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Evaluation of *Moringa oleifera* Aqueous Extract for Antinociceptive and Anti-Inflammatory Activities in Animal Models

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Abstract

Moringa oleifera L. (Moringaceae) is known to possess high nutritional value and is used in a folklore medicine to treat various ailments related to pain and inflammation. The aim of the present study was to evaluate the antinociceptive and anti-inflammatory effects of the aqueous extract of the leaves of *M. oleifera* in laboratory animals, using the writhing, hot-plate and formalin tests as the antinociceptive assays, and carrageenan-induced paw edema test as the anti-inflammatory assay. The extract (10, 30 and 100 mg/kg) exhibited significant ($P < 0.05$) antinociceptive activity, which occurred in a dose-dependent manner, in all tests used. The extract also exhibited significant ($P < 0.05$) anti-inflammatory activity in a dose-dependent manner. Furthermore, the extract antinociceptive activity was suggested to be modulated via opioid receptors at the central, but not peripheral, antinociceptive level, based on the ability of 5 mg/kg naloxone to reverse the extract activity in the hot-plate, but not the writhing test. In conclusion, *M. oleifera* leaves possess peripherally non-opioid mediated and centrally opioid mediated antinociceptive and anti-inflammatory activities. This study also confirms the traditional uses of *M. oleifera* in the treatment of ailments, particularly those related to pain and inflammation.

Keywords: *Moringa oleifera*, aqueous extract, antinociceptive activity, anti-inflammatory activity, opioid-mediated, dose-independent.

Introduction

Moringa oleifera L. (Moringaceae), known as "kelor" to the Malays, is widespread in India, south-east Asia, Africa, and central America (Anwar et al., 2007). The plant has been used in African folk medicine to treat rheumatic and articulary pain (Ndiaye et al., 2002). The plant is known to possess high nutritional value, because of the presence of protein, vitamins and various phenolic compounds (Anwar et al., 2007; Manguro & Lemmen, 2007), and has a diverse range of medicinal uses, which include antioxidant and anticarcinogenesis (Bharali et al., 2003), anti-inflammatory, antispasmodic and diuretic (Caceres et al., 1992), antiulcer, antibacterial, and antifungal (Caceres et al., 1991), and wound healing (Rathi et al., 2006).

Several types of bioactive compounds have been isolated from the leaves of *M. oleifera*. The leaves, in particular, have been reported to contain hypotensive compounds of the glycoside type (i.e., niazinin A, niazinin B, niazimicin, niaziminin A, and niaziminin B) (Caceres et al., 1991). Other than that, the methanol extract of *M. oleifera* leaves has also been reported to contain various types of flavonol glycosides, as well as kaempferol, rutin, and quercetin (Manguro & Lemmen, 2007). Although study on the anti-inflammatory activity of the leaves of *M. oleifera* has been reported elsewhere, the antinociceptive activity of the plant was not reported. Thus, the present study was carried out to evaluate the antinociceptive and anti-inflammatory activities of *M. oleifera* using various animal models.

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Materials and Methods

Plant collection

M. oleifera leaves were collected in October 2005 from their natural habitat in Kampung Felda Nitar 1, Mersing, Johor, Malaysia and identified by Mr. Shamsul Khamis, a botanist from the Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. A voucher specimen (SK1007/05) was deposited in the Laboratory of Natural Products, IBS, UPM, Serdang, Selangor, Malaysia.

Preparation of *M. oleifera* aqueous extract (MPAE)

The leaves were oven-dried at 50°C for two days and then ground into powder form. The powdered leaves were boiled with distilled water (1:10 ;w/v) at 80°C for 12 hours. The mixture was then filtered to remove the plant residue, and the supernatant (MPAE) obtained was freeze-dried and kept at −20°C prior to use. Immediately before use the crude-dried MPAE was prepared in the dose range of 10, 30, and 100 mg/kg by re-dissolving the MPAE in dH_2O.

Experimental animals

Male Balb/C mice (25–30 g) and Sprague-Dawley rats (200–250 g) were used in this study. All animals were obtained from the Animal House of the Faculty of Veterinary Medicine, UPM. They were housed in standard cages, with five mice per cage and three rats per cage, at a temperature of 22°± 2°C and 12/12 h light-dark cycle. The animals had free access to food and water. The experimental procedures were carried out in strict compliance with the Animal Ethics Committee rules and regulations followed in this Institute and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

Evaluation of Pharmacological Activities

Antinociceptive assays

Acetic acid-induced writhing test

The acetic acid-induced abdominal writhing test was performed as described by Collier et al. (1968) with slight modification (Sulaiman et al., 2004). The test was performed to assess the ability of MPAE to affect the nociceptive response induced by noxious chemical stimulus. Briefly, five groups of six mice were pre-treated via intraperitoneal (i.p.) injection with normal saline (0.9% NaCl), 100 mg/kg acetylsalicylic acid (ASA), or the MPAE (10, 30, and 100 mg/kg), respectively, and 30 min later, 0.6% (v/v) acetic acid was administered (i.p.). The number of writhings, resulting from the injection of acetic acid, consists of a contraction of the abdominal region together with a stretching of the hind limbs, and was counted cumulatively over the period of 30 min after the acetic acid administration. Antinociceptive activity was indicated by the reduction in the mean of the number of writhings in the test groups compared with the control group. The percentage of antinociception was calculated based on the formula described by Dambisya and Lee (1995) as:

$$\%\ \text{of antinociception} = \frac{(\text{control group mean}) - (\text{test group mean})}{(\text{control group mean})} \times 100\%$$

Hot-plate test

The hot-plate test was performed according to the method described by Eddy and Leimbach (1953) with slight modification (Zakaria et al., 2005). The test was used to determine the antinociceptive activity of MPAE against the nociceptive response evoked by heat-induced noxious stimulus. The temperature of the hot-plate (Model 7280, Ugo Basile, Italy) was maintained at 50 ± 2°C. Briefly, five groups of six mice were pre-treated (i.p.) with normal saline, 5 mg/kg morphine, or the MPAE (10, 30, and 100 mg/kg), respectively. Thirty min after the administration of the test solutions, the animals were placed into the perspex cylinder on the heated surface and the time between placement and the reactivity towards pain (i.e., licking of the paw or jumping) was recorded as the latency to discomfort. Latency to a discomfort reaction (licking paws or jumping) was determined before and after drug administration. The cut-off time of 20 s was chosen to avoid tissue injury. Mice with baseline latencies between 4–7 s were used in this study. The latency was recorded before (BF) and 30, 60, 90, 120, 150, 180, 210, and 240 min following the i.p. administration of the test solutions. The prolongation of the latency times of the test solutions compared to the values of the control group was used for statistical comparison.

Formalin test

The procedure described by Hunskaar and Hole (1987) was adopted for the formalin test with slight modifications. Nociceptive response was induced by administering 25 μl of 25% (v/v) formalin in the subplantar region of the right hind paw. Briefly, six groups of six rats were pre-treated (i.p.) with normal saline, 100 mg/kg ASA, 5 mg/kg morphine, or the MPAE (10, 30, and 100 mg/kg). Thirty min later, the respective animals were administered with formalin (intraplantar) and immediately placed into a transparent plexiglass observation chamber. The amount of time each rat spent licking or biting the injected paw, which are indicators of pain, was measured for the duration of 30 min following the formalin administration. The early phase (0–5 min), which represents neurogenic pain response, and the late phase (15–30 min), which represents an inflammatory

pain response was measured after the formalin injection (Hunskaar & Hole, 1987).

Determination of opioid receptor involvement in the MPAE antinociception

Two groups of six mice were used in the study to determine the involvement of opioid receptor in the modulation of MPAE antinociceptive activity using the acetic acid-induced writhing and hot-plate tests, respectively. The mice were pretreated (i.p.) with 5 mg/kg naloxone, a non-selective opioid receptor antagonist, followed 15 min later with the administration of 100 mg/kg MPAE (i.p.). Thirty min after the administration of MPAE, the mice were subjected to the writhing or hot-plate tests.

Anti-inflammatory assay

Carrageenan-induced paw edema

The anti-inflammatory activity of MPAE was assessed using the method described by Winter et al. (1962) with slight modifications (Sulaiman et al., 2004). A 0.1 ml aliquot of 1% (w/v) sterile λ carrageenan was administered (intraplantar) into the right hind paw to induce paw edema, 30 min after pre-treatment (i.p.) of rats (n = 6) with normal saline, 100 mg/kg ibuprofen, or the MPAE (10, 30, and 100 mg/kg). The measurement of paw volume changes of the test and control groups due to carrageenan administration were determined using a plethysmometer (Model 7140, Ugo Basile, Italy). The paw volume was measured before (initial volume) and at the interval of 1, 2, 3, 4, and 5 h after (final volume) the carrageenan administration. The edema volume was determined using the following formula:

$$\text{Edema volume} = \text{final volume-initial volume}$$

Statistical analysis

All data were expressed as mean ± SEM (standard error of mean). The statistical significance between treatment group and control was assessed using one way analysis of variance (ANOVA) followed by Tukey test using Sigma Stat version 2.0 program where $P < 0.05$ was considered as the limit of significance.

Results

Antinociceptive profiles of the MPAE

The antinociceptive activity of MPAE assessed using the acetic acid-induced writhing test is shown in Table 1. The 10, 30, and 100 mg/kg MPAE exhibited a significant ($P < 0.05$) antinociceptive activity in a dose-dependent manner. The percentage of antinociception for the three doses ranges between 48–93% with the 10 mg/kg MPAE producing approximately 50% antinociception. In addition, the 30 mg/kg MPAE was found to produce an activity that was as effective as the 100 mg/kg ASA.

Table 1. Effect of the *M. oleifera* aqueous extract on acetic acid-induced writhing test in mice.

	Dose (mg/kg; i.p.)	No. of writhings	Inhibition %
Control (NaCl)	—	27.3 ± 1.0	
M. oleifera aqueous extract	10	14.2 ± 1.4*	48.0
	30	10.2 ± 0.9*	62.6
	100	1.8 ± 0.8*	93.4
M. pterygosperma aqueous extract (100 mg/kg, i.p.) + naloxone (5 mg/kg, i.p.)	—	2.0 ± 0.7*	92.7
Acetylsalicylic acid	100	9.7 ± 0.5*	64.5

N = 6. Values are mean ± Standard error of the mean (SEM). *P < 0.05 significantly different from control.

The antinociceptive activity of MPAE assessed using the hot-plate test is shown in Table 2. The extract also exhibited a significant ($P < 0.05$) antinociceptive activity, which is seen in a dose-dependent manner. Interestingly, the 10 mg/kg MPAE did not produce any significant activity whereas the 100 mg/kg MPAE produced an activity that was comparable to the 5 mg/kg morphine. The onset and offset of activity for 30 and 100 mg/kg MPAE was 60 and 30 min, and 210 and 240 min, respectively. Other than that, the latency of discomfort for the control group (normal saline-treated) at the interval time of 240 min was found to be significantly higher than the data recorded prior (BF) to the experiment.

The antinociceptive activity of MPAE assessed using the formalin test is shown in Table 3. The 10, 30 and 100 mg/kg MPAE was also found to produce significant ($P < 0.05$) antinociceptive activity in a dose-dependent manner in both phases of the test. The percentage of antinociception for the early and late phases ranged from approximately 49–80% and 19–100%, respectively. The ED_{50} values for MPAE in the former and latter phases were expected to be approximately 10 and 18 mg/kg, respectively. Once again, the 100 mg/kg MPAE was found to produce almost similar strength of activity when compared to 5 mg/kg morphine.

Involvement of opioid receptor in the MPAE antinociceptive activity

Based on the data obtained (Tables 1 and 2), the administration of naloxone did not reverse/block the extract antinociceptive activity. This finding directly indicates that the MPAE antinociceptive activity did not involve modulation of the opioid receptor.

Table 2. Effect of *M. oleifera* aqueous extract on the hot-plate test in mice.

	Dose (mg/kg)	Latency time (min)								
		0	30	60	90	120	150	180	210	240
Control (NaCl)	—	4.6 ± 0.2	5.4 ± 0.4	5.4 ± 0.6	4.8 ± 0.5	5.6 ± 0.5	4.2 ± 0.4	5.7 ± 0.8	5.4 ± 0.7	6.3 ± 0.5
M. oleifera aqueous	10	4.9 ± 0.1	6.7 ± 0.3	6.2 ± 0.3	6.2 ± 0.9	6.3 ± 0.4	6.2 ± 0.5*	6.7 ± 0.4	6.7 ± 0.4	6.0 ± 0.3
extract	30	4.7 ± 0.2	6.4 ± 0.5	7.5 ± 0.6*	7.1 ± 0.6	7.4 ± 0.5*	7.5 ± 0.5*	7.0 ± 0.6	7.7 ± 0.8*	7.5 ± 0.6
	100	5.5 ± 0.2*	10.6 ± 0.4*	10.7 ± 0.4*	11.1 ± 0.2*	10.7 ± 0.5*	11.4 ± 0.3*	10.8 ± 0.2*	11.0 ± 0.1*	10.3 ± 0.2*
M. oleifera aqueous extract (100 mg/kg) + naloxone (5 mg/kg)	—	5.1 ± 0.3	8.3 ± 0.3*	8.4 ± 0.1*	7.7 ± 0.2*	7.4 ± 0.1*	8.0 ± 0.3*	8.1 ± 0.3*	8.1 ± 0.2*	8.3 ± 0.2*
Morphine	5	5.52 ± 0.4*	10.21 ± 1.2*	12.39 ± 2.2*	12.30 ± 1.2*	10.98 ± 1.4*	10.45 ± 1.2*	9.83 ± 1.3*	8.92 ± 1.5*	9.46 ± 1.6*
Morphine + naloxone		5.73 ± 0.4*	5.61 ± 0.4*	5.78 ± 0.6*	5.51 ± 1.1*	5.54 ± 1.0*	5.42 ± 0.5*	5.57 ± 0.4*	5.39 ± 0.8*	5.50 ± 1.3*

N = 6. Values are mean ± SEM. *$P < 0.05$ significantly different from control.

Table 3. Effect of *M. oleifera* aqueous extract in the early and late phases of the formalin test in mice.

		Licking time (sec)		Inhibition %	
	Dose (mg/kg	Early phase	Late phase	Early phase	Late phase
Control (NaCl)	—	92.4 ± 7.3	57.6 ± 2.3		
M. oleifera aqueous extract	10	47.3 ± 10.6*	46.4 ± 11.0*	48.8	19.4
	30	38.6 ± 0.5*	1.6 ± 1.6*	58.2	97.2
	100	18.9 ± 4.9*	0.0 ± 0.0	79.5	100.0
Acetylsalicylic acid	100	88.0 ± 3.1*	3.2 ± 0.6*	4.8	94.4
Morphine	5	16.22 ± 0.9*	4.85 ± 0.7*	82.4	91.6

N = 6. Values are mean ± SEM. *P < 0.05 significantly different from control.

Anti-inflammatory activity of MPAE

The anti-inflammatory activity of MPAE assessed using the carrageenan-induced paw edema is shown in Tables 4 and 5. The MPAE, at all doses used, exhibited a significant ($P < 0.05$) and dose-dependent anti-inflammatory activity, with activity observed until the end of the experiment. However, the MPAE anti-inflammatory activity, although significant ($P < 0.05$), was less effective when compared to the standard anti-inflammatory drug, 100 mg/kg ibuprofen.

Discussion

The present study demonstrated the potential antinociceptive effect of the aqueous extract of *M. oleifera* leaves when assessed in chemical (i.e., acetic acid-induced writhing and formalin tests) and thermal (i.e., hot-plate test) models of nociception. In addition, the extract was also found to possess anti-inflammatory activity when assessed using the carrageenan-induced paw edema test.

The acetic acid-induced writhing test is a highly sensitive and very useful tool to elucidate the analgesic property of foundation drugs and drugs under development. Acetic acid is thought to act indirectly by inducing the release of endogenous mediators, which then stimulate the nociceptive neurons sensitive to non-steroidal anti-inflammatory drugs (NSAIDS) and opioids (Collier et al., 1968). The writhing test confirms the peripheral analgesic property of compounds/extracts, including the MPAE, and suggests the binding of the extract to peripheral receptors in the peritoneum (Bentley et al., 1983). Thus, it is thought that the analgesic potential shown by compounds/extracts in the acetic acid-induced writhing test is significant but not specific (Chan et al., 1995). Several tests (i.e., the hot-plate and formalin tests) should be performed before drawing a conclusion on the mechanisms of action that might contribute to the observed antinociceptive activity of MPAE.

The hot-plate test is a specific test carried out to verify involvement of a central mechanism with compounds/drugs showing antinociceptive activity (Pini et al., 1997). Compounds/extracts, like MPAE, that show central antinociceptive activity by increasing the latency to discomfort in the hot-plate test, are suggested to act like centrally mediated drugs (Hosseinzadeh & Younesi, 2002) by activating the periaqueductal gray matter (PAG) to release endogenous peptides (i.e., endorphin or enkephalin). These endogenous peptides descend the spinal cord and function as inhibitors of the pain impulse transmission at the synapse in the dorsal horn (Katzung, 2005).

In addition, the ability of MPAE to affect the early and late phases of the formalin test, which is use to clarify the peripheral and central antinociceptive activities of compounds/extracts, also indicates its potential as a centrally acting antinociceptive agent (Chan et al., 1995). Both phases have their own characteristics that can be use as tools to assess the antinociceptive potential as well as to elucidate the mechanisms of antinociception (Tjølsen et al., 1992). According to Hunskaar and Hole (1987), the early phase represents a direct irritant effect of formalin on sensory fibers, while the late phase represents

Table 4. Effect of *M. oleifera* aqueous extract on carrageenan-induced paw edema in rats.

		Paw volume (ml)				
	Dose (mg/kg)	1 h	2 h	3 h	4 h	5 h
Control (NaCl)	—	0.42 ± 0.07	0.63 ± 0.07	0.86 ± 0.03	0.86 ± 0.11	0.90 ± 0.11
M. oleifera aqueous extract	10	0.10 ± 0.01*	0.20 ± 0.01*	0.29 ± 0.01*	0.34 ± 0.01*	0.38 ± 0.01*
	30	0.10 ± 0.00*	0.17 ± 0.01*	0.27 ± 0.01*	0.33 ± 0.01*	0.29 ± 0.01*
	100	0.10 ± 0.01*	0.14 ± 0.01*	0.21 ± 0.02*	0.25 ± 0.01*	0.21 ± 0.01*
Ibuprofen	100	0.10 ± 0.01*	0.12 ± 0.01*	0.15 ± 0.01*	0.10 ± 0.02*	0.11 ± 0.02*

N = 6. Values are mean ± SEM. *P < 0.05 significantly different from control.

Table 5. Percentage of inhibition of edema volume by *M. oleiferra* aqueous extract and ibuprofen.

		% of inhibition				
	Dose (mg/kg)	1 h	2 h	3 h	4 h	5 h
M. oleifera aqueous extract	10	76.2	52.3	31.0	19.0	9.5
	30	76.2	59.5	35.7	21.4	31.0
	100	76.2	66.7	50.0	40.5	50.0
Ibuprofen	100	76.2	81.0	82.6	88.4	87.78

response likely secondary to the development of inflammatory process and the release of algesic mediators. According to Shibata et al. (1989) and Santos et al. (1994), drugs acting centrally (i.e., narcotics/opioids) inhibit both phases of the formalin test while those acting peripherally (i.e., NSAIDs) inhibit only the late phase, respectively. Other than that, the ability to affect the writhing, hot-plate and formalin tests reflect the MPAE effectiveness in blocking/reducing the inflammatory-mediated and non-inflammatory-mediated types of pain (Pini et al., 1997) or pain that is caused by a combination of neurogenic- and inflammatory-mediated processes (Amanlou et al., 2005), respectively. Further, the ability of MPAE to inhibit chemically- and thermally-induced nociceptive processes also demonstrated its potential to influence the peripheral and central antinociceptive mechanisms, which is a characteristic of strong analgesics (Hunskaar & Hole, 1987).

On the other hand, the ability of MPAE to reduce the volume of paw edema suggests the extract ability to modulate the inflammatory processes, and thus confirmed the folklore used of *M. oleifera* generally, in the treatment of inflammatory swelling, ulcers, sores, glandular swelling, wounds and cuts as described earlier. Although the folklore use of the *M. oleifera* leaves is associated with the glandular swelling, wounds and cuts, the fact that the antinociceptive and anti-inflammatory activities were somehow associated with those claims seems to support the finding. It is generally known that prostaglandins play an important role in nociceptive and inflammatory processes (Katzung, 2005; Marieb, 2000), the ability of MPAE to exhibit those activities could be associated partly with the manipulation of prostaglandins actions.

Generally, several mechanisms of antinociception could be linked to the observed antinociceptive activity of MPAE. The fact that MPAE peripheral and central activities were not inhibited/reversed by naloxone lead us to exclude the involvement of opioid mechanism in the modulation of the extract antinociceptive activity. Although MPAE was found to show a characteristic of strong analgesics like opioid drugs, it is plausible to suggest that the mechanism/pathway taken by the extract to produce the activity did not involve activation of the opioid receptors. This could be an interesting factor in our quest for new analgesic compounds with fewer side effects as a substitute for opioids, which have been known to cause various unwanted side effects (Katzung, 2005). Although other possible non-opioid mechanisms (i.e., serotonergic, dopaminergic, adrenergic, etc.) could take part in modulating the MPAE activity, we would rather focus on the inhibition of the prostaglandins and cyclooxygenase (COX) actions at the moment. Uzcátegui et al. (2004) have reported earlier on the presence of centrally synthesized prostaglandins or COX, and the ability to inhibit either prostaglandins or COX could definitely contribute to the central antinociceptive activity as seen with the MPAE in the present study. Since MPAE affect the writhing test and the second phase of the formalin test, the ability of MPAE to inhibit/block the action of peripheral prostaglandins or COX could also be suggested to help explain the extract antinociceptive activity. This suggestion could also be supported by the anti-inflammatory activity exerted by the extract as the carrageenan-induced inflammatory process is a COX-dependent response, which is more effectively controlled with the arachidonate COX, rather than the arachidonate lipooxygenase, inhibitors (Gamache et al., 1986).

Several types of bioactive compounds have been isolated from various parts of *M. oleifera*. The leaves, in particular, have been reported to contain hypotensive compounds of the glycoside type (i.e., niazinin A, niazinin B, niazimicin, niaziminin A, and niaziminin B) (Caceres et al., 1991). Other than that, the methanol extract of *M. oleifera* leaves has also been reported to contain various types of flavonol glycosides, as well as kaempferol, rutin, and quercetin (Manguro & Lemmen, 2007). Based on the reported compounds isolated from *M. oleifera* leaves, several mechanisms of action related to those types of compounds could be used to explain the observed antinociceptive and anti-inflammatory activities of the extract. Flavonoids, in particular, have been reported to be effective blockers of nitric oxide synthase type 2 (NOS-2) action (Olszanecki et al., 2002), which in turn induced NO synthesis, and NO has earlier been associated with the antinociceptive (Ferreira et al., 1991; Machelska et al., 1997) and anti-inflammatory (Kim et al., 2004) mechanisms. In addition, flavonoids also inhibited protein tyrosine kinases action that is involved in the NOS-2 expression at the molecular level (Oblak et al., 2000). Meotti et al. (2005) have reported on the flavonoids' ability to affect the L-arginine/NO pathway, which is one of the important pathways in the antinociceptive mechanism, because of its ability to block the protein kinase C pathway. Chen and Pace-Asciak (1996) have earlier demonstrated the vasodilation properties of certain flavonoids, which is important in the antinociceptive and anti-inflammatory processes. This finding is supported by the Naseri et al. (2005) report on the connection between the activation of muscarinic receptors and the release of NO from endothelial cells during vasorelaxation processes. Other than that, flavonoids have been shown to inhibit the action of phospholipase A2 and phospholipase C, which involve in the inflammatory processes (Middleton et al., 2000). Meanwhile, Robak et al. (1998) have earlier reported on the ability of flavonoids to modulate the

induction of NOS-2 via indirect inhibition of the cyclooxygenase and/or lipoxygenase pathways. With regard to the *M. oleifera* extract anti-inflammatory property, it is reasonable to suggest the inhibitory action of flavonoids on the nuclear factor-kappaB (NF-κB) as one of the mechanisms involved (Nam, 2006). As a conclusion, the aqueous extract of *M. oleifera* leaves possess the non-opioid-mediated peripheral and opioid-mediated central antinociceptive, anti-inflammatory and antipyretic activities, which require further extensive study. This study also confirms the folklore medicinal uses of the plant to treat various ailments related to pain and inflammatory processes.

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