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Anti-inflammatory properties of hydroalcoholic extracts of Argentine Puna plants

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ABSTRACT

The aim of this study is to evaluate the activity of thirteen hydroalcoholic extracts obtained from aerial parts of plants from the Argentine Puna on pro-inflammatory enzymes and inflammatory mediators.
Eleven extracts were non-cytotoxic on RAW 264.7. Data obtained suggest the capacity of these Argentine Puna plant extracts to inhibit the production of inflammatory mediators (nitric oxide and prostaglandin) at different levels. The plant extracts can affect enzyme expression and/or enzyme activity, and they can also act by NO scavenging. Each extract exerts its anti-inflammatory effect through different mechanisms. The inhibitory ability on pro-inflammatory enzymes by these hydroalcoholic extracts supports their potential use as sources of natural anti-inflammatory agents. Moreover, all extracts were non-toxic on *Artemia salina* toxicity test. The consumption of dietary supplements prepared with these plant species could be used to prevent the development of chronic inflammatory pathologies.

1. Introduction

The Puna, a highland region of the central Andes (3300–5000 m above sea level) that extends from southern-central Peru to northern Argentina and Chile, is characterized by a low relative humidity, high solar radiation levels and a wide variation of temperature during the day and the night (Cabrera, 1968). In spite of the hostile environment, about 1500 plant species grow in this region. The plants have developed adaptive mechanisms to survive in this aggressive environment, like the synthesis of secondary metabolites that, at the same time, have important beneficial properties to human health (García & Beck, 2006). The dominant plant species include Asteraceae, Fabaceae, Poaceae, Solanaceae and Verbenaceae. Plants play an important role in the daily life of the inhabitants of this region. These species are employed in construction, as food, medicine, forage, fuel and elements in spiritual activities (Villagrán, Romo, & Castro, 2003).

The plants selected for this study are used as medicine by the Puna people, and many of their popular uses are associated with the treatment of inflammatory processes. These herbal species are mainly prepared by maceration, infusion and decoction of the plants' aerial parts (Abad & Bermejo, 2007; Rodríguez, Aceñolaza, & Zamboni, 2013; Zampini, Isla, & Schmeda-Hirschmann, 2009). Some of their functional properties have been described (Cuello, Alberto, Zampini, Ordoñez, & Isla, 2011; D'Almeida, Alberto, Quispe, Schmeda-Hirschmann, & Isla, 2012; Nuño et al., 2012; Zampini, Isla, & Schmeda-Hirschmann, 2009; Zampini et al., 2008; Zampini et al., 2009).

Inflammation is an unspecific physiological response of the body to a harm produced by endogenous or exogenous agents. It's a necessary self-limited response of the body to a chemical, mechanical or biological harm induced in the tissue, thus acting as a defensive barrier of the organism. Controlling inflammation is of major importance in the treatment of illnesses associated with chronic inflammations, such as in arthritis, osteoarthritis, sclerosis, arteriosclerosis, Alzheimer, diabetes, insulin-resistance, obesity, allergies, asthma, chronic bronchitis, cancer, tuberculosis, retinitis, psoriasis, lung fibrosis, and chronic gastritis, among others (McGeer & McGeer, 2001; Sinicrope & Gill, 2004). A large number and variety of anti-inflammatory drugs are used to control the symptoms and to prevent the further development of these illnesses into a worse state of body damage. Current anti-inflammatory drugs can inhibit inflammation as curative agents. Nevertheless, these

Abbreviations: COX, cyclooxygenase; DMSO, dimethylsulfoxide; DTNB, 5,5′ dithiobis-2-nitrobenzoic acid; DMAC, 4-dimethylaminocinnamaldehyde; DMEM, Dulbecco's modified Eagle's medium; DNPH, 2,4-dinitrophenylhydrazine; DW, dry weight; ELISA, enzyme-linked inmunosorbent assay; GAE, gallic acid equivalents; NO, nitric oxide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LTs, leukotrienes; LOX, lipoxygenase; MTT, 3-(4,5-tetrazolium dimetiltiazol-2-il)-2,5-diphenil; NE, naringenin equivalents; PB_2E, procyanidin B_2 equivalents; PBS, phosphate buffered saline; PGs, prostaglandins; PVDF, polyvinylidene fluoride; QE, quercetin equivalents; SP, soluble principle; TNFα, tumor necrosis factor alpha.

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conventional drugs have not been successful in the treatment of chronic inflammatory disorders because they present some side effects that cause damage to the body when consumed over long periods of time, which worsens the general condition of the patient and generates an increase in health costs in order to prevent and treat its side effects (Babasaheb et al., 2012). Hence, there is a need to look for compounds without side effects to obtain adequate and safer treatment of chronic sicknesses.

Botanical dietary supplements, also called botanical nutraceuticals or herbals, can be defined as plant-derived materials with medical benefits aimed at disease prevention or treatment. Epidemiological studies have shown the effect of plant-derived food consumption in the prevention and treatment of inflammatory conditions like cardiovascular and neurodegenerative diseases, and cancer (Yao et al., 2004). The natural compounds, present in these food products, can be responsible for this health-promoting activity. One of the on-going research candidates are plant constituents used in food and traditional medicine. Thus, the aim of this research is to obtain hydroalcoholic extracts from Puna plants that are able to inhibit the activity and expression of proinflammatory enzymes.

2. Materials and methods

2.1. Chemical reagents

Folin–Ciocalteau reagent, aluminum chloride, quercetin dihydrate, naringenin, gallic acid, dexamethasone, nimesulide, dimethylaminocinnamaldehyde, 2,4-dinitrophenylhydrazine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), Griess reagent, fetal bovine serum (FBS), *Escherichia coli* lipopolysaccharide (LPS), anti-β-actin polyclonal antibody, anti-rabbit IgG and anti-mouse IgG secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS) were from Gifco Life Technologies (Paisley, Renfrewshire, UK). The anti-iNOS, anti-COX-2 polyclonal antibodies were from Cayman (Ann Arbor, MI, USA).

2.2. Plant material

Selected plant species were collected in the Puna region of northwestern Argentina (Salta, Jujuy, Tucumán and Catamarca) at different altitudinal levels, between 2600 and 4800 masl. The botanical identification of the plants was done by Dr. Ana Soledad Cuello and the voucher specimens were conserved in the Miguel Lillo Foundation's (LIL) and Instituto de Estudios Vegetales' Herbariums. The aerial parts were used in all the experiments.

2.3. Preparation of extracts

The air-dried plant material was macerated in hydroalcoholic solution (20 g per 100 mL of ethanol 17°) for 7 days under shaking (40 cycles/min) at room temperature. Afterwards, extracts were filtered using a Whatman no. 1 filter paper (Sigma-Aldrich).

With the aim to carry out a chemical and biological characterization, the extracts were evaporated "*in vacuo*" (40 °C), the residual water was freeze-dried by lyophilization. The resulting dried extracts were suspended with DMSO to obtain stock solutions of 50 mg/mL and stored at 4 °C in the dark.

2.4. Phytochemical screening

2.4.1. Qualitative analysis

Qualitative screening was carried out to determine the existence of the main chemical groups: cardiac glycosides (Teke et al., 2010), coumarins (Teke et al., 2010), flavonoids (Mojab, Kamalinejad, Ghaderi, & Vahidipour, 2003), tannins (Adegboye, Akinpelu, & Okoh, 2008), free anthraquinones (Onwukaeme, Ikuegbvweha, & Asonye, 2007), saponins (Ayoola et al., 2008), terpenoids, steroids and alkaloids (Adegboye et al., 2008).

2.4.2. Quantitative analysis

Total phenolic compound content was determined according to the Folin–Ciocalteau method (Singleton, Orthofer, & Lamuela-Raventos, 1999). The reaction mixture, containing 5 μL of each extract, 2 mL of distilled water, 200 μL of Folin–Ciocalteu reagent and 800 μL of sodium carbonate (15.9%, w/v), was heated at 50 °C for 5 min in a water bath. Absorbance was measured at 765 nm. Results were expressed as μg of gallic acid equivalents per mg of dry weight (μg GAE/mg DW) (R^2 0.997, p 0.05).

Non-flavonoid phenols were measured by the determination of the total phenol content remaining after the precipitation of the flavonoids with acidic formaldehyde (Zoecklein, Fugelsang, Gump, & Nury, 1990). Results were expressed as μg GAE/mg DW.

The flavone and flavonol content were determined according to Popova, Silici, Kaftanoghu, and Bankova (2005). A mixture of 10 μL of each extract, 100 μL of 5% $AlCl_3$, and absolute methanol for a total volume of 5 mL was maintained for 10 min at room temperature. Then, the absorbance was measured at 425 nm. Flavonoid content was expressed as μg quercetin equivalents per mg of dry weight (μg QE/mg DW) (R^2 0.999, p 0.05).

Content of flavanones and dihidroflavanones was measured by Nagy and Grançai (1996) method with slight modifications. 100 μL of each extract react with 2 mL of 1% 2,4-dinitrophenylhydrazine (DNPH) and 70% methanol. It was incubated for 50 min at 50 °C. After cooling at room temperature, 300 μL of this reaction mixture was mixed with 700 μL of 10% KOH in 70% methanol. It was incubated for 2 min at room temperature and then centrifuged at 10,000 ×g for 7 min. 2.5 mL of absolute methanol was added to 0.5 mL of the supernatant and the absorbance was measured at 495 nm. Naringenin was used as standard and the results were expressed as μg of naringenin equivalents per mg of dry weight (μg NE/mg DW) (R^2 0.990, p 0.05).

The total condensed tannin (proanthocyanidins) content was determined with 4-dimethylaminocinnamaldehyde (DMAC) according to Prior et al. (2010). Each extract reacted with 450 μL of 0.1% DMAC and the total volume was complete with acidified ethanol 0.1%. The mixture was put to react for 20 min at 30 °C. During this time, a blue-green complex was formed and its OD was measured using a spectrometer at 640 nm. Procyanidin B_2 was used as standard drug, and results were expressed in μg of procyanidin B_2 equivalents per mg of dry weight (μg PB_2E/mg DW) (R^2 0.989, p 0.05).

2.5. Effect on pro-inflammatory mediators

2.5.1. Inhibition of cyclooxygenase (COX) activity in a cell free system

The inhibitory activity of the plant extracts on COX-2 was measured using a COX inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's instructions, based on measuring prostaglandin (PG) by ELISA. A human recombinant COX-2 enzyme was used to form PG from arachidonic acid. The assay to obtain 100% COX activity was performed with DMSO as solvent control. The inhibitory assays were developed in the presence of 200 μg/mL plant extracts or nimesulide (0.25–2.0 μM, commercial anti-inflammatory). Enzyme control was performed with COX that had been inactivated by being placed in boiling water for 3 min. The intra- and inter-assay coefficients of variation were 5 and 10%, respectively. The effect of the different plant extracts on pro-inflammatory mediators was evaluated by calculating the inhibition percentage of PGE_2 production.

2.5.2. Cell culture

RAW 264.7 cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The murine macrophages were

maintained in DMEM supplemented with 10% FBS, 1% antibiotic (100 μg/mL streptomycin and 100 U/mL penicillin) at 37 °C with humidified air containing 5% CO_2. Cells were grown in flasks and sub-cultured by scraping when they reached 80–90% confluence with a 1:3 or 1:6 ratio in fresh medium. The cells were then subjected to the treatment described below.

2.5.3. Cytotoxicity assay

Cell viability was assessed using the MTT assay as described previously (Mossman, 1983). To summarize, RAW 264.7 cells were seeded into a 96-well plate at a density of 2.0×10^5 cells per well and incubated at 37 °C for 24 h. The cells were then treated with various concentrations of the extracts (0–200 μg/mL). After additional 24-h incubation at 37 °C, the medium was removed and 100 μL of MTT (0.5 mg/mL in PBS) was added to the wells and was incubated until blue deposits were visible. The formazan salts formed were dissolved by adding 200 μL of DMSO in each well. The absorbance was then measured at 490 nm using a plate reader (Labsystems Multiskan MCC/340, Labsystems, Helsinki, Finland). The percentage of cell viability was calculated by using the cell viability of the control group, which received vehicle (100% viability).

2.5.4. Inhibition of nitric oxide (NO) production

A total of 2×10^5 RAW 264.7 cells were seeded into a 96-well plate and incubated for 2 h at 37 °C with 5% CO_2. Then, the cells were stimulated by adding 1 μg/mL of LPS with or without plant extracts (50 to 200 μg/mL), dexamethasone (0.4 to 4.0 μg/mL) or vehicle (DMSO, 0.1% of final concentration). Cells were further incubated at 37 °C under 5% CO_2 during 20 h. At the end of each experiment, supernatants were collected. To evaluate the inhibitory activity of plant extracts on NO production, the culture media was assayed using Griess reaction (Granger, Taintor, Boockvar, & Hibbs, 1996). Briefly, an equal volume of Griess reagent was mixed with culture supernatant and the color developed was measured at 540 nm using the microplate reader. Percentage of the NO inhibition was calculated by the relation between NO levels of LPS-stimulated cells and treated with plant extracts, and cells treated with vehicle (control).

2.5.5. Analysis for inhibition of nitric oxide synthase (iNOS) and COX-2 expression

To measure the iNOS and COX-2 expression, a Western blotting technique was used. RAW 264.7 cells (2×10^6) were cultured in the presence or absence of LPS (1.0 μg/mL) and with/without test samples (0–200 μg/mL) or dexamethasone (10 μM, 3.92 μg/mL) during 20 h. Afterwards, the cells were washed twice with ice-cold PBS and lysed with Laemmli buffer on ice. Cell extracts were obtained by centrifugation at 14,000 ×g at 4 °C for 10 min. The proteins were separated on SDS-PAGE and transferred into PVDF membranes. The membranes were immediately blocked with 5% (w/v) non-fat dry milk in PBS at room temperature for 1 h. The membranes were incubated with polyclonal antibodies anti-iNOS (1: 4000), anti-COX-2 (1:8000) or anti-β-actin (1:10000) at 4 °C overnight. After three washings in PBS containing 0.05% Tween 20 for 10 min, the PVDF membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG antibody for iNOS and COX-2 or goat anti-mouse IgG for β-actin at room temperature for 1 h. After three washings in PBS containing 0.05% Tween 20 during 10 min, the antibody labeling was visualized using chemiluminescent reagent (Inmobilon™ Western HRP Substrate, Millipore, Billerica, MA, USA), according to the manufacturer's instructions. Pre-stained blue protein markers were used for molecular weight determination. The immunoreactive bands were visualized with a LAS 3000 mini system (Fujifilm, Tokyo, Japan). Western blot quantification was carried out with Multi Gauge software (Fujifilm). The quantification of COX-2 and iNOS proteins was performed as described (Fallarini et al., 2009), by calculating the ratio between COX-2 or iNOS with β-actin protein expression; the latter was selected as reference house-keeping protein.

2.6. Nitric oxide scavenging activity

To measure the extracts' capacity to depurate the nitric oxide we used Kumaran and Karunakaran's (2007) method with some modifications. Briefly, different concentrations of the extracts (0–400 μg/mL) were mixed in a 96-well flat-bottomed plate with 30 μL of sodium nitroprusside 10 mM, and sodium phosphate buffer (0.1 M, pH 7.5) to a final volume of 150 μL. The reaction mixture was incubated for 1 h under light at room temperature. Finally, 150 μL of Griess reagent was added to each well to measure at 550 nm the amount of nitrite produced. Ascorbic acid (10–100 μg/mL) was used as positive control.

2.7. Acute toxicity test

The acute toxicity levels of the extracts were studied using *Artemia salina* as test organism (Svensson, Mathiasson, Martensson, & Bergatröm, 2005).

To obtain *A. salina* larvae, its cysts were hatched in artificial seawater ($NaCl$, $MgCl_2.6H_2O$, Na_2SO_4, $CaCl_2.2H_2O$, KCl). After 24 h of incubation at 25 °C, the larvae have hatched and are in their most sensitive state. Between 10 and 12 larvae were transferred to microplates containing fresh medium and 200 to 1500 μg/mL of each extract. Solvent control (DMSO) without extract and a positive control (sodium dichromate) were also assayed. All the plates were incubated for 24 h at 25 °C. The total number of immobile larvae was counted for each concentration and the immobility was calculated. The larvae were considered immobile if they did not show any forward movement for 10 s.

2.8. Statistical analysis

All assays were conducted at least three times with three different sample preparations. Each experimental value is expressed as the mean ± standard error of the mean (S.E.M.). The scientific statistic software InfoStat (Student Version 2011) was used to evaluate the significance of differences between groups. Comparisons between groups were done using a one-way ANOVA with Tukey post-test at a confidence level of 95%. The criterion of statistical significance was taken as $p \leq 0.05$.

3. Results

The extraction yields of the plant species expressed as mg of soluble principles per g of dry weight (mg SP/g DW) varied between 82.3 ± 2.1 and 184.9 ± 19.1 mg SP/g DW, with the levels of extraction of soluble principles oscillating from 4.11 to 14.36 mg SP/mL. The highest yields of extraction was observed for *Tessaria absinthioides* and *Parastrephia* spp.

3.1. Phytochemical screening

As shown in Table 1, the qualitative analysis of the crude extracts revealed the presence of phytochemicals from different chemical groups. All the extracts showed a strong positive reaction for flavonoids and condensed tannins. On the other hand, coumarins were present in the majority of the extracts but triterpenes and saponins were less extended among the plants studied. Alkaloids were only present in *Parastrephia lucida*. *Tetraglochin cristatum Tetraglochin cristatum* tested negative for free anthraquinone derivatives while *T. absinthioides*, *Acantholippia deserticola* and *Junellia seriphiodes* showed absence of cardiac glycosides and anthraquinone derivatives.

The quantitative analysis of total phenolic content showed the highest yield for *T. cristatum* (221.3 ± 4.6 μg GAE/mg), followed by *Ephedra multiflora* (178.2 ± 0.3 μg GAE/mg) (Table 2). A higher percentage of total phenolic compounds in *E. multiflora* corresponded to flavonoids compounds, being especially abundant in condensed tannins. *J. seriphioides* showed the highest flavanones-dihydroflavanones content. *Parastrephia lepidophylla* and *T. absinthioides* showed the highest

Table 1
Phytochemical screening of the studied plant species.

Family	Scientific name	Alkaloids		Cardiac glycosides		Coumarins	Flavonoids	Tannins	Saponins	Triterpenes/steroids	Free anthraquinone derivatives	
		Dragendorff	Mayer	Kedde	Keller-Kiliani						Bornträger	Mg $(AcO)_2$
Asteraceae	*Baccharis boliviensis* (Wedd.) Cabr.	−	−	+	+	+	+	+	+	+	+	+
	Baccharis incarum (Wedd.) Perkins	−	−	+	+	−	+	+	+	+	+	+
	Chiliotrichiopsis keidelii Cabrera	−	−	+	+	+	+	+	+	+	+	+
	Chuquiraga atacamensis Kuntze	−	−	+	+	+	+	+	−	+	+	+
	Nardophyllum armatum (Wedd.) Reiche	−	−	+	+	−	+	+	+	−	+	+
	Parastrephia lepidophylla (Meyen) Cabr.	−	−	+	+	+	+	+	−	−	+	+
	Parastrephia lucida (Meyen) Cabr.	+	+	+	−	+	+	+	+	−	+	+
	Parastrephia phyliciformis (Meyen) Cabr.	−	−	+	+	+	+	+	−	−	+	+
	Tessaria absinthioides (Hook. & Am.) DC	−	−	−	−	+	+	+	+	−	−	−
Ephedraceae	*Ephedra multiflora* (Phil. ex Stapf.)	−	−	+	−	+	+	+	−	−	+	+
Rosaceae	*Tetraglochin cristatum* (Britton) Rothm.	−	−	+	−	−	+	+	−	−	−	−
Verbenaceae	*Acantholippia deserticola* (Phil.) Moldenke	−	−	−	−	−	+	+	+	+	−	−
	Junellia seriphioides (Gillies & Hook.) Moldenke	−	−	−	−	−	+	+	−	−	−	−

(+) Detectable; (−) non detectable.

content of flavones and flavonols. The species of *Parastrephia* genus, *Chiliotrichiopsis keidelii* and *T. cristatum* were more abundant in non-flavonoid phenolics than the other plant species.

3.2. Effect on inflammatory mediators

Fig. 1 shows the inhibition of COX-2 activity by 200 μg/mL of all plant extracts tested in a cell free system. *J. seriphioides* showed maximum inhibitory effect, around 50%. This could be due to the highest content on flavanones and dihydroflavanones in comparison to the other plant species (Table 2). The 50% inhibition was reached by the reference drug, nimesulide, at 1.25 μM (0.39 μg/mL). In the case of *P. lepidophylla*, a significant decrease in COX-2 activity (47%) was also observed.

The toxicity of plant extracts against RAW 264.7 cells was studied. Only two extracts (*T. cristatum* and *A. deserticola*) were toxic at the assayed concentration range (50–200 μg/mL) on cell systems. Eleven

Table 2
Quantification of phenolic compounds of the Puna plant species extracts.

Family	Scientific name	Total phenolics (μg GAE/mg DW)	Non flavonoid phenolics (μg GAE/mg DW)	Flavones and flavonols (μg QE/mg DW)	Flavanones and dihydroflavanones (μg NE/mg DW)	Condensed tannins (μg PB_2E/mg DW)
Asteraceae	*B. boliviensis*	$110.3 \pm 2.8^{b,c,d}$	101.7 ± 0.4^{e}	$19.2 \pm 3.1^{b,c,d}$	$23.8 \pm 1.0^{a,b}$	0.50 ± 0.02^{a}
	B. incarum	64.2 ± 1.0^{a}	$53.0 \pm 0.1^{b,c}$	$12.9 \pm 2.6^{a,b,c}$	$27.6 \pm 1.5^{a,b}$	0.34 ± 0.04^{a}
	C. keidelii	$158.5 \pm 10.2^{e,f,g}$	139.5 ± 2.4^{g}	$12.4 \pm 0.2^{a,b}$	$35.6 \pm 2.3^{b,c}$	0.38 ± 0.02^{a}
	Ch. atacamensis	63.8 ± 1.3^{a}	$62.4 \pm 0.2^{c,d}$	6.3 ± 1.6^{a}	$41.3 \pm 4.4^{b,c}$	1.64 ± 0.01^{d}
	N. armatum	$102.3 \pm 0.5^{b,c}$	91.9 ± 1.6^{e}	5.9 ± 0.1^{a}	$51.0 \pm 6.3^{c,d,e}$	1.30 ± 0.09^{c}
	P. lepidophylla	$156.8 \pm 0.3^{e,f,g}$	133.2 ± 2.3^{g}	27.9 ± 1.9^{e}	$24.1 \pm 4.8^{a,b}$	1.07 ± 0.01^{b}
	P. lucida	$143.1 \pm 1.6^{d,e,f}$	122.1 ± 4.7^{f}	$16.4 \pm 0.1^{b,c}$	$41.3 \pm 4.4^{b,c}$	0.32 ± 0.01^{a}
	P. phyliciformis	$159.5 \pm 6.2^{e,f,g}$	152.7 ± 2.7^{h}	$20.4 \pm 0.2^{c,d,e}$	$31.2 \pm 0.9^{a,b,c}$	0.40 ± 0.02^{a}
	T. absinthioides	$127.3 \pm 4.7^{c,d,e}$	99.3 ± 0.5^{e}	$24.9 \pm 1.8^{d,e}$	$44.3 \pm 0.9^{b,c,d}$	0.45 ± 0.02^{a}
Ephedraceae	*E. multiflora*	178.2 ± 0.3^{g}	46.1 ± 1.2^{b}	$20.1 \pm 0.1^{b,c,d}$	13.7 ± 3.7^{a}	33.72 ± 0.01^{h}
Rosaceae	*T. cristatum*	221.3 ± 4.6^{h}	$132.4 \pm 0.5^{f,g}$	$13.0 \pm 0.3^{a,b,c}$	$49.6 \pm 2.1^{c,d,e}$	5.46 ± 0.01^{f}
Verbenaceae	*A. deserticola*	66.9 ± 0.7^{a}	65.1 ± 2.0^{d}	6.6 ± 0.1^{a}	$40.6 \pm 0.1^{b,c}$	1.35 ± 0.07^{c}
	J. seriphioides	$77.0 \pm 2.1^{a,b}$	65.4 ± 0.4^{d}	$12.8 \pm 1.1^{a,b,c}$	66.7 ± 8.0^{e}	2.36 ± 0.01^{e}

GAE: gallic acid equivalents, QE: quercetin equivalents, NE: naringenin equivalents, PB_2E: procyanidin B2 equivalents. Values are reported as mean ± S.E.M. Different letters in the same column show significant differences among each treated group, according to Tukey test ($p \leq 0.05$).

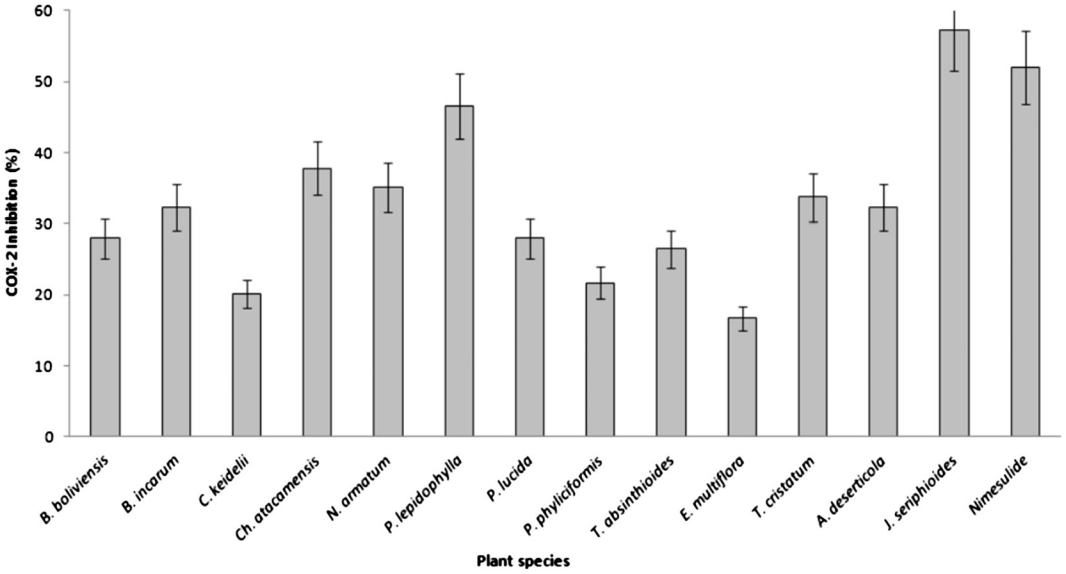

Fig. 1. Effect of 200 μg/mL plant extracts and 0.4 μg/mL nimesulide on COX-2 enzyme activity assayed in a cell free system. Data are the mean ± S.E.M. of three different experiments.

plant extracts were not toxic (viability 95%) until 200 μg/mL. A 0.1% of DMSO didn't modify the cell viability in our experiments.

The induction of RAW 264.7 cells into an inflammatory state by treatment with LPS caused significant increase in NO production. Pretreatment of cells with the non-toxic extracts inhibited NO overproduction in a concentration-dependent manner (Fig. 2). The species of the Asteraceae family were the most effective, specially the species of the genus *Parastrephia*, such as *P. lucida*, which was the most active followed by *P. lepidophylla* and *P. phyliciformis* (IC_{50} = 55, 82 and 125 μg/mL, respectively). *B. incarum*, *B. boliviensis* and *T. absinthioides* showed IC_{50} values of 167, 195 and 160 μg/mL, respectively. Of the eleven plant extracts assayed, *J. seriphioides* was the only one that not shown inhibitory effect on NO release. Dexamethasone, used as a positive control, showed an IC_{50} for NO production of 2.1 μg/mL, which was lower than the values obtained for the extracts.

As shown in Fig. 3, the iNOS and COX-2 protein expression was not detected in non-stimulated RAW 264.7 cells, whereas treatment with LPS induced high levels of these proteins. The iNOS and COX-2 protein expression was significantly decreased by *P. lucida*, *P. phyliciformis*, *Baccharis incarum* and *T. absinthioides* (70, 59, 58 and 63% for iNOS and 38, 33, 43, and 44% for COX-2, respectively) using 200 μg/mL, while *P. lepidophylla* only diminished iNOS level by 54%. COX-2 expression was not affected by *P. lepidophylla* and *J. seriphioides*; however, they were able to directly inhibit COX-2 activity (Fig. 1). The other plant species didn't inhibit the iNOS and COX-2 expression (data not shown). Data obtained suggest that the inhibition of NO and PG production by some Puna plant extracts in macrophages occurs at the level of enzyme expression and/or enzyme activity. The positive control dexamethasone (3.92 μg/mL) decreased 80 and 92% iNOS and COX-2 expression, respectively. In all experiments, the housekeeping protein β-actin was expressed equally, irrespective of the treatment.

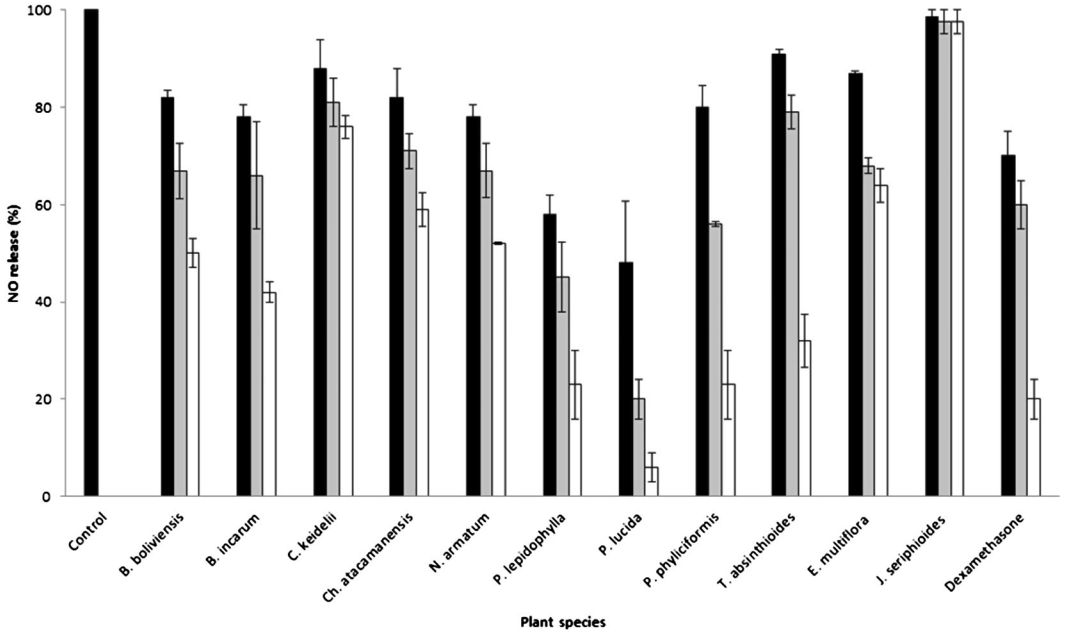

Fig. 2. Effects of 50 (■), 100 (■) and 200 (□) μg/mL plant extracts and 0.4 (■), 2.0 (■) and 4.0 (□) μg/mL dexamethasone on nitrite (NO) production in RAW 264.7 cell cultures. Data are the mean ± S.E.M. of at least three different experiments run in sextuplicate. Cells treated with 0.1% DMSO alone were considered as control.

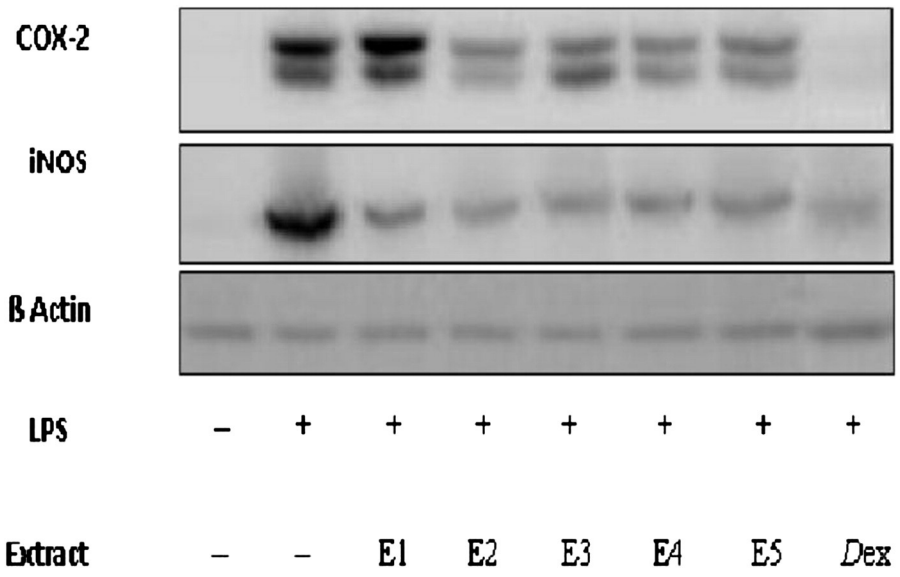

Fig. 3. Effects of 200 μg/mL plant extracts (E1: extract of *P. lepidophylla*, E2: extract of *P. lucida*, E3: extract of *P. phyliciformis*, E4: extract of *B. incarum*, E5: extract of *T. absinthioides*) and 3.92 μg/mL dexamethasone (Dex) on LPS-induced iNOS and COX-2 expression in RAW 264.7 cells. Cells were pretreated with LPS with (+) and without (−) plant extracts during 20 h. Cell lysates were separated on SDS-PAGE. iNOS, COX-2 and β-actin were detected by Western blot analysis.

While the plant extracts inhibit the activity and/or expression of iNOS enzyme, they could also exhibit an antioxidant effect; therefore we examined their ability to scavenge the NO generated by the enzyme. Fig. 4 shows the results of all plants extracts' scavenging activity. According to the percentage of NO scavenging at 200 μg/mL *C. keidelii*, *E. multiflora*, *Nardophyllum armatum*, *P. lepidophylla*, *P. phyliciformis* and *T. cristatum* exhibited a moderate (30–43%) activity, while *B. incarum*, *B. boliviensis*, *T. absinthioides* and *J. seriphioides* showed a low activity (10–25%). *A. deserticola* and *Chuquiraga atacamensis* were not able to scavenge NO radicals. Ascorbic acid (positive control) reached a 96% of NO scavenging at a concentration of 100 μg/mL.

4. Discussion

Inflammatory and pathogenic conditions activate COX and LOX, the key enzymes in the synthesis of prostanoids and eicosonoids from polyunsaturated fatty acids, which are involved in various inflammatory and allergic disorders (Martin et al., 2004).

There are two major isoforms of COX. The inducible isoform (COX-2) is found in major concentration in tissues where there is an inflammatory response, while the constitutive isoform (COX-1) seems to be more related to physiological processes. Inhibition of COX activity is the mechanism by which non-steroidal anti-inflammatory drugs exert

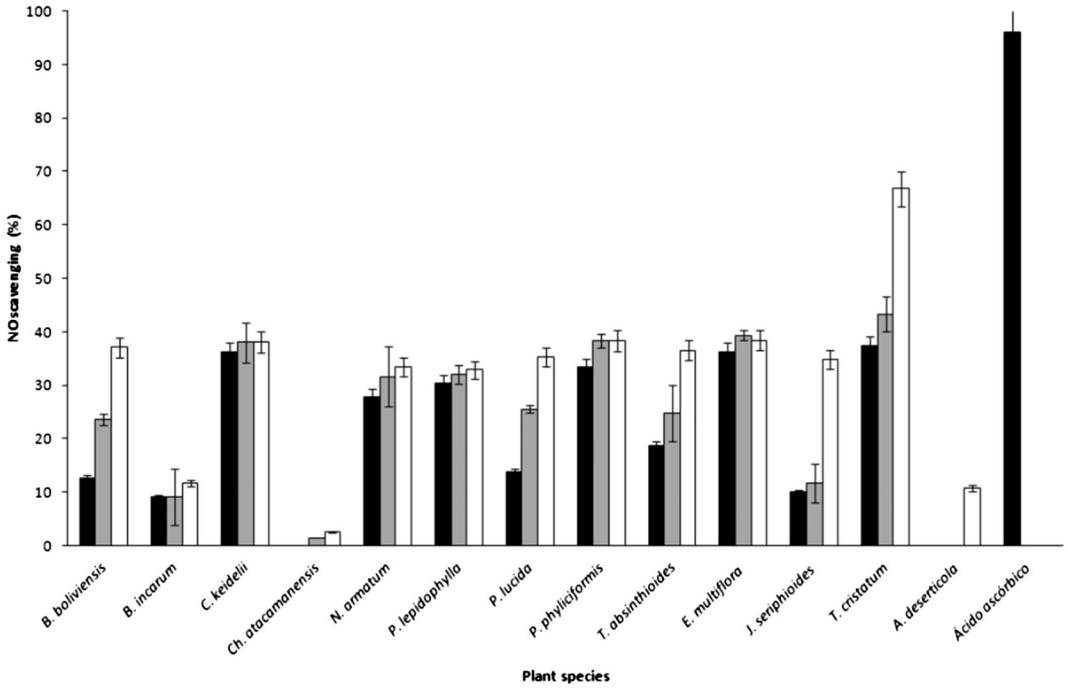

Fig. 4. Nitric oxide scavenging effect of 100 (■), 200 (▩) and 400 (□) μg/mL of plant extracts in a free cell system and 100 μg/mL (■) of ascorbic acid. Data are the mean ± S.E.M. of at least three different experiments.

their analgesic, antipyretic, anti-inflammatory, and antithrombotic effects (Lee, Mukhtar, Bickers, Kopelovich, & Athar, 2003).

NO is one of the inflammatory mediators that is synthesized by iNOS in macrophages and is induced by different inflammatory stimuli such as bacterial endotoxic LPS and inflammatory cytokines (Achike & Kwan, 2003). Although NO is required in immunological defense mechanisms and to maintain the dilation of blood vessels, higher concentrations of NO can cause oxidative damage, septic shock, necrosis, increase the edema and exacerbates the allergic response, which, in turn, is involved in various illness processes, such as gastroenteritis, rheumatoid arthritis, asthma and osteoarthritis (Bae, Kim, & Lee, 2012).

Since some extracts are inhibitors of NO and PG production, we further investigated their effects on the expression levels of iNOS and COX-2. Several studies have suggested that over-expression of either inducible enzymes COX-2 or iNOS might be intimately involved in the pathogenesis of diseases including inflammation, cancer, multiple sclerosis, Parkinson's syndrome, and Alzheimer's disease (Hong et al., 2002). Therefore, compounds that inhibit the activity or expression of COX-2 or iNOS might be an important therapeutic target to treat various inflammatory diseases or for cancer chemoprevention.

Up to the present, several biological activities of plant species of the Argentine Puna such as antioxidant, antibacterial, and antifungal were studied (Cuello et al., 2011; D'Almeida et al., 2012; Nuño et al., 2012; Zampini, Cuello, et al., 2009; Zampini et al., 2008). However, there are few reports on their anti-inflammatory activity. In a previous work, we determined the ability of the *B. incarum*, *Baccharis boliviensis*, *Ch. atacamensis* and *P. lucida* ethanolic extracts (60-80° ethanol) to inhibit COX-1 and COX-2 activities (Alberto, Zampini, & Isla, 2009; D'Almeida et al., 2013) and *Fabiana* species to inhibit lipoxygenase (LOX) (Cuello et al., 2011).

A wide range of biological effects have been attributed to dietary polyphenols underlying their potential interest for an approach based on nutritional prevention. Phenolic compounds are widely considered to contribute to health benefits in humans. They are considered to possess anti-inflammatory properties and, therefore, were proposed as an alternative natural approach to prevent or treat chronic inflammatory diseases like chronic intestinal inflammation, cancer, diabetes, cardiovascular and neurodegenerative diseases (Sergent, Piront, Meurice, Toussaint, & Schneider, 2010). There is a need to find new and safe compounds capable of contributing to the prevention or even the treatment of inflammatory diseases. Phenolic compounds, as an alternative natural source, offer a great hope for these diseases (Sergent et al., 2010). The hydroalcoholic extracts studied in this work were particularly rich in different phenolic compounds.

Some flavonoids, such as luteolin, 3′,4′-dihydroxyflavone, galangin, morin and apigenin were found to be inhibitors of COX, while some flavones/flavonols/isoflavones, mainly flavones, considerably inhibited NO production (Kim, Son, Chang, & Kang, 2004). Some of these compounds have been previously isolated and identified in *B. incarum*, *B. boliviensis* and *P. lucida* (Abad Martinez, Latourrette Bessa, & Bermejo, 2005; Calle, Yupanqui, Flores, & Almanza, 2012; D'Almeida et al., 2013; Zampini, Isla, & Schmeda-Hirschmann, 2009).

D'Almeida et al. (2013) demonstrated that *P. lucida* extract inhibits arachidonic acid metabolism via several enzymes (COX, LOX and phospholipase A_2). The compounds with anti-inflammatory activity identified by a bio-guided isolation were 5,4′-dihydroxi-7-methoxyflavanone, apigenin, apigenin methyl ether and apigenin trimethyl ether, methyl and dimethyl ethers from quercetin, kaempferol and luteolin methyl ether, ferulic acid esters, cinnamic acid and vanillin.

In the present study, we have examined the anti-inflammatory capacity of hydroalcoholic extracts obtained from aerial parts of the Argentine Puna plants. *J. seriphioides* and *P. lepidophylla* exert a preferential effect on COX-2 activity but not on the enzyme expression, while *P. lucida* and *B. incarum* extracts exert not only an inhibitory effect on COX-2 activity but also on the protein expression, similar to *P. phyliciformis* and *T. absinthioides* extracts.

It's also remarkable that all *Parastrephia* species, have significant effect on iNOS expression as well as NO scavenging capacity, while *B. incarum* and *T. absinthioides* exert a preferential effect on protein expression. On the other hand, some of the species studied seem to act mainly in inhibiting the enzyme activity (*Ch. atacamensis*) or by scavenging the NO generated (*C. keidelii*, *N. armatum*, *E. multiflora*).

None of the extracts were toxic in the range of the concentration evaluated by *A. salina* test, and the LD_{50} (concentration that kills 50% of the *A. salina* larvae) wasn't reached, while potassium dichromate exhibited a LD_{50} of 29.97 μg/mL. According to Nguta et al. (2011), extracts with LD_{50} values above 1000 μg/mL are considered to be non-toxic.

5. Conclusions

Data obtained suggests the capacity of these Argentine Puna plant extracts to inhibit the production of inflammatory mediators (nitric oxide and prostaglandins) at different levels (enzyme expression and/or enzyme activity), and they can also act by NO scavenging. The inhibitory ability on pro-inflammatory enzymes by these hydroalcoholic extracts support the potential use of these plant species as dietary supplements that could be used to prevent the development of chronic inflammatory pathologies. Moreover, our results support the traditional use of Puna plants as sources of natural anti-inflammatory agents.

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