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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