, as well as on the 2nd phase of the formalin test, but with no effect on the hot plate test. These authors also found that the ethanolic extract inhibited the carrageenan-induced edema and leukocyte migration into the peritoneal cavity. However, all these effects were recorded at dose intervals (100 to 400 mg/kg) much higher than those used in the present study. It is important to mention that 100 mg/kg were the highest dose used by us, which would correspond to a human equivalent dose (HED) of 19 mg/kg, for a 70 kg person (Nair and Jacob, 2016). Besides, the recommended dose of oxaceprol, an L-proline derivative, as NMP, is 200 or 400 mg three times a day, corresponding to 600 or 1200 mg/day, respectively (HED = 8.5 or 17.0 mg/kg, for a 70 kg person).

Our results indicate that NMP attenuated carrageenan-induced inflammation, by reducing neutrophil migration and MPO release. The injection of carrageenan into the pleural space leads to pleurisy and PMN infiltration. Models of carrageenan-induced pleurisy have been widely employed for investigating the pathophysiology of acute inflammation and evaluating the efficacy of drugs in inflammation (Cuzzocrea et al., 1999). We observed that the carrageenan-induced infiltration of neutrophils into the mouse peritoneal cavity was, in great part, inhibited by previous NMP or INDO treatments. Furthermore, NMP and INDO significantly inhibited MPO release from human neutrophils. MPO is considered a hallmark of neutrophils infiltration in inflammation. Neutrophils are highly found in inflammatory diseases where they cause tissue damage through antigen presentation and secretion of cytokines, chemokines, prostaglandins and leukotrienes (Wright et al., 2010). Importantly, although neutrophils are major effectors of acute inflammation, these cells also contribute to chronic inflammatory conditions and adaptive immune responses (Kolaczkowska and Kubes, 2013). Thus, by decreasing neutrophils infiltration, NMP could be a potential candidate for both acute and chronic inflammatory conditions.

Others (Pereira et al., 2013), by using the cyclophosphamide-induced cystitis in rats, observed that the ethanol extract of the inner bark of S. obtusifolium decreased MPO activity in bladder tissue, accompanied by lipoperoxidation, not supporting, thus, the use of this species for the treatment of cystitis. Evidences (Yildrim et al., 2004) indicate that oxidants may be important in the pathogenesis of cyclophosphamide-induced cystitis. In addition, its pathophysiological mechanisms include transcription factors, cytokines and free radicals, among other factors (Korkmaz et al., 2007). Recently (Figueiredo and Lima, 2015), the anthocyanins present in the fruit of S. obtusifolium were found to be a rich source of antioxidants, what could justify the use of this species in the prevention
or treatment of inflammatory conditions as the cyclophosphamide-induced cystitis.

In the present study, we demonstrated that NMP treatments significantly attenuated iNOS and COX-2 immunoreactivities in the carrageenan-induced edematous paw. Evidences indicate that NO acts as a pro-nociceptive mediator in inflammation, in experimental models, including carrageenan-induced inflammation. PGs are also well established inflammatory mediators. Furthermore, COX enzymes catalyze the biosynthesis of arachidonic acid to PGH2, the committed step in PG formation (Toriyabe et al., 2004). Several in vivo and in vitro studies have suggested an interaction of NO and PG, in which the production of PG E2 is augmented further in the presence of NO. Inhibition of NO production by iNOS inhibitors has been found to decrease the production of PG E2 (Salvemini et al., 1995; Chen and Levine, 1999). It is thought that this NO effect is due to its ability to activate the COX enzyme. In inflammation, the functional relation between NO and COX pathways suggests that NO exacerbates the inflammatory process, through the generation of additional PG production (Salvemini et al., 1993).

Furthermore, evidences indicate that the administration of a NOS-nonselective inhibitor suppresses COX-2 expression in carrageenan-induced inflammation, and iNOS induces a massive NO production at the inflammatory site, contributing to COX-2 upregulation (Toriyabe et al., 2004). Others (Chun et al., 2004) demonstrated NO-induced COX-2 upregulation, through the transcription factor NF-kB, a known regulator of both COX-2 and iNOS expressions. The nuclear factor NF-kB pathway is considered a proinflammatory signaling pathway, based on its role in the expression of proinflammatory genes, including cytokines, chemokines and adhesion molecules (Lawrence, 2009).

In the present study, we observed that NMP decreased NF-kB and TNF-alpha immunoreactivities. It has been previously demonstrated (Cuzzocrea et al., 1999) that iNOS and COX-2 expressions are mediated by TNF-alpha and IL-1. NF-kB is known to upregulate signaling proteins, such as inducible nitric oxide synthase (iNOS). Consequently, under normal conditions, NF-kB activation will prevent TNF-alpha induced cell death in hepatocytes and other primary cells, and NF-kB-regulated signaling pathways become the predominant biologic response to TNF-alpha. Key regulators of NF-alpha signaling pathways are ROS, which can regulate both apoptotic signaling and NF-kB transcription (Han et al., 2009). Furthermore, evidences (Saud et al., 2005; Nandi et al., 2010) indicate that TNF-alpha modulates iNOS expression in some inflammation models in vivo.

Previously (Jonac et al., 1996; Bauer et al., 1999; Herrmann et al., 2000; Veihelmann et al., 2001; Krüger et al., 2007), oxaceprol, another proline derivative (N-acetyl-L-hydroxyproline), as NMP, was shown to present anti-inflammatory properties. Oxaceprol is considered an atypical inhibitor of inflammation, since it acts by inhibiting leukocyte adherence and infiltration in inflammatory processes (Jonac et al., 1996; Parnham, 1999; Veihelmann et al., 2001). Clinical studies (Krüger et al., 2007) suggest that oxaceprol is a...
potential candidate for the therapy of osteoarthritis, with efficacy equivalent to that of diclofenac. However, the oxaceprol action mechanism, although similar to that of NMP, differs, in the sense that while oxaceprol does not inhibit prostaglandin synthesis, NMP does, as demonstrated by the decrease in COX-2 immunoreactivity.

In conclusion, in the present study we showed, for the first time, the antinociceptive and mainly the anti-inflammatory properties of a natural l-proline derivative, isolated from S. obtusifolium. This compound inhibited the increased immunoreactivities for iNOS, COX-2, TNF-alpha and NF-kB observed in edematous paws from untreated mice, inhibitions which are certainly related to the drug effect. Furthermore, additional studies, as those involving pharmacokinetic and toxicokinetic profiles, would be important in order to include NMP in translational studies, as a potential new anti-inflammatory drug.

Contributions of authors

Pedro Everson Alexandre de Aquino and Lucas Antônio Duarte Nicolau performed the in vivo experiments. Edilberto Rocha Silva did the plant collection, and advised the chemical study, including spectral analyses. Nayara Coriolano de Aquino and Sabrina Matias dos Santos carried out all the isolation and purification of the compound. Talita Rocha Magalhães and Luzia Kalyne Almeida Moreira Leal were responsible for the in vitro experiment. Kelly Rose Tavares Neves performed the immunohistochemical assays. Glaucce Socorro de Barros Viana carried out the statistical analyses, coordinated and wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2016.11.010.

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