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Effect of the Hydroalcoholic Extract of *Bidens pilosa* L. on Leukocytes Mobilization

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Abstract: This study investigated the effect of a crude Hydroalcoholic Extract (HE) obtained from aerial parts of *Bidens pilosa* L. on *in vitro* and *in vivo* leukocyte mobilization, using models of chemotaxis and pleurisy induction by carrageenan in rats, respectively. Leukocytes treated with 100 and 200 g mL^{-1} of HE had a reduction of chemotaxis in 35.5 and 52.2%. Rats treated orally at dose of 250 mg kg^{-1} showed 60.5% reduction of total cells in pleural exudate. HE induced reduction of leukocyte mobilization in both *in vitro* and *in vivo* assays, which suggests a potential anti-inflammatory effect.

Key words: *Bidens pilosa,* leukocytes, Asteraceae, *in vitro*, *in vivo*

INTRODUCTION

Bidens pilosa L. (Asteraceae) is a plant widely found in tropical and subtropical regions all over the world (Tan *et al*., 2000; Chiang *et al*., 2004). Commonly known as hairy beggar-ticks or Spanish needles, it is considered a noxious and invasive weed in cultivated lands in Brazil, where it is popularly called picão or picão-preto (Brandao *et al*., 1997; Grombone-Guaratini *et al*., 2005).

Silva *et al*. (2011) summarize several classes of secondary metabolites had information on the 198 natural products isolated from different parts of *B. pilosa*. Some of its bioactive compounds and extracts fractions have been reported to have anti-bacterial, antifungal, antiparasitic, anti-hyperglycemic, anti-angiogenic, antioxidant and anti-inflammatory activity (Geissberger and Sequin, 1991; Jager *et al*., 1996; Rabe and van Staden, 1997; Alvarez *et al*., 1999; Pereira *et al*., 1999; Wu *et al*., 2004; Chiang *et al*., 2004; Tobinaga *et al*., 2009; Bairwa *et al*., 2010).

Within this context, the present study aimed to investigate the effect of the hydroalcoholic extract of aerial parts of *B pilosa* on leukocytes mobilization.

MATERIALS AND METHODS

Plant preparation: *B. pilosa* was collected in Vale dos Sinos region, in Rio Grande do Sul State, Southern Brazil. A voucher of this plant was deposited at Herbarium of the Botanic Department, under the number ICN 674. The dried and powdered aerial parts (about 500 g) were macerated in ethanol/water (80: 20, v/v) with occasional stirring for 7 days at room temperature. The solution obtained after filtration was concentrated under reduced pressure (40°C) to obtain 230 g of a dark solid. Further chemical analysis of the ethanol crude extract revealed the presence of phenolic compounds, flavonoids, alkaloids and steroids.

Animals: Male Wistar rats weighing 180-200 g were housed at room temperature (22-25°C) and fed standard rodent diet and water *ad libitum*. Care and handling of the animals were in agreement with internationally accepted procedures and approved by our institutional committee following the International Guiding Principles for Biomedical Research Involving Animals.

Chemotaxis assay: The chemotaxis assay was performed using a chamber as described by Boyden (1962) with changes introduced by Zigmond *et al*. (1981) and Snyderman and Goetzl (1981). Polymorphonuclear Neutrophils (PMN) were obtained 4 h after the injection of 20 mL of sterile 1% glycogen (w/v) in the peritoneum of Wistar rats. The isolated leukocytes were suspended in Hanks' Balanced Salt Solution (HBSS), pH 7.4 and diluted to obtain a leukocyte density of about 3.5×10^{6} cells mL^{-1}. To obtain the chemotactic stimulant, plasma was incubated at 37°C for 30 min with a 65 μg mL^{-1} solution of LPS from *Escherichia coli* and then diluted in HBSS. The

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cell suspension was transferred to different tubes, treated separately with crude extract to a final concentration of 50, 100 and 200 M, indomethacin at 100 M (positive control) and control cells suspended in vehicle (HBSS). The cells were incubated at 37°C in water bath for 1 h. After that, leukocyte suspensions were placed in the upper wells and separated from the chemotactic stimulant in the lower compartment by an 8.0 m nitrocellulose filter (Millipore, USA). The chamber was incubated at 37°C in humidified air with 5% CO_2 for 1 h. After incubation, the top plate, gasket and filter were removed. The filter was air-dried and stained with panoptic staining. The cells that had migrated through to the underside of the filter were counted in duplicate in five high-power fields using a 100×objective.

Pleurisy induction: Rats were anaesthetized and 0.1 mL of a 1 mg mL^{-1} solution of carrageenan was injected intrapleurally, as described by Spector (1956). Four hours later, the animals were sacrificed by exsanguination through severed carotid and jugular veins and the pleural cavity was exposed. Exudates were collected and the cavity was flushed with Phosphate-Buffered Saline (PBS). The total number of leukocytes in the pleural exudate was counted in a Neubauer chamber. Slides of the cell exudate were also prepared and differential cell counting was performed. Leukocyte accumulation in the peripheral blood was also determined. Rats were first anesthetized and blood samples were obtained from the tail before and 4 h after carrageenan injection for differential leukocyte counting. The animals were divided in four groups: two crude extract groups (125 and 250 mg kg^{-1}), one indomethacin (10 mg kg^{-1}) and one control (saline) group. All animals were treated orally 60 min before the induction of inflammation.

Statistical analysis: Data are described as Mean±SEM. The differences between control and treatment tests were analyzed using the Student t test for chemotaxis and pleurisy models. One-way ANOVA and the Tukey's test were used to analyze the significance of differences between means.

RESULTS AND DISCUSSION

Inflammation is a pathophysiologic response of mammalian tissues to a variety of hostile agents, such as infectious organisms, toxic chemical substances, physical injury, or tumor growth, which leads to local accumulation of plasma fluids and blood cells (Takeda and Akira, 2005).

Cell migration has been the focus of research for more than a century because of its role in several important physiological and pathological processes. During inflammatory responses, immune cells migrate from the periphery into the injury site in response to locally released chemotactic agents (Broughton *et al*., 2006). This is usually a beneficial process; however, there can be negative consequences, such as when inflammation leads to a chronic immune response (Norman and Kubes, 2005; Moser and Willimann, 2004).

Table 1: Effect of Hydroalcoholic Extract (HE) of *Bidens pilosa* on LPS-induced chemotaxis of rat polymorphonuclear cells

Treatment	Migration (m)
Control	108.0±10.6
Indomethacin 100 (g mL^{-1})	11.4±3.4$^{A\#}$
HE 50 (g mL^{-1})	112.8±7.3
HE 100 (g mL^{-1})	69.6±13.3^{A}
HE 200 (g mL^{-1})	51.6±11.0^{A}

n = 10 lectures/field Mean±SEM, Student's t-test: Compared with control group: $^{A}p<0.01$ ANOVA/Tukey: $^{\#}p<0.01$

Consequently, pharmaceuticals that can modulate immune response, particularly those that act on cells that participate in inflammatory responses, are an important resource and potential drugs should be tested *in vitro* and *in vivo* using sensitive and reproducible assays (Kishimoto *et al*., 2006; De Lucca *et al*., 2005). Several methods have been used to measure the *in vitro* chemotactic response of cells. Variations of the Boyden chamber assay Boyden (1962) are the most commonly used. In these assays, the cells are placed on a microporous membrane over a source of chemotactic agent. As the cells detect a concentration gradient of a chemotactic agent diffusing from below, they migrate through the membrane to its underside. Migrating cells are detected on the reverse side of the membrane after staining. Responding cells are usually counted as an endpoint assay at a predetermined time point.

The evaluation of chemotactic response of cells treated with *B. pilosa* extract revealed antimigratory activity in cells treated with *B. pilosa* crude extract at doses of 100 and 200 M and 35.5 and 52.2% inhibition of leukocyte migration (Table 1). Present study was the first to investigate the effect of *B. pilosa* crude extract on leukocyte motility that was induced to undergo chemotactic migration in a consistent and highly reproducible *in vitro* assay.

However, the results of *in vitro* assays are not always reliable because of the absence of pharmacokinetic factors, which justifies the conduction of *in vivo* assays. Therefore, a pleurisy assay was conducted with Wistar rats. Intrapleural administration of carrageenan into the pleural space leads to pleurisy, an inflammation characterized by immediate recruitment of polymorphonuclear cells (PMN). Carrageenan is a high-molecular-weight sulfated polysaccharide, capable of inducing the release of mediators of vascular changes

Table 2: Effect of hydroalcoholic extract (HE) of *Bidens pilosa* in total number of leukocytes in peripheral blood before and after induction of inflammation by carrageenan administration and cell profile in pleural exudate

	Leukocytes in peripheral blood×10^6 cells mL^{-1}			
Treatment	TL (before)	TL (after)	Neu (before)	Neu (after)
Control	8650.33±3478.2	112108.00±1853.19**	1259.67±484.05	4680.25±1508.29**
Indomethacin 10 (mg kg^{-1})	6566.67±1504.88	8250.00±1459.79*^{A}	1891.67±573.06	4443.16±1687.42**
HE 125 (mg kg^{-1})	7700.00±1349.10	11158.33±3402.12**	1452.08±397.19	5637.25±1163.50**
HE 250 (mg kg^{-1})	9691.67±2455.08	12541.67±871.45**	1953.10±425.70^{A}	6277.92±1495.21**

	Leukocytes in peripheral blood ×10^6 cells mL^{-1}			Pleural exudate ×10^6 cells mL^{-1}	
Treatment	Ly (before)	Ly (after)	Total cells	PMN	MN
Control	7135.0±2167.39	6341.75±2002.88	2.63±1.03	2.02±1.11	0.61±0.25
Indomethacin 10 (mg kg^{-1})	4446.00±1290.52^{A}	3584.42±960.36$^{B\#}$	1.87±0.95^{A}	0.81±0.44^{A}	1.06±0.71^{B}
HE 125 (mg kg^{-1})	6121.42±1375.33	5127.25±2746.64	2.25±1.36	1.33±1.36	0.92±0.70
HE 250 (mg kg^{-1})	7456.17±2203.82	5761.33±1196.40*	1.04±0.37$^{B\#}$	0.23±0.21$^{A\#}$	0.81±0.27

Each value is the mean for 7-9 animals. (Mean±SEM). TL: Total number of leukocytes, Neu: Neutrophils, Ly: Lymphocytes, PMN: Polymorphonuclear cells, MN: Mononuclear cells. Student's t-test: Compared with control group: $^{A}p<0.05$; ^{B}p .01. Compared between before and after inflammatory induction process *p<0.05; **p<0.01. ANOVA/Tukey: p<0.01

associated with acute inflammation (Corsini *et al*., 2005), such as histamine, thromboxane A2, leukotrienes, cytokines and nitric oxide release (Eum *et al*., 2003).

Recruitment of leukocytes from circulation to sites of inflammation involves numerous soluble factors that mediate communication and interaction between circulating leukocytes and vascular endothelium (Luster, 1998). Of these soluble mediators, chemokines play a pivotal role in the process of adhesion and directional migration of leukocytes. Chemokines are produced by a variety of cell types, such as those of hematopoietic and nonhematopoietic origins, in response to antigens, polyclonal stimulants, cell irritants and cytokines.

A study that used *B. pilosa* infusion found enhanced cytokine production by whole blood (Abajo *et al*., 2004). Other *in vitro* and *in vivo* studies used methanol extracts at 100 g mL^{-1} and 10 mg mL^{-1} concentrations and found suppression of human lymphocyte proliferation (Pereira *et al*., 1999). The data obtained showed the potent immunosuppressive action of *B. pilosa* extracts, which suggests its use as an anti-inflammatory drug. Moreover, Tan *et al*. (2000) found significant inhibitory activity in the synthesis of prostaglandins by *B. pilosa* ethanol extract in an *in vitro* study.

The pleurisy assay in rats revealed an increase in the number of peripheral neutrophils in all groups after the administration of the inflammatory agent (Table 2). Animals treated with indomethacin had a lower number of leukocytes than the control group after carrageenan administration. The differential count showed no significant differences were found between groups before or after inflammation, or between study and control groups. A significant reduction of lymphocytes was found after pleurisy induction in animals treated with 250 mg kg^{-1} of *B. pilosa*. Animals that received indomethacin also had a lower number of lymphocytes than the control group but before the induction of inflammation. No significant differences were found between treatments before and after pleurisy. The pleural exudate cell profile showed a significant decrease (about 28.9%) in number of migrated leukocytes in animals treated with indomethacin when compared with the control group. There was a predominance of Mononuclear cells (MN) and their number was greater than in the control group. Polymorphonuclear (PMN) cells were 40% of the cells found in the control group and a significant reduction was found. Animals treated with 125 mg kg^{-1} of plant extract did not show a significant response to treatment and no leukocyte mobilization to the pleural cavity was found. Animals that received 250 mg kg^{-1} doses had a decrease of about 60.5% of leukocyte migration to pleural exudate and about 88.6% decrease in number of PMN cells when compared with controls, which suggests a potential anti-inflammatory activity of *B. pilosa* at this dose. Kankaanranta *et al*. (1994) suggested that the suppression of neutrophil migration might control inflammatory responses, a mechanism of action found in certain nonsteroidal anti-inflammatory drugs.

The wide pharmacological applications of *B. pilosa* can be attributed to its chemical compounds, especially polyacetylenes and flavonoids, which are active anti-inflammatory agents (Pereira *et al*., 1999). The metabolism and bioavailability of phenolic compounds *in vivo* seem to correlate well with their *in vitro* anti-inflammatory properties. Polyacetylenes isolated from *B. camphylotheca* inhibited prostaglandin biosynthesis (Redl *et al*., 1994).

The Shinoda test showed that *Bidens pilosa* extracts are positive for flavonoids (Markham, 1982) and several studies that reported on the anti-inflammatory activity of flavonoids have been cited by Middleton and Kandaswami (1994). Additionally, phenolic, alkaloid,

steroid and lactonic compounds were also found. These findings are in agreement with those reported by Oliveira *et al.* (2004) and Alvarez *et al.* (1999). However, tannins and saponins were not found, as reported by Tan *et al.* (2000) and Alvarez *et al.* (1999). This difference in chemical composition may be assigned to edaphic and climatic variations.

In this study, the *in vitro* and *in vivo* anti-inflammatory properties of the crude extract of *Bidens pilosa* were demonstrated and findings support its general use in folk medicine.

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