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Antinociceptive action of the extract and the flavonoid quercitrin isolated from *Bauhinia microstachya* leaves

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Abstract

This study examined the antinociceptive effect of *Bauhinia microstachya* (Leguminosae), a native plant widely distributed in the South of Brazil, in several chemical and mechanical models of pain. The methanolic extract (ME) from *B. microstachya* (3–30 mg kg^{-1}, i.p.) and the isolated compound quercitrin (1–10 mg kg^{-1}, i.p.), given 30 min earlier, produced a dose-dependent inhibition of acetic-acid-induced visceral pain in mice, with a mean ID50 value (dose necessary to reduce the nociceptive response by 50% relative to the control value) of 7.9 and 2.4 mg kg^{-1}, respectively. The ME of *B. microstachya* (3–100 mg kg^{-1}, i.p., 30 min earlier) also caused a dose-dependent inhibition of capsaicin-induced pain, with a mean ID50 value of 18.8 mg kg^{-1}. Moreover, the ME (3–100 mg kg^{-1}, i.p., 30 min earlier) produced marked inhibition of both phases of formalin-induced pain, with mean ID50 values for the neurogenic and the inflammatory phases of 30.3 and 17.2 mg kg^{-1}, respectively. In addition, the ME of *B. microstachya* (3–300 mg kg^{-1}, i.p., 30 min earlier) inhibited, in a graded manner, the hyperalgesia induced by bradykinin (3.2 μg/paw), substance P (13.5 μg/paw), carrageenan (300 μg/paw), capsaicin (100 μg/paw) and adrenaline (100 ng/paw) in the rat paw, with mean ID50 values of 20.5, 17.9, 101.8, 54.2 and 99.7 mg kg^{-1}, respectively. Taken together, these data demonstrate that ME of *B. microstachya* elicited a pronounced antinociceptive action against several chemical and mechanical models of pain in mice and rats. The precise mechanism responsible for the antinociceptive effect of the extract still remains unclear, but seems to be partly related to modulation of the release or action of pro-inflammatory mediators involved in the models of pain used. Finally, the flavonoid quercitrin isolated from this plant appears to contribute for the antinociceptive property of the methanolic extract.

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Introduction

Despite the progress made in recent years in the development of pain therapy, there is still a need for effective and potent analgesics, especially for the treatment of chronic pain. In this regard, it has been widely shown that many plant-derived compounds present significant analgesic effects (Calixto et al 2000). Thereby, they represent potential molecules for the development of new drugs, especially designed for the treatment or control of chronic inflammatory and painful states. These antinociceptive substances include alkaloids, terpenoids, flavonoids and others. For this reason, plant-derived substances had, and certainly will continue to have, a relevant place in the process of drug discovery, particularly in the development of new analgesic drugs (Calixto et al 2000).

The plants of the genus *Bauhinia* consist of approximately 300 species that grow in tropical areas. Preliminary phytochemical studies on this genus have revealed that it is mainly constituted of steroidal glycosides, triterpenes, lactones and flavonoids (Meyre-Silva et al 2001). *Bauhinia microstachya* is a native plant popularly known as escada-de-macaco, cipó-escada or escada-de-jabuti, which grows in the South of Brazil. Its leaves and barks are used in folk medicine against several disorders, including inflammation, infections, diabetes, respiratory- and urinary-tract disorders and dolorous processes (Meyre-Silva et al 2001). Pharmacological and biochemical studies have confirmed these properties (Willain Filho et al 1997; Meyre-Silva et al 2001; Vasconcelos et al 2004). Moreover, some *Bauhinia* species also have traditional use as

antidote to poison (Shivarajan & Balchandran 1994), even against scorpion stings (Lans et al 2001). Recently, Menezes et al (2004) demonstrated that the hydroalcoholic extract of *B. microstachya* produced significant and potent in-vitro antioxidant activity against DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radicals and the phosphomolybdenum complex. In addition, preliminary studies conducted by our group have demonstrated that the methanolic extract and some flavonoids obtained from *B. microstachya* had antinociceptive activity in mice (Meyre-Silva et al 2001).

In this study we have attempted to extend our previous finding, evaluating in greater detail the antinociceptive properties of the methanolic extract (ME) of *B. microstachya* in chemical and mechanical models of nociception in mice and rats. In addition, we have also analysed the possible antinociceptive effect of the flavonoid isolated from this plant.

Materials and Methods

Preparation of the methanolic extract and isolation of the active compound

Plant material was collected in February 2000, at Urussanga, State of Santa Catarina, Brazil, and was classified by Dr Ademir Reis (Department of Botany, Federal University of Santa Catarina, Florianópolis, Brazil). A voucher specimen was deposited at the Barbosa Rodrigues Herbariun (Itajaí, Brazil), under number VC Filho 021. Air-dried leaves of *Bauhinia microstachya* (600 g) were powdered and extracted with methanol (5 L) at room temperature for approximately two weeks. After solvent removal, the extract was concentrated under reduced pressure and successively partitioned with *n*-hexane, dichloromethane (DCM), ethyl acetate (EA) and butanol (*n*-BuOH) as described previously. The ethyl acetate fraction (2.83 g) showed the most suitable phytochemical profile and good analgesic activity in preliminary analysis and for this reason was chromatographed using a silica gel column eluted with a mixture of $CHCl_3$–MeOH with increasing polarity. Elution with $CHCl_3$–MeOH (7:3 v/v) yielded a compound (183 mg), identified as quercetin 3-*O*-rhamnoside (quercitrin) by comparing physical and spectral data with those of published values (Meyre-Silva et al 2001).

Drugs

The drugs used were: formalin, acetic acid (Merck, A.G., Darmstadt, Germany), carrageenan, substance P, bradykinin, capsaicin, diclofenac and adrenaline (Sigma, St Louis, MO, USA). All drugs and the methanolic extract were dissolved in 0.9% NaCl solution, with the exception of capsaicin, which was dissolved in absolute ethanol, and quercitrin, which was dissolved in Tween 80. The final concentration of both ethanol and Tween 80 did not exceed 5% and did not cause any effect per se.

Animals

Experiments were conducted using Wistar rats (180–210 g) and Swiss mice (25–35 g) of both sexes, housed at $22 \pm 2^{\circ}C$ under a 12-h light–dark cycle (lights on at 0600 h) and with free access to food and water. Rats and mice (male and female were homogeneously distributed among groups) were acclimatized at the laboratory for at least 1 h before testing and were used only once throughout the experiments. The experiments were performed after approval of the protocol by the Institutional Ethics Committee and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann 1983). The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Abdominal constriction response caused by intraperitoneal injection of acetic acid

The abdominal constrictions were induced according to procedures described previously (Santos et al 1998) and resulted in contraction of the abdominal muscle together with a stretching of the hind limbs in response to an intraperitoneal injection of acetic acid (0.6%) just before the test. Mice were pre-treated with the ME (3–30 mg kg^{-1}, i.p.) or with quercitrin (1–10 mg kg^{-1}, i.p.) of *B. microstachya* or with diclofenac (3–30 mg kg^{-1}, i.p.) 30 min before the irritant injection. Control mice received a similar volume of the appropriate vehicle (10 mL kg^{-1}, i.p.) used to dilute the ME. After the challenge, the mice were individually placed into glass cylinders (20 cm diameter), and the abdominal constrictions were counted cumulatively over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of abdominal constrictions (i.e., the difference between control mice (pre-treated with vehicle) and mice pre-treated with the ME, quercitrin or diclofenac).

Formalin-induced nociception

The procedure used was essentially the same as that described previously (Santos & Calixto 1997; Santos et al 1998). Mice received 20 μL of a 2.5% formalin solution (0.92% formaldehyde) made up in saline, injected intraplantarly in the ventral surface of the right hindpaw. Mice were observed from 0–5 min (neurogenic phase) and 15–30 min (inflammatory phase) and the time spent licking the injected paw was recorded with a chronometer and considered as indicative of nociception. Mice received the ME of *B. microstachya* (3–100 mg kg^{-1}, i.p.) 30 min beforehand. Control mice received vehicle (10 mL kg^{-1}, i.p.). Following intraplantar injection of formalin, the mice were immediately placed in a glass cylinder (20 cm diameter), and the time spent licking the injected paw was recorded for both the early and late phase of this model.

Capsaicin-induced nociception

In an attempt to provide more direct evidence concerning the possible antinociceptive effect of the ME in neurogenic pain, we also investigated whether the ME of *B. microstachya* inhibited capsaicin-induced nociception in mice paw. The procedure used was similar to that described previously

(Sakurada et al 1992; Santos & Calixto 1997). After the adaptation period, 20 μL of capsaicin (1.6 μg/paw, prepared in saline) was injected intraplantarly in the ventral surface of the right hindpaw. Mice were individually observed for 5 min following capsaicin injection. The amount of time spent licking the injected paw was recorded with a chronometer and was considered as indicative of nociception. Mice were treated with ME of *B. microstachya* (3–100 mg kg^{-1}, i.p.) 30 min before capsaicin injection. Control mice received vehicle by the intraperitoneal (10 mL kg^{-1}) route.

Hyperalgesia in the rat paw

The possible anti-hyperalgesic effect of *B. microstachya* was evaluated using the procedures previously described (Randall & Selitto 1957; De Campos et al 1996). Rats were pre-treated intraperitoneally with the ME (10–300 mg kg^{-1}) or vehicle (10 mL kg^{-1}, control group), 30 min before injection of 0.1 mL of carrageenan (300 μg/paw), adrenaline (100 ng/paw), substance P (13.5 μg/paw), capsaicin (100 μg/paw) or bradykinin (3.2 μg/paw) or saline, in the right hindpaw. The hyperalgesia was evaluated 30 min later, except for carrageenan, which was assessed at 3.5 h. The nociceptive threshold (of squeak response or paw withdrawal) was assessed by applying increasing pressure to the dorsal site of inflamed or control paws, using an analgesy meter (Ugo Basile, Milan, Italy) according to the method of Randall & Selitto (1957), with minor modifications. The analgesy meter was graduated from 0 to 750 g, and the threshold was expressed as load (g) tolerated. When bradykinin was used, rats were pre-treated with the angiotensin-converting enzyme inhibitor captopril (5 mg kg^{-1}, s.c.) 1 h before the experiments, to prevent its degradation (De Campos et al 1996).

Statistical analysis

The results were presented as mean ± s.e.m., except the ID50 values (i.e., the dose of extract, quercitrin or diclofenac necessary to reduce the nociceptive response by 50% relative to the control value), which were reported as geometric means accompanied by their respective 95% confidence limits. The ID50 value was determined by linear regression from individual experiments using linear regression GraphPad software (GraphPad software, San Diego, CA). The statistical significance of differences between groups was detected by analysis of variance followed by Newman–Keuls' test. A value of $P < 0.05$ was considered as indicative of significance.

Results

Abdominal constriction response caused by intraperitoneal injection of acetic acid

The ME of *B. microstachya* (3–30 mg kg^{-1}) and the purified compound, quercitrin (1–10 mg kg^{-1}), given by the intraperitoneal route 30 min beforehand, produced a dose-related inhibition of acetic-acid-induced abdominal constrictions in mice (Table 1), with mean ID50 values (and their 95% confidence limits) of 7.9 (4.8–12.8) and 2.4 (1.4–8.9) mg kg^{-1} and the inhibitions observed were 94 ± 4% and 75 ± 2% for the ME and quercitrin, respectively. The treatment of mice with diclofenac (3–30 mg kg^{-1}, i.p., 30 min beforehand) also produced marked and dose-related inhibition of acetic-acid-induced writhing response. The calculated mean ID50 value and inhibition were 12.1 (9.4–15.6) mg kg^{-1} and 93 ± 7%. However, diclofenac was 1.5- to 5.0-fold less potent than quercitrin and the ME obtained from *B. microstachya* in attenuating acetic-acid-induced pain (Table 1).

Formalin-induced nociception

The results presented in Table 2 show that the ME of *B. microstachya* (3–100 mg kg^{-1}, i.p., 30 min beforehand), also caused significant inhibition of both neurogenic (0–5 min) and inflammatory (15–30 min) phases of formalin-induced nociception in mice. The calculated mean ID50

Table 1 Effect of the methanolic extract (ME) and quercitrin isolated from *Bauhinia microstachya* or diclofenac administered intraperitoneally against acetic-acid-induced writhing response in mice

Treatment	Dose (mg kg^{-1})	No. of writhes
ME	0	57.9 ± 4.4
	3	37.6 ± 4.0***
	10	22.4 ± 2.7***
	30	4.5 ± 1.9***
Quercitrin	0	53.4 ± 5.6
	1	33.0 ± 6.9*
	3	23.5 ± 5.4**
	10	11.2 ± 3.0***
Diclofenac	0	42.2 ± 1.5
	3	37.7 ± 2.4
	10	19.9 ± 5.4***
	30	2.4 ± 2.1***

Data are expressed as mean ± s.e.m., n = 6–12. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control (0).

Table 2 Effect of the methanolic extract (ME) of *Bauhinia microstachya* administered intraperitoneally against the neurogenic (0–5 min) and inflammatory (15–30 min) phases of formalin-induced licking in mice

ME dose (mg kg^{-1})	Licking (s)	
	0–5 min	15–30 min
0	58.3 ± 3.8	128.4 ± 8.7
3	54.8 ± 4.5	127.8 ± 18.6
10	53.5 ± 2.6	71.9 ± 6.2***
30	35.7 ± 5.5**	54.9 ± 6.3***
100	16.7 ± 4.2***	30.5 ± 10.3***

Data are expressed as mean ± s.e.m., n = 6–12. **$P < 0.01$, ***$P < 0.001$ vs control group (0).

Table 3 Effect of the methanolic extract (ME) of *Bauhinia microstachya* administered intraperitoneally against capsaicin-induced licking in mice

ME (mg kg^{-1})	Licking (s)
0	48.4 ± 2.8
3	41.0 ± 2.2*
10	25.6 ± 1.5***
30	16.9 ± 2.5***
100	14.0 ± 3.4***

Data are expressed as mean ± s.e.m., n = 6–12. *$P < 0.05$, ***$P < 0.001$ vs control (0).

values for these results were: 30.3 (26.2–34.9) and 17.2 (11.5–25.6) mg kg^{-1} and the inhibitions observed were 73 ± 7% and 86 ± 8% for the neurogenic and inflammatory phases, respectively.

Capsaicin-induced nociception

The intraperitoneal administration of the ME of *B. microstachya* (3–100 mg kg^{-1}) 30 min beforehand also produced a dose-dependent attenuation of capsaicin-induced neurogenic pain in mice (Table 3), with a mean ID50 value of 18.8 (13.9–25.5) mg kg^{-1} and the inhibition observed was 71 ± 7%.

Hyperalgesia in the rat paw

When assessed using the Randall–Selitto model, the ME (10–100 mg kg^{-1}, i.p., 30 min before) dose-dependently and completely reversed the hyperalgesic effect caused by intraplantar injection of capsaicin (100 μg/paw), substance P (13.5 μg/paw) and bradykinin (3.2 μg/paw) (Figure 1A–C), with mean ID50 values of 54.2 (42.6–68.9), 17.9 (8.9–33.9) and 20.5 (14.2–29.5) mg kg^{-1} and inhibitions of 97 ± 3, 100 and 100%, respectively. Moreover, the ME at the same doses also reversed in a dose-related manner the hyperalgesic effect caused by carrageenan (300 μg/paw) and adrenaline (100 ng/paw) (Figure 2A, B), with mean ID50 values of 101.8 (71.0–145.9) and 99.7 (75.6–131.6) mg kg^{-1} and inhibitions observed of 100 and 73 ± 7%, respectively. Furthermore, at the ID50 level, the ME was about 1.8- to 5.6-fold more potent in inhibiting the hyperalgesia response caused by capsaicin, substance P and bradykinin.

Discussion

Preliminary studies accomplished by our group have recently demonstrated that the ME of *B. microstachya* and the purified compound identified as quercitrin, administered intraperitoneally, produced significant antinociception against acetic-acid-induced visceral nociception in mice (Meyre-Silva et al 2001). In this study, we have confirmed and extended the initial observations by demonstrating that the ME of this plant, administered by the intraperitoneal route in mice, produced a dose-related and marked antinociception in several models of chemical nociception, namely acetic-acid-induced visceral pain and capsaicin- and formalin-induced licking. The isolated compound, quercitrin, given systemically (i.p.), was also capable of inhibiting the abdominal constrictions produced by acetic acid in mice. Furthermore, the intraperitoneal administration of the ME was able to reverse, in a dose-related manner and with distinct potency, the mechanical hyperalgesia in the rat paw induced by carrageenan, capsaicin, substance P, bradykinin and adrenaline.

In this study, we have confirmed that the ME and the isolated compound, quercitrin, both from *B. microstachya* leaves, given systemically, produced a dose-related inhibition of the number of abdominal constrictions elicited by acetic acid; quercitrin was at the ID50 level, about 3.3-fold more active than the ME in attenuating the writhing response caused by acetic acid. Furthermore, the ME and quercitrin were 1.5- to 5.0-fold more potent than diclofenac (used as positive control) in attenuating acetic-acid-induced pain.

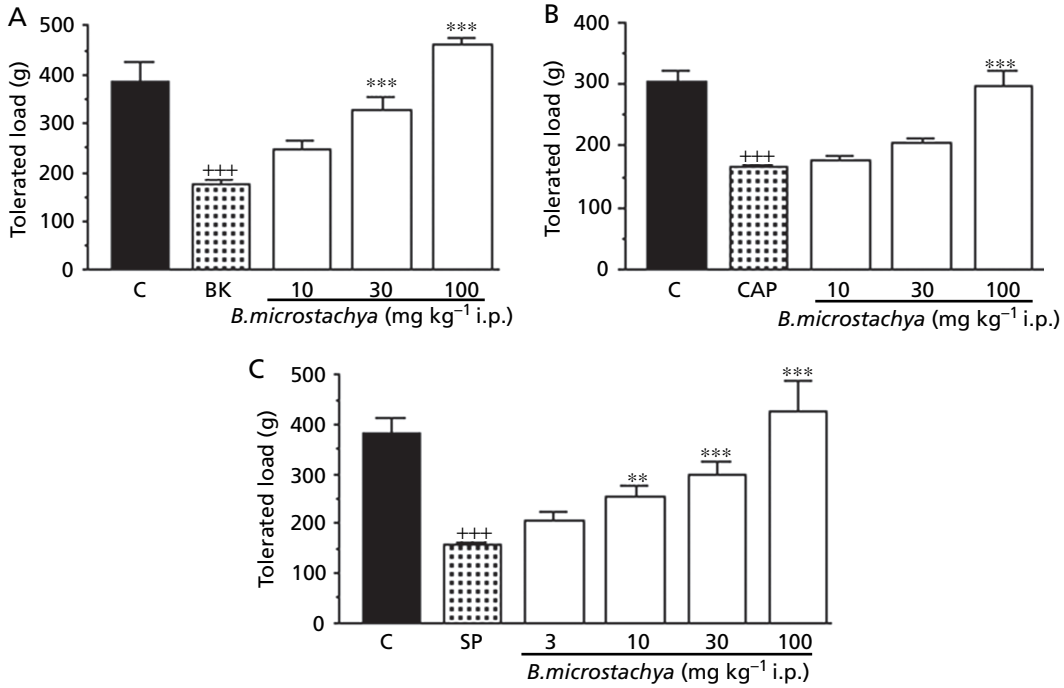

Figure 1 Effect of the methanolic extract of *Bauhinia microstachya* administered intraperitoneally on bradykinin (3.2 μg/paw, A), capsaicin (100 μg/paw, B) and substance P (13.5 μg/paw, C) induced hyperalgesia in the rat paw. Data represent the mean of 6–10 rats and the vertical lines indicate the s.e.m. The closed columns indicate the control values (saline-injected paws) and the hatched column indicates the bradykinin (BK), substance P (SP) and capsaicin (CAP) injected paws, in the absence of the extract. $^{**}P < 0.01$, $^{***}P <$ 0,001 compared with flogistic agents (BK, SP or CAP (hatched columns)) (one-way analysis of variance followed by Newman–Keuls test); $^{+++}P <$ 0,001 compared with control (closed columns).

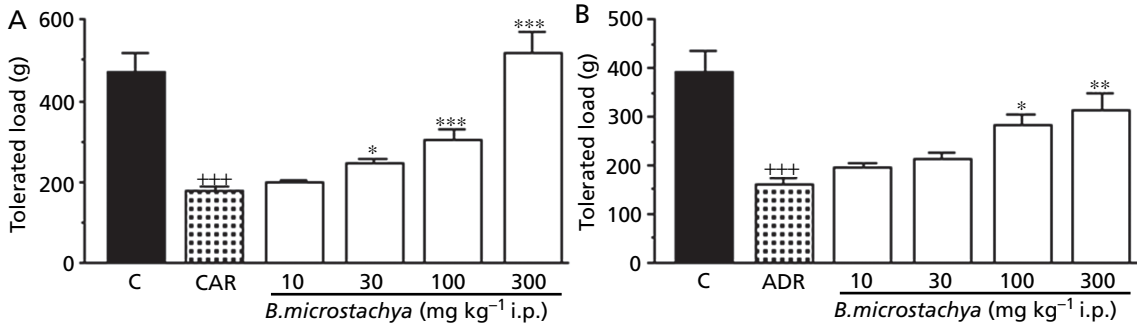

Figure 2 Effect of the methanolic extract of *Bauhinia microstachya* administered intraperitoneally on carrageenan (300 μg/paw, A) and adrenaline (100 ng/paw, B) induced hyperalgesia in the rat paw. Data represent the mean of 6–10 rats and the vertical lines indicate the s.e.m. The closed columns indicate the control values (saline-injected paws) and the hatched column indicates the carrageenan (CAR) and adrenaline (ADR) injected paws, in the absence of the extract. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with flogistic agents (CAR or ADR (hatched columns)) (one-way analysis of variance followed by Newman–Keuls test); $^{+++}P < 0.001$ compared with control groups (closed columns).

The acetic-acid-induced writhing reaction in mice, described as a typical model for visceral inflammatory nociception, has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents (Tjølsen & Hole 1997). The major transmission pathway for inflammatory pain has been documented as that comprising peripheral polymodal receptors, such as vanilloid, bradykinin, prostaglandin and tachykinin receptors, among others, around small vessels that signal to the central nervous system (CNS) via sensory afferent C-fibres entering the dorsal horn (Kumazawa et al 1996; Ikeda et al 2001). Moreover, Ribeiro et al (2000) showed that the nociceptive response caused by acetic acid is also dependent on the release of TNF-α, interleukin-1β and interleukin-8 via modulation of macrophages and mast cells localized in the peritoneal cavity.

Results of this study also showed, for the first time, that the ME exhibited potent and graded antinociception when administered by the intraperitoneal route in two models of neurogenic pain, the early phase of the formalin test and

against capsaicin-induced licking. It is well known that non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, paracetamol, indometacin and diclofenac, are largely ineffective or cause very weak inhibition against both models. In addition, NSAIDs can attenuate, in a dose-related manner, the second phase of formalin-induced licking (Hunskaar & Hole 1987; Malmberg & Yaksh 1992; Santos et al 1998). It has also been reported that morphine, some tachykinin receptor antagonists, non-selective excitatory amino acid antagonists and both B_1 and B_2 bradykinin receptor antagonists are able to inhibit the neurogenic and inflammatory components of capsaicin and formalin tests (De Campos et al 1996; Santos & Calixto 1997).

Our results also demonstrated that the ME of *B. microstachya* was equipotent, at the ID50 level, in inhibiting the nociception caused by both formalin (both phases) and capsaicin. These results support the view that the mechanisms by which the ME exerts its antinociceptive effect do not differ largely with respect to their action on pain transmission in response to intraplantar injection of formalin or capsaicin. In line with this view, behavioural and eletrophysiological studies have shown that several mediators, such as kinins, excitatory amino acids, prostaglandins, neuropeptides and nitric oxide, among others, play an important role in the nociceptive response caused by the formalin and capsaicin models of pain (Hunskaar & Hole 1987; Malmberg & Yaksh 1992; Sakurada et al 1992; De Campos et al 1996; Santos & Calixto 1997).

Another interesting finding of this study was the demonstration, for the first time, that the ME of *B. microstachya*, given intraperitoneally, was able to reverse, in a dose-related manner, substance P-, bradykinin-, carrageenan-, capsaicin- and adrenaline-induced hyperalgesia in the rat paw. Several inflammatory mediators produce nociception by peripheral and spinal sensory fibre sensitization through protein kinase activation, including PKC, PKA and mitogen-activated kinases (Scholz & Woolf 2002). Evidence now suggests that cyclooxygenase products derived from the arachidonic acid pathway could mediate the mechanical hyperalgesia produced by carrageenan (De Campos et al 1996). Moreover, bradykinin- and substance P-induced overt nociception and mechanical hyperalgesia are mediated by the peripheral activation of PKC and the vanilloid receptor (Ferreira et al 2004). On the other hand, intraplantar capsaicin seems to induce nociception action via direct activation of peripheral vanilloid receptor (Santos & Calixto 1997; Ferreira et al 2004). On top of this, the nociception caused by carrageenan, adrenaline, acetic acid, formalin and capsaicin is also sensitive to PKA or PKC inhibitors or gene deletion (Malmberg et al 1997; Khasar et al 1999). Therefore, the ability of quercitrin and the ME to inhibit bradykinin-, capsaicin-, substance-P- and adrenaline-induced hyperalgesia could show an interaction with kinase pathways or with their respective receptors.

Chemical studies carried out with this ME allow us to isolate and identify a flavonoid in *B. microstachya*, which seems to contribute, at least in part, to the antinociceptive properties reported for the ME. By comparing physical and spectral data with those of published values (Meyre-Silva et al 2001), it was possible to identify and elucidate its structure as being quercitrin (quercetin 3-O-rhamnoside). Flavonoids are polyphenolic compounds of low molecular weight that can inhibit several enzymes, including those involved in arachidonic acid metabolism (Landolfi et al 1984). Flavonoids like quercitrin are found in all plants, and all vegetable-consuming animals, including man, are exposed to dietary flavonoids contained in fruits, flowers, seeds, coffee and tea (Sanchez de Medina et al 1996). Quercitrin is one of the glycoside forms of quercetin, one of the most common flavonoids in plants (Galvez et al 1994). Several pharmacological activities have been demonstrated both in-vivo and in-vitro for these flavonoids, including anti-inflammatory (Del Carmen Recio et al 1995), anti-thrombotic (Landolfi et al 1984), anti-diarrhoeic (Galvez et al 1995) and anti-colitis (Sanchez de Medina et al 1996; Camuesco et al 2004) activities. The pharmacological mechanism for these compounds has been related to inhibitory actions on several enzymes (Landolfi et al 1984), such as NO synthase (Camuesco et al 2004) or actions as free radical scavengers and inhibitors of peroxidation (Mora et al 1990). However, further studies are necessary to chemically and pharmacologically isolate and characterize other active principle(s) present in *B. microstachya*.

Conclusion

In summary, this study has confirmed previous studies in which quercitrin and the ME obtained from *B. microstachya* elicited significant and dose-related antinociception in chemical models of nociception in mice. The ME was also capable of reducing, greatly, the mechanical hyperalgesia in rats induced by several flogistic agents. Furthermore, the isolated flavonoid, quercitrin, seems, at least in part, to contribute to the explanation of the antinociceptive properties of the ME. However, more pharmacological and chemical studies are necessary to characterize the precise mechanism(s) responsible for the antinociceptive action, and also to identify other active compounds present in *B. microstachya*.

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