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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 (Cavendish et al., 2015; 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

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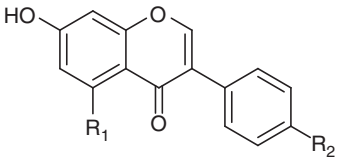
(with three leaflets), and the flowers are dark pink. This clover blooms in spring, and it is cold resistant. *Trifolium riograndense* is especially interesting to forage plant breeders because of its tolerance to acidic and aluminum-rich soils (Burkart, 1987), a condition quite common in this region.



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

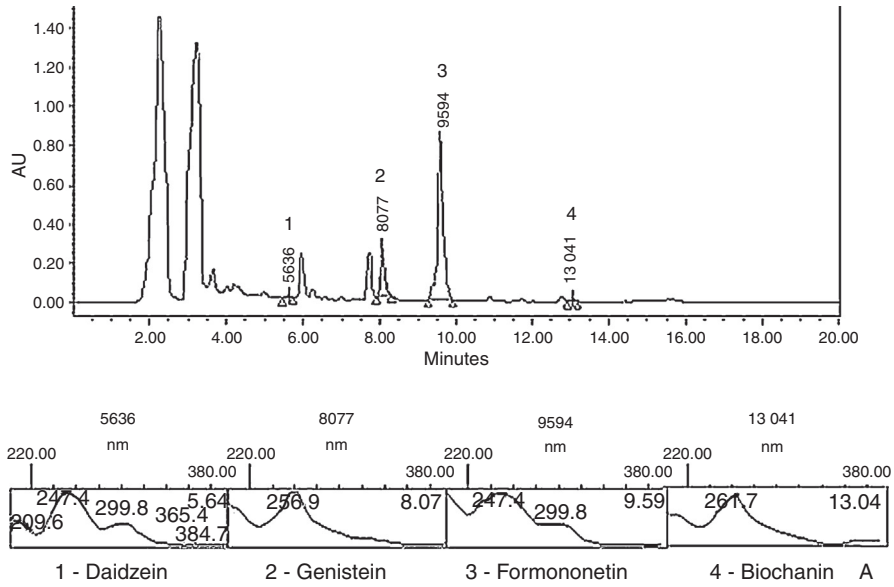


Fig. 1. Chromatogram and UV spectra (260 nm) of the isoflavones daidzein, genistein, formononetin, and biochanin A found in *Trifolium riograndense*.

and test group of five animals each. *T. riograndense* dry extract was resuspended in saline and administered orally 1 h before subplantar injection of carrageenan (0.1 ml of a suspension at 5 mg/ml) using a single dose of 100 mg/kg body weight for each group of samples ($n = 5$). The control group received equivalent volumes of the vehicle. The activity was compared with the effect of the positive control indomethacin (99% purity; Sigma) administration (10 mg/kg in saline, *p.o.*).

Male Wistar rats were anaesthetized with sodium pentobarbital (40 mg/kg, *i.p.*) and injected subplantarly into one of the hind paws with 0.1 ml of 0.5% λ-carrageenan type IV solution in isotonic saline (Sigma chemical Co., St. Louis, MO). The contralateral paw was injected with 0.1 ml saline solution and used as control. Edema was measured using a digital plethysmometer Ugo Basile (model 7140, Italy) at 1, 2, 3 and 4 h after carrageenan injection. Edema volume was expressed for each animal as the percentage change in rat paw volume after carrageenan injection compared with placebo group.

Statistical analysis

Results are expressed as the mean ± SEM and were tested for significance using Student's *t*-test. Probability values (p) of less than 0.05 were taken to indicate statistical significance.

Results and discussion

In this HPLC system, daidzein (**3**), genistein (**4**), formononetin (**2**) and biochanin A (**1**) were eluted with the following retention time ranges: 5.646, 8.077, 9.594, and 13.041 min, respectively (Fig. 1). R^2 values for the least-square regression equations fitted to the standard curves were as follows: daidzein (0.9983), genistein (0.9999), formononetin (0.9996), and biochanin A (0.9997).

The concentrations of the four investigated isoflavones in different parts from *T. riograndense* are summarized in Table 1. Total isoflavone concentration was 18.30 mg/g of dry plant material. In leaves, total isoflavone concentration was 7.331 mg/g of dry plant. The root presented the lowest isoflavone concentration, 2.806 mg/g. The main isoflavone found in all parts of the plant was formononetin (16.683 mg/g) followed by biochanin A (1.207 mg/g).

The isoflavone concentration found in *Trifolium riograndense* is high, when compared with other species of the Leguminosae family. For example, the concentration of isoflavones in soy seeds, the most consumed source of isoflavone in the world, varies between 0.5 and 2.0 mg/g, while the lowest isoflavone content found in *T. riograndense* was the roots with 2.806 mg/g and the highest was in the leaves with 7.331 mg/g (USDA, 2002).

Table 1
Isoflavone contents (milligram per gram of dry weight; arithmetic means of the analytical data are given; number of replicates: 3) in different parts of *Trifolium riograndense*.

Plant organ	Isoflavones (mg/g)				
	Daidzein	Genistein	Formononetin	Biochanin A	Total
Leaves	0.063	0.167	6.623	0.478	7.331
Flowers	0.059	0.015	3.180	0.188	3.442
Stolons	0.065	0.033	4.348	0.275	4.721
Roots	0.000	0.008	2.532	0.266	2.806
Total	0.187	0.223	16.683	1.207	18.300

Previous research has quantified isoflavones in other *Trifolium* species. Ramos et al. (2008) analyzed five populations of red clover (*T. pratense*) and observed that aglycone content varied between 0.008 and 0.091 mg/g expressed in daidzein, 0.05 and 0.131 mg/g in genistein, 6.568 and 23.462 mg/g in formononetin, and 2.499 and 10.337 mg/g in biochanin A. In general, the daidzein (**3**) and genistein (**4**) contents of *T. riograndense* are higher than in *T. pratense*, while the concentration of biochanin A (**1**) in *T. riograndense* is lower (1.207 mg/g).

A screening of 57 *Trifolium* species for isoflavone concentration showed that several present extremely high amounts of these compounds. From this point of view, eleven species, *T. lappaceum, T. phleoides, T. hirtum, T. alpestre, T. medium, T.subterraneum, T. heldreichianum, T. pratense, T. isodon, T. miegeanum*, and *T. scabrum*, are interesting, with isoflavone contents ranging from 10.39 to 88.38 mg/g. Most of the other 46 *Trifolium* species were either free of isoflavones or presented very low contents of these compounds (Oleszek et al., 2007).

Previously, other *Trifolium* species have low concentration of isoflavones. Wu et al. (2003) determined isoflavone content in *T. repens, T. hybridum*, and *T. campestre*. Total isoflavones in different parts of these species were found to be much lower: 0.21–0.35 mg/g in *T. repens*, 0.07–0.40 mg/g in *T. hybridum*, and 0.003–0.006 mg/g in *T. campestre*.

Table 2
In vitro chemotactic response of neutrophils treated with the suspension of dry extract of *Trifolium riograndense* isoflavones.

Sample	Concentration (μg/ml)	Distance migrated (μm)	% Inhibition
Control		115 ± 2	100
Trifolium riograndense	100	10 ± 1*	91
	50	9 ± 1[a]	92
	25	8 ± 1[a]	93
	10	8 ± 1[a]	93
	5	7 ± 1[a]	94
	1	13 ± 1[a]	89
	0.5	76 ± 4[a]	34
	0.2	120 ± 1[a]	0
Daidzein	10	25 ± 1[a]	78
Genistein	10	55 ± 3[a]	52
Formononetin	10	51 ± 4[a]	55
Biochanin A	10	9 ± 1[a]	92

The results of distance migrated are mean ± SEM.
[a] $p < 0.005$, significantly different from control by Student's *t*-test.

Regarding the anti-inflammatory activity of dry extract of leaves *T. riograndense*, we have inhibitory activity of the extract against neutrophil migration is shown in Table 2. All extracts concentrations (0.2–50.0 μg/ml) showed a dose-dependent inhibition of neutrophil migration. Except for the 0.2 μg/ml extract, all other extracts inhibited the phenomenon by more than 90% ($p < 0.005$). In addition, isolated isoflavones were also assayed, all of which significantly inhibited neutrophil migration, especially biochanin A (**1**), which was able to inhibit chemotaxis by 92% followed by 78% of daidzein (**3**). However, the major isoflavone, formononetin (**2**), did not present the strongest anti-inflammatory activity, and the anti-inflammatory capacity of the extract of *T. riograndense* is mainly related to the class of the isoflavone compounds present as observed in this study.

Indeed, all of these isoflavones have been object of anti-inflammatory investigation and the results are in agreement with the literature. Previous study has demonstrated that biochanin A antagonizes the IL-1β-induced catabolic effects through its anti-inflammatory activity by the modulation of NFκB signaling, resulting in potent anti-inflammatory, anti-catabolic, and antioxidative effects through antagonistic effects against IL-1b in primary rat chondrocytes. Such results suggest that biochanin A may be an important phytoestrogen to prevent osteoarthritis (Oh et al., 2016). In other study, biochanin A showed protective effect on LPS/GalN-induced liver injury, by the protection against LPS/GalN-induced liver injury by activating the Nrf2 pathway and inhibiting NLRP3 activation (Liu et al., 2016b). Formononetin (**1**) demonstrated a reduction in some inflammatory mediators such as nuclear factor κB (NF-κB) and IL-1β *in vitro* (Wang et al., 2012). In addition, this compound was able to decrease the levels of TNF-α and IL-6 (Li et al., 2014) and improve superoxidase dismutase activity (Ma et al., 2013), demonstrating an anti-inflammatory and antioxidant activities associated with neuron and lung protective effects *in vivo*. In order to study the effect of daidzein for the treatment of bone loss, this compound was tested on the expression of the osteoblast-produced bone regulatory factors OPG, RANKL and IL-6 in human osteoblastic MG-63 cells. The results showed that daidzein increased the levels of OPG and decreased those of RANKL and IL-6 (Sun et al., 2016). Genistein was also submitted for investigation of its anti-inflammatory activity and the results confirmed an important decrease in the TNF-α and IL-6 levels (Incir et al., 2016).

The suppression of neutrophil functions is one of the ways to control inflammatory conditions. The relationship between traditional use of a plant and an inflammatory process has been studied using several species. The Boyden chamber is a simple and efficient method to determine whether an isolated compound or extract has the ability to inhibit neutrophil chemotaxis (Suyenaga et al., 2011).

Table 3
Effect of oral administration of *Trifolium riograndense* extract on rat paw edema induced by carrageenan ($n = 5$ animals).

Treatment	Volume of paw edema (ml) ± SEM (% inhibition)			
	1 h	2 h	3 h	4 h
Control	1.19 ± 0.15	1.44 ± 0.23	2.13 ± 0.17	1.69 ± 0.26
Indomethacin 10 mg/kg	0.12 ± 0.23[a] (89.9%)	0.47 ± 0.27[a] (67.4%)	0.72 ± 0.35[a] (66.2%)	0.48 ± 0.48 (71.6%)
T. riograndense 100 mg/kg	0.38 ± 0.24a (68.1%)	0.66 ± 0.26 (54.2%)	1.13 ± 0.19[a] (46.9%)	0.58 ± 0.26[a] (65.7%)

[a] $p < 0.05$, significantly different from control by Student's *t*-test.

The results obtained in the analysis of neutrophil chemotactic migration prompted us to evaluate the *in vivo* antiedematogenic activity of the extract of *T. riograndense*. In the rat paw edema, the inflammatory effect induced by injection of carrageenan 0.5%, produced edema after 60 min. The previous administration (60 min) of the extract of *T. riograndense* 100 mg/kg induced significant inhibition, when compared with the control ($p < 0.05$) (Table 3). As shown in Table 3, the extract inhibited paw edema already within the first hour of the experiment (54.2–68.1% of inhibition).

Considering the results obtained in the anti-inflammatory assays, the findings indicated that the extract obtained from the leaves showed anti-inflammatory and antiedematogenic proprieties due to reduction of acute edema.

Isoflavones have attracted attention due to their role in the amelioration of postmenopausal symptoms, cardiovascular diseases, cognitive function, and breast and prostate cancers (Verheus et al., 2007). Isoflavone-based nutraceuticals are the most assayed polyphenol supplements. The interest in isoflavones as dietary components and their scarcity in Western diets as compared to Asian diets, where they are abundant due to soy consumption, has resulted in increasing demand for new plant sources of these compounds (Wang et al., 2013).

The model of chemotaxis is simulated by *in vitro* leukocyte migration from the intravascular space into the tissue (Hofbauer et al., 1998). The chemotaxis assay evaluates the decrease in motility of leukocyte chemotactic agents ahead, and considers the space traversed by leukocyte migration as a measure of activity. It should be emphasized that the reading of cell migration is observed *in vitro* and therefore is not a measure of the distance migrated *in vivo*, though both parameters are highly correlated. Based on the knowledge that neutrophils, in particular leukocytes, play an important role in the inflammatory process, it may be suggested that inhibition of their migration may be responsible for part of the anti-inflammatory activity. The suppression of neutrophil function can control the inflammatory response being applied as a mechanism of action of certain anti-inflammatory drugs (Rioja et al., 2000).

This test demonstrated the action of dry extract from *T. riograndense* leaves on leukocytes migration, proving the occurrence of chemotaxis inhibition. This effect on the migration caused by the extract of this species of clover can be attributed to isoflavones. The fact that the extract of *T. riograndense* caused greater inhibition on leukocyte chemotaxis, when compared with the isolated isoflavones, suggests the occurrence of synergism, in which the action of two or more isoflavones may cause a more intense effect.

It should be emphasized that besides the isoflavones analyzed in this work, other molecules of this class of flavonoids may be present, contributing to the activity observed here. Therefore, it becomes necessary to undertake a more complete chemical investigation to characterize any possible correlation between the substances identified and the biological activity observed.

The paw edema induced by carrageenan follows a model of acute inflammation that consists of two phases: the first, which was detected after around 1 h and was called the fast phase, with release of histamine and serotonin, and the second stage, called late, with the mediators (kinins, prostaglandins) released after 2 and 3 h, respectively (Vinegar et al., 1969; Di Rosa et al., 1971). In a model of induced arthritis, *Trifolium resupinatum* var. *microcephalum* was shown to have activity in rat paw edema (Sabudak et al., 2008). Chemical analyses showed that *T. 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 property. However, the cellular mechanisms involved in this activity deserve further study. Other tests, *in vivo* and *in vitro*, are necessary to confirm the results, and further investigations could indeed establish probable mechanisms of action.

Conflicts of interest

The authors declare no conflicts of interest.

Author's contribution

GPRP, CBM, GRD and PCC carried out the phytochemical process. GPRP and GRD made the chromatographic assays. MAA and JASZ contributed in writing the manuscript. MAA, EESS and GRD performed the biological analysis. MDA helped in the collection and identification of the plant material. MAA and EESS contributed to the critical reading the manuscript. All authors have approved the final version for publishing.

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