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Inhibition of pro-inflammatory enzymes by medicinal plants from the Argentinean highlands (Puna)

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ABSTRACT

Ethnopharmacological relevance: Human groups in the Argentinean Andes highlands (Puna) selected native plants as anti-inflammatory agents. The indications of use are mainly to relieve pain, as infusions, ethanolic extracts or plasters.

Aim of the study: The objective of the study was to assess the effect of hydroalcoholic extracts from native highland plants as anti-inflammatory agents according to the traditional indications of use. The chemical profile of the three most active species was analyzed by HPLC-ESI-MS to get an insight into the constituents and the effects observed according to the ethnopharmacological information.

Materials and methods: Hydroalcoholic extracts from 13 Argentinean Puna plants used as anti-inflammatory were evaluated as inhibitors of the pro-inflammatory enzymes phospholipase A_2 ($sPLA_2$), lipoxygenase (LOX), hyaluronidase, and for their capacity to stabilize red blood cells membrane. In addition, the extracts were evaluated to determine their reducing power, iron chelating capacity and $ABTS^{\bullet+}$ radical scavenging effect. The chemical profiles of the most active species were analyzed by HPLC-ESI-MS.

Results: Among the species investigated, *Ephedra multiflora* was the most active as LOX inhibitor (IC_{50}:132 μg/mL), by reducing the non-heme iron group and by scavenging radicals. The IC_{50} values of the reference compounds caffeic acid and naproxen were 57.0 and 14.0 μg/mL, respectively. *Parastrephia lucida* showed the highest $sPLA_2$ inhibitory effect (63% of inhibition at 200 μg/mL). Under the same experimental conditions, the IC_{50} of the reference compound acetylsalicylic acid was 65 ± 1 μg/mL. *Tessaria absinthioides* exhibited the best inhibition towards hyaluronidase with an IC_{50} of 93.2 ± 4.3 μg/mL. Under the same experimental conditions, the reference compounds quercetin and indomethacin presented IC_{50} values of 340.0 ± 17.0 and 502.0 ± 10.0 μg/mL, respectively.

Among the most active species, 13 compounds were tentatively identified by HPLC-ESI-MS in *E. multiflora* and *P. lucida,* and 12 compounds in *T. absinthioides*. The constituents included caffeoyl- and feruloylquinic acid derivatives, flavonoids, simple phenolics and sesquiterpene glycosides.

Conclusions: Six out of the 13 species investigated showed a moderate to strong effect towards the enzyme $sPLA_2$ (> 40% inhibition at 200 μg/mL) while three species presented a strong activity against LOX with IC_{50} < 250 μg/mL and three were very active against hyaluronidase. Most of the crude drug extracts were able to stabilize the red blood cells membrane, preventing their lysis. The compounds identified in the extracts explain, at least in part, the activity found in the samples. The effect observed for the most active species supports their traditional use as anti-inflammatory agents. However, more studies should be undertaken to disclose the potential of the Puna plants as anti-inflammatory crude drugs.

1. Introduction

The Andes highlands, known as Puna, extends throughout southern Peru, west of Bolivia, north of Chile and northwest of Argentina at altitudes ranging from 3300 to 5000 m above sea level. The Argentinean Puna comprises part of Salta, Jujuy, Tucumán and

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Table 1
Scientific name, common name, voucher number (kept at the Herbario de la Fundación Miguel Lillo, Tucumán, Argentina, LIL) and traditional uses of the plant species investigated. The aerial parts of all the plant species are used to prepare infusion, decoctions and tinctures with a medicinal purpose.

Plant family	Scientific name	Common name	Collection place	Voucher number	Traditional use
Asteraceae	*Baccharis boliviensis* (Wedd.) Cabr.	Lejía, tola chica, tola, tolilla	Punta de la Peña, Catamarca; 26° 1′S 67°20′O	607936/LIL	Muscular and gastric pain, antiseptic, anti-inflammatory and gastroprotective. Used to splint broken bones (Villagrán et al., 2003)
	Baccharis incarum (Wedd.) Perkins	Lejía, tola	Punta de la Peña, Catamarca; 26° 1′S 67°20′O	607934/LIL	Antiseptic, anti-inflammatory, anti-pyretic, anti-diarrheic, gastroprotective, to relieve rheumatism and stomach pain, liver and prostate illness, to heal wounds, burns and ulcers (Cuello, 2006; Alberto et al., 2009).
	Chiliotrichiopsis keidelii Cabrera	Tola vizcacha, vizcachera tola, trompo, tola, tola tuni, lejía ploma	Aguas Calientes, Salta; 25° 41′S 66°46′O	610652/LIL	Antiseptic (Zampini et al. 2009b).
	Chuquiraga atacamensis Kuntze	Monte de suri, lengua de gallina, azafrán, quebrolla, San Pedro	Punta de la Peña, Catamarca; 26° 1′S 67°20′O	607929/LIL	Abortive, for ovary and womb illness. For flu, inner fevers, and cooling (Villagrán et al., 2003; Cuello, 2006).
	Nardophyllum armatum (Wedd.) Reiche	Suri-Yanta	Quebrada Seca, Catamarca; 26° 1′S 67°14′O	607928/LIL	For flu, cough, indigestion and diarrhea (Alonso and Desmarchelier, 2015)
	Parastrephia lepidophylla (Wedd.) Cabrera	Tola, tola de vaca	Laguna de Pozuelo, Jujuy; 23°30′S 66°55′O	68979/LIL	In patches, plasters and cataplasms for broken bones and to relieve pain, to lose weight. Used as anti-inflammatory, antiseptic and in a mixture with *Chuquiraga atacamensis* for flu, fever and colds (Villagrán et al., 2003).
	Parastrephia lucida (Meyen) Cabrera	Romero, tola, chachacoa, tola de río, tola de agua	Punta de la Peña, Catamarca; 26° 1′S 67°20′O	607923/LIL	To make patches, plasters and cataplasms for broken bones and bruises. To relieve pain, febrifuge, antiseptic and anti-inflammatory (Ariza Espinar and Novara, 2005; García and Beck, 2006).
	Parastrephia phyliciformis (Meyen) Cabrera	Tola, tola de río	Huaca Huasi, Tucumán; 26°39.30′S 65°44.23′O	487802/LIL	Anti-inflammatory for toothache, to make plasters and cataplasms to heal broken bones. To relieve pain and as antiseptic (Ariza Espinar and Novara, 2005; Villagrán et al., 2003).
	Tessaria absinthioides (Hook. & Arn.) DC	Pájaro bobo, sorona, hierba de zorra	El Peñón, Catamarca; 26° 28′S 67°15′O	607895/LIL	To treat rheumatism, prostate illness, cancer, and to lower cholesterol (Villagrán et al., 2003; Cuello, 2006).
Ephedraceae	*Ephedra multiflora* Phil. Ex Stapf.	Tramontana	Punta de la Peña, Catamarca; 26° 1′S 67°20′O	607941/LIL	Aerial parts are chewed for toothache. Used to stop bleeding and for "air pain" (Villagrán et al., 2003; Pérez, 2006).
Rosaceae	*Tetraglochin cristatum* (Britton) Rothm	Horizonte, canguia, rancha-rancha	Huaca Huasi, Tucumán; 26°39.30′S 65°44.23′O	610669/LIL	Antiseptic (García and Beck, 2006).
Verbenaceae	*Acantholippia deserticola* (Phil.) Mold.	Rica rica	Laguna Blanca, Catamarca; 26°30′S 66°40′O	607917/LIL	For cold, flu, diarrhea, inflammation, fevers, gastrointestinal inflammation, dyspepsia, liver disorders, against malaria and as sedative (Pérez, 2006).
	Junellia seriphioides (Gillies et Hook.) Mold.	Monte de lagarto, leña de lagarto, rosita, perilla	El Bolsón, Catamarca; 27°55′00″S 65°52′60″O	610854/LIL	Diuretic, analgesic, for stomach pain, indigestion, fever, flu, cough and bladder inflammation (Villagrán et al., 2003; Cuello, 2006).

Catamarca provinces, in an altitudinal level ranging between 2600 and 4800 m.a.s.l. It is a cold and arid region characterized by high radiation levels, strong winds and an important fluctuation of temperature. The vegetation is dominated by an alternation of grasses and shrubs steppes (tolares) that are used by the Puna inhabitants as a source of food, medicine, forage and building (García and Beck, 2006).

In northwestern Argentina, there is a rich tradition in the use of native medicinal and food plants that can be traced back to the old cultures settled in the area before Spanish conquest. At present times, the population of the Puna has mostly quechua and aymara roots and their knowledge about the natural resources has been transmitted orally throughout generations. Puna inhabitants use local medicinal plants as a complement to modern medicine. They are commercialized in plastic bags (some 30 g of crude drug) in the region and in fruit and herbal markets in many cities of northwestern Argentina (Barbarán, 2008; Pérez, 2006).The use of plants in infusions, decoction, poultices or cataplasms to relieve inflammation and/or pain caused by diverse etiological agents is common in the highlands.

Although necessary as a defensive barrier of our organism, inflammation is associated to several chronic diseases of major medical importance, including sclerosis, atherosclerosis, allergies, asthma (Dwyer et al., 2004), Alzheimer, cancer, diabetes, obesity arthritis, osteoarthritis, psoriasis, chronic gastritis, ulcers, among others (Roy et al., 2012). Assays that determine the effect of crude drugs on inflammation markers such as eicosanoids (through inhibition of its producing enzyme), interleukins, cytokines like tumor necrosis factor-α (TNF-α), nitric oxide, C-reactive proteins in serum, platelet aggregation factor (PAF), among others, are used to determine the potential of medicinal plants as anti-inflammatory agents (Strömstedt et al., 2013).

Previous studies carried out in our laboratory have shown that the Puna plants analysed in this study are able to inhibit the activity and the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), and to scavenge nitric oxide produced by the aforementioned enzyme (Alberto et al., 2009; D'Almeida et al., 2012; Torres Carro et al., 2015). Therefore, more studies have been carried out to asset their potential as anti-inflammatory crude drugs. The aim of this work was to assess the inhibitory effect of selected Puna plants on pro-inflammatory enzymes (lipoxygenase, phospholipase and hyaluronidase), according to the traditional use as anti-inflammatory agents, and to provide evidence on the possible effectiveness of the Argentinean highland crude drugs as anti-inflammatory agents.

2. Materials and methods

2.1. Chemicals

Soybean lipoxygenase, caffeic acid, potassium ferricyanide and dimethyl aminobenzaldehide were purchased from Sigma-Aldrich (MO, USA). Hyaluronidase, linoleic acid and dimethyl sulfoxide (DMSO) were obtained from Merck (Germany). Potasium hyaluronidate was purchased from Calbiochem, (USA). Triton X-100 was supplied by Fluka Chemical Corp. (USA). 1,2-diheptanoylthio-glycerophosphocholine (1,2 dHGPC), secretory phospholipase A_2 ($sPLA_2$) from bee venom and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Cayman Chemical Co. (MI, USA). Trichloroacetic acid, potassium persulfate and $FeSO_4$ were from Cicarelli (Sta. Fe, Argentina). 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) was from Fluka. $FeCl_3$ was from Biopak (Bs.As., Argentina). Other chemicals were of analytical grade quality.

2.2. Plant material

The plants included in this study were collected at different altitudinal levels in the highlands of the Puna phytogeographic region of northwestern Argentina. The plants were selected according to literature and field data. The collects were carried out in March 2010. The scientific and common names, voucher number and traditional uses with references are summarized in Table 1. The aerial parts of the plants were used for these studies as indicated by traditional healers. The voucher specimens are kept in the Herbarium of Fundación Miguel Lillo (LIL), Tucumán, Argentina.

2.3. Extraction

The air-dried plant material was powdered and extracted by maceration with EtOH:H_2O 20:80, in a 20 g powdered plant per 100 mL of solvent ratio. The maceration was performed for 7 days under shaking (40 cycles/min) at room temperature. After filtering, the extracts were taken to dryness under reduced pressure and then lyophilized. The % w/w extraction yields from dry plant material was as follows: *Baccharis boliviensis* (15.65), *Baccharis incarum* (13.72), *Chiliotrichiopsis keidelii* (15.32), *Chuquiraga atacamensis* (10.67), *Nardophyllum armatum* (12.68), *Parastrephia lepidophylla* (17.78), *Parastrephia lucida* (15.46), *Parastrephia phyliciformis* (16.09), *Tessaria absinthioides* (18.49), *Ephedra multiflora* (12.17), *Tetraglochin cristatum* (8.23), *Acantholippia deserticola* (12.33) and *Junellia seriphioides* (8.73). For the different assays, the lyophilized extracts were dissolved in DMSO to obtain stock solutions of 50 mg/mL, which were stored at 4 °C in the dark.

2.4. In vitro anti-inflammatory activity

2.4.1. Inhibition of secretory phospholipase A_2

$sPLA_2$ plays an important role in inflammation as the first enzyme in the arachidonic acid (AA) pathway. This enzyme catalyzes the hydrolysis of membrane phospholipids, releasing AA and a lysophospholipid. Once the AA is released from the cellular membrane, it is later oxidized by COX and LOX enzymes to produce eicosanoids. This is a rate-limiting step of the biosynthesis of inflammation mediators (Balsinde et al., 1999). Therefore, $sPLA_2$ would be a promising target for new anti-inflammatory drugs and there lays the importance of searching for $sPLA_2$ specific inhibitors.

The $sPLA_2$ activity was determined using 1,2-diheptanoylthio-glycerophosphocholine (1,2-dHGPC) and Triton X-100 as substrates according to Torres Carro et al. (2016). To reconstitute the substrate to a final concentration of 1.25 mM, a buffer Tris–HCl (10 mM, pH 8) with $CaCl_2$ (10 mM), KCl (100 mM) and Triton X-100 (0.3 mM) was used. The reaction mixture contained 50 µL of buffer Tris–HCl (10 mM, pH 8), 10 µL of DTNB (10 mM), 10 µL of enzyme $sPLA_2$ (1 mg/mL) and 200 µg/mL of extract or commercial anti-inflammatory drug (acetylsalicylic acid, 20–80 µg/mL). The reaction started with the addition of 150 µL of 1,2 dHGPC (1.66 mM) and was maintained for 20 min at 25 ºC. The absorbance was read at 414 nm after 20 min using a microplate reader (Absorbance Microplate Reader Biotek ELx808™).

2.4.2. Inhibition of lipoxygenase

This enzyme is a relevant target in inflammatory processes. LOX activity is associated to many chronic diseases like asthma, allergic disorders, liver fibrosis, atherosclerosis, among others (Dwyer et al., 2004). Since COX and LOX share the same substrate (AA), inhibiting COX pathway leads to a shift towards the production of leukotriene (pro-inflammatory mediator) by 5-LOX, due to increased substrate availability (Hudson et al., 1993). As a result, it is necessary to search for dual inhibitors to treat chronic inflammation effectively.

LOX activity was determined spectrophotometrically according to Taraporewala and Kauffman (1990). The method is based on the enzymatic oxidation of linoleic acid to its hydroperoxide. To determine hydroperoxide, soy lipoxygenase-1 (948 U/mL) was incubated with linoleic acid (50 µM) in sodium borate buffer (200 mM, pH 9.0) for 4 min at 25 °C. The absorbance at 234 nm was measured using a Spectronic Unicam (Genesys) spectrophotometer. The inhibitory as-

says were performed in presence of extracts at different concentrations (50–400 μg/mL). The effect was evaluated by calculating the percentage of inhibition of hydroperoxide production from the ΔOD (optical density) values at 234 nm after 3 min of incubation. The compounds concentration leading to a 50% inhibition of hydroperoxide release (IC_{50}) was calculated by regression analysis using a concentration-inhibition response curve. Caffeic acid (up to 100 g/mL) and naproxen (up to 25 μg/mL) were used as reference compounds.

2.4.3. Inhibition of hyaluronidase

Hyaluronic acid is an important component of the extracellular matrix of soft connective tissue, synovial liquid, vitreous humor, among others. It is degraded by hyaluronidase, which has been found to be overexpressed in chronic diseases like rheumatoid arthritis and osteoarthritis. It is also strongly related to allergic conditions, inflammation, migration of cancer, etc. (Shibata et al., 2002). On inflammation, hyaluronidase increases tissues permeability and favors the spreading of the inflammatory response throughout the affected organ. Moreover, its products of degradation have angiogenic properties, stimulate pro-inflammatory cytokines and the immune system action, and induce the expression of inflammation-related genes (Stern, 2004).

Hyaluronidase activity was evaluated following the method of Lee et al. (1993), by estimating the amount of N-acetyl glucosamine (NAGA) released by hyaluronidase from its substrate, sodium hyaluronidate. The reaction mixture contained different volumes of sodium acetate buffer (0.2 M, pH 4.5) to a final volume of 70 μL, 5 μL of hyaluronidase (57 U) and 20 μL of $CaCl_2$ (0.125 M); it was pre-incubated for 10 min at 37 °C. Fifty microliters of sodium hyaluronidate (1.33 mg/mL) were then added in presence and absence of different extract concentrations (100–800 μg/mL) and the reaction mixture was incubated for 30 min at 37 °C. The reaction was stopped by the addition of 50 μL of potassium tetraborate 0.4 M, followed by heating at 100 °C for 3 min. The reaction mixture was cooled and 1.5 mL of p-dimetylamino benzaldehyde (1 mg/mL) was added. Then, the mixture was incubated at 37 °C for 15 min and the variation of the absorbance was monitored at 550 nm using a microplate reader (Absorbance Microplate Reader Biotek ELx808^{TM}). Indomethacin and quercetin were employed as reference drugs (50–200 μg/mL). The inhibitory concentration of enzymatic activity by 50% (IC_{50}) was calculated by interpolation of the concentration-enzyme activity curves.

2.4.4. Stabilization of human red blood cells membrane (HRBC)

It is important to prevent and limit the release of lysosomal enzymes to the extracellular matrix because of a damage on neutrophils membrane, in a bid to reduce a further harm to the affected tissue and to limit the inflammatory response. The human red blood cells membrane (HRBC) method is widely used as an *in vitro* evaluation of anti-inflammatory property because of the red blood cells membrane similarity to lysosomal membrane. Therefore, it is expected that an extract capable of preventing red blood cell lysis could be able to alleviate some symptoms of inflammation (Azeem et al., 2010; Saleem et al., 2011).

2.4.4.1. Human red blood cell suspension. Blood was collected from healthy volunteers and was mixed with an equal volume of a sterile Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride). The blood was centrifuged and the red cells were washed with isotonic NaCl solution (0.85%, pH 7.2) four times. Afterward, a 10% v/v suspension of red cells was made using an isotonic NaCl solution and was stored at 4 °C.

2.4.4.2. Cell membrane stabilization assay. The assay was performed as described by Torres Carro et al. (2016) to study the anti-inflammatory activity of plant extracts. Indomethacin and dexamethasone were used as standard drugs. Different concentration of the extracts (400–1500 μg/mL) were mixed with 333 μL of sodium phosphate buffer (0.15 M, pH 7.4), 200 μL of HRBC suspension and deionized water for a total volume of 1500 μL. DMSO was used as solvent control instead of the extracts. All the assay mixtures were incubated at 37 °C for 30 min and then centrifuged at 650x*g* for 1 min. The hemoglobin content in the supernatant was estimated at 550 nm using a microplate reader (Absorbance Microplate Reader ELx808^{TM}). The percentage of HRBC membrane stabilization was calculated using the following formula:

$$\%\ \text{Lysis prevention} = 100 - [(A_0 - As)/A_0] \times 100$$

where A_0 is the absorbance of the control without extract and an equivalent amount of DMSO, and As is the absorbance in presence of the extract. DMSO concentration in the mixture was less than 3%.

2.5. Antioxidant activity

Most of LOX inhibitors exert their action on the enzyme active site by chelating the iron, by reducing it to its ferrous form, or by scavenging electrons participating in the redox cycle of iron. Therefore, LOX inhibitors can be classified as redox active compounds, iron ligand inhibitors and non-redox inhibitors (Werz and Steinhilber, 2005).

2.5.1. Iron III to Iron II reductive capacity

Regulation of LOX activity is associated to a cyclic redox reaction of a non-heme iron group located in its active site. When the enzyme is in a resting state, the iron is in its reduced form (Fe^{2+}), but is quickly oxidized to its ferric state (Fe^{3+}) by hydroperoxides, which allows LOX to enter into a catalytic cycle in which the iron acts as an electron acceptor and donor (Radmark et al., 2007).

The extracts capacity to reduce Fe (III) to Fe (II) was assessed spectrophotometrically according to D'Almeida et al. (2013). Potassium ferricyanide 1% (416 μL) was mixed with different concentrations of the extracts (50-400 μg/mL), and phosphate buffer (0.1 M; pH 6.3) was added to reach a final volume of 1 mL. The mixture of reaction was incubated for 20 min at 50 °C and then 416 μL of 10% trichloroacetic acid was added to stop the reaction. Finally, the mixture was centrifuged for 10 min at 650x*g*. An aliquot of the supernatant (416 μL) was mixed with 416 μL of distilled water and 83 μL of 0.1% $FeCl_3$,and was incubated for 10 min at room temperature. The absorbance was measured at 700 nm using a Spectronic Unicam (Genesys) spectrophotometer. The absorbance values were used to determine the concentration at which the absorbance is 0.5. The 50% of reducing power (RC_{50}), expressed as micrograms per milliliter (μg/mL), was calculated by interpolation from a linear regression analysis (D'Almeida et al., 2013). Ascorbic acid (2–16 μg/mL) and gallic acid (0.4–3.5 μg/mL) were used as positive controls.

2.5.2. Iron chelating activity

The chelation of ferrous ions by natural products was determined according to D'Almeida et al. (2013). Briefly, 6 μL of 2 mM $FeSO_4$ were added to different concentration of the extracts (200–500 μg/mL) or positive control Na_2EDTA (5–20 μg/mL) and ultrapure water to a final volume of 143 μL. The reaction was initiated by the addition of 7 μL of 5 mM ferrozine solution which forms a colored complex with Fe^{2+}. The mixture was shaken and maintained at room temperature for 10 min. The absorbance was measured using a microplate reader (Thermo Scientific Multiskan GO) at 562 nm. The percentage of inhibition of complex formation was calculated using the following formula:

$$\%\ \text{Inhibition} = 100 - [(A_0 - As)/A_0] \times 100$$

where A_0 is the absorbance of the control without extract and an equivalent amount of DMSO, and As is the absorbance in presence of

the extract.

2.5.3. ABTS free radical scavenging activity

There is a proven relationship between inflammatory diseases and oxidative stress, since inflammatory processes generate a large amount of free radicals. It is well known that many pathological processes are caused by free radicals; such is the case of cardiovascular disorders, cancer, diabetes mellitus, rheumatoid arthritis, acute and chronic inflammatory processes, osteoporosis, ulcers, sunburn, neurodegenerative diseases, among others (Sies, 2013). Hence, the importance of finding natural products with anti-inflammatory activity that could also act as free radical scavengers. The $ABTS^{\bullet+}$ free radical scavenging assay is a widely used method to determine the total antioxidant activity of fluids.

The antioxidant capacity was evaluated using the improved $ABTS^{\bullet+}$ method described by D'Almeida et al. (2013). One hundred microliters of an $ABTS^{\bullet+}$ solution (absorbance of 0.7 at 750 nm) was added to different concentrations of the extracts (10–200 μg/mL) to a final volume of 200 μL. The absorbance was measured at 750 nm with a microplate reader (Thermo Scientific Multiskan GO). The percentage of scavenging was measured after 6 min of reaction, using the following formula:

$$\% \text{ scavenging} = 100 - [(A_0 - As)/A_0] \text{ x } 100$$

where A_0 is the absorbance of the control without extract and an equivalent amount of DMSO, and As is the absorbance in presence of the extract. The SC_{50} is defined as the concentration of the extracts, in micrograms per milliliter (μg/mL), necessary to scavenge 50% of the ABTS free radicals. Quercetin (2–20 μg/mL) and ascorbic acid (0.3–3 μg/mL) were used as positive controls.

2.6. Hemolysis

In previous studies, we evaluated the toxic effect of these selected plant species on the brine shrimp *Artemia salina*. None of them was toxic up to a concentration of 1000 μg/mL (Torres Carro et al., 2015). Nonetheless, more assays are needed to confirm these findings. The hemolytic activity assay has been used as an indicator of general toxicity since it provides information about the interaction of bioactive molecules and the cell membrane (Araújo deOliveira et al., 2009).

To determine the effect of the extracts on erythrocytes, we used a slightly modified version of the method described by Ahmad and Aquil (2007). Different concentration of the extracts (200–1000 μg/mL) were mixed with 334 μL of phosphate buffer (0.15 M, pH 7.4), 168 μL of HRBC suspension (prepared according to the procedure described in 2.4.4.1) and different volumes of a 0.85% NaCl solution for a total volume of 1500 μL. A 0% hemolysis control was also done by replacing the extracts for DMSO as solvent control (final concentration less than 3%). For the 100% hemolysis control, deionized water was added instead of isotonic NaCl solution. Triton X-100 (50–125 μg/mL) was used as positive control. All the assay mixtures were incubated at 37 °C during 30 min, and then centrifuged at 650x*g* for 1 min. The hemoglobin content in the supernatant was measured at 550 nm using a microplate reader (Absorbance Microplate Reader ELx808^{TM}). The percentage of hemolysis was calculated using the following formula:

$$\% \text{ of hemolysis} = 100 - [(A_0 - As)/A_0] \text{ x } 100$$

where A_0 is the absorbance of the control without extract and an equivalent amount of DMSO, and As is the absorbance in presence of the extract.

2.7. Chemical profiling

The most active extracts were analyzed by HPLC-ESI-MS/MS to get an insight into the constituents of the crude drugs. Mass spectra were recorded using an Agilent 1100 (Agilent Technologies Inc, CA, USA) liquid chromatography system connected through a split to an Esquire 4000 Ion Trap LC/MS system (Bruker Daltoniks GmbH, Germany). Ionization was performed at 3000 V, assisted by nitrogen as nebulizing gas at 27.5 psi and as drying gas at 350 ºC and a flow rate of 8 L/min. Negative ions were detected using full scan (m/z 20–2200) and normal resolution (scan speed 10,300 $m/z/s$; peak with 0.6 FWHM/m/z). The trap parameters were set in ion charge control (ICC) using manufacturer default parameters, and maximum accumulation time of 200 ms.

The mass spectrometric conditions for analysis were: electrospray needle, 4000 V; end plate offset, -500 V; skimmer 1, 56.0 V; skimmer 2, 6.0 V; capillary exit offset, 84.6 V; capillary exit, 140.6 V. Collision induced dissociation (CID) spectra were obtained with a fragmentation amplitude of 1.00 V (MS/MS) using helium as the collision gas and was automatically controlled through Smart Frag option. A MultoHigh 100 RP 18-5μ (250x4.6 mm) column (CS-Chromatographie Service GmbH, Langerwehe, Germany), maintained at 25 °C, was used for the HPLC analysis. Approximately 5 mg of each extract was dissolved in 1 mL MeOH:H_2O (1:1 v/v), filtered through a 0.45 μm PTFE filter (Waters) and submitted to HPLC-ESI-MS/MS analysis. The compounds were monitored at 254 nm. The HPLC analyses were performed using a linear gradient solvent system consisting of water (A), acetonitrile (B) and 1% formic acid (C) as follows: 30% A, 20% B and 50% C, over 20 min, followed by 18% A, 12% B and 70% C for 35 min. The flow rate was 1 mL/min. The volume injected was 20 μL.

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 deviation (SD). The 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 and discussion

3.1. In vitro anti-inflammatory activity

3.1.1. Inhibition of secretory phospholipase A_2 ($sPLA_2$)

The extracts from the selected Puna plants were assessed for their inhibitory effect towards the enzyme $sPLA_2$ at a concentration of 200 μg/mL. The results, expressed as percentage of enzyme inhibition, are summarized in Fig. 1. The most active samples were *P. lucida* and *B. boliviensis* with 63% and 53% inhibition, respectively. Other plants, including *P. lepidophylla, P. phyliciformis, B. incarum* and *T. absinthioides,* inhibited the enzyme in a range of 40–48%. All the active species belong to Asteraceae plant family. Under our experimental conditions, the reference drug acetylsalicylic acid showed an IC_{50} of 65 ± 1 μg/mL.

3.1.2. Inhibition of lipoxygenase (LOX)

The activity of the different extracts against LOX is summarized in Fig. 2. The IC_{50} values of the samples ranged between 132.6 ± 2.0 and 389.2 ± 12.3 μg/mL (Table 2). The most active species was *E.multiflora*, with an IC_{50} value of 132 μg/mL. Under the same experimental conditions, the IC_{50} values of caffeic acid and naproxen were 57.0 and 14.0 μg/mL, respectively. When compared to the reference compounds, the activity of *E. multiflora* should be regarded as high.

3.1.3. Inhibition of hyaluronidase

The effect of the Puna plant extracts on the enzyme hyaluronidase is shown in Fig. 3, while the IC_{50} values of the most relevant species is summarized in Table 2. The most active species was *T. absinthioides*, with an IC_{50} value of 93.2 ± 4.3 μg/mL, which was about 8-fold lower

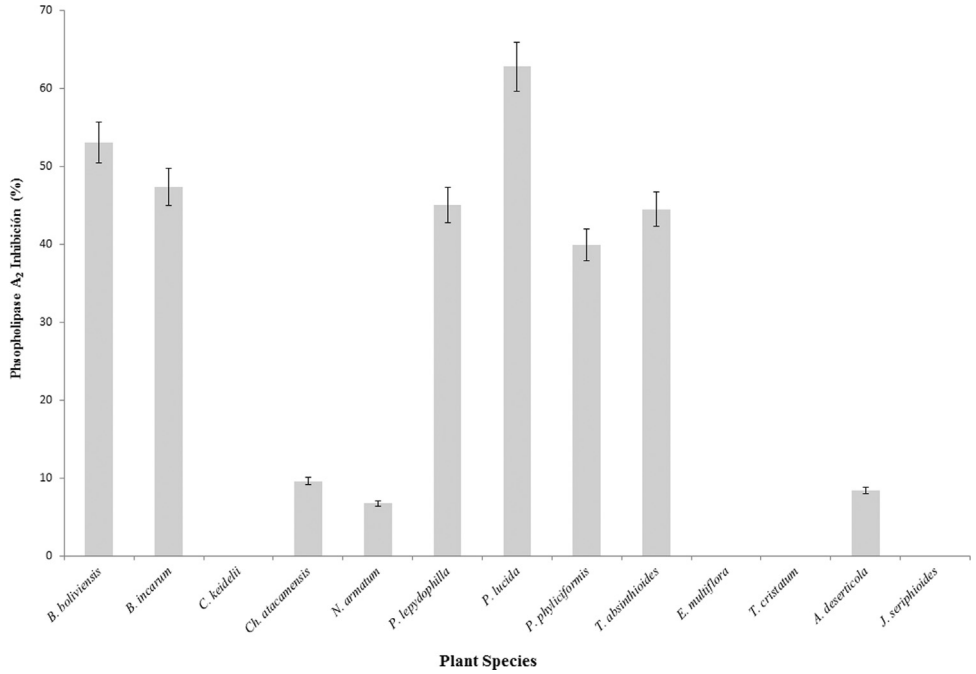

Fig. 1. Secretory phospholipase A_2 inhibition by the hydroalcoholic plant extracts used as anti-inflammatory in the Argentinean Puna. Extracts were assayed at 200 μg extract/mL. Data are presented as mean percent inhibition ± SD of three different experiments. Acetylsalicylic acid (IC_{50} of 65 ± 1 μg/mL) was used as reference compound. For the plant identification, please, see Table 1.

than the reference drug indomethacin (502.0 ± 10.0 μg/mL), and 3.6-fold lower than quercetin (340.0 ± 17.0 μg/mL). *E. multiflora* also exhibited a good inhibitory capacity with an IC_{50} value of 398.4 ± 4.7 μg/mL. Shibata et al. (2002) used the anti-allergic drug disodium cromoglycate (DSCG) and the polyphenols catechin and epigallocatechin gallate (EGCG) as efficient inhibitors of hyaluronidase activity. Their IC_{50} values were 140, 180 y 90 μg/mL, respectively. According to this report, *T. absinthioides* is more active than DSCG and catechin, and has a similar activity than EGCG, which is interesting for a crude extract.

3.1.4. Stabilization of human red blood cells membrane (HRBC)

The results of the HRBC stabilization assay are presented in Fig. 4. The most active species were the Asteraceae plant species *P. lepidophylla*, *P. phyliciformis*, *B. incarum* and *B. boliviensis*, and the Verbenaceae *J. seriphioides*. This plant species showed similar or higher values of stabilization than extracts of other plant species with anti-inflammatory activity from India (Azeem et al., 2010; Saleem et al., 2011). The IC_{50} values of the most active species are shown in Table 2. Indomethacin at 0.1, 0.2 and 0.4 mg/mL, showed 28%, 38% and 32% protection, respectively, and was not protective at 1.5 mg/mL. Dexamethasone was less active, showing at 0.1, 0.2 and 0.4 mg/mL,

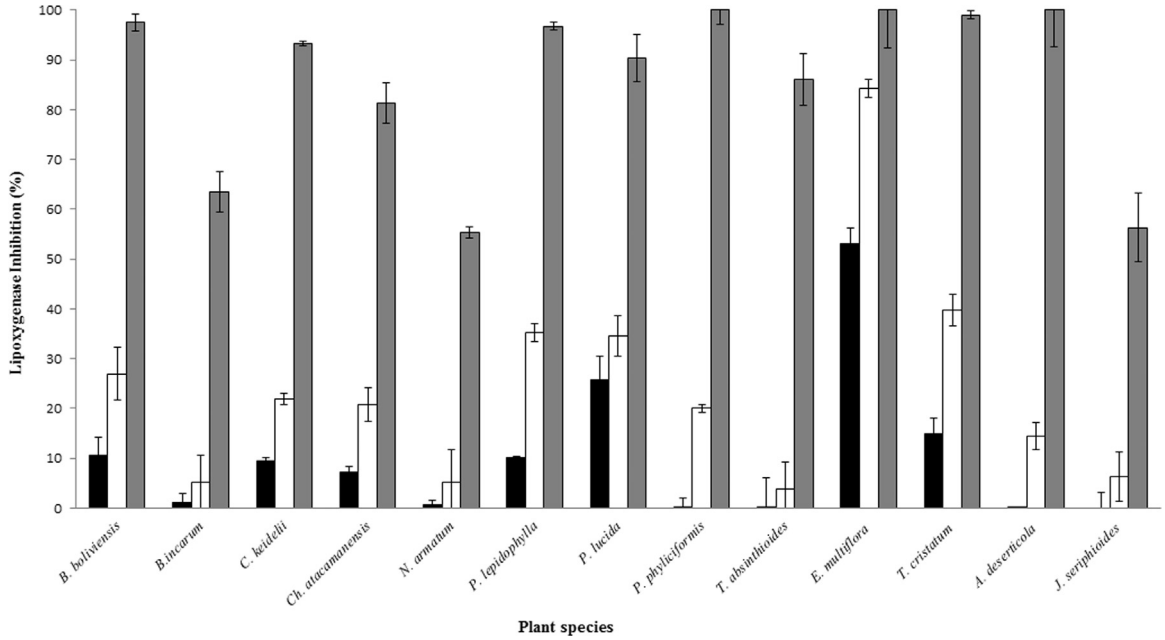

Fig. 2. Lipoxygenase inhibition by the hydroalcoholic plant extracts used as anti-inflammatory in the Argentinean Puna. Percentage of inhibition at 150 μg/mL (□), 200 μg/mL (▒) and 400 μg/mL (■). Caffeic acid (IC_{50} 57.0 ± 2.8 μg/mL) and naproxen (IC_{50} 14.0 ± 0.5 μg/mL) were used as positive controls. Data are presented as mean percent inhibition ± SD of three different experiments. For the plant identification, please, see Table 1.

Table 2
LOX inhibition mechanism of hydroalcoholic extracts of Argentinean Puna plants.

Scientific name	Reducing power RC_{50} (μg/mL)	Iron chelating capacity Q (%)	$ABTS^{\bullet+}$ radical scavenging SC_{50} (μg/mL)	LOX IC_{50} (μg/mL)	Hyaluronidase IC_{50} (μg/mL)	HRBC stabilization IC_{50} (mg/mL)
Baccharis boliviensis	72.5 ± 0.1^{e}	$35.1 \pm 2.7^{a,b,c,d}$	69.6 ± 1.5^{g}	$270.9 \pm 5.6^{e,f}$	-	1.29 ± 0.07^{b}
Baccharis incarum	158.8 ± 3.6^{f}	$13.9 \pm 5.1^{a,b}$	101.0 ± 1.4^{i}	367.1 ± 5.0^{h}	-	1.15 ± 0.08^{b}
Chiliotrichiopsis keidelii	46.8 ± 2.5^{d}	$38.9 \pm 6.2^{a,b,c,d}$	46.5 ± 0.7^{d}	$271.3 \pm 4.5^{e,f}$	-	0.82 ± 0.03^{a}
Chuquiraga atacamensis	191.6 ± 0.4^{g}	$43.8 \pm 1.6^{c,d}$	75.5 ± 0.7^{h}	$303.3 \pm 6.3^{f,g}$	-	-
Nardophyllum armatum	$38.3 \pm 1.0^{b,c,d}$	$19.5 \pm 0.8^{a,b,c}$	30.0 ± 0.1^{b}	382.7 ± 6.0^{h}	-	-
Parastrephia lepidophylla	$29.3 \pm 1.9^{b,c}$	12.5 ± 5.9^{a}	25.7 ± 0.4^{b}	219.5 ± 2.1^{d}	-	$1.08 \pm 0.01^{a,b}$
Parastrephia lucida	62.2 ± 2.7^{e}	$34.4 \pm 5.1^{a,b,c,d}$	57.0 ± 0.1^{e}	$253.5 \pm 6.9^{d,e}$	-	-
Parastrephia phyliciformis	$30.8 \pm 2.4^{b,c}$	$35.0 \pm 3.0^{a,b,c,d}$	27.5 ± 0.1^{b}	$253.1 \pm 0.1^{d,e}$	-	$1.09 \pm 0.04^{a,b}$
Tessaria absinthioides	$40.3 \pm 0.5^{c,d}$	$15.5 \pm 5.9^{a,b,c}$	$43.3 \pm 3.0^{c,d}$	319.5 ± 5.6^{g}	93.2 ± 4.3^{a}	-
Ephedra multiflora	28.3 ± 0.9^{b}	$20.4 \pm 0.1^{a,b,c}$	26.2 ± 0.4^{b}	132.6 ± 2.0^{c}	398.4 ± 4.7^{c}	-
Tetraglochin cristatum	$29.7 \pm 0.6^{b,c}$	$41.9 \pm 6.0^{b,c,d}$	38.2 ± 1.1^{c}	217.8 ± 4.1^{d}	$367.2 \pm 3.3^{b,c}$	-
Acantholippia deserticola	181.9 ± 4.7^{g}	57.2 ± 9.4^{d}	64.0 ± 3.5^{f}	$277.5 \pm 8.0^{e,f}$	-	-
Junellia seriphioides	333.3 ± 0.1^{h}	$38.4 \pm 8.5^{a,b,c,d}$	131.1 ± 0.9^{j}	389.2 ± 12.3^{h}	873.0 ± 9.1^{e}	1.23 ± 0.05^{b}
Ascorbic acid	5.4 ± 0.03^{a}	-	1.7 ± 0.4^{a}	-	-	-
Gallic acid	1.6 ± 0.01^{a}	-	-	-	-	-
Quercetin	-	-	3.6 ± 0.4^{a}	-	340.0 ± 17.0^{b}	-
Na_2EDTA	-	14.0 ± 0.3 (IC_{50} μg/mL)	-	-	-	-
Caffeic acid	-	-	-	57.0 ± 3.99^{b}	-	-
Naproxen	-	-	-	14.0 ± 0.70^{a}	-	-
Indomethacin	-	-	-	-	502.0 ± 10.0^{d}	-

Comparative iron reducing concentration (RC_{50}), iron chelating capacity (at 500 μg/mL), ABTS free radical scavenging concentration (SC_{50}), LOX inhibition concentration (IC_{50}), hyaluronidase (IC_{50}) and human red blood cell membrana stabilization (HRBC) (IC_{50}) of the hydroalcoholic plant extracts investigated. Values (mean ± SD, n=3) in the same column followed by a different letter are significantly different (Tukey test, $p \leq 0.05$).

stabilization values of 13%, 9% and 4%, respectively.

3.2. Antioxidant activity

3.2.1. Iron III to Iron II reductive capacity

All plant extracts reduced Fe^{3+} in a dose-dependent manner. The most active species were *E. multiflora*, *T. cristatum* and *P. lepidophylla*, with RC_{50} values of 28.3 ± 0.9, 29.7 ± 0.6 and 29.3 ± 1.9 μg/mL, respectively. Even though they were less active than positive controls ascorbic acid and gallic acid, at the IC_{50} values obtained for LOX, 100% of the iron would be reduced.

3.2.2. Iron chelating activity

All the plant extracts exhibited a low capacity to scavenge Fe^{2+} (Table 2), meaning they might inhibit LOX activity preferentially by reducing its non-heme iron group and/or by scavenging the electrons of the iron redox cycle. At the highest concentration evaluated (500 μg/mL), *A. deserticola* was the most active species as Fe^{2+} scavenger, with

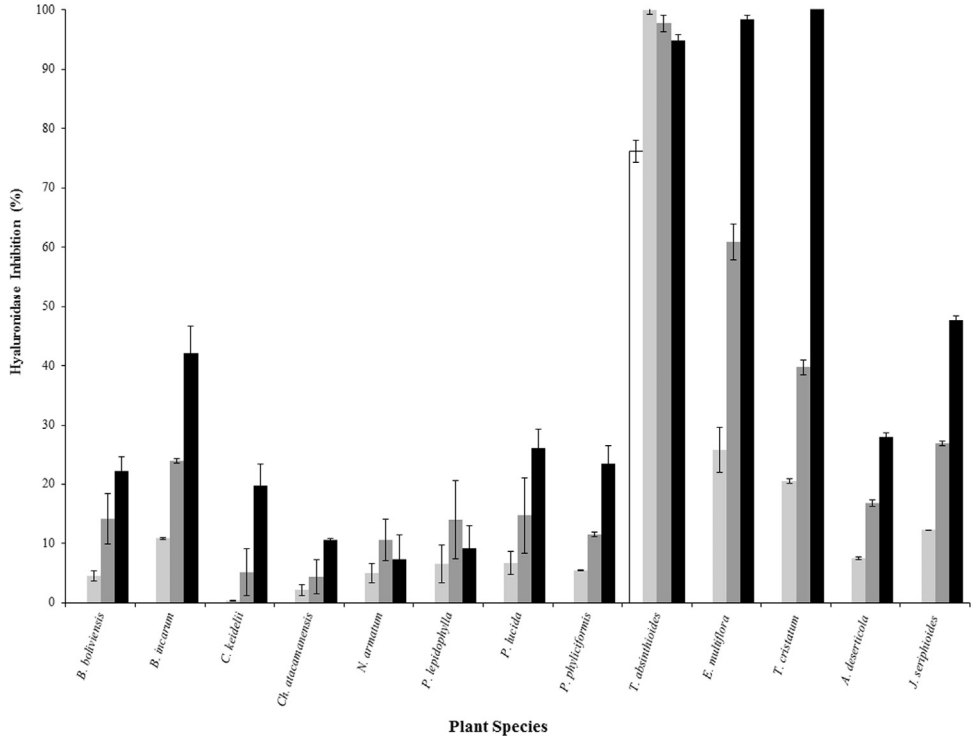

Fig. 3. Hyaluronidase inhibition by the hydroalcoholic plant extracts used as anti-inflammatory in the Argentinean Puna. Percentage of inhibition at 150 μg/mL (□), 200 μg/mL (▒), 400 μg/mL (▓) and 800 μg/mL (■). Indomethacin (IC_{50} 502.0 ± 7.1 μg/mL) and quercetin (IC_{50} 340.0 ± 12.0 μg/mL) were used as reference drug. Data are presented as mean percent inhibition ± SD of three different experiments. For the plant identification, please, see Table 1.

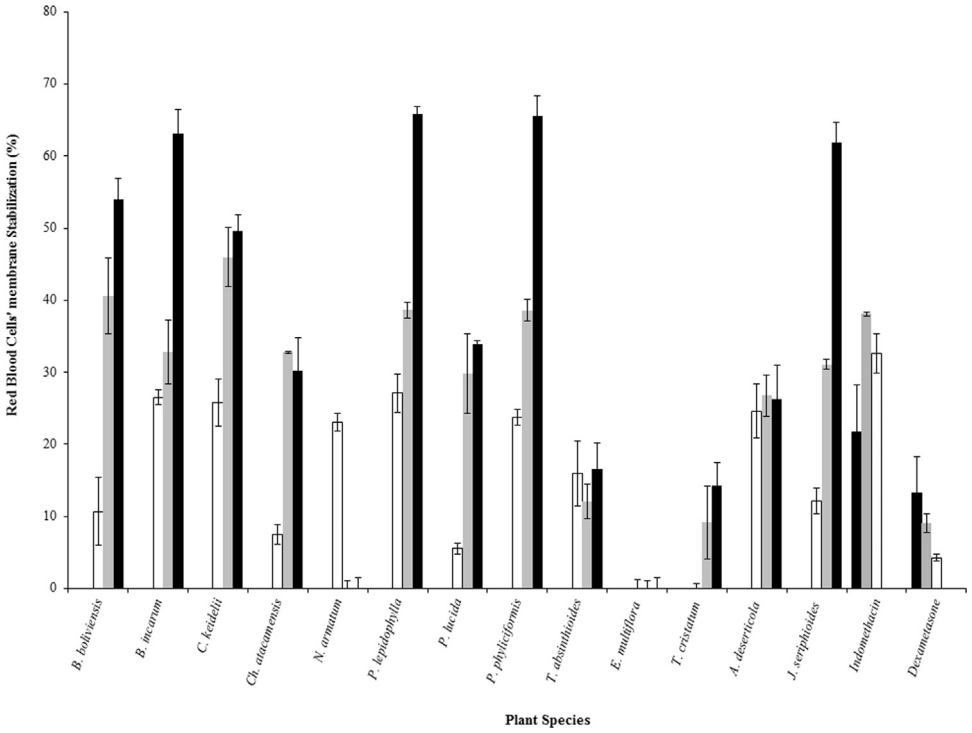

Fig. 4. Stabilization of red blood cells membrane by the hydroalcoholic plant extracts used as anti-inflammatory agents in the Argentinean Puna. Percentage of inhibition at 0.4 mg/mL (□), 0.8 mg/mL (▒) and 1.5 mg/mL (■) for the extracts; and 0.1 mg/mL (■), 0.2 mg/mL (▒) and 0.4 mg/mL (□) for indomethacin and dexamethasone. Data are presented as mean percent inhibition ± SD of three different experiments. For the plant identification, please, see Table 1.

an inhibition of 57.2 ± 9.4%.

3.2.3. ABTS free radical scavenging activity

As shown in Table 2, all plant extracts were able to scavenge $ABTS^{\bullet+}$ radical, being the most active species *P. lepidophylla*, *P. phyliciformis* and *E. multiflora* (SC_{50} of 25.7 ± 0.4, 27.5 ± 0.1 and 26.2 ± 0.4 μg/mL, respectively). Previous studies have demonstrated the $ABTS^{\bullet+}$ radical scavenging capacity of aqueous, ethanolic and methanolic extracts of some of these species (Zampini et al., 2008, 2009a, 2009b).

3.3. Hemolysis

The extracts from the selected plants were assessed for their hemolytic effect. Eleven out of the thirteen species studied did not show any hemolytic activity up to a concentration of 1000 μg/mL, except for *N. armatum* and *J. seriphioides*, which exhibited a HC_{50} (concentration that produce the hemolysis of 50% of the cells) of 680.6 ± 10.9 and 445.8 ± 7.3 μg/mL, respectively. The HC_{50} were still higher than the positive control Triton X-100 (HC_{50}= 114.5 ± 0.7 μg/mL).

3.4. Chemical profiling

Based on the new results and a previous report (Torres Carro et al., 2015), we selected for chemical profiling by HPLC-ESI-MS/MS the most active species in inhibiting different stages of the inflammatory response *in vitro*. The selected plants were *P. lucida* (inhibits $sPLA_2$, iNOS expression and COX-2 activity and expression), *E. multiflora* (inhibits LOX) and *T. absinthioides* (inhibits hyaluronidase, COX-2 and iNOS expression). The HPLC-ESI-MS/MS analysis of the samples (Fig. 5, Table 3) allowed the tentative identification of 13 compounds in *E. multiflora*, 13 in *P. lucida* and 12 in *T. absinthioides*. The constituents included *O*- and *C*-glycosyl flavonoids, caffeoylquinic acid derivatives, simple phenolics and for *T. absinthioides,* also flavonoid and sesquiterpene sulphates.

Selected ion chromatograms were used to identify the main constituents and related compounds in the extracts. The ions at *m/z* 353 and 191 were used for the caffeoylquinic derivatives, while 317, 301 and 285 were used for myrcetin, quercetin and kaempferol/luteolin derivatives, respectively. Detection of sulphate was by the neutral loss of 80 amu. The tentative identification of the constituents in the polar extracts investigated by HPLC-MS/MS is described below.

3.4.1. Ephedra multiflora

Some 13 compounds were detected/tentatively assigned in the extract investigated. Compounds A, B and C with $[M-H]^-$ ion at *m/z* 451, 421 and 425 amu, respectively, shows losses of 210, 180 and 184 amu, leading to the base peak at m/z 241. The identity of the compounds remains to be established and will need isolation and NMR studies for identification. The phenolics **1**, **2** and **12** were assigned based on the characteristic $[M-H]^-$ ion at *m/z* 289 for **1** and **2** and 575 for **12**, respectively. Fragmentation of compound **12** leads to the catechin/epicatechin ion at *m/z* 289 and was assigned as catechin/epicatechin dimer (**12**), while compounds **1** and **2** were identified as catechin/epicatechin. The mass spectra of compounds **4**, **6**, **7**, **8**, **11** and **13** with a $[M-H]^-$ ion at *m/z* 639, 609, 623, 609, 625 and 463, respectively, shows neutral loss of glucuronic acid (176 amu) and a hexose (162 amu) for **4**, rhamnose (146 amu) and hexose for **6** and **8**, rhamnose and glucuronic acid for **7**, two hexoses for **11** and one hexose for **13**, leading to the base peak at *m/z* 301, in agreement with quercetin (Q) glycosides. The compounds were tentatively identified as Q-glucuronate hexoside (**4**), Q-rhamnoside hexoside (**6**), Q-rhamnoside glucuronate (**7**), Q-rhamnoside hexoside isomer (**8**), Q-dihexoside (**11**), and Q hexoside (**13**). The compound **3**, with a $[M-H]^-$ ion at *m/z* 625, shows consecutive loss of hexose and rhamnose, leading to the *m/z* ion at 317 and was assigned as myricetin hexoside rhamnoside. The compounds **5** and **10** with *m/z* ions at 447 and 431 amu, were identified as C-glycosyl flavones on the basis of the characteristic losses of 120, 90 and 30 amu from the phenolic moiety and were identified as orientin/isoorientin and vitexin/isovitexin, respectively (Cuyckens and Claeys, 2004). The mass spectrum of compound **9** shows the loss of 308 amu (rhamnose and hexose), leading to the base peak at *m/z* 285, compatible with luteolin rhamnoside hexoside.

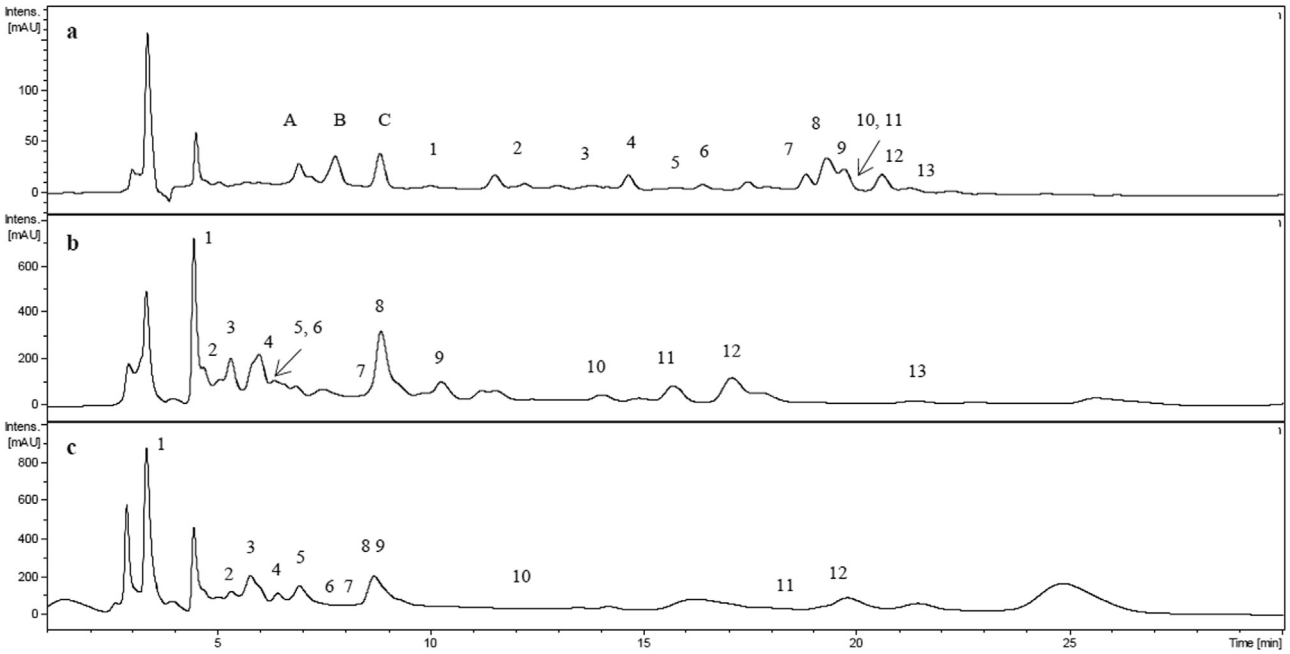

Fig. 5. HPLC-MS chromatograms of hydroalcoholic extracts of: **(A)** *Ephedra multiflora*. Compounds: A: unknown; B: unknown; C: unknown; 1: Catechin/epicatechin; 2: Catechin/epicatechin; 3: Myricetin hexoside rhamnoside; 4: Quercetin glucoronate hexoside; 5: Orientin/isoorientin; 6: Quercetin rhamnoside hexoside; 7: Quercetin rhamnoside glucuronate; 8: Quercetin rhamnoside hexoside; 9: Luteolin rhamnoside hexoside; 10: Vitexin/Isovitexin; 11: Quercetin dihexoside; 12: (epi) Catechin dimer; 13: Quercetin hexoside. **(B)** *Parastrephia lucida*. Compounds: 1: Caffeoylquinic acid; 2: 4-methoxycinnamic acid hexoside pentoside; 3: Caffeoylquinic acid isomer; 4: Hydroxycoumaric acid hexoside pentoside; 5: Dicaffeoylquinic acid; 6: Dicaffeoylquinic acid pentoside; 7: Quinic acid rhamnoside 3-(3,4-dimethoxyphenyl) propanoate; 8: Quinic acid rhamnoside; 9: Quinic acid glucuronate; 10: Rutin (Quercetin rutinoside); 11: Kaempferol pentoside hexoside; 12: Kaempferol hexoside; 13: Quercetin. **(C)** *Tessaria absinthioides*. Compounds: 1: Dicaffeoylquinic acid; 2: Protocatechuic acid; 3: Caffeoylquinic acid; 4: Vanillic acid sulphate; 5: Feruloyl caffeoylquinic acid sulphate; 6: Sesquiterpene hexoside; 7: Sesquiterpene ester; 8: Taxifolin dimethylether sulphate; 9: Quercetin sulphate; 10: Sesquiterpene pentoside sulphate; 11: Isorhamnetin/Rhamnetin; 12: Quercetin dimethylether. Axis: x-axis: retention time (min); y axis: absorbance (for A) and intensity (mAU) for B and C.

3.4.2. Parastrephia lucida

The chemical profiling of *P. lucida* allowed the tentative identification of 13 compounds in the extract, including four caffeoylquinic acid derivaties and four flavonoids. The compounds **1** and **3** presented a $[M-H]^-$ ion at *m/z* 353 amu and the loss of the caffeoyl moiety, leading to a base peak at *m/z* 191 and were identified as isomeric caffeoylquinic acids. Compounds **5** and **6** showed the neutral loss of a caffeoyl unit and a caffeoyl and pentose fragments, leading to the base peak at *m/z* 353 and were assigned as dicaffeoylquinic acid **5** and dicaffeoylquinic acid pentoside **6**, respectively. The compounds **2** and **4** were assigned as phenyl propanoids based on the neutral loss of hexose and pentose for both compounds, leading to a base peak at *m/z* 176 and 165, respectively, being tentatively identified as 4-methoxycinnamic acid hexoside pentoside **2** and hydroxycoumaric acid hexoside pentoside **4**, respectively. The compound **7** showed a $[M-H]^-$ ion a 529 amu and the neutral loss of 192 amu, leading to a m/z 337 ion, being tentatively assigned as quinic acid rhamnoside 3-(3,4-dimethoxyphenyl) propanoate **7**. The compounds **8** and **9**, with *m/z* ions at 337 and 367 amu, shows the neutral loss of a rhamnose and glucuronic acid, respectively, leading to a base ion at *m/z* 191. They were tentatively identified as quinic acid rhamnoside **8** and quinic acid glucuronate **9**. The compounds **10** and **13** were assigned as quercetin rutinoside and quercetin, respectively, based on the *m/z* ion at 609 for **10**, with loss of a rutinose moiety, leading to the base peak at *m/z* 301, in agreement with quercetin. The constituents **11** and **12**, with *m/z* ions at 579 and 447 amu, respectively, shows the loss of a pentose and hexose for **11** and a hexose for **12**, leading to the base peak at *m/z* 285, in agreement with kaempferol. The compounds were identified as kaempferol pentoside hexoside **11** and kaempferol hexoside **12**, respectively.

3.4.3. Tessaria absinthioides

The mass spectra of compounds **1** and **3** shows a deprotonated ion at *m/z* 515 for **1** and a pseudo-molecular ion at *m/z* 707 for **3**, leading to the fragment at *m/z* 353 and 191, in agreement with caffeoylquinic acids. The compounds were identified as dicaffeoylquinic acid **1** and caffeoylquinic acid **3**, respectively. The compound **2** was identified as protocatechuic acid based on the *m/z* ion at 153 amu and the MS^2 ion at *m/z* 109, in agreement with a reference sample of the compound. The compound **4** showed the neutral loss of a sulphate from the deprotonated ion at *m/z* 247, followed by the loss of CO_2 and was assigned as vanillic acid sulphate. The compound **5** with a deprotonated ion at *m/z* 627, shows consecutive loses of a sulphate (80 amu) and a 194 amu fragment, compatible with ferulic acid, leading to the ions at *m/z* 353 and 191, in agreement with caffeoylquinic acid. The compound was tentatively assigned as a feruloyl caffeoylquinic acid sulphate.

The compounds **6**, **7** and **10** shows the neutral loss of a hexose and water for **6**, a fragment of 102 amu for **7** and a pentose and sulphate for **10**, leading to a daughter ion at *m/z* 249 for **6** and **7** and 241 for **10**, respectively and were tentatively assigned to a sesquiterpene hexoside **6**, a sesquiterpene ester **7** and sesquiterpene pentoside sulphate **10**, respectively. The identity of the terpenes and the compounds as well remain to be established after isolation and full structural elucidation using NMR and other techniques. The mass spectrum of compound **8** shows an M+H ion at *m/z* 411, which shows the neutral loss of a sulphate (80 amu), leading to the *m/z* ion at 331 amu, which further loss a methyl group, compatible with a sulphate derivative of a dimethyl ether of taxifolin. The compounds **9**, **11** and **12** were identified as the flavonoids quercetin sulphate and the methyl derivatives of quercetin isorhamnetin/rhamnetin and quercetin dimethylether, respectively, based on the neutral loss of sulphate (80 amu) for **9**, a methyl group for **11** and **12**, leading to the *m/z* ions at 301, 300 and 315 amu, respectively.

Table 3
Compounds identification in hydroalcoholic extracts of *Ephedra multiflora*, *Parastrephia lucida* and *Tessaria absinthioides* by HPLC-MS.

Plant species	Compound	Rt (min)	$[M-H]^-$	MS/MS	Tentative identification
Ephedra multiflora	A	6.4	451	241	Unknown
	B	6.9	421	241	Unknown
	C	7.8	425	241	Unknown
	1	9.5	289	245, 205	Catechin/epicatechin
	2	12.5	289	245	Catechin/epicatechin
	3	13.7	625	463, 317	Myricetin hexoside rhamnoside
	4	14.4	639	463, 301	Quercetin glucuronate hexoside
	5	16.0	447	429, 357, 327	Orientin/isoorientin
	6	17.0	609	447, 301	Quercetin rhamnoside hexoside
	7	18.4–18.5	623	447, 301	Quercetin rhamnoside glucuronate
	8	19.4	609	447, 301	Queretin rhamnoside hexoside
	9	20.2–20.4	593.5	431, 285	Luteolin rhamnoside hexoside
	10	20.4	431	341, 311	Vitexin/Isovitexin
	11	20.7	625	463, 301	Quercetin dihexoside
	12	20.8–21.0	575	449, 289	(epi) Catechin dimer
	13	21.9	463	301	Quercetin hexoside
Parastrephia lucida	1	4.3	353	191, 135	Caffeoylquinic acid
	2	5.0	471	339, 176	4-methoxycinnamic acid hexoside pentoside
	3	5.6–5.8	353	191	Caffeoylquinic acid isomer
	4	6.1–6.3	459	165	Hydroxycoumaric acid hexoside pentoside
	5	6.3	515	353	Dicaffeoylquinic acid
	6	6.9	647	353	Dicaffeoylquinic acid pentoside
	7	8.3	529	337	Quinic acid rhamnoside 3-(3,4-dimethoxyphenyl) propanoate
	8	8.6–8.7	337	191	Quinic acid rhamnoside
	9	9.6–9.8	367	191	Quinic acid glucuronate
	10	14.5–15.0	609	301	Rutin (Quercetin rutinoside)
	11	15.4	579	447, 285	Kaempferol pentoside hexoside
	12	17.2–17.4	447	285	Kaempferol hexoside
	13	21.6	301	124	Quercetin
Tessaria absinthioides	1	3.2	515	353	Dicaffeoylquinic acid
	2	5.6	153	109	Protocatechuic acid
	3	5.7–5.9	707	353, 191	Caffeoylquinic acid
	4	6.5–6.6	247	167, 123	Vanillic acid sulphate
	5	6.9–7.0	627	547, 353, 273, 191	Feruloyl caffeoylquinic acid sulphate
	6	7.2–7.5	429	267, 249	Sesquiterpene hexoside
	7	7.3	351	249	Sesquiterpene ester
	8	8.3–8.5	411	331, 316	Taxifolin dimethyl ether sulphate
	9	8.4	381	301	Quercetin sulphate
	10	12.5	453	373, 241	Sesquiterpene pentoside sulphate
	11	18.6	315	300	Isorhamnetin/Rhamnetin
	12	19.1	331	315.9	Quercetin dimethyl ether

3.5. Phytochemical constituents and bioactivity of the most active species

Crude plant drugs showing anti-inflammatory activity are relevant in the traditional medicine of the Andes highlands (Puna). Previous studies have disclosed the antimicrobial and antioxidant properties of selected Puna plants (Alberto et al., 2009; Zampini et al., 2009a,b). The capacity of these Puna plants to inhibit the pro-inflammatory enzyme COX-2 and inducible nitric oxide synthase (iNOS) activity and expression was recently reported (Torres Carro et al., 2015). In this study, we further investigated the anti-inflammatory activity in Puna species to determine their inhibitory capacity on other important pro-inflammatory enzymes like $sPLA_2$, LOX and hyaluronidase, as well as their possible mechanism of inhibition of LOX by assessing their ability to scavenge radicals and reduce and/or chelate the Fe^{3+}/Fe^{2+} of the enzyme active site. We also proceeded with the identification of the constituents of the most active species by HPLC-ESI-MS/MS.

In the present study, the most active species towards the enzyme LOX was *E. multiflora*. Most of the constituents occurring in the polar extract of the plant were tentatively identified as quercetin, myricetin and luteolin glycosides as well as the flavonoid *C*-glycosides orientin/isoorientin and vitexin/isovitexin (Fig. 5, Table 3).

The enzyme inhibitory effect of the extract can be explained, at least in part, by some compounds reported in this work. The anti-inflammatory and antioxidant capacity of flavonoids present in *P. lucida* extract has been reported by D'Almeida et al. (2013), and for the ones identified in *B. incarum* the report was made by Zampini et al. (2009a) and by Ribeiro et al. (2014). Luteolin was also recognized as inhibitor of $sPLA_2$ and COX-2 (D'Almeida et al., 2013).

The compounds identified in *P. lucida* and *T. absinthioides* were mainly caffeoylquinic acid derivatives. In *P. lucida* we also identified flavonoids; while in *T. absinthioides* simple phenolics, flavonoids and sesquiterpene derivatives were found (Table 3). The *in vitro* anti-inflammatory potential of *P. lucida* might be related to the presence of caffeoylquinic acid derivatives and flavonoids such as quercetin and rutin, whose anti-inflammatory activity has already been reported (Chen et al., 2001; Shan et al., 2009). Quercetin and its derivatives are well known for their beneficial effects on human health. They are able to inhibit the activity and expression of various enzymes associated to the inflammatory response, including the enzymes of the AA pathway, iNOS and hyaluronidase (García-Mediavilla et al., 2007; Kim et al., 2005). There is a strong correlation between inflammation and oxidative stress; therefore, quercetin capacity to scavenge radicals, inhibit lipid peroxidation, reduce Fe^{3+} and chelate Fe^{2+} would potentiate its anti-inflammatory capacity (Fernandez et al., 2002; Kim et al., 2005). Some compounds identified in *P. lucida* and *T. absinthioides* extracts (quercetin in both extracts and rutin in *P. lucida*) have an inhibitory effect on $sPLA_2$, which might explain the capacity of these species to inhibit this enzyme (Yarla et al., 2015; Lindahl and Tagesson, 1997).

From a chemotaxonomic point of view, the genus *Parastrephia* is rich in phenolic acids (Torres Carro et al., 2015). D'Almeida et al.

(2013) carried out a bio-guided fractionation of *P. lucida* methanolic extract (less polar than the present extract), and the most active fractions against sPLA$_2$ contained mainly methoxyflavones and flavonols bearing two oxygen functions in the B-ring; being the main compound luteolin-7-methylether. These fractions also contained tetrahydroxymethoxy flavone, 5,4'-dihydroxy-7-methoxyflavanone in lower quantity and traces of a tremetone derivative.

In our sample of *T. absinthioides*, flavonoids, vanillic acid, protocatechuic acid, caffeoylquinic acid derivatives and feruloyl caffeoylquinic acid derivatives were identified by HPLC-ESI-MS. The inhibitory effect on COX-2, hyaluronidase, and iNOS expression of the crude drug could be explained by the presence of the above mentioned compounds as reported by Masella et al. (2012) for protocatechuic acid, Rhee et al. (2008) for taxifolin, Shan et al. (2009) for chlorogenic acid and Chen et al. (2001) for quercetin and rutin. In the extract of *T. absinthioides*, several sulfate derivatives were detected. According to Piazzon et al. (2012), the sulfate derivatives from ferulic and caffeic acid were less efficient as antioxidants as the unconjugated phenolics.

Sesquiterpene glycosides and a sesquiterpene ester were tentatively identified in the extract of *T. absinthioides* by HPLC-MS. However, the identity of the compounds remains to be established after isolation and full structural elucidation. This is the first report on the occurrence of glycosyl sesquiterpenes in *T. absinthioides*. Glycosyl sesquiterpenes and the methyl ester of dicaffeoylquinic acid have been isolated from the aerial parts of *T. integrifolia* by Ono et al. (2000). Sanz et al. (1997) also reported the isolation of 2-deoxytessaric acid, ilicic acid, 3-oxo-4,1-(13)-eudesmadien-12-oic acid, 3β,5α-dihydroxycostic acid and tessaric acid from *T. absinthioides*.

4. Conclusion

In the present work, hydroalcoholic extracts from 13 plants used mainly as anti-inflammatory agents in the Argentinean highlands were assessed for phospholipase A$_2$, LOX and hyaluronidase inhibition as well as for their capacity to stabilize red blood cell membranes. In addition, the same extracts were evaluated for their reducing power, iron chelating activity and ABTS radical scavenging effect. The present work reinforces the potential of the Puna plants as anti-inflammatory crude drugs for the treatment of chronic diseases and complements previous studies on other biological targets. The information obtained allowed a better understanding of the ethnopharmacological background in the plant selection as anti-inflammatory agents in the South American highlands. Even though modern medicine has reached this difficult to access region, medicinal plants are still their principal medicinal resource. The plants investigated in this work, have been used for centuries by Puna inhabitants to treat a variety of inflammatory diseases; an ancestral custom that has been kept and transmitted throughout generations until present times. Most of the crude extracts assessed were able to inhibit sPLA$_2$ activity, being the most active *P. lucida*, with percentages of inhibition higher than 50%. All of the extracts assayed inhibited LOX activity, with *E. multiflora* showing the lowest IC$_{50}$, while *T. absinthioides* exhibited an important inhibitory capacity on hyaluronidase activity, which was higher than that of the anti-inflammatory drugs used as positive controls. Finally, *P. lepidophylla* and *P. phyliciformis* were the most potent plant species at protecting red blood cells' membrane. Some 12 flavonoids and 10 phenolic acids were identified in *P. lucida*, *E. multiflora* and *T. absinthioides*, with higher inhibition of different pro-inflammatory enzymes. In summary, a better picture on the anti-inflammatory effect of selected Argentinean Puna plants was obtained, giving support to the traditional indications of use.

Conflict of interest

The authors have declared that there is no conflict of interest.

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