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Original article

Anti-inflammatory activity and chemical analysis of extracts from *Trifolium riograndense*

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ABSTRACT

Aiming to investigate new therapeutic agents with fewer side effects, the number of studies about natural products has increased. Phenolic compounds comprise a well-studied class of abundant plant-derived compounds, whose anti-inflammatory activity has been described. Isoflavones are phenolic compounds that occur mainly in the Leguminosae family, and can be found in many species, such as *Trifolium riograndense* Burkart, Leguminosae (clover). In this study an HPLC method was used to determine and quantify four isoflavones (genistein, daidzein, formononetin, and biochanin A) in hydrolyzed leaf, flower, stolon, and root extracts of *T. riograndense*. *In vivo* anti-inflammatory activity was investigated using the rat paw edema method and *in vitro* chemotaxis model with a dry extract from the leaves, which had the highest amount of isoflavones. The major isoflavone found in all parts of the plant was formononetin. The chemotaxis assay revealed that the different concentrations (0.2–50 μg/ml) of the dry extract significantly inhibited neutrophil migration in a concentration-dependent manner (more than 90%). In the rat paw edema test, oral administration of clover extract 100 mg/kg was able to significantly inhibit the edema formation induced by carrageenan. In conclusion, chemical analyses showed that *Trifolium riograndense* is a plant rich in isoflavones and a new interesting option as isoflavone source. The results of the biological tests taken together show that the extract of *T. riograndense* has anti-inflammatory effect in rodents.

Introduction

Several classes of secondary metabolites are known to have anti-inflammatory activities, such as terpenes, alkaloids and phenolic compounds. Among these, flavonoids are the compounds with the widest variety of activities reported, being the anti-inflammatory property attributed to the ability of the compounds to inhibit both cyclooxygenase and the 5-lipoxygenase metabolic pathway of arachidonic acid (Winekenstadde et al., 2015; Honmore et al., 2016). Furthermore, studies have shown that flavonoids are able to increase capillary permeability and exert an inhibitory effect on protein exudation and leukocyte migration (Liu et al., 2016).

Isoflavones, a class of phytoestrogens, are plant metabolites structurally similar to the steroidal estrogen 17-β-estradiol. These compounds have become the object of widespread attention as potential therapeutic agents, particularly in women's health contexts. In addition to their estrogenic activity, these compounds have been associated with prevention of breast and prostate cancer as well as cardiovascular disease and inflammatory conditions (Ji et al., 2016; Sahpaz et al., 2016; Zhang et al., 2016).

The *Trifolium* taxon is one of the most important genera of the Leguminosae family, due to its agricultural value and the considerable number of constituent species (about 230) (Gillet et al., 2001). Most studies carried out to characterize isoflavone levels and quantify biological activities were performed with *Trifolium pratense* L. (red clover). The species contains related isoflavone glycosides, mainly the aglycones biochanin A (**1**) and formononetin (**2**), besides smaller amounts of daidzein (**3**) and genistein (**4**) glycosides (Lemeziene et al., 2015; Tava et al., 2015). However, the literature does not cite studies on *Trifolium riograndense* Burkart, Leguminosae. This clover species native to southern Brazil, especially the northern region of Rio Grande do Sul state is an herbaceous perennial plant that grows to 50 cm in height. The leaves are trifoliate (with three leaflets), and the flowers are dark pink. This clover

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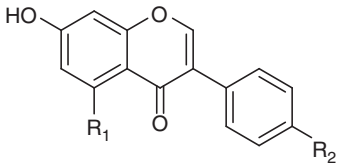
blooms in spring, and it is cold resistant. *Trifolium riograndense* is especially interesting to forage plant breeders because of its tolerance to acidic and aluminum-rich soils (Burkart, 1987), a condition quite common in this region.



1 R_1=OH; R_2=OCH_3
2 R_1=H; R_2=OCH_3
3 R_1=R_2=OH
4 R_1=H; R_2=OH

The aim of this work was to quantify the isoflavone aglycones daidzein, formononetin, genistein, and biochanin A in different organs of *Trifolium riograndense* (leaf, stolon, flower, and root) using High Performance Liquid Chromatography (HPLC), and to evaluate the *in vivo* and *in vitro* anti-inflammatory activity of a dry extract prepared with *T. riograndense* leaves.

Materials and methods

Plant material

Trifolium riograndense Burkart, Leguminosae, was collected during its flowering stage, in November 2007, in several cities in north Rio Grande do Sul state, Brazil. The plant material was identified by the botanist Dra. Silvia T. S. Miotto and a voucher specimen was deposited at the Herbarium in the ICN Herbarium, UFRGS, Porto Alegre, Brazil (number 157822). The leaves, stolons, flowers, and roots of the gathered plants were sorted and dried in an oven at 100 °C for 1 h. Next, the plant material was ground using a mortar and pestle.

Chemicals and reagents

Daidzein, genistein, carrageenan and indomethacin were purchased from Sigma–Aldrich; formononetin and biochanin A were purchased from Fluka. Acetonitrile (HPLC grade) was obtained from Merck; HCl, methanol, dichloromethane, and ethanol were purchased from Vetec; and trifluoroacetic acid (analytical grade) was obtained from Nuclear.

Preparation of the extracts for HPLC analysis

Each sample was prepared and analyzed in triplicate. Initially, 10 mg of pulverized plant was extracted with 4 ml of 6 M HCl and incubated at 100 °C for 15 min in water bath under magnetic stirring. After cooling, the residue was filtrated and extracted with 15 ml of dichloromethane (three times). The extract was concentrated under reduced pressure, dissolved in 10 ml of methanol. The extract was filtrated through a 0.45 μm membrane before injection in the HPLC system (Ramos et al., 2008).

HPLC analysis of extracts for isoflavone content

The HPLC analyses were performed according to Ramos et al. (2008), on a Waters Alliance 2695 chromatograph with a diode array detector (UV/VIS Waters 2487). The system was equipped with a C18 reverse-phase column (Nova-Pak, 4 μm, 3.9 × 150 mm) with guard-column and operated at room temperature. Elution of isoflavones was performed using a linear gradient system, and the mobile phase consisted of acetonitrile:water:trifluoroacetic acid (20:80:0.01 (v/v/v)) (A) and acetonitrile:trifluorocetic acid (100:0.1 (v/v)) (B). The gradient profile was: 0–10 min from 0 to 40% B, 10–11 min 40% B, 11–12 min from 40 to 100% B. At the end of each run, 100% A was used for 6 min to restore the initial conditions. The flow-rate was 0.7 ml/min. The detection wavelength was 260 nm.

The identification of isoflavones was performed by comparing the UV profiles and retention times with chemical reference substances. Standard curves were generated for the four isoflavones (daidzein, formononetin, genistein, and biochanin A). The area under the curve for each isoflavone of the extract was determined, and these areas were used to calculate the percent weight of isoflavones in the samples, based on standard curve, linear regression, and amount injected in the column. Relative Standard Seviations (RSD) for area values from triplicate injections were calculated as: RSD = [(mean − standard deviation)/mean] × 100, and the samples' RSD had to be <5% to be considered valid data.

Dry extract preparation

The dry extract was obtained by soaking the leaves of *T. riograndense* dried and crushed with 40% ethanol at room temperature three times, each time for three days. The ethanol extract was partitioned with dichloromethane. The solvent was removed under reduced pressure and the resulting concentrated extract was dissolved in water and subjected to lyophilization to produce the dry extract.

Animals

Wistar rats (180–220 g) were obtained from the Breeding Laboratory, UFRGS, Brazil. The animals were housed four per cage in a temperature controlled room with free access to food and water. This study was approved by the Ethical Committee from Universidade Federal do Rio Grande do Sul (protocol number: 2007981).

Anti-inflammatory activities

Chemotactic migration

Chemotactic migration was measured according to the method described by Suyenaga et al. (2011). A total of seven rats were used in this assay. For obtaining rat polymorphonuclear neutrophils, 20 ml of sterile 1% glycogen (w/v) were injected into the peritoneum of one Wistar rat and 4 h later, the animal was killed by decapitation and the leukocytes collected. *T. riograndense* dry extract was dissolved in rat leukocytes solution to the concentrations of 100, 50, 25, 10, 5, 1, 0.5, and 0.2 μg/ml, and incubated at 37 °C for 30 min. Plasma collected from six rats was incubated at 37 °C for 30 min with 65 μg/ml of LPS (lipopolysaccharide from Escherichia coli) and diluted in Hanks buffer to a 20% solution (v/v). The reference drugs biochanin A (**1**), formononetin (**2**), daidzein (**3**), and genistein (**4**) (10 μg/ml) were also dissolved in Hanks buffer.

The leukocyte/samples were added in the upper wells of the chamber, separated by an 8.0 μm nitrocellulose filter (Millipore, USA) from the chemotactic stimulant (LPS) present in the bottom compartment. The chamber was kept at 37 °C for 1 h and, after that, the leucocytes migration through the filter was measured by using an optical microscope. The distance from the top of the filter to the farthest plane of focus containing two cells allowed the evaluation of leukocyte migration. Measurements were taken from five fields across each one of duplicate filters and the results expressed as mean ± standard error of the mean (SEM).

Carrageenan-induced paw edema in rats

Anti-inflammatory activity was evaluated by the carrageenan-induced rat paw edema test, as described by Winter et al. (1962). A total of fifteen rats were divided into control, positive control

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