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Antiinflammatory and antinociceptive activities of *Blechnum occidentale* L. extract

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ABSTRACT

Aim of study: Blechnum occidentale L. is a terrestrial fern that ranges from the United States to South America, and is employed in Brazilian folk medicine. In the present study we investigated the antinociceptive and antiinflammatory activities of the methanolic extract of *Blechnum occidentale* L. (MEB) in animal models of pain and inflammation to support its medicinal use in treatment of inflammatory and pulmonary diseases, urinary infections and liver diseases.

Materials and methods: The antinociceptive activity of MEB was evaluated using the writhing, formalin, and tail flick tests. The antiinflammatory activity of MEB was evaluated in carrageenan-induced paw oedema and neutrophil migration. In order to discard possible non-specific muscle relaxant or sedative effects of MEB, mice motor performance was evaluated in the rota rod test and its toxicity evaluated over 14 days.

Results: Intraperitoneal (IP) administration of MEB (0.01–100 mg/kg) produced a dose-related antinociception on acetic acid-induced writhing in mice. Oral administration of MEB, at a different range of doses (100–400 mg/kg), also produced significant antinociceptive effect on the writhing test. Furthermore, treatment with MEB (100 and 200 mg/kg IP) inhibited significantly both the early and late phases of formalin-induced hypernociception in rats. In contrast, treatment with MEB (100 and 200 mg/kg IP) did not prevent the thermal nociception in the tail flick test. The IP administration of MEB (100 and 300 mg/kg) significantly reduced the paw oedema induced by carrageenan. Moreover, systemic treatment with MEB (11–300 mg/kg) reduced the neutrophil migration in the carrageenan-induced migration to the peritoneal cavity. In the rota rod test, MEB-treated mice did not show any significant motor performance alterations with the dose of 300 mg/kg. In addition, over the study duration of 14 days, there were no deaths or toxic signs recorded in the mice given 100 or 1000 mg/kg of MEB.

Conclusion: The results described here are the first report of pharmacological studies of *Blechnum occidentale* L. and indicate that this plant has antinociceptive and antiinflammatory activities which support its folk medicine use.

1. Introduction

The clinical treatment of inflammatory diseases is dependent on nonsteroidal or steroidal chemical therapeutics (Rainsford, 2007). Nonsteroidal antiinflammatory drugs (NSAID) reduce the pain and inflammation by blocking the metabolism of arachidonic acid by cyclooxygenase enzyme (COX), and thereby the production of prostaglandin (Vane, 1971). Since prostaglandins are cytoprotective, long-term administration of NSAID may induce gastro-intestinal ulcers, bleeding, and renal disorders due to their non-selective inhibition of both isoforms of the COX enzyme, the constitutive (COX-1) and the inducible (COX-2) isoforms (Robert, 1976; Peskar, 1977; Tapiero et al., 2002). On the other hand, fully selective and reversible COX-2 inhibitors with reduced gastro-intestinal toxicity have been associated with adverse car-

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diovascular effects (Dogné et al., 2005). Furthermore, the use of steroidal drugs as antiinflammatory agents is also becoming highly controversial due to their multiple side effects (Schäcke et al., 2002; Reinke et al., 2002). Therefore, developing new agents with more powerful analgesic and antiinflammatory activities and with lesser side effects is, at present, of great interest.

Since ancient times, pteridophytes have been considered an excellent source of medicines, as Dioscorides and Galeno stated in their manuscripts (Barros and Andrade, 1997), and have been used to treat different types of pain (Cambie and Ash, 1994; Christensen, 1997; Gogoi, 2002; Bresciani et al., 2003). Some species of *Blechnum*, an example of pteridophyte genus, are employed in folk medicine worldwide. *Blechnum occidentale* L. is a terrestrial and/or rupestrial fern that ranges from the United States to South America, including Colombia, Venezuela, Guayanas, Ecuador, Peru, Bolivia, Brazil, Paraguay, Chile, and Argentina (Moran, 1995). In Brazil this fern has been used to treat inflammatory and pulmonary diseases, urinary infections and liver diseases (Barros and Andrade, 1997). However, few works about pteridophyte medicinal properties have been reported, especially in oriental countries where traditional medicine employs many species of herbs and is largely used by a large number of people. In fact, to the best of our knowledge, this is the first report of pharmacological studies of *Blechnum occidentale* L. As part of our continuous interest in Brazilian native plants and in order to support the use of this fern in folk medicine, the present study was undertaken to establish the antiinflammatory and antinociceptive properties of the methanolic extract of *Blechnum occidentale* L. (MEB). In addition, we evaluated acute toxicity and motor performance alterations associated with MEB.

2. Materials and methods

2.1. Plant material

Plant specimens were collected in the Atlantic Forest region at Salvador, Bahia State, Brazil, in August, 2006, in authorized areas by IBAMA (Brazilian Institute for the Environment and Natural Resources) and received botanic identification by Dr. Fabiana R. Nonato. A voucher specimen has been deposited at the Herbarium of Universidade Estadual de Feira de Santana, Bahia, Brazil (HUEFS 142950).

2.2. Preparation of the methanolic extract

The air-dried and powdered blades of *Blechnum occidentale* (37.6 g) were exhaustively extracted with methanol. The obtained extracts were filtered and evaporated under reduced pressure, on a rotary evaporator at 40–45 °C, to yield 5.6 g (15%, w/w) of crude methanolic extract.

2.3. Animals

Experiments were performed on male Wistar rats (180–200 g) or Swiss Webster mice (30–35 g) from the Animal Facilities of Centro de Pesquisas Gonçalo Moniz. Animals, individually housed at 24 ± 1 °C, under a 12:12 h light–dark cycle (lights on at 07:00 a.m.), with free access to chow and tap water until the day of the experiment, when only water was made available to them. Each animal was used only once. Animal care and handling procedures were in accordance with International Association for the Study of Pain guidelines for the use of animals in pain research (Zimmermann, 1983) and Institutional Animal Care and Use Committee – FIOCRUZ 26/2009-1.

All efforts were made to minimize the number of animals used and any discomfort. All behavioral testing was performed between 8:00 a.m. and 4:00 p.m.

2.4. Nociceptive tests

In the present study we used the term hypernociception rather than hyperalgesia or allodynia to define the decrease in the nociceptive withdrawal threshold, since the pain perception in animals is not obvious.

2.4.1. Writhing test

The intraperitoneal and oral antinociceptive doses of MEB were determined in mice using the writhing test. Acetic acid (0.8%, v/v, 10 ml/kg) was injected into the peritoneal cavities of mice, which were placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 30 min after the stimulus injection (Collier et al., 1968). The writhing response consists of a contraction of the abdominal muscle together with a stretching of the hind limbs. The antinociceptive activity was expressed as the writhing scores over 30 min.

2.4.2. Formalin test

Rats were placed in an open Plexiglas observation chamber for 30 min to accommodate to their surroundings, and then removed for formalin administration. Rats were gently restrained while the dorsum of the hind paw was subcutaneously administered with 50 μl of formalin 1% (1:100 dilution of stock formalin solution, 37% formaldehyde in 0.9% saline) using a 30 gauge needle. Following injection, the rat was returned to the observation chamber for a 60 min observation period. A mirror was placed behind the chamber to enable unhindered observation of the formalin-injected paw. Rats were observed from 0 to 10 min (early phase) and from 15 to 60 min (late phase) and a nociception score was determined for each period by counting the number of flinches of the injected limb during the observation time (Dubuisson and Dennis, 1977). Flinches were discrete and easily quantifiable.

2.4.3. Tail flick test

The tail flick test (Analgesiometer, Insight, Brazil) in rats was conducted as described elsewhere (D'Amour and Smith, 1941), with minor modifications. Each animal was placed in a ventilated tube with the tail laid across a wire coil which was at room temperature (23 ± 2 °C). The coil temperature was then raised by the passage of electric current and the latency for the tail withdrawal reflex was measured. The heating was applied to a portion of the ventral surface of the tail 4 cm from the tip. Each trial was terminated after 6 s to minimize the probability of skin damage. Tail flick latency was measured before and 40 min after the MEB or saline administration.

2.5. Paw oedema induced by carrageenan

The volume of the mice paw was measured with a plesthismometer (Ugo Basile, Comerio, Italy) before (Vb, baseline) the intraplantar administration of carrageenan (200 μg) and 2, 4, 24 and 48 h after (Vt), as described previously (Winter et al., 1962). The amount of paw swelling was determined for each mouse and the difference between Vt and Vb was taken as the oedema value.

2.6. Leukocyte migration

The leukocyte migration test in mice was conducted as described elsewhere (Ramos et al., 2005). Carrageenan (2 mg/cavity) was injected into the peritoneal cavities in mice, and leukocyte migration was evaluated 4 h after stimulus. The animals were sacrificed,

and the peritoneal cavity cells were harvested with 3 ml PBS containing 1 mM EDTA. Total cell counts were performed in a Neubauer chamber and differential cell counts (100 total cells) were obtained using panotic-stained cytospin preparations. The differential count was performed under light microscope, and the results were presented as number of neutrophils per cavity, according to standard morphological criteria. The results were expressed as the number of neutrophils/cavity.

2.7. Acute toxicity

The method described by Lorke (1983) with slight modification was used to determine the safety of the MEB. Briefly, normal healthy male mice were divided into groups of five mice in each cage. MEB (100 and 1000 mg/kg) or vehicle was intraperitoneally administered. Access to food and water, toxic symptoms and the general behavior of mice were observed continuously for 1 h after the treatment and then intermittently for 4 h, and thereafter over a period of 24 h. The mice were further observed for up to 14 days following treatment for any signs of toxicity and mortality.

2.8. Motor function assay: rota rod

To evaluate the possible non-specific muscle-relaxant or sedative effects of MEB, mice were submitted to the rota rod task (Vaz et al., 1996). Rota rod apparatus (Insight, Ribeirão Preto, Brazil) consisted of a bar with a diameter of 3 cm, subdivided into four compartments. The bar rotated at a constant speed of five revolutions per minute. The animals were selected 24 h previously by eliminating those mice which did not remain on the bar for two consecutive periods of 120 s. Animals were treated with Diazepan (10 mg/kg IP), MEB (200 mg/kg IP) or vehicle and 30 min after were placed on a rotating rod. The latency to falling was measured up to 120 s. The results are expressed as the average time(s) the animals remained on the rota rod in each group.

2.9. Drugs

Indomethacin, dexamethasone and carrageenan were obtained from Sigma Chemical Company (St. Louis, MO, USA). Diazepam was obtained from Cristália (Itapira, São Paulo, Brazil). Indomethacin was dissolved in Tris–HCl 0.1 M pH 8.0 plus saline. Dexamethasone (1 mg/ml) was dissolved in ethanol (10% in normal saline). Remaining drugs and MEB were dissolved directly in saline. The drugs were administrated by oral (PO), intraperitoneal (IP) or subcutaneous (SC) routes.

2.10. Data analysis

Data are presented as means ± SEM of measurements made on five animals in each group. Comparisons across three or more treatments were made using one-way ANOVA with Tukey's post hoc test or repeated measures two-way ANOVA with Bonferroni's post hoc test, when appropriate. All data were analyzed using the Prism 4 computer software (GraphPad, San Diego, USA). Statistical differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Antinociceptive effect of MEB

The antinociceptive activity of MEB was evaluated by using the writhing test. Intraperitoneal administration of MEB (0.01–100 mg/kg), 30 min before the acid injection, produced a significant ($P < 0.01$) and dose-related inhibition of acetic acid-induced abdominal constrictions in mice (Fig. 1A). Indomethacin (10 mg/kg IP), a standard NSAID used as positive control, also produced significant inhibition of acetic acid-induced writhing response. Moreover, oral administration of MEB (100–400 mg/kg), 40 min before the acid injection, produced a significant ($P < 0.05$) inhibition of acetic acid-induced abdominal constrictions in mice (Fig. 1B). Fig. 2 shows the effect of MEB on the formalin-induced hypernociception in rats. Injection of formalin in control animals induced a biphasic flinching response, with the early phase ranging from 0 to 10 min (Fig. 2A) and the late phase from 15 to 60 min (Fig. 2B) after the injection. Treatment with MEB (100 and 200 mg/kg) by intraperitoneal route 30 min before the formalin caused a antinociceptive effect ($P < 0.001$) in both the early and late phases of formalin-induced hypernociception in rats. Indomethacin (5 mg/kg IP) produced a significant ($P < 0.001$) antinociceptive effect in rats submitted to the formalin test. In a different way, MEB (100 and 200 mg/kg IP) did not alter the latency response to the tail flick test. In contrast, morphine (5 mg/kg SC) caused a significant increase in the latency response (data not shown).

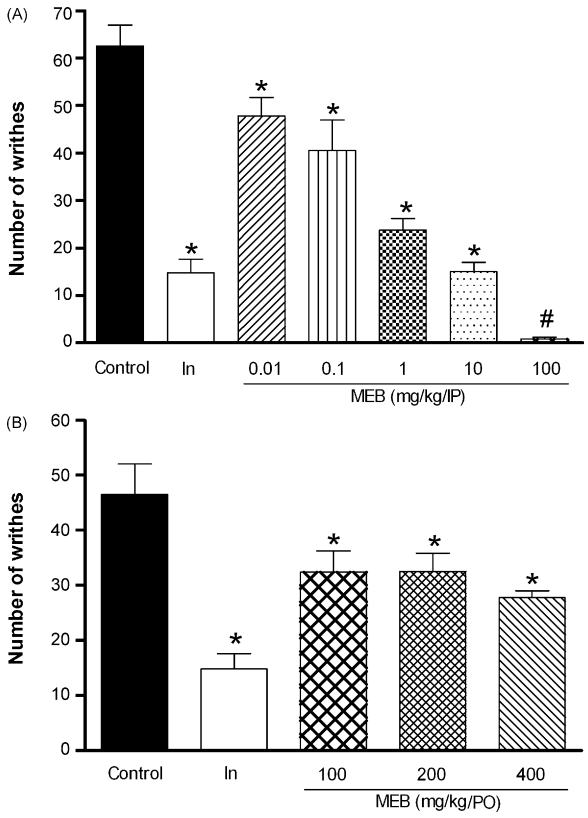

Fig. 1. Effects of oral or intraperitoneal administration of *Blechnum occidentale* L. methanolic extract (MEB) on acetic acid-induced writhing in mice. (A) Mice were treated with MEB (0.01, 0.1, 1, 10 and 100 mg/kg) or control (saline) by intraperitoneal route 30 min before acetic acid 0.8% (injected at time zero). (B) Mice were orally treated with MEB (100, 200 and 400 mg/kg) or saline (control group) 40 min before acetic acid 0.8%. Indomethacin (In; 10 mg/kg IP) was the reference drug. Data are expressed as means ± S.E.M.; $n = 5$ mice per group. *Significantly different from control group; #significantly different from the remaining groups ($P < 0.05$) as determined by ANOVA followed by Tukey's test.

3.2. Antiinflammatory effects of MEB

The antiinflammatory effects of MEB were initially evaluated in the paw oedema model in mice. The results in Fig. 3A indicate that the administration of MEB (100 and 300 mg/kg IP) 30 min before carrageenan reduced significantly ($P < 0.01$) the oedema at 2, 4, 24 and 48 h after the carrageenan injection. Furthermore, the administration of MEB at the same doses 4 h before car-

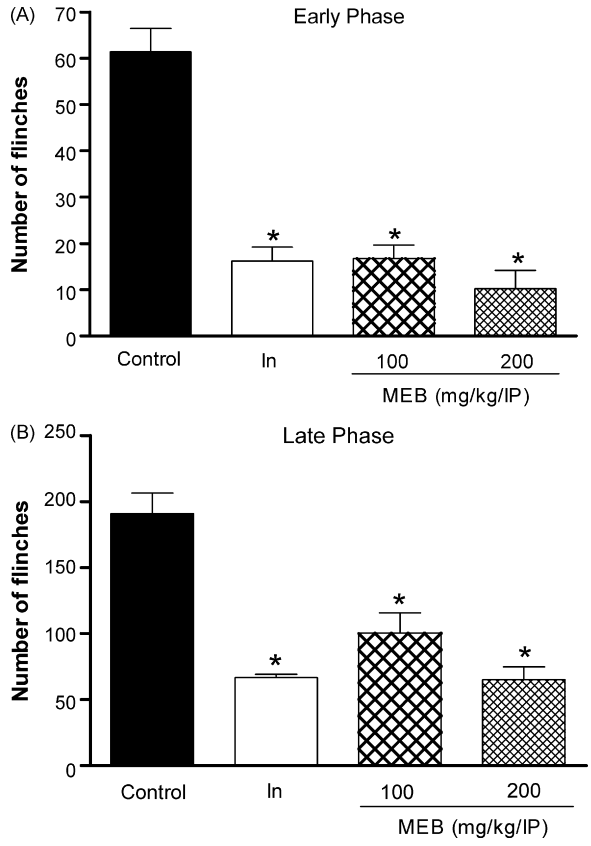

Fig. 2. Effects of *Blechnum occidentale* L. methanolic extract (MEB) treatment on inflammatory pain induced by formalin in rats. Inflammatory pain was induced by subcutaneous intraplantar injection of formalin 1% (50 μl). (A and B) Effects of MEB on the early and late phases of formalin-induced flinches in rats, respectively. Wistar rats were treated with MEB (100 and 200 mg/kg) or saline (control group) by intraperitoneal administration 30 min before formalin (injected at time zero). Indomethacin (In; 5 mg/kg IP) was the reference drug. Data are expressed as means ± S.E.M.; $n = 5$ mice per group. *Significantly different from control group ($P < 0.05$) as determined by ANOVA followed by Tukey's test.

rageenan also reduced significantly ($P < 0.01$) the oedema 2, 4 and 24 h, but not 48 h after the carrageenan injection (Fig. 3B). The oedema induced by intraplantar injection of carrageenan in mice was strongly inhibited at 2, 4, 24 and 48 h by pre-treatment (4 h before) with dexamethasone (0.7 mg/kg SC). MEB was also capable of reducing the neutrophil migration in the carrageenan-induced

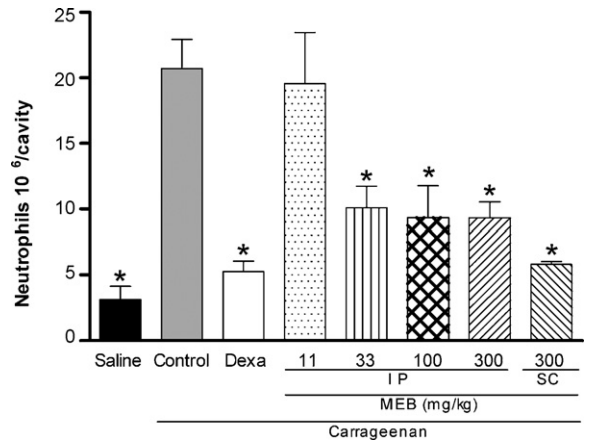

Fig. 4. Effects of *Blechnum occidentale* L. methanolic extract (MEB) treatment on carrageenan-induced neutrophil migration to peritoneal cavity. Mice were treated with MEB (11, 33, 100 and 300 mg/kg IP), MEB (300 mg/kg SC), dexamethasone (0.7 mg/kg SC) or saline (control group) 4 h before carrageenan (2 mg/cavity) intraperitoneal stimulus. Peritoneal exudates were collected 4 h after carrageenan. Total counts were performed with a cell counter and differential cell counts were carried out on light microscope. Results are expressed as means ± S.E.M. of number of neutrophil 10^6/cavity ($n = 5$). *Significantly different from control group ($P < 0.01$) as determined by ANOVA followed by Tukey's test.

neutrophil migration to the peritoneal cavity (Fig. 4). Mice were treated with MEB (11, 33, 100, 300 mg/kg IP and 300 mg/kg SC) or dexamethasone (0.7 mg/kg SC) 4 h before the intraperitoneal injection of carrageenan (2 mg/cavity). The neutrophil migration to the peritoneal cavity was evaluated 4 h after carrageenan. MEB, as well as the reference drug dexamethasone, significantly inhibited the carrageenan-induced neutrophil migration ($P < 0.01$).

3.3. Acute toxicity of MEB

Over the study duration of 14 days, there were no deaths recorded in the groups of mice given 100 or 1000 mg/kg IP of MEB. During the observation period, MEB administration did not produce any variations in the general appearance or toxic signs in the animals.

3.4. Effect of MEB in the motor performance

In the rota rod test, MEB-treated mice did not show any significant motor performance alterations with the dose of 300 mg/kg IP

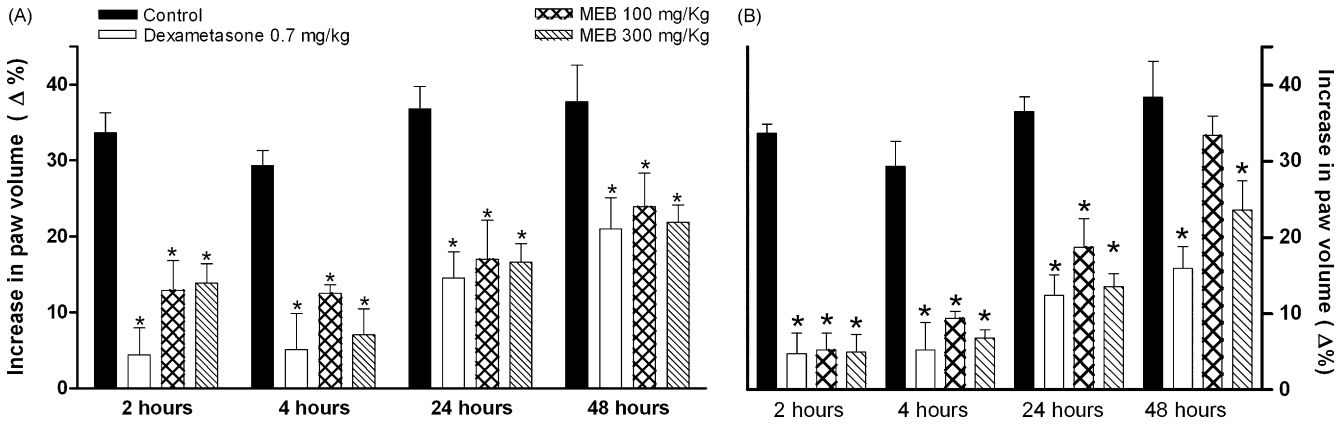

Fig. 3. Effects of *Blechnum occidentale* L. methanolic extract (MEB) treatment on carrageenan-induced oedema. Oedema was induced by intraplantar injection of carrageenan (200 μg/25 μl/paw) in mice. MEB (100 and 300 mg/kg) or saline (control group) was administered by intraperitoneal route 30 min (A) or 4 h (B) before carrageenan (injected at time zero). Dexamethasone (0.7 mg/kg SC), the reference drug, was administered 4 h before carrageenan. Data are expressed as means ± S.E.M.; $n = 5$ mice per group. *Significantly different from control group ($P < 0.05$) as determined by two-way ANOVA followed by Bonferroni's test.

(113.7 ± 6.3 s) if compared to control (105.4 ± 8.1 s). As expected, the central nervous system depressant diazepam (10 mg/kg IP) reduced the time of mice on the rota rod after 30 min of treatment with this standard drug (6.1 ± 1.9 s).

4. Discussion

The purpose of this work was to establish the scientific basis for the folk use of *Blechnum occidentale* L. The present study demonstrates, for the first time, that systemic administration of the MEB, at doses that did not produce any motor performance alteration, produced consistent antinociceptive and antiinflammatory effects in different models of pain and inflammation. The antinociceptive effect of MEB was observed in acetic acid-induced writhing and formalin tests, but not in the tail flick test. Moreover, MEB produced antiinflammatory effect on carrageenan-induced paw oedema and neutrophil migration.

Intraperitoneal administration of MEB produced a dose-related antinociception when assessed in acetic acid-induced writhing in mice. Oral administration of MEB was less potent and efficacious than its intraperitoneal administration in preventing the nociception induced by acetic acid. In fact, the bioavailability of active substances may be decreased when given orally, due to the instability in gastric and intestinal fluids and/or poor absorption in the gastro-intestinal tract (Kofi-Tsekpo, 1994). The writhing test has long been used as a screening tool for the assessment of analgesic or antiinflammatory properties of new substances (Collier et al., 1968). This method presents a good sensitivity; however, it shows poor specificity. To avoid misinterpretation of the results, in the present study we confirmed the antinociceptive effect of MEB in a model of inflammatory pain, the formalin test, which has two distinctive phases that can possibly indicate different types of pain (Hunskaar and Hole, 1987). The early and late phases of formalin test have obvious differential properties, and therefore this test is useful not only for assessing the analgesic substances but also for elucidating the mechanism of analgesia (Shibata et al., 1989). The early phase, named non-inflammatory pain, is a result of direct stimulation of nociceptors and reflects centrally mediated pain; the late phase, named inflammatory pain, is caused by local inflammation with a release of inflammatory and hyperalgesic mediators (Hunskaar and Hole, 1987). In the present study we found that MEB produced antinociceptive activity both in the early and late phases of formalin test. Considering the inhibitory property of MEB on the second phase of formalin, we suggest that its antinociceptive activity is due, at least in part, to an antiinflammatory action. In line with this idea, the treatment with MEB did not prevent the nociception in the tail flick test. The thermal model of the tail flick test is considered to be a spinal reflex, but could also involve higher neural structures and this method identifies mainly central analgesics (Jensen and Yaksh, 1986; Le Bars et al., 2001). The fact that MEB produced antinociception in all nociceptive models tested, except in the tail flick test, suggests that it does not block the neural transmission of pain, like morphine does. Moreover, disproving relaxing or motor deficit effects, MEB treatment, at the therapeutic doses, did not affect the motor performance of the mice as tested in the rota rod test. This result corroborates the antinociceptive effect of MEB suggested by the nociceptive tests.

With the goal of proving the antiinflammatory property of MEB, we evaluated the effects of MEB treatment on the carrageenan-induced paw oedema in mice. This method was chosen for this study since it is the most prominent experimental model in search for new antiinflammatory drugs and evaluation of antiinflammatory effect of natural products (Sugishita et al., 1981; Posadas et al., 2004; Asres et al., 2005). We found that the administration of MEB reduced significantly the carrageenan-induced paw oedema. This result reinforces the idea that the MEB possesses peripheral action, probably related to the arachidonic acid cascade (Le Bars et al., 2001). The injection of carrageenan in mice produces a typical biphasic oedema associated with the production of several inflammatory mediators such as histamine, serotonin, bradykinin, prostaglandins, nitric oxide, and cytokines (Levy, 1969; Di Rosa et al., 1971; Vinger et al., 1987; Henriques et al., 1987; Nantel et al., 1999; Posadas et al., 2004; Rocha et al., 2006). It has been demonstrated that a different profile of inflammatory mediators involved with the first and second phases of the carrageenan-induced paw oedema in mice. Siqueira-Junior et al. (2003) demonstrated that treatment with the COX-1 inhibitor reduced of the early phase of paw oedema. Moreover, COX-2 is up-regulated only in the second phase (Posadas et al., 2004). Conversely, it has been shown that nitric oxide and TNF-alpha are implicated in the two phases of carrageenan-evoked mouse paw oedema (Posadas et al., 2004; Bucci et al., 2005; Rocha et al., 2006). According to the result of our study, MEB was able to effectively inhibit the oedema in both the earlier and in the later phases, suggesting that MEB inhibits different chemical mediators of inflammation. In addition, as these effects are the result of a single administration of the extract, it is possible that a relatively long-lasting antiinflammatory action of MEB. We also found that the full antiinflammatory effect of MEB was observed 4 h after its administration. This delayed antiinflammatory effect could indicate that it is dependent on the modulation of transcriptional factors and *de novo* synthesis of proteins involved in the inflammatory response. In fact, the major therapeutic effects of glucocorticoids, the most clinically used antiinflammatory drugs, are dependent on the interaction with responsive genes, which ultimately lead to the inhibition of pro-inflammatory protein transcription (Barnes, 2006). Confirming the relevant antiinflammatory activity of MEB we observed that the neutrophil migration to peritoneal cavity was strongly reduced by the extract. The cell migration inhibition in models of inflammation has been considerate a convincing indicator of antiinflammatory activity (Bradley et al., 1982; Faurschou and Borregaard, 2003).

Taken together, the results presented herein strongly suggest that *Blechnum occidentale* L. possesses analgesic and antiinflammatory effects, supporting the use of this plant species in folk medicine. Furthermore, the acute toxicity does not show any symptoms, changes in behavior or mortality at 1 g/kg doses that indicate a therapeutic safety for the doses pharmacologically active. The precise mechanisms through which MEB exerts its action are currently under investigation, but possibly it could be related to the arachidonic acid cascade and/or modulation of pro-inflammatory molecules production.

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