Anti-inflammatory and anti-nociceptive effects of *Zeyheria montana* (Bignoniaceae) ethanol extract

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In this work, the analgesic and anti-inflammatory activities of Zeyheria montana *Mart. ethanol leaf extract were investigated at doses of 75, 150 and 300 mg/kg body weight. In the analgesic assay, against a chemical stimulus in mice, acetic acid-induced writhes were significantly inhibited by the extract at doses of 75 mg/kg (67.27%), 150 mg/kg (49.38%) and 300 mg/kg (82.87%). Also, a vigorous decrease in hyperalgesia was observed when measured after 2 h and 6 h of lipopolysaccharide stimulation of rats for all doses of extract tested.* Z. montana *extract, at doses of 75 and 300 mg/kg, caused very slight central analgesia in rats submitted to thermal stimulus, particularly noticeable at 30 min following treatment. The anti-inflammatory activity of* Z. montana *extract on carrageenan-induced oedema in rats was evaluated. The oedema development, measured at 180 min following carrageenan intraplantar injection, was significantly reduced by all tested doses: 75 mg/kg (33.30%), 150 mg/kg (45.80%) and 300 mg/kg (75.00%). The* LD_{50} *value was greater than 2000 mg/kg. These results demonstrated that the ethanol extract from* Z. montana *leaf possesses anti-nociceptive and anti-inflammatory activities, which could be of relevance for the pharmacological control of pain and inflammatory processes.*

Key words: anti-inflammatory - anti-nociceptive - *Zeyheria montana*

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most important drugs used in the reduction of pain and inflammation. Their efficacy has been confirmed by a wide number of experimental and clinical studies (Kean & Buchanan 2005, Zochling et al. 2006, Ong et al. 2007). Despite their efficacy, the use of NSAIDs induces serious adverse effects when administrated chronically, with the gastrointestinal tract being the most affected system (Ofman et al. 2002). For this purpose, an increasing number of studies are being carried out in search of new therapeutics from medicinal plants, especially those with proclaimed popular use as anti-inflammatory and analgesic agents (Gupta et al. 2006).

Zeyheria montana Mart. (Bignoniaceae) [syn. *Zeyheria digitalis* (Vell). Hoehne], commonly known as *"*bolsa de pastor*"*, is a plant species native to the Brazilian "cerrado". *Z. montana* leaf and root extracts are conventionally used in Brazilian folk medicine for the treatment of ulcers, skin tumours and inflammatory diseases (Jacome et al. 1999, Bertoni et al. 2007). Phytochemical analyses of *Z. montana* leaf extract showed the presence of terpenoids and flavonoids, while root and stem extracts are particularly rich in lapachol (Almeida et al. 1990, Machado et al. 2006). Indeed, the amount of lapachol produced by *Z. montana* is about 19 times higher than in *Tabebuia avallenedae*, a plant species also from the Bignoniaceae family and traditionally known as the main source of this compound (Almeida et al. 1990, Jacome et al. 1999). Previous results have validated the popular use of *T. avallenedae* in the treatment of inflammatory and infectious disease, as well as an inhibitor of tumour growth, which has been mainly linked to the action of lapachol (Goel et al. 1987, Almeida et al. 1990, Favaro et al. 1990, Miranda et al. 2001). Moreover, the terpenoid ursolic acid has been isolated from *Z. montana* and is associated with *Z. montana*'s anti-tumour activities (Varanda et al. 1992).

Therefore, based on ethnopharmacological information, the aim of this work was to evaluate the analgesic and anti-inflammatory effects of *Z. montana* ethanol leaf extract.

MATERIAL AND METHODS

Drugs - Acetic acid was purchased from Chemco, Campinas, SP, Brazil; Aspirin from Bayer, São Paulo, SP, Brazil; Carrageenan from Sigma, St. Louis, MO, USA; indomethacin (Indocid^{R}) from Merck Sharp-Dolme, UK; and Tramadol, from Biosintética, São Paulo, SP, Brazil. Polyoxyethylene Sorbitan Monolaurate (Tween^{R} 20) was from USB (Cleveland, Ohio, USA) and solvents from Synth (São Paulo, SP, Brazil).

Animals - Pharmacological and toxicological experiments were carried out using Wistar albino male rats (150-250 g) or Swiss albino male mice (18-25 g) bred at the University of São Paulo (Ribeirão Preto, SP, Brazil) and at ANILAB Company (Paulínia, SP, Brazil), respectively. Animals were kept in cages under a 12 h light/12 h dark cycle at 21 ± 3°C, with food and water *ad libitum* and acclimatised to the laboratory room for 2-3 h prior to pharmacological experiments, which were performed under sound-attenuated conditions and at a controlled temperature (20-22ºC). All experiments were performed

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following the guidelines of the Brazilian Society of Neurosciences and received the approval of the Ethical Committee at the Universidade de Ribeirão Preto (UNAERP) (protocol n. 102/08).

Plant collection and extraction procedure - *Z. montana* leaves were collected in Franca, São Paulo, Brazil, and identified at the Instituto de Botânica at University of Campinas (Campinas, SP, Brazil). A voucher specimen (803) is kept in the Herbarium of UNAERP. Dried and powdered leaves (1 kg) were macerated in 95% ethanol (5.0 L) for 24 h at 25°C and the filtrate was concentrated, lyophilised and resuspended in distilled water prior to use.

High pressure liquid chromatography (HPLC) profile of Z. montana leaf ethanol extract - In order to obtain the chromatographic profile of *Z. montana* leaf ethanol extract, HPLC analysis was performed on a Shimadzu LC10ADvp system equipped with a Supelco LC18 column (SupelcosilTM RP-18, 250 x 4.6 mm id), coupled to a diode array detector, monitored at 210 nm to triterpenes and 280 nm to flavonoids. A two-solvent gradient system of water-acetic acid 0.1% (A) and methanol (B) was used. Ethanol extract (5 mg/mL) and triterpene standards (betulin, betulinic acid, oleanolic acid and ursolic acid) were eluted with 85% of B, at 1.0 mL/min under isocratic conditions. The gradient program for flavonoid analysis, using Zeyherin A and B as standards for comparison to the ethanol extract (5 mg/mL), consisted of three periods: (i) 0-20 min, 40-85% B, (ii) 20-22 min, 85-40% B and (iii) 22-28 min, 40% B, at a flow-rate of 1.0 mL/min. The identification of triterpenes and flavonoids in the ethanol extract was done by comparing their retention times and spectral data with those of standard compounds.

Anti-nociceptive activity: acetic acid-induced abdominal constrictions in mice - *Z. montana* leaf extract (75, 150 or 300 mg/kg, ip), aspirin (100 mg/kg, po) and saline (100 µL, ip) was administrated 60 min prior to the ip injection of 100 µL of 1% acetic acid in male Swiss mice that were divided into control (n = 7) and experimental groups (n = 7). After a latency time of 5 min, the number of writhing and stretching movements were counted over a period of 30 min and expressed as a percentage of analgesic activity according to the following equation: % analgesic activity = N - N^1/N x 100, where N is the number of abdominal contractions of animals treated with saline and N^1 is the number of abdominal contractions of animals treated with *Z. montana* extract or aspirin.

Anti-nociceptive activity: hyperalgesia test (Hargreaves) - *Z. montana* leaf extract (75, 150 and 300 mg/kg, ip), aspirin (100 mg/kg, po) or saline (100 µL, ip) was administered 60 min prior to the intraplantar injection of lipopolysaccharide (LPS) (250 ng/paw) in male Wistar rats that were divided into control (n = 6) and experimental groups (n = 6). Briefly, the hyperalgesia was evaluated as previously described (Hargreaves et al. 1988, Costello & Hargreaves 1989), where the hyperalgesia intensity was assessed by the variation in the time of animal reaction (index of hyperalgesia, IH) following the equation: IH = (To/Tn) - 1, with To and Tn being the basal time of animal reaction before LPS injection and the time values measured at 2 h and 6 h after the LPS stimulation, respectively.

Anti-nociceptive activity: nociceptive response induced by a thermal stimulus - Experiments were carried out following the method of Hargreaves et al. (1988) with modifications. Rats, distributed into control (n = 9) and experimental (n = 9) groups, were individually placed in an acrylic box under a mobile infrared heating lamp at 55 + 1.0°C (Ugo Basile equipment, Varese, Italy). The basal time reaction, the time elapsed until the animal reacted (shaking and/or lifting its right hind-paw), was observed over a period of 60 min prior to treatments. Animals were injected with *Z. montana* leaf extract (75, 150 or 300 mg/kg, ip), tramadol (100 mg/kg, ip) or saline (100 µL, ip) 60 min prior to the thermal stimulus. The latency time for the animal response was recorded at 30, 60, 120 and 180 min.

Anti-inflammatory activity: inhibition of carrageenan-induced paw oedema - The anti-inflammatory effects of *Z. montana* extract were measured according to Winter et al. (1962). Right hind-paw volume of each animal in control (n = 7) or in experimental (n = 7) groups was measured prior to any treatments using a digital Plethysmometer 7140 (Ugo Basile, Verese, Italy). *Z. montana* extract (75, 150 or 300 mg/kg, ip), indomethacin (10 mg/kg, ip) or saline (100 µL, ip) was administered to animals 60 min before the injection of 50 µL of an aqueous solution of carrageenan (01%, w/v) into the subplantar region of right hind-paw. Moreover, paw volumes were measured after carrageenan injection at times of 30, 60, 120 and 180 min. In this work, paw volume determinations were the average value of three repeated measures. Percentage of oedema development inhibition was expressed using the following equation: % oedema development inhibition = [(Vt - V0) control - (Vt - V0)treated]/(Vt - V0) × 100, where Vt is the average paw volumes for each group and V0 average paw volumes for each group prior to treatments, according to Lanhers et al. (1991).

Toxicological assays - Male Swiss mice, distributed into five groups of six animals each, were treated with *Z. montana* leaf extract (125, 250, 500, 1000 or 2000 mg/kg, po) containing 2.5% Tween-20 (v/v). Controls received 0.1 mL of saline also containing 2.5% Tween-20 (v/v) (po). Signs of toxicity were recorded for 24 h and LD_{50} was calculated from the plotted data. Ulcerogenic effect of *Z. montana* leaf extract (75, 150 or 300 mg/kg, po) was determined as described by Volterra et al. (1974), with modifications.

Statistical analyses - Statistical significance was determined by ANOVA following Student's *t* test. Results were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Since geographic distribution, environmental and plant physiological conditions may contribute to the great morphological and chemical diversity of plant species (Zoghbi et al. 1998), a chromatographic profile of *Z. montana* ethanol leaf extract, as compared to flavonoid

and triterpene standards used as phytochemical markers, is provided in Figs 1 and 2. This information should be of relevance in the future to find the active compound(s) in the *Z. montana* extract.

As showed in Table I, *Z.montana* leaf extract caused a reduction in the number of acetic acid-induced abdominal constrictions at doses of 75 mg/kg (67.27%), 150 mg/kg (49.38%) and 300 mg/kg (82.87%). A reduction in acetic acid-induced writhes (64.83%) was also caused by a 100 mg/kg dose of aspirin, however, this effect was inferior to those obtained by extract doses of 75 and 300 mg/kg. Acid acetic-induced abdominal constrictions are a useful experimental tool in the testing of new analgesic drugs. The abdominal injection of acetic acid in mice has been attributed to the release of arachidonic acid via the cyclooxygenase enzyme. Also, it has been suggested that the inhibition of prostaglandin synthesis by this pathway is remarkably efficient as an anti-nociceptive mechanism in visceral pain (Franzotti et al. 2002). Since all tested doses of *Z. montana* leaf extract were able to inhibit the acetic acid-induced writhes, the peripheral analgesic activity exhibited by this extract may be related to the inhibition of prostaglandin production. Peripheral analgesic activity of *Z. montana* extract was also confirmed by the hyperalgesia test (Hargreaves) (Fig. 3). All evaluated doses were able to inhibit hyperalgesia to a similar extent as aspirin. At two and six hours after LPS stimulation, the degrees of hyperalgesia reduction were, respectively, 91.60% and 85.70% (75 mg/kg), 83.30% and 57.10% (150 mg/kg), 83.30% and 85.70% (300 mg/kg).

Thermal stimuli are frequently used in the investigation of new opioid analgesic compounds and in differentiating non-narcotic analgesics from narcotic ones (Turner 1965, Plummer et al. 1996). Only a very slight central analgesic effect was observed in rats treated with *Z. montana* extract. The latency time for the animal writhing/stretching response was discreetly increased by extract doses of 75 and 300 mg/kg, 30 min following treatment, while tramadol showed its greatest analgesic effect after 60 min (Table II). Thus, *Z. montana* leaf extract seems to have a very weak central analgesia effect, whereas it is clear that it has peripheral analgesic activity (Table I).

The carrageenan-induced rat paw oedema is prompted by an early event involving the release of high concentrations of histamine and serotonin (Vinegar et al. 1969). The late phase of the inflammatory process, after the first 60 min of oedema induction, is characterised by the presence of prostaglandins (PGI_2), bradykinin and maximal oedema volume (Di Rosa et al. 1971, Gilman 1985). At the doses and time duration assayed, *Z. montana* leaf extract significantly reduced oedema development after carrageenan injection (Fig. 4). The administration of *Z. montana* leaf extract before the injection of the flogistic agent, at doses of 75 mg/kg, 150 mg/kg and 300 mg/kg,

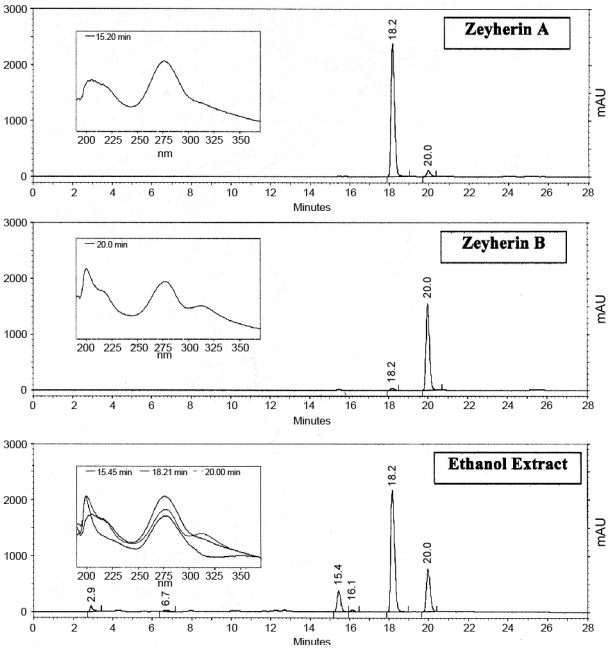

Fig. 1: chromatographic profile of the zeyherin A (18.1 min) and zeyherin B (19.9 min) and *Zeyheria montana* ethanol extracts at 280 nm.

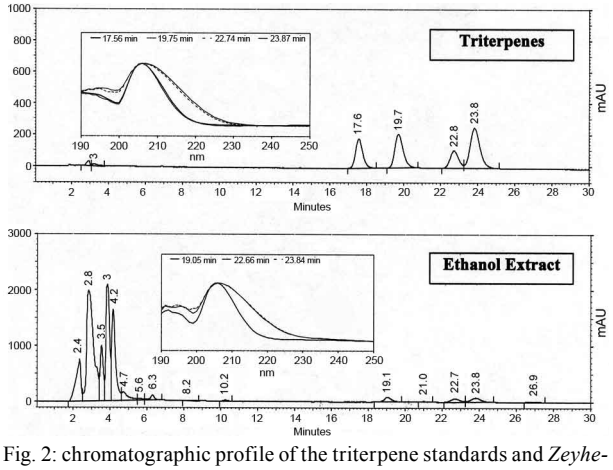

Fig. 2: chromatographic profile of the triterpene standards and *Zeyheria montana* ethanol extracts at 210 nm. Betulin (17.5 min), betulinic acid (19.7 min), oleanolic acid (22.7 min) and ursolic acid (23.8 min).

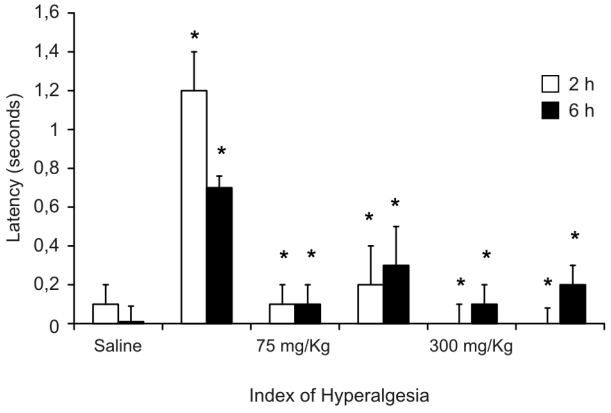

Fig. 3: results show the latency time for hind paw withdrawn at 2h/6h after lipopolysaccharide (LPS) stimulation. Each bar is the mean ± s.e.m. from six animals for group. Asterisk indicates: $p < 0.01$ when compared to saline/LPS group (ANOVA following Student's *t* test). Antinociceptive effect of *Zeyheria montana* leaf extract in hyperalgesia test (Hargreaves).

prevented the development of oedema in 15.30%, 23.00% and 38.40% of animals, respectively, after 30 min and in 17.60%, 35.20% and 47.00%, of animals, respectively, after 60 min. After 120 and 180 min, doses of 75 mg/kg (30.00% and 33.30%, respectively), 150 mg/kg (20.00% and 45.80%, respectively) and 300 mg/kg (50.00% and 75.00%, respectively) caused a more pronounced level of protection against oedema development. Indomethacin also exhibited effective anti-inflammatory activity, inhibiting the paw oedema development in 38.40%, 47.00%, 65.00% and 70.80% of animals after 30, 60, 120 and 180 min, respectively. Since *Z. montana* leaf extract exhibited a marked anti-inflammatory effect during the early and late phases of oedema, it is possible that compounds present in this extract play a role in decreasing the histamine/serotonin release rate, as well as could act as bradykinin/prostaglandin synthesis inhibitors.

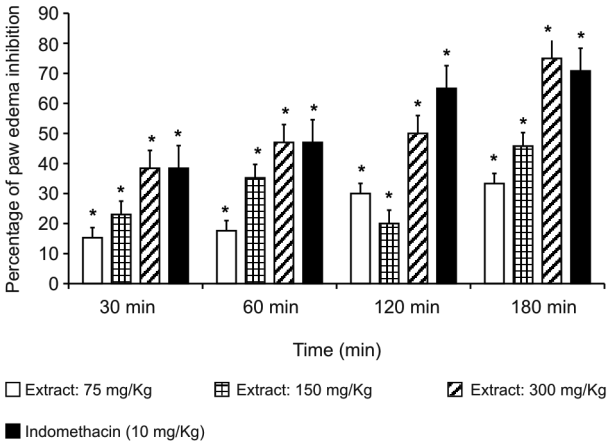

Fig. 4: anti-inflammatory effect of *Zeyheria montana* leaf extract in carrageenan-induced rat paw edema test. Results show the % of paw edema inhibition. Asterisk indicates ($p < 0.05$) when experimental group was compared to saline control group.

No signs of toxicity, such as diarrhoea, motor impairment, ataxia, hyperexcitability or alterations on respiratory frequency or piloerection, were noted in the control or experimental animals. Also, no gastric ulcerogenic effect was observed in controls or in animals treated with *Z. montana* extract (75, 150, 300 mg/kg, po), while an ulcerogenic index of 16.2 ± 6.6 was observed in animals treated with indomethacin (48 mg/kg, po) (data not shown). The LD50 (at 24 h) was greater than 2000 mg/kg, indicating the safety of *Z. montana* leaf extract.

Bertoni et al. (2003) reported the isolation of the flavones zeyherin a (8-hydroxi-5,6,7-trimethoxyflavone) and zeyherin b (6-hydroxi-5,6,7-trimethoxyflavone) from *Z. montana* leaf extract. Those substances share a high chemical similarity with a flavone from *Zeyheria tuberculosa*, described by Kutney and Hanssen (1971), which exhibits significant anti-herpes virus activity (Hayashi et al. 1997). In addition, the pentacyclic triterpenes betulinic acid, ursolic acid and oleanolic acid, with validated anti-inflammatory activity, were found in *Z. montana* leaves (Bertoni et al. 2007). Since an essential database on the chemical profile of *Z. montana* leaf extract is established, in which flavones and triterpenes are the major constituents, this information should be considered for future purification of analgesic and

TABLE I

Antinociceptive effect of *Zeyheria montana* leaf extract on acetic acid-induced abdominal constrictions in mice

Treatments	Dose mg/kg	Number of animals	Number of abdominal constrictions (30 mim)	Abdominal constrictions percent inhibition (%)
Acetic acid control	00	7	93.42 ± 6.47	0.00
Extract	75	7	30.57 ± 10.90[a]	67.27
Extract	150	7	47.28 ± 12.88[a]	49.38
Extract	300	7	16.00 ± 5.16[a]	82.87
Aspirin	100	7	32.85 ± 6.69[a]	64.83

a: experimental groups compared to saline control group ($p < 0.05$). Values are means ± SEM.

TABLE II

Antinociceptive effect of *Zeyheria montana* leaf extract against a thermal stimulus

Treatments	Dose mg/kg	0 h	30 min	60 min	90 min	120 min
Control	0	13.33 ± 0.90	14.86 ± 2.46	18.06 ± 2.90	17.88 ± 3.41	15.86 ± 2.88
Extract	75	16.90 ± 1.08	20.13 ± 2.25[a]	18.31 ± 2.20	19.91 ± 2.12	19.18 ± 3.14
Extract	150	13.75 ± 1.25	20.18 ± 2.77	19.45 ± 3.96	17.73 ± 2.26	18.78 ± 2.96
Extract	300	18.39 ± 1.45	21.90 ± 2.94[a]	17.23 ± 2.94	18.30 ± 2.12	18.26 ± 2.74
Tramadol	100	15.70 ± 2.06	24.90 ± 2.87[a]	26.15 ± 2.70	24.41 ± 3.35[a]	21.50 ± 2.31

a: experimental groups compared to saline control group ($p < 0.05$). Values are means ± SEM.

anti-inflammatory active compounds from this natural source. Our results indicate that *Z. montana* leaf extract has both effective peripheral analgesic activity and pronounced anti-inflammatory activity, even when compared to known drugs, such as aspirin and indomethacin. Furthermore, the extract exhibited a low LD_{50} value. Finally, the obtained results point out the potential of *Z. montana* leaf extract for the pharmacological control of pain and inflammatory processes.

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