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Involvement of the L-arginine-nitric oxide pathway in the antinociception caused by fruits of *Prosopis strombulifera* (Lam.) Benth.

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ABSTRACT

Ethnopharmacological relevance: Prosopis strombulifera (Lam.) Benth. is a rhizomatous shrub that grows in the north and central zone of Argentina. In folk medicine, the fruits of this plant have been used as an astringent, anti-inflammatory and odontalgic agent and anti-diarrheic.
Aim of the study: To investigate the antinociceptive effect of ethanol (EE), chloroform (CE) and ethyl acetate (EtOAcE) extracts of *Prosopis strombulifera* fruits and the involvement of the L-arginine-nitric oxide pathway in this effect.
Materials and methods: The antinociceptive effects of the EE, CE and EtOAcE of *Prosopis strombulifera* fruits were evaluated *in vivo* using the formalin-induced pain test in mice with aspirin and morphine as reference antinociceptive compounds. The participation of the L-arginine-nitric oxide pathway in the antinociceptive effect was investigated in the same animal model using L-arginine as a nitric oxide (NO) precursor. The *in vitro* inhibitory effect of the extracts on LPS-induced nitric oxide production and iNOS expression was investigated in a J774A.1 macrophage-derived cell line.
Results: CE (300 mg/kg), in contrast to EE and EtOAcE, caused significant inhibition ($p < 0.05$) of the *in vivo* nociceptive response. Moreover, CE (100–1000 mg/kg, p.o.) produced a dose-dependent inhibition of the neurogenic and the inflammatory phases of the formalin test with inhibition values (at 600 mg/kg) of $42 \pm 7\%$ and $62 \pm 7\%$, respectively. CE inhibition was more potent in the inflammatory phase, with an ID_{50} of 400.1 (252.2–634.8) mg/kg. The antinociception caused by CE (600 mg/kg, p.o.) was significantly attenuated ($p < 0.05$) by i.p. treatment of mice with L-arginine (600 mg/kg). In addition, CE (100 μg/mL) produced significant *in vitro* inhibition ($p < 0.001$) of LPS-induced NO production, which was not observed with EE and EtOAcE at the same concentration. The inhibition of NO production by CE (10–100 μg/mL) was dose-dependent, with an IC_{50} of 39.8 (34.4–46.1) μg/mL, and CE significantly inhibited LPS-induced iNOS expression in J774A.1 cells.
Conclusions: This study supports, in part, the ethnomedical use of *Prosopis strombulifera* fruits by showing that its CE produces moderate antinociception *in vivo*. The findings also provide scientific information for understanding the molecular mechanism involved in the analgesic effect of this plant.

Abbreviations: CE, chloroform extract; DMSO, dimethylsulfoxide; EE, ethanol extract; EtOAce, ethyl acetate extract; FBS, fetal bovine serum; HRP, horseradish peroxidase; IC_{50}, 50% inhibitory concentration; ID_{50}, 50% inhibitory dose; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; L-NOARG, N^{ω}-nitro-L-arginine; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide; NO, nitric oxide; NOS, nitric oxide synthases; p.o., *per os*; PBS, phosphate-buffered saline; s.c., subcutaneous; SDS, sodium dodecyl sulfate; TBS, tris-buffered saline.

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1. Introduction

The genus *Prosopis* belongs to the Leguminosae family, subfamily Mimosaceae, and comprises 44 species distributed mainly in arid and semiarid tropical and subtropical countries. In America, *Prosopis* species can be found from the southwestern part of the United States to the Argentinian Patagonia, and is characteristic of the Chaco phytogeographical province in South America (Tapia et al., 2000). *Prosopis strombulifera* (Lam.) Benth. is one of 31 species of *Prosopis* L. in Argentina. It is a rhizomatous shrub of less than 1.5 m in height, and grows in the north and central zone of the country, from Salta in the north to Rio Negro in the south, and San Juan in the west to Buenos Aires (Ariza Espinar et al., 2006). This species is popularly known as "retortuño", "retortón" or "mastuerzo". In folk medicine, the fruits of this plant have been used as an astringent, anti-inflammatory and odontalgic agent and anti-diarrheic (Ariza Espinar et al., 2006; Ratera and Ratera, 1980; Toursarkissian, 1980).

Previous phytochemical studies carried out by our group on the aerial parts (leaves) of this species have revealed the presence of several flavonoids: luteolin, luteolin-7-glucoside, vitexin, isovitexin, quercitrin, rhamnosyl vitexin and rutin (Gianinetto et al., 1975). Other scientific studies about the biological activity of aqueous extract obtained from *Prosopis strombulifera* fruits have reported antibacterial properties (Anesini and Perez, 1993; Perez and Anesini, 1994). However, to date, there are no studies supporting the antinociceptive properties of *Prosopis strombulifera* fruits. Hence, the aim of this study was to investigate the antinociceptive effect of *Prosopis strombulifera* fruit extracts and the involvement of the L-arginine-nitric oxide pathway in this effect.

2. Materials and methods

2.1. Plant material

Fruits of *Prosopis strombulifera* were collected in Mendoza province, in November 2009 and identified by experts from Instituto Multidisciplinario de Biología Vegetal (IMBIV) UNC-CONICET. A voucher specimen was deposited at the CORD (UNC Botanical Museum) as reference material (CORD 1285).

The plant material was dried at room temperature and powdered. The powder (495 g) was extracted with ethanol (4000 mL) by soxhlet and the solvent was evaporated under reduced pressure to obtain the crude ethanol extract [EE, yield 130.3 g (26.32%, w/w)]. EE was suspended in boiling water and, after cooling to room temperature, the solution was partitioned with chloroform and ethyl acetate. The yield obtained after removing the solvent under vacuum was 2.64 g (0.53%, w/w) and 1.93 g (0.39%, w/w) of chloroform extract (CE) and ethyl acetate extract (EtOAcE), respectively. Additionally, a phytochemical screening of *Prosopis strombulifera* fruits was performed as described in a previous work to screen for tannins, alkaloids, saponins, flavonoids, carbohydrates and steroids (Pinto Vitorino et al., 2004).

2.2. Chemical reagents

The following substances were used: formalin and morphine hydrochloride (Merck, AG, Darmstadt, Germany); aspirin, N^{ω}-nitro-L-arginine (L-NOARG), L-arginine hydrochloride, D-arginine hydrochloride, LPS (from *Escherichia coli*) and MTT were purchased from Sigma–Aldrich (St. Louis, MO, USA). FBS was obtained from Gibco (Invitrogen, Carlsbad, CA, USA), and RPMI-1640 medium and penicillin–streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Rabbit anti-iNOS and rabbit anti-calregulin polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated donkey anti-rabbit IgG antibody was obtained from Amersham (Buckinghamshire, UK). Other chemicals were of analytical grade. The different extracts obtained from *Prosopis strombulifera* and aspirin were dissolved in saline plus DMSO. The final concentration of DMSO did not exceed 5% and did not cause any effect *per se*. Other drugs were dissolved in RPMI-1640 medium or sterile normal saline according to the assay.

2.3. Animals

Experiments were conducted using Swiss mice (25–35 g) of both sexes, housed at 22 ± 2 °C under a 12 h light/dark cycle (lights on at 6:00 a.m.) and with access to food and water *ad libitum*. Animals (male and female mice homogeneously distributed among the groups) were habituated to laboratory conditions for at least 2 h before testing. Experiments were performed between 09:00 and 16:00 h. Each animal was used only once during the study. The experiments were approved by the Ethics Committee for Animal Research of the Federal University of Santa Catarina (Protocol number PP00608) and were performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain on conscious animals (Zimmermann, 1983). The number of animals and the intensity of the noxious stimuli were the minimum necessary to obtain reliable data.

2.4. Nociception induced by formalin

The procedure used was essentially the same as previously described (Santos and Calixto, 1997; Santos et al., 1999). Mice received 20 μL of a 2.5% formalin solution (0.92% formaldehyde) in saline and were injected intraplantarly in the ventral surface of the right hindpaw. Animals were immediately placed in a glass cylinder (20 cm diameter) and observed for 0–5 min (neurogenic phase) and 15–30 min (inflammatory phase). The time spent licking the injected paw was recorded with a chronometer and considered to be indicative of nociception.

In order to investigate the antinociceptive effect of the different extracts of *Prosopis strombulifera*, animals received CE, EE and EtOAcE (300 mg/kg, p.o.) 60 min before the formalin injection. Control animals were treated with vehicle (10 mL/kg, p.o.). Aspirin (400 mg/kg, p.o.) and morphine (5 mg/kg, s.c.) administered 60 min and 30 min before formalin injection, respectively, were used as reference antinociceptive compounds.

In a separate series of experiments, mice were treated with vehicle (10 mL/kg, p.o.) or different concentrations of CE (100–1000 mg/kg, p.o.) 60 min before the formalin injection with the aim of evaluate the CE dose–response.

The role played by the L-arginine-nitric oxide pathway in the antinociception caused by CE from *Prosopis strombulifera* fruits was investigated in a separate series of experiments. Thus, mice were pretreated with vehicle (10 mL/kg, i.p.), L-arginine (600 mg/kg, i.p., a NO precursor) or D-arginine (600 mg/kg i.p., an inactive isomer of L-arginine) and after 20 min, each experimental group received vehicle (10 mL/kg, p.o.), L-NOARG (75 mg/kg, i.p., a NOS inhibitor) or CE (600 mg/kg, p.o.), 30, 30 and 60 min before formalin injection, respectively.

2.5. Cell culture

The J774A.1 macrophage-derived cell line was cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (50 units/mL), and streptomycin (50 μg/mL) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.6. MTT assay

J774A.1 cells (1×10^5) were cultured in 96 well-plates, as described in Section 2.5. After 24 h, cells were pre-incubated with different concentrations of EE, CE and EtOAcE (100 and 200 μg/mL) for 24 h. Then, 100 μL of MTT solution (5 mg/mL) in PBS were added to each well and incubated at 37 °C for 4 h, and cells were lysed with HCl 0.01N–10% SDS (100 μL). After an incubation of 18 h at room temperature, the optical density was measured at 595 nm using an ELISA microplate reader (Bio-Rad Laboratories).

2.7. Measurement of NO production

J774A.1 cells (2.5×10^5) were cultured in 24 well-plates as described in Section 2.5. After 24 h, cells were pre-incubated with the different extracts (EE, CE, and EtOAcE) at increasing concentrations (10–100 μg/mL) for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Nitrite accumulation, an indicator of NO production, was measured in the conditioned culture medium by the Griess reaction (Ortega et al., 2010; Schulz et al., 1999). Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 30% (v/v) acetic acid and 0.1% (w/v) naphthyl ethylenediamine in 60% (v/v) acetic acid], incubated at room temperature for 30 min in the dark, and the absorbance at 540 nm was measured in an ELISA microplate reader (Bio-Rad Laboratories). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was calculated from a standard curve using freshly prepared sodium nitrite in culture medium. Data were expressed at the total μM nitrite produced, and the percentage of inhibition was calculated as follows: % inhibition = $[(Ac - Ae)/Ac] \times 100$, where *Ac* and *Ae* represent the absorbance of the groups treated with LPS alone and with LPS plus extract, respectively.

In order to evaluate the dose–response effect for those extracts showing a significant NO inhibition at 100 μg/mL concentration, cells were treated as described above and the concentrations of extracts were selected to cover the range of inhibition (0–100%).

2.8. Western blot analysis

To evaluate the iNOS expression, J774A.1 cells (2.5×10^5) were cultured in 24 well-plates as described in Section 2.5, incubated with different concentrations of CE (20–100 μg/mL) for 1 h and then stimulated with LPS (1 μg/mL) for 24 h of incubation. Then, cells were washed twice with ice-cold PBS and lysated in 120 μL of ice-cold sample buffer [Tris–HCl (62.5 mM pH 6.8), 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol (DTT), and 0.01% (w/v) bromophenol blue]. Equal amounts of protein of the cell lysates in sample buffer were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% polyacrylamide) for 2 h at 120 V, and then transferred to nitrocellulose membranes at 100 V for 60 min at 4 °C. These membranes were incubated in 5% (w/v) skim milk in 0.1% Tween 20-TBS (TBS-T20) for 2 h, washed twice with TBS-T20 and then incubated with a rabbit anti-iNOS polyclonal antibody (1/200) overnight at 4 °C. After washing, the membranes were incubated with HRP-conjugated secondary antibody for 2 h at room temperature, and the peroxidase activity was revealed using enhanced chemiluminescence (ECL)-detection reagents (Pierce, Rockford, IL) and Kodak BioMax Light Film. Rabbit anti-calregulin polyclonal antibody (1/200) was used to determine the loading protein, and the protein bands of iNOS and calregulin on X-ray films were scanned and densitometrically analyzed using image software (UVP Vision Works®LS Image Acquisition and Analysis Software, Upland, CA, USA).

2.9. Statistical analysis

Data were expressed as mean ± S.E.M. The statistical significance of differences between groups was detected by ANOVA followed by the Newman–Keuls' test. *p* values less than 0.05 ($p < 0.05$) were considered to be indicative of significance. The ID_{50} values (the dose of extract necessary to reduce the response by 50% relative to the vehicle group) and the IC_{50} value (the concentration of extract necessary to reduce the NO production by 50%) were reported as geometric means accompanied by their respective 95% confidence limits and were determined by nonlinear regression from individual experiments. All statistical analyses and graphic design were carried out using GraphPad Prism Version 4 (GraphPad software, San Diego, CA, USA).

3. Results

3.1. Phytochemical screening

The qualitative phytochemical analysis of *Prosopis strombulifera* fruits indicated the intense presence of flavonoids, tannins and carbohydrates. Saponins and steroids were present in minor quantity and alkaloids were absent.

3.2. Effect of the extracts of Prosopis strombulifera fruits on the formalin test in mice

The results presented in Table 1 show that the oral administration of CE, but not of EE and EtOAcE, at the dose of 300 mg/kg, given 60 min beforehand, did not produce any irritation by itself (result not shown), but caused a significant inhibition of the neurogenic phase (0–5 min) and inflammatory phase (15–30 min) of formalin-induced licking. The reference opioid analgesic, morphine (5 mg/kg, s.c.), administered 30 min before formalin injection, produced a significant inhibition of both phases of the formalin test. In contrast, the non-opioid non-steroidal analgesic, aspirin (400 mg/kg, p.o.), given 60 min before the assay, was able to significantly reduce only the inflammatory phase of formalin-induced pain.

In addition, the results depicted in Fig. 1(A and B) show that CE (100–1000 mg/kg, p.o.) produced a dose-related inhibition of both the neurogenic (0–5 min) and inflammatory (15–30 min) phases of formalin-induced licking. However, the antinociceptive effect was significantly more pronounced against the second phase of this model of pain. The mean ID_{50} values calculated for these effects were >1000 and 400.1 mg/kg (252.2–634.8 mg/kg, 95% confidence limits) and the inhibitions observed were 42 ± 7% and 62 ± 7% at a dose of 600 mg/kg, for neurogenic and inflammatory phases, respectively.

3.3. Involvement of the L-arginine-nitric oxide pathway on the antinociceptive effects of CE of fruits of Prosopis strombulifera

The results presented in Fig. 2 show that the 20 min prior treatment of animals with the NO precursor L-arginine (600 mg/kg, i.p.), but not with D-arginine (600 mg/kg, i.p.), completely reversed the antinociceptive response caused by CE (600 mg/kg, p.o.) and L-NOARG (75 mg/kg, i.p., used as positive control), when analyzed against both the neurogenic (0–5 min) and inflammatory (15–30 min) phases of formalin-induced licking (Fig. 2A and B).

3.4. Effect of EE, CE and EtOAcE from Prosopis strombulifera fruits on cell viability of J774.A1 macrophages

The cell metabolic activity of J774.A1 macrophages was examined by using MTT reduction as an indirect measurement of cell viability. EE, CE and EtOAcE, at concentrations of 100 μg/mL, did not

Table 1
Effects of oral treatment with EE, CE and EtOAcE of *Prosopis strombulifera* fruits on formalin-induced nociception.

Treatment	Dose	Formalin test			
		0–5 min		15–30 min	
		Reaction time (s)	Inhibition (%)	Reaction time (s)	Inhibition (%)
Vehicle	10 mL/kg	82.2 ± 8.3	–	241.0 ± 18.6	–
EE	300 mg/kg	79.5 ± 5.1	–	231.3 ± 45.9	–
CE	300 mg/kg	45.5 ± 5.6*	45.0 ± 7.0	121.3 ± 19.7**	50.0 ± 8.0
EtOAcE	300 mg/kg	61.7 ± 10.5	25.0 ± 13.0	192.8 ± 21.8	20.0 ± 9.0
Aspirin	400 mg/kg	75.6 ± 3.0	8.0 ± 3.6	81.2 ± 16.7**	66.3 ± 6.9
Morphine	5 mg/kg	22.5 ± 4.8**	72.6 ± 5.8	15.1 ± 25.1**	93.7 ± 10.4

Mice (n = 4–7 per group) were treated with extract 60 min before formalin injection and results were expressed as mean ± S.E.M. Aspirin and morphine were used as reference antinociceptive compounds. *p < 0.05, **p < 0.01, significantly different from vehicle group.

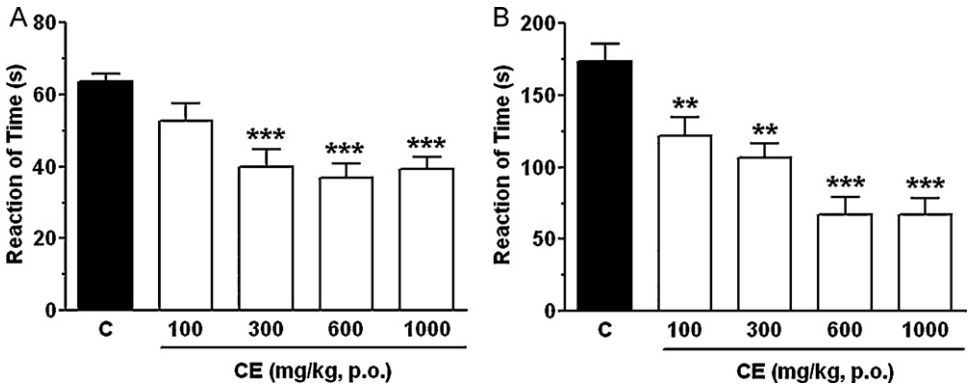

Fig. 1. Effects of CE of *Prosopis strombulifera* fruits (100–1000 mg/kg, p.o.) on the neurogenic phase (panel A) and the inflammatory phase (panel B) of formalin-induced licking in mice. Each column represents the mean ± S.E.M. (n = 7–10). Control values (C) indicate the administration of vehicle (saline plus DMSO, 10 mL/kg), and the asterisks denote the significance levels when compared with the control group; **p < 0.01, ***p < 0.001.

significantly decrease the cell metabolic activity of J774A.1 control cells (data not shown).

3.5. Effect of EE, CE and EtOAcE from fruits of Prosopis strombulifera on LPS-induced NO production

J774 A.1 macrophages were stimulated with LPS for 24 h in order to induce NO synthesis. The production of NO was estimated from the accumulation of nitrite in the medium, using the Griess reagent. The LPS-stimulated J774 A.1 cells produced 47.2 ± 5.1 μM of nitrite over a 24 h period (Table 2). When the cells were incubated with different concentrations of EE, CE and EtOAcE (10 and 100 μg/mL), only CE showed significant inhibition at 100 μg/mL (88.3 ± 4.0%, Table 2). L-NOARG (a NOS inhibitor) significantly inhibited NO production (Table 2). Considering that CE inhibited NO production, a concentration–response effect was studied. CE inhibited NO production, in a concentration-dependent manner, in LPS-activated J744A.1 cells, with an IC_{50} value of 39.8 μg/mL (34.4–46.1 μg/mL, 95% confidence limits) (Fig. 3).

3.6. Inhibitory effect of CE on the iNOS expression in J774A.1 cells

The effect of CE on iNOS enzyme protein contents was examined by Western blot. Fig. 4A shows that CE inhibited iNOS protein levels, in a concentration-dependent manner. A quantitative analysis by densitometry demonstrated that CE (100 μg/ml) produced a significant decrease in LPS-induced iNOS protein levels (Fig. 4B).

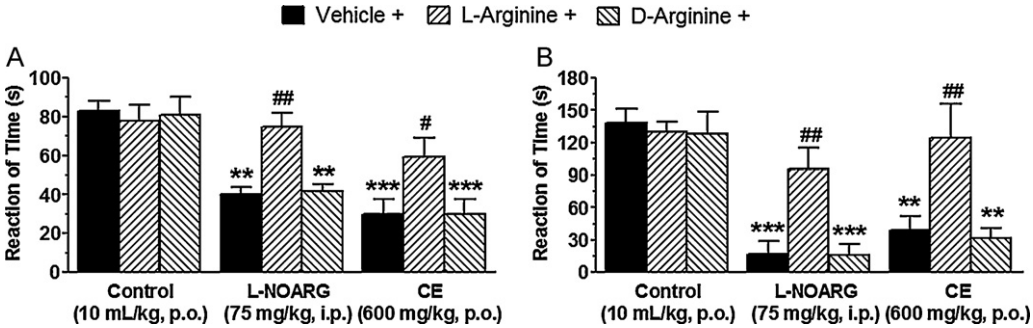

Fig. 2. Effects of the pre-treatment of mice with L-arginine (600 mg/kg, i.p.) or D-arginine (600 mg/kg, i.p.) on the antinociceptive profiles of the CE (600 mg/kg, p.o.) and L-NOARG (75 mg/kg, i.p.) against the neurogenic phase (panel A) and the inflammatory phase (panel B) of formalin-induced licking in mice. Each column represents the mean ± S.E.M. (n = 5–8). Control groups indicate the administration of vehicle, L-arginine or D-arginine alone, and the asterisks denote the significance levels when comparing the L-NOARG and CE treatments with the respective control group **p < 0.01 and ***p < 0.001; (#) indicates the significance levels when comparing the L-NOARG and CE treatments with their respective vehicle group, #p < 0.05 and ##p < 0.01 (one-way ANOVA followed by Newman–Keuls' test).

Table 2
Inhibition of LPS-induced NO production by EE, CE, and EtOAcE extracts from *Prosopis strombulifera* fruits in J774A.1 cells.

Treatment	Extract concentration (μg/mL)	NO production	
		Nitrite (μM)	Inhibition (%)
Control	–	3.6 ± 0.9	–
LPS (1 μg/mL)	–	47.2 ± 5.1	–
LPS + EE	10	49.6 ± 0.5	–
	100	42.9 ± 3.8	9.3 ± 8.0
LPS + CE	10	43.8 ± 2.2	7.4 ± 4.7
	100	5.5 ± 1.9***	88.3 ± 4.0
LPS + EtOAcE	10	51.1 ± 2.3	–
	100	39.5 ± 3.2	16.5 ± 6.7
LPS + L-NOARG (2 mM)	–	32.9 ± 1.4*	30.3 ± 3.0

Results were expressed as mean ± S.E.M. of three different experiments. *$p < 0.05$, ***$p < 0.001$, significantly different from LPS group.

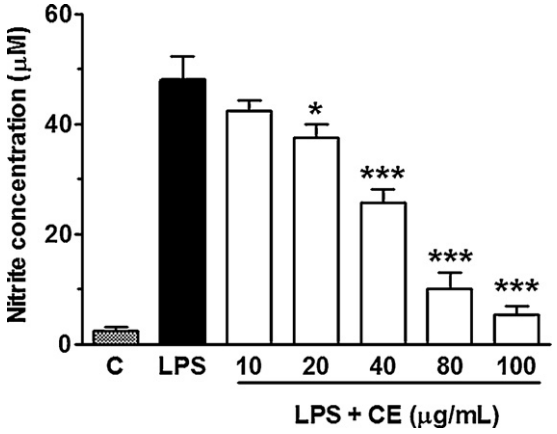

Fig. 3. Concentration-dependent inhibition of LPS-induced NO production by CE. J774A.1 cells were incubated with LPS (1 μg/mL) for 24 h and different concentrations of CE (10, 20, 40, 80 and 100 μg/mL) were assayed. Data were expressed as nitrite concentration representing the mean ± S.E.M. of three different experiments. *$p < 0.05$; ***$p < 0.001$, significantly different from LPS group.

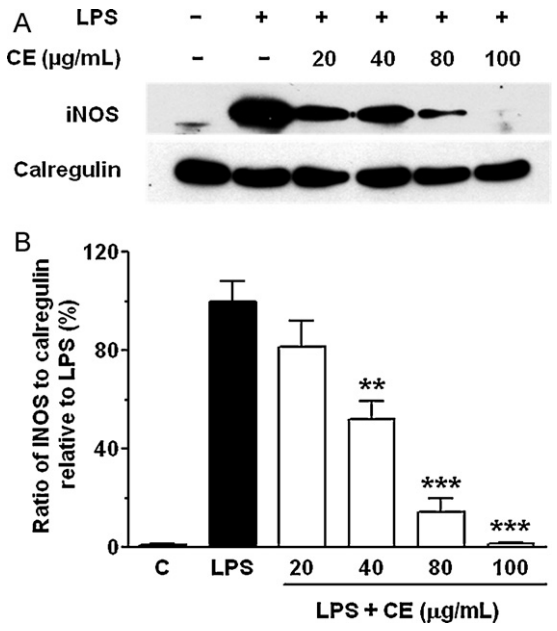

Fig. 4. Inhibition of LPS-induced iNOS protein expression by CE. J774A.1 cells were incubated with LPS (1 μg/mL) for 24 h, and different concentrations of CE (20, 40, 80 and 100 μg/mL) were assayed. (A) Western blot analysis of iNOS protein and calregulin (loading control). (B) Densitometric analysis of iNOS and calregulin bands. The results are presented as the mean ± S.E.M. of three different experiments. **$p < 0.01$; ***$p < 0.001$, significantly different from LPS group.

4. Discussion

Previous chemical reports of aerial parts of this species (leaves) revealed the presence of several flavonoids (Gianinetto et al., 1975). Similarly, in the present study, the phytochemical screening of *Prosopis strombulifera* fruits revealed a significant presence of flavonoids among other constituents. Taking into account this phytochemical information, the fruits of this species were extracted with various organic solvents differing in their polarity, obtaining the ethanol, the chloroform and the ethyl acetate extracts, which were then evaluated *in vivo* for their antinociceptive effects using the formalin-induced pain test. This experimental model of nociception has two distinct phases which may indicate different types of pain: the first and the second phase correspond to the neurogenic and inflammatory pain response, respectively (Guimaraes et al., 2010; Tjolsen et al., 1992). The formalin-test results demonstrated that oral administration of CE, but not EE and EtOAcE, at the dose of 300 mg/kg, caused a significant inhibition of the neurogenic ($p < 0.05$) and inflammatory ($p < 0.01$) phases. However, the CE effect was more pronounced against the inflammatory phase, suggesting an anti-inflammatory action as a putative mechanism for the antinociceptive effect of the extract. In addition, experimental evidence indicates that nitric oxide, among other inflammatory mediators, participates in the second phase of the formalin-induced pain test (da Matta et al., 2011; Garcia et al., 2004; Tjolsen et al., 1992). Considering the important inhibitory effect of CE on the second phase of the formalin test, the involvement of the L-arginine-nitric oxide system in the antinociceptive response produced by CE of *Prosopis strombulifera* fruits was evaluated and confirmed.

NO plays an important role in the development and maintenance of pain (Jin et al., 2010; LaBuda et al., 2006). Also, various evidence suggests that the inhibition of NO synthesis attenuates inflammatory pain (Boettger et al., 2007; De Alba et al., 2006). NO is synthesized from L-arginine by three structurally distinct isoforms of the NOS family: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Boettger et al., 2007; Salerno et al., 2002). Among the three NOS isoforms, iNOS plays an important role in pain conditions with an inflammatory component (Dudhgaonkar et al., 2008; LaBuda et al., 2006). Of the three extracts, only CE showed a significant decrease ($p < 0.001$) in NO production in LPS-activated J744A.1 in a dose-dependent manner. Also, CE produced a dose-dependent downregulation of the iNOS protein expression in LPS-stimulated J774A.1 cells. The *in vitro* anti-inflammatory activity of CE provides scientific information about the probable molecular mechanism involved in inhibition of the inflammatory nociceptive response by CE.

Taken together, the results of this study may indicate the ethnopharmacological relevance of *Prosopis strombulifera* fruits use as an antinociceptive/anti-inflammatory agent. However, no relevant scientific information about the manner and the amount in which the plant is consumed in Argentinean traditional medicine is known at present. So, further ethnopharmacological studies are necessary to improve understanding of the relationship between the traditional use of *Prosopis strombulifera* fruits and their *in vivo* and *in vitro* biological effects with respect to pain and inflammation.

The phytochemical compounds responsible for the *Prosopis strombulifera* antinociceptive and anti-inflammatory effects are unknown. The phytochemical screening of *Prosopis strombulifera* fruits indicated the intense presence of polyphenols, such as flavonoids and tannins, which have been demonstrated to have antinociceptive and anti-inflammatory activity on experimental animals and in *in vitro* assays (Birt, 2006; de Queiroz et al., 2010; Filho et al., 2008; Middleton et al., 2000; Souza et al., 2007). These data support the idea that flavonoids and tannins may contribute to the antinociceptive effect caused by CE of *Prosopis strombulifera*

fruits. Pharmacological and biochemical studies are under way to find out the active constituents responsible for the observed biologic properties of the fruits of this species.

In conclusion, the major findings of this study are: (1) CE of *Prosopis strombulifera* fruits has *in vivo* antinociceptive activity by inhibiting the neurogenic and inflammatory phases of the formalin model, (2) the L-arginine-NO pathway is involved in the analgesic effect exerted by CE *in vivo*, and (3) CE inhibits LPS-induced NO production and iNOS expression in J774A.1 cells *in vitro*. The inhibition of NO synthesis may be one of the molecular mechanisms involved in the CE inhibitory effect of the inflammatory nociceptive response. Furthermore, these data support, at least in part, the ethnomedical use of *Prosopis strombulifera* fruits.

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