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In vivo and *in vitro* anti-inflammatory activity of red clover *Trifolium pratense* dry extract

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Article

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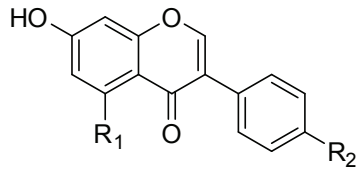
Abstract: Red clover *Trifolium pratense* L., Fabaceae, contains four isoflavones, mainly formononetin and biochanin A, and in smaller concentrations, daidzein and genistein. These compounds have gained a lot interest due to its human health benefits, such as estrogenic and progestogenic activities, antioxidant, anti-cancer and others. The objective of this study was to determine *in vivo* and *in vitro* anti-inflammatory activity of red clover dry extract. The *in vitro* anti-inflammatory activity was assayed by the technique using the Boyden chamber method, evaluating the leukocyte migration inhibition (chemotaxis). The *in vivo* anti-inflammatory activity was tested by a carrageenan-induced rat paw edema test. The results of anti-inflammatory *in vitro* test showed that there was a significant inhibition of leukocyte migration at the concentrations of 100, 50, 25, 10 and 5 µg/mL of red clover dry extract, these doses resulted in 94.73, 95.39, 94.73, 84.68 and 78.75% of inhibition for each dose, respectively. The anti-inflammatory in vivo test resulted in a significant activity in both tested doses (100 and 50 mg/kg of red clover dry extract) and at each tested time. The average percentage of edema inhibition was 63.37%. The findings of this study suggested that red clover extract might be suitable for the treatment of inflammatory diseases.

Introduction

Trifolium pratense L., Fabaceae, (red clover) is one of several botanical dietary supplements that are being marketed for use in alleviation of hot flashes and other menopausal symptoms (Mu et al., 2009). Animal's studies, clinical trials and epidemiologic observations suggest a potential of dietary phytoestrogens in the prevention of "Western Diseases" (*e.g.* atherosclerosis, cardiovascular diseases, osteoporosis, different kind of cancer) (Cornwell et al., 2004; Geller & Studee, 2006). Isoflavones, such as daidzein, genistein, formononetin and biochanin A, are the most important group of phytoestrogens. These compounds occur in soy, soy products, kudzu and red clover (Cornwell et al., 2004). Due to the reported beneficial health effects of phytoestrogens the demand for food supplements rich in isoflavones is strongly increasing (Krenn & Paper, 2009).

Red clover contains mainly glycosides of the isoflavones formononetin and biochanin A and has gained a lot of interest. In humans these glycosides are hydrolyzed and resulting formononetin (**1**) and biochanin A (**2**), which are metabolized to daidzein (**3**) and genistein (**4**) (Krenn & Paper, 2009).



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

Many new drugs derived from plant secondary metabolites have been applied for the treatment and/or prevention of various diseases. Investigations about natural products have recently regained prominence with the increasing of understanding of their biological significance and increasing recognition of their origin and structural diversity. Most clinically important medicines are steroidal or non-steroidal anti-inflammatory chemical therapeutics for treatment of inflammatory-related diseases. Though these have potent activity, long-term administration is required for treatment of chronic disease.

Furthermore, these drugs have various and severe adverse effects. Therefore, naturally occurring agents, with high effectiveness and very few side-effects, are desirable as substitutes for chemical therapeutics (Menichini et al., 2009).

Inflammation is a basic pathological mechanism that underlies in a variety of diseases. The inflammatory reaction involves complex interactions between inflammatory cells neutrophils, lymphocytes, and monocytes/macrophages) and vascular cells (endothelial and smooth muscle cells) (Albertini et al., 2007).

Acute rat paw inflammation is characterized by migration of inflammatory cells into the microvascular system and fluid entering the interstitial tissue. These events are induced by inflammatory mediators which bind to specific receptors on inflammatory and endothelial cells (Doerschuk et al., 1999; Saito et al., 2002).

Chemotaxis is the phenomenon in which the direction of a cell's locomotion is determined by an extracellular gradient of chemicals and plays a critical role in many diverse physiological processes, including the recruitment of leukocytes to sites of infection, trafficking of lymphocytes throughout the human body, and patterning of neuronal cells in the developing nervous system. Considering that chemotaxis plays an important role in the development and physiology of our body, improperly guided cell movements can cause several pathological conditions, including tumor growth, cancer metastasis and inflammatory diseases, such as asthma, arthritis and atherosclerosis (Koizumi et al., 2007; Liehn et al., 2006; Silva et al., 2007).

Once the investigation of chemotactic ability plays an important role in the investigation of new anti-inflammatory agents, in this study the anti-inflammatory effects of red clover dry extract were investigated *in vitro*, by the inhibition of leukocyte chemotaxis, employing a modified Boyden chamber, and in vivo, with carragenan-induced paw edema in rats model.

Material and Methods

Plant material

Seeds from *Trifolium pratense* L., Fabaceae, red clover (population Nova Santana), were planted in an experimental field at Eldorado do Sul-RS, Brazil (30º05'02" S, 51º36'58" W and elevation 40 m). Leaves from these plants were harvested after five months. These leaves were identified by Dr. S. T. S. Miotto, Universidade Federal do Rio Grande do Sul. A voucher specimen (ICN 136089) is deposited at the Herbarium ICN Bioscience Institute, Universidade Federal do Rio Grande do Sul, Brazil.

Dry extract preparation

The dry extract was obtained from dried red clover leaves that were macerated three times with ethanol 40%, and after the ethanolic extract was separated with dichloromethane and then the solvent was evaporated and resuspended with water:ethanol (95:5, v:v). After that, this solution was frozen and lyophilizated. The isoflavones from dry extract were quantified by a previously validated method by our group (Ramos et al., 2008).

Animals

Male Wistar rats (180-220 g) were obtained from Breeding Laboratory, UFRGS, Brazil. The animals were housed five per cage in a room with controlled temperature with free access to food and water. This study was approved by the Ethical Comitee from Universidade Federal do Rio Grande do Sul (protocol number: 2007723).

Anti-inflammatory activities

Chemotactic migration

Chemotactic migration was measured by the method described previously by Suyenaga et al. (2011). Prior to the chemotactic assay, red clover dry extracts was dissolved in rat leukocytes solution to obtain the concentrations of 100, 50, 25, 10 and 5 µg/mL, and incubated at 37 ºC for 1 h. Plasma collected from rats was incubated at 37 ºC for 30 min with 65 µg/mL of LPS (lipopolysaccharide from *Escherichia coli*), the plasma was diluted in Hanks buffer at 20% concentration (v/v).

Chemotactic migration of leukocytes through an 8 µm nitrocellulose filter, towards the chemotactic stimulant (LPS treated plasma), was measured, after incubation for 1 h at 37 ºC, using the micrometer on the fine-focus knob of the microscope. The distance from the top of the filter to the farthest plane of focus still containing two cells, in five microscopic fields, allowed the evaluation of leukocyte migration. All experiment was carried out in duplicate.

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). Red clover dry extract was ressuspended in saline, resulting a suspension, that was 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 and 50 mg/kg for each group of animals. The placebo group received equivalent volumes of the vehicle (saline).

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-Aldrich 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.

The activity was compared with the effect of indomethacin (99% purity; Sigma) administration (10 mg/kg, *p.o.*; Sigma-Aldrich Chemical Co.).

Statistical analysis

Data were expressed by mean±SEM and analyzed by one way analysis of variance (ANOVA) followed by the Tukey multiple range test considering $p \leq 0.05$ as significant for chemotactic assay; and two way ANOVA followed by the Tukey multiple range test considering $p \leq 0.05$ as significant for carrageenan-induced paw edema test.

Results and Discussion

The red clover dry extract was analyzed, by a HPLC method previously validated by our group for the quantification of isoflavones (Ramos et al., 2008), and this analysis revealed that this dry extract had around 200 mg/g of isoflavones, sum of concentration of daidzein (6.64 mg/g), genistein (5.84 mg/g), formononetin (84.46 mg/g) and biochanin A (111.72 mg/g).

In the *in vitro* anti-inflammatory model, red clover dry extract was tested using the Boyden chamber method (Zigmond & Hirsch, 1973; Suyenaga et al., 2011), which is based on a chamber that has two compartments filled and separated by a microporous membrane. This assay simulated the chemotaxis of the leukocytes toward to chemotactic agent. After the appropriate incubation time, the membrane between the two compartments is fixed and stained, and the number of cells that migrated to the lower portion, in which is the LPS, is determined with a microscope.

Neutrophils are highly specialized leukocytes that play an important role in innate immunity and are the main kind of leukocytes present in the acute phase of the inflammation. During an infection or damage tissue, neutrophils respond to inflammatory and chemoattractant factors by their migration through the microvascular endothelium, extracellular matrix and mucosal epithelium at inflammatory sites (Liu et al., 2004).

Through our results is possible to verify the inhibition of leukocyte migration in all the tested concentrations (5 to 100 µg/mL) of red clover dry extract (Table 1). The mean and the standard error were calculated and the data were statistically analyzed by ANOVA (analysis of variance), followed by Tukey test, using confidence interval of 95%.

Table 1. *In vitro* chemotaxis migration of neutrophils treated with red clover dry extract. Chemotaxis is presented as mean±SEM of leukocyte migration.

Sample	Migrated distance (µm)±SEM	% inhibition
Positive control**	121.40±1.90	0.00
100 µg/mL	6.40±1.84*	94.73
50 µg/mL	5.60±1.58*	95.39
25 µg/mL	6.40±1.58*	94.73
10 µg/mL	18.60±2.74*$^{\#+a}$	84.68
5 µg/mL	25.80±2.74*$^{\#+ab}$	78.75

**Positive control: lipopolysaccharide from *Escherichia coli*; *$p \leq 0.001$ compared to positive control; $^{+}p \leq 0.001$ compared to 100 µg/mL dose; $^{\#}p \leq 0.001$ compared to 50 µg/mL dose; $^{a}p \leq 0.001$ compared to 25 µg/mL dose; $^{b}p \leq 0.001$ compared to 10 µg/mL dose.

Neutrophils treated with 100, 50, 25, 10 and 5 µg/mL of red clover dry extract, reduced at all doses, significantly ($p \leq 0.001$) the migration of them through chamber filled with plasma treated with LPS, the percentage of inhibition for the doses described above were, respectively, 94.73, 95.39, 94.73, 84.68, 78.75% (Table 1), when compared to the control. When the doses are compare between then, is possible to observe that there was a significant difference ($p \leq 0.001$) between all doses compared with the doses of 5 and 10 µg/mL, no significant differences were found between the doses of 100, 50 and 25 µg/mL. Thus, there is no need to use doses higher than 25 µg/mL.

The results of the oral administration of the red clover dry extract suspension for the inhibition of rat pew edema induced by carrageenan are shown in the Table 2. The results for the rat paw edema are expressed as mean±standard error of mean (SEM). The mean and standard error were calculated and the data were statistically analyzed by ANOVA (analysis of variance), followed by Tukey test using a confidence interval of 95%.

The red clover dry extract is composed of approximately 200 mg of isoflavones per gram of dry extract. The Table 2 presents the results obtained from the oral administration of a suspension in water of the dry extract of red clover in relation to the negative control and indomethacin (positive control). It can be observed that the extract showed significant antiedematogenic activity, within the first hour after the suspension administration for both tested doses. The dose of 100 mg/kg after two hours showed a significant difference when compared to indomethacin. Two hours after administration of doses

is also possible to observe difference between doses. It can be also observed that the extract showed significant antiedematogenic activity at all doses and times tested.

Table 2. Effect of red clover dry extract orally administrated on carrageenan-induced rat paw edema (n=7 animals).

Treatment	Mean edema size (mL)±SEM (% inhibition)			
	1h	2h	3h	4h
Control	1.46±0.23	1.64±0.27	2.27±0.10	1.98±0.20
Indomethacin (10 mg/kg)	0.73±0.23** (50%)	0.99±0.22** (40%)	0.86±0.22* (62%)	0.60±0.13* (70%)
Red Clover (50 mg/kg)	0.65±0.13* (55%)	0.75±0.11** (54%)	0.77±0.07* (66%)	0.96±0.18* (51%)
Red Clover (100 mg/kg)	0.47±0.09* (68%)	0.43±0.08*#+ (74%)	0.67±0.15* (70%)	0.62±0.12* (69%)

*$p\leq0.001$ compared to control; **$p\leq0.01$ compared to control; #$p\leq0.05$ compared to indomethacin; +$p\leq0.05$ between doses.

Considering that de red clover dry extract, used for the preparation of the suspension is constituted mainly by isoflavones aglycones, it is suggested that the demonstrated activity is related to these compounds, or the synergism of these main compounds among them or with other present in lower concentrations.

The antiedematogenic activity after the first hour of dosing the extract can be explained by the fact that the absorption of the aglycones is quick and extensive (Izumi et al., 2000; Beck et al., 2005).

The anti-inflammatory activity related to flavonoids is well known and studied. This activity is due to the inhibition of some enzymes involved in inflammation and/or the cellular signaling pathways such as cyclooxygenase (COX) where some flavonoids act by the COX and 5-lipoxygenase enzymes inhibition (Selloum et al., 2003).

Through the results analyzed, the *in vitro* and *in vivo* anti-inflammatory activities highlighted the anti-inflammatory activity of red clover dry extract. So, the findings of this study suggest that more research in this area should be developed, because the results were promising.

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