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Anti-Inflammatory and Antinociceptive Effects of *Mitragyna speciosa* Korth Methanolic Extract

W.M. Shaik Mossadeq[a, c] M.R. Sulaiman[a] T.A. Tengku Mohamad[a] H.S. Chiong[a]
Z.A. Zakaria[d] M.L. Jabit[e] M.T.H. Baharuldin[b] D.A. Israf[a]

Departments of [a]Biomedical Sciences and [b]Human Anatomy, Faculty of Medicine and Health Sciences, and [c]Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, [d]Faculty of Pharmacy, Universiti Teknologi MARA, Shah Alam, and [e]Technical Services Centre, MARDI, Kuala Lumpur, Malaysia

Key Words
Mitragyna speciosa · Anti-inflammatory · Antinociceptive · Opioid system

Abstract
Objectives: To determine the anti-inflammatory and antinociceptive activities of *Mitragyna speciosa* Korth methanol extract in rodents. ***Materials and Methods:*** Anti-inflammatory activity was evaluated using carrageenan-induced paw edema and cotton pellet-induced granuloma tests in rats. Antinociceptive activity was measured using the writhing test and the hot plate test in mice, and the formalin test in rats. All drugs and extracts were diluted in dH_2O and administered through the intraperitoneal route. Results were analyzed using one-way ANOVA followed by Dunnett's test for multiple comparisons among groups. ***Results:*** Results showed that intraperitoneal administration of the extract at doses of 100 and 200 mg/kg produced significant dose-dependent activity in all of the nociceptive models evaluated ($p < 0.05$). With the formalin test, the antinociceptive activity in mice was inhibited only at the highest dose of the extract (200 mg/kg). The study also showed that intraperitoneal administration of the methanol extract of *M. speciosa* (100 and 200 mg/kg) significantly and dose-dependently suppressed the development of carrageenan-induced rat paw edema ($p < 0.05$). In the chronic test, however, significant reduction in granulomatous tissue formation in rats was observed only at the highest dose of the methanol extract of *M. speciosa* (200 mg/kg, $p < 0.05$). ***Conclusion:*** The present study suggests the presence of potent antinociceptive and anti-inflammatory principles in the extract, supporting its folkloric use for the treatment of these conditions.

Introduction

Mitragyna speciosa Korth, a member of the Rubiaceae family, is a tropical plant that is widely found in the rainforests of Malaysia and in the central and southern regions of Thailand. The leaves of the *M. speciosa* Korth tree, known as 'biak-biak' or 'ketom' in Malaysia and as 'kratom' in Thailand, are often chewed, smoked or made as tea and have been traditionally used by many laborers to increase work efficiency and tolerance of hard work [1]. In Malaysia's folk medicine, the leaves are used to treat diarrhea, fever, asthma, as cough suppressant and for

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Prof. Dr. Mohd Roslan Sulaiman
Department of Biomedical Sciences, Faculty of Medicine and Health Sciences
Universiti Putra Malaysia, 43400 Serdang, Selangor (Malaysia)
Tel. +60 3 8947 2603, Fax +60 3 8947 2585
E-Mail mrs@medic.upm.edu.my or mrs4969@gmail.com

some users, to prolong sexual intercourse. In addition, it is also used for deworming, as cure for stomach ailments and as a substitute for opium or morphine in the treatment of drug addicts [2, 3]. Over 20 alkaloids have been isolated from *M. speciosa* leaves, with mitragynine reported to be the major alkaloid that is responsible for the substance's opioidergic effect [4, 5]. Pharmacologically, *M. speciosa* has been shown to possess antitussive, antinociceptive, anti-inflammatory and antidiarrheal properties [1, 6–8]. Despite the reported antinociceptive and anti-inflammatory activities of *M. speciosa* methanol extract (MSM), previous studies focused on the activities of its alkaloids. Therefore, the aim of the present study was to investigate the medicinal properties of MSM with regard to its anti-inflammatory and antinociceptive effects, so as to provide some pharmacological evidences for its folkloric uses.

Materials and Methods

Plant Material

The fresh mature leaves of *M. speciosa* were collected from undisclosed locations in Selangor and Perlis, Malaysia. The leaves were identified and authenticated taxonomically by a botanist, Ms. Radhiah Zakaria, at the Herbarium Laboratory, Faculty of Forestry, Universiti Putra Malaysia (UPM), Serdang, Selangor, where a voucher specimen (ALS 001) was deposited for future reference.

Preparation of the Extract

MSM was prepared as previously described [9]. Briefly, the leaves (1,000 g) were dried at room temperature for 10 days, pulverized into a coarse dry powder (<1 mm from our observation) and extracted with 95% methanol in the ratio of 1:10 (w/v) by cold maceration for 72 h. The extract was evaporated to a dark brown semisolid mass (yield 12%, w/w) under reduced pressure and kept at –20°C prior to use.

Phytochemical Analysis

Phytochemical screening of the MSM was performed to detect the presence of different classes of constituents, such as alkaloids, flavonoids, saponins, steroids and triterpenoids, using the following reagents and chemicals: alkaloids with Mayer and Dragendorff's reagents, flavonoids with NaCl and HCl, tannins with 1% gelatin and 10% NaCl solutions, saponins with frothing test and finally, steroids and triterpenoids with Liebermann-Burchard test.

Animals

Adult male Sprague-Dawley rats (150–200 g) and male Balb C mice (20–30 g) were used throughout these experiments. The animals were maintained in a room with a 12-hour light-dark cycle for at least 7 days before the experiment to allow acclimatization. The animals were provided with food and water ad libitum. All experiments were performed according to the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals [10] and approved by the Ethics Committee on Animal Experimentation, Faculty of Medicine and Health Sciences, UPM.

Writhing Test

The test was performed according to Zakaria et al. [11], with slight modifications [12]. Sixty mice which were equally divided into six groups (n = 10) were intraperitoneally (i.p.) pretreated with MSM (50, 100, 200 mg/kg), 0.9% NaCl (control), acetylsalicylic acid (ASA, 100 mg/kg) or morphine (5 mg/kg). In an attempt to investigate the participation of the opioid system in the antinociceptive activity of this plant, two separate groups of mice consisting of 10 mice per group were pretreated with the nonselective opioid receptor antagonist naloxone (5 mg/kg, i.p.), which was injected 10 min before the administration of the extract (200 mg/kg, i.p.) or morphine (5 mg/kg, i.p.). After 30 min, 0.6% (v/v) solution of acetic acid was injected i.p. (10 ml/kg). The number of abdominal constrictions together with the stretching of one or both hind legs occurring between 5 and 30 min after acetic acid injection was recorded.

Formalin Test

This procedure was essentially similar to that described previously by Zakaria et al. [11]. Sixty rats were equally divided into six groups (n = 10). In this model, formalin (2.5%, 50 μl) was injected via the intraplantar route into the right hind paw of rats 30 min after the i.p. administration of 0.9% NaCl (10 ml/kg, control), MSM (50, 100, 200 mg/kg), ASA (100 mg/kg), or morphine (5 mg/kg). The amount of time the animal spent licking or biting the injected paw was measured between 0 and 5 min (phase 1, neurogenic) and 15–30 min (phase 2, inflammatory) after the injection of formalin.

Hot Plate Test

The test was performed as previously described [11]. In this model, 60 mice were equally divided into six groups (n = 10). Thirty minutes after pretreatment with either 0.9% NaCl (control), MSM (50, 100, 200 mg/kg, i.p.), ASA (100 mg/kg), and morphine (5 mg/kg), the mice were placed on a heated metal plate (Ugo Basile, model 7280) maintained at 53 ± 1°C and the response latency for nociceptive behavior, e.g. shaking, licking the paw or jumping, was recorded. Mice were removed from the hot plate immediately after the response. Response latencies were measured at 0-, 30-, 60-, 120-, 180-, and 240-min intervals after substance administration, with a cutoff time of 20 s to avoid tissue injury. In order to investigate the participation of the opioid system in the analgesic property of this plant, two separate groups of mice consisting of 10 mice per group were pretreated with the nonselective opioid receptor antagonist naloxone (5 mg/kg, i.p.), which was injected 10 min before the administration of the extract (200 mg/kg, i.p.) or morphine (5 mg/kg, i.p.) and the experiment was repeated.

Carrageenan-Induced Paw Edema Test

The carrageenan-induced rat paw edema was assessed by the method described by Loro et al. [13]. Paw edema was measured with a plethysmometer (model 7140, Ugo Basile, Italy). The basal volume of the right hind paw was determined before administration of any drug. Eight animals per group were pretreated with MSM (50, 100 and 200 mg/kg, i.p.). Thirty minutes later, edema

was induced with 0.1 ml of 1% (w/v) solution of carrageenan, injected into the subplantar region of the rat hind paw. Control animals received 0.9% NaCl (10 ml/kg), whereas positive control animals received ASA (100 mg/kg) under the same experimental conditions. The volumes of the injected paws were measured immediately after injection (0 h) and then every hour until 5 h after induction of edema. The results are presented as the paw volume variation in relation to basal values.

Cotton Pellet-Induced Granuloma Test

The method of Okoli et al. [14] was employed, with slight modifications. Forty rats were equally divided into five groups (n = 8). On day 1, the rats were pretreated with MSM (50, 100 and 200 mg/kg, i.p.). Control animals received either 0.9% NaCl or equal volume of ASA (100 mg/kg). Thirty minutes after pretreatment, a sterilized cotton pellet (30 ± 1 mg) was subcutaneously introduced in the dorsum of rats anesthetized with Avertin (10 ml/kg, i.p.). The rats were treated with a single injection of ASA, 0.9% NaCl or MSM (50, 100 and 200 mg/kg) daily for 7 consecutive days. On day 8, the animals were sacrificed, the pellets dissected out and granulomas dried at 60°C overnight to determine the final dry weight. The difference between the initial (30 mg) and final dry mass was considered as the weight of the granulomatous tissues produced.

Statistical Analysis

The results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Dunnett's test for multiple comparisons among groups. Values with $p < 0.05$ were considered to be statistically significant.

Results

Phytochemical Analysis

A phytochemical screening of MSM indicated the presence of the following secondary metabolites: alkaloids and flavonoids in high concentration, saponins in moderate concentration, while tannins and sterols were detected in a low concentration. The extract, however, was devoid of triterpenes.

Effect of MSM on Acetic Acid-Induced Writhing

The results of the acetic acid-induced writhing test in mice are given in table 1. At doses of 100 and 200 mg/kg i.p., MSM inhibited the writhing responses of mice caused by the intraperitoneal administration of acetic acid. The maximal inhibition of the writhing response was 52.3% with the dose of 200 mg/kg, slightly lower compared to inhibition by ASA (55.5%) at a dose of 100 mg/kg.

Effect of MSM on the Formalin Test

MSM (200 mg/kg) and morphine significantly inhibited both phases of the formalin test showing a pain inhibition of 37.4 and 36.0% in the early phase (0–5 min) and 46.3 and 53.9% in the late phase (15–60 min), respectively. In contrast, ASA inhibited only the second phase of the formalin response (table 2).

Effect of MSM in the Hot Plate Test

Morphine and MSM (200 mg/kg) caused a significant increase in the response latency time to thermal stimulation in mice (table 3). This effect started 30 min after treatment and persisted throughout the 240-min duration of the experiment.

Table 1. Effect of MSM on acetic acid-induced abdominal writhing test in mice

Group	Dose, mg/kg, i.p.	Writhings	Inhibition, %
Control (NaCl 10 ml/kg, i.p.)		134.7 ± 12.3	–
MSM	50.0	105.6 ± 12.6	21.6
	100.0	81.3 ± 7.9*	39.6
	200.0	64.2 ± 11.3*	52.3
ASA	100.0	59.9 ± 10.8*	55.5
Morphine	5	25.9 ± 9.1	80.7
Morphine+naloxone	5+5	53.7 ± 10.51	60.1
MSM+naloxone	200+5	77.2 ± 3.9	42.0

Values are mean ± SEM (n = 10). * $p < 0.05$ significantly different from control (ANOVA followed by Dunnett's test). Control (134.7 ± 38.75).

Table 2. Effect of MSM on formalin-induced pain in mice

Treatment	Total time spent licking, s			
	0–5 min	inhibition, %	15–30 min	inhibition, %
Control (NaCl 10 ml/kg, i.p.)	64.6 ± 6.5	–	205.5 ± 12.8	–
MSM, mg/kg, i.p.				
50.0	57.6 ± 5.4	10.8	171.3 ± 20.3	16.6
100.0	57.6 ± 5.5	10.8	131.0 ± 19.7*	36.2
200.0	40.4 ± 4.6*	37.4	109.0 ± 14.1*	46.3
ASA, mg/kg, i.p.				
100.0	54.0 ± 4.9	16.4	144.9 ± 22.4*	29.5
Morphine, mg/kg, i.p.				
5.0	41.4 ± 3.51*	36.0	94.8 ± 15.7*	53.9

Values are mean ± SEM in seconds (n = 10). * $p < 0.05$ compared to the control group (ANOVA followed by Dunnett's test).

Table 3. Effect of MSM on the hot plate test in mice

	Dose, mg/kg	Latency time, s					
		0	30	60	120	180	240
Control	–	4.92 ± 0.24	5.37 ± 0.54	5.82 ± 0.81	5.49 ± 0.72	5.26 ± 0.71	4.99 ± 0.60
MSM	50	5.57 ± 0.13	6.37 ± 0.52	7.66 ± 0.31	7.79 ± 0.38*	6.45 ± 0.43	4.89 ± 0.34
	100	5.12 ± 0.23	6.49 ± 0.56	7.91 ± 0.62*	7.51 ± 0.80*	6.41 ± 0.53	5.12 ± 0.51
	200	4.59 ± 0.17	6.64 ± 0.47	6.70 ± 0.5*	8.40 ± 0.50*	7.57 ± 0.44*	6.68 ± 0.44*
ASA	100	5.14 ± 0.23	6.00 ± 0.39	6.07 ± 0.63	5.64 ± 0.56	6.37 ± 0.34	6.35 ± 0.59
Morphine	5	5.17 ± 0.22	8.07 ± 0.60*	9.19 ± 0.64*	9.64 ± 0.62*	8.59 ± 0.46*	6.88 ± 0.57*
Morphine+naloxone	5+5	4.89 ± 0.22	5.39 ± 0.36**	6.55 ± 0.38**	8.57 ± 0.72**	6.47 ± 0.52**	5.92 ± 0.40**
MSM+naloxone	200+5	4.98 ± 0.21	5.50 ± 0.42**	5.61 ± 0.26**	7.71 ± 0.62**	5.88 ± 0.41**	4.88 ± 0.28**

Values are mean ± SEM (n = 10). * $p < 0.05$ compared to the control. ** $p < 0.05$ compared to the group receiving appropriate drug/extract at the same dose without naloxone (Dunnett's test).

Table 4. Effect of MSM on carrageenan-induced hind paw edema in rats

	Dose, mg/kg	Edema, ml				
		1 h	2 h	3 h	4 h	5 h
Control	–	0.48 ± 0.05	0.47 ± 0.06	0.56 ± 0.05	0.47 ± 0.03	0.48 ± 0.04
ASA	100	0.27 ± 0.06 (44)*	0.43 ± 0.07 (9)	0.28 ± 0.05 (50)*	0.36 ± 0.05 (23)	0.39 ± 0.06 (19)
MSM	50	0.24 ± 0.05 (50)*	0.44 ± 0.04 (6)	0.39 ± 0.05 (30)	0.35 ± 0.04 (26)	0.49 ± 0.05 (NI)
	100	0.15 ± 0.04 (69)*	0.25 ± 0.04 (47)*	0.27 ± 0.05 (52)*	0.31 ± 0.03 (34)	0.49 ± 0.07 (NI)
	200	0.16 ± 0.04 (67)*	0.15 ± 0.04 (68)*	0.21 ± 0.06 (63)*	0.19 ± 0.06 (60)*	0.18 ± 0.07 (63)*

Values are mean ± SEM, while those in parentheses represent percent inhibition of edema (n = 8). NI = No inhibition. * $p < 0.05$ compared to the control group.

Effect of MSM in Carrageenan-Induced Paw Edema

Subplantar injection of carrageenan in control animals produced a local edema that increased progressively to a maximum intensity 3 h after the injection and then gradually declined with time (table 4). On the other hand, MSM at doses of 100 and 200 mg/kg caused significant ($p < 0.05$) inhibition of the development of paw edema with an activity higher than that of ASA (100 mg/kg), with maximal percent of inhibition during the first 3 h after challenge. In addition, pretreatment with ASA only exhibited a significant inhibitory action on paw edema at 1 and 3 h after carrageenan injection, decreasing edema formation by 44 and 60%, respectively. Nevertheless, the group treated with 200 mg/kg MSM showed the best activity, reducing edema by 60 and 63%, respectively, 4 and 5 h after carrageenan injection, even when the inhibitory effects of the other treatments progressively declined.

Effect of MSM on Cotton Pellet-Induced Granuloma

Investigation of the effect of MSM on the proliferative phase of inflammation revealed that daily administration of MSM (200 mg/kg) significantly ($p < 0.05$) inhibited the growth of granuloma tissue, provoking an inhibitory effect (44.9%) greater than that of ASA (25.4%) when compared to the control group. In comparison, daily treatments of MSM (50 and 100 mg/kg) showed only weak to moderate inhibitory effect with 16.9 and 21.6% inhibition, respectively (table 5).

Discussion

In the present study, the antinociceptive and anti-inflammatory effects of the *M. speciosa* leaves were investigated in various related models in vivo. It was demon-

Table 5. Effect of MSM on cotton pellet-induced granuloma test in rats

Treatment	Dose, mg/kg, i.p.	Granuloma weight, mg	Inhibition, %
Control	–	90.5 ± 0.010	–
ASA	100	67.5 ± 0.003*	25.4
MSM	50	75.2 ± 0.005	16.9
	100	71.0 ± 0.004	21.6
	200	49.8 ± 0.003*	44.9

Values are mean ± SEM (n = 8). * $p < 0.05$ compared to the control group.

strated that MSM (100 and 200 mg/kg, i.p.) significantly inhibited the mice's writhing response in the acetic acid-induced abdominal constriction test. It has been postulated that acetic acid, which was used to induce writhing, acts indirectly by releasing endogenous mediators that stimulate pain nerve endings. Increased levels of PGE_2 and $PGF_{2\alpha}$ as well as in lipoxygenase, liberation of sympathetic nervous system mediators in the peritoneal fluid and the release of cytokines, such as TNF-α, interleukin-1β and interleukin-δ, by resident peritoneal macrophages and mast cells have been reported to be responsible for pain sensation caused by i.p. administration of acetic acid [15–17]. The results also showed that ASA, known to inhibit cyclo-oxygenase [18], causes significant inhibition. On the basis of this result, it can be assumed that the mode of action of this activity might involve a peripheral mechanism probably mediated via inhibition of lipoxygenases and/or cyclo-oxygenase activity. However, the drawback of this model is that other drugs can cause a similar effect, such as adrenergic antagonist and muscle relaxants, leading to possible false-positive results [19]. Due to this, the formalin and hot plate tests were selected to continue this investigation, since they are more specific and it is possible to identify two distinct phases of nociception.

Formalin-induced nociception is a well-described model and can be consistently inhibited by typical analgesic and anti-inflammatory drugs, including morphine and ASA [12]. In this model, MSM (200 mg/kg) and morphine inhibited the first and the second phase, while ASA inhibited only the second phase of the formalin test. Considering the inhibitory property of MSM on the first and second phases of the formalin test, we might suggest that the extract contains active principles acting both centrally and peripherally, which also implies that the extract possesses both antinociceptive and anti-inflammatory activity. Furthermore, the central analgesic effect of MSM is supported by the results observed in the hot plate test, a specific test used to elucidate central antinociceptive properties of pain-relieving agents such as opioid-derived analgesic drugs [20]. In the hot plate model, morphine and MSM (200 mg/kg) caused a significant increase in the response latency time to the thermal stimulus, thus confirming the central activity of the extract. In addition, the results also showed that pretreatment with a nonselective opioid receptor antagonist, naloxone, reversed the antinociceptive effect of MSM as well as morphine in the hot plate test. These findings clearly suggest that the antinociceptive effect of MSM is mediated by activation of the opioid system, which is in agreement with the previous findings [3, 5].

Carrageenan-induced rat paw edema is one of the conventional tests used to evaluate the acute phase of the anti-inflammatory effect of drugs and natural products [21]. Carrageenan-induced inflammation is biphasic in nature. The first phase is attributed to the release of histamine and serotonin; the second phase results mainly from the potentiating effects of bradykinin on mediator release and also of prostaglandins, producing edema after the mobilization of leukocytes [22].

With respect to the first phase, the release of histamine and other mediators produced increased vascular permeability surrounding the site of damaged tissue resulting in edema at the site. Therefore, inhibition of increased vascular permeability and subsequent exudation will, to some extent, implicate the extent of inflammatory reaction produced at the site of injury. In this model, the subplantar injection of carrageenan in control animals produced local edema, which increased progressively to reach maximal intensity 3 h after the injection, after which the effect gradually declined with time. However, MSM (100 and 200 mg/kg) inhibited the development of paw edema more than ASA, demonstrating maximum inhibition during the first 3 h after challenge, and continued to do so even when the inhibitory effects of the other treatments progressively declined. This suggests that the extract may suppress the early phase of edema, possibly by inhibiting the synthesis, release or actions of the various hyperalgesic mediators which are known to mediate acute inflammation induced by phlogistic agents and thus produce reduced sensitivity to pain receptors [23]. However, the inhibitory activity produced by the extract at a dose of 200 mg/kg for a period of 4 h may be attributed to the action of arachidonic acid and

its metabolites, which at this stage produces edema dependent on neutrophil mobilization [24]. To gain further insight into the chronic anti-inflammatory effects induced by the extract, the granulomatous tissue induction model was employed. This procedure induced an inflammatory process which involves proliferation of modified macrophages, fibroblasts as well as the multiplication of blood vessels producing a highly vascularized and reddened mass known as granulation tissue. In this model, daily administration of MSM (200 mg/kg) inhibited the growth of granuloma tissue, provoking an inhibitory effect greater than that of ASA when compared to the control group. A putative mechanism associated with this activity may be due to the inhibition of the synthesis of many mediators involved in the formation of fibrovascular tissue, including chemokines, cytokines and eicosanoids [25–27]. It is also unclear whether the enhancement of immune response at this stage, if any, may play a role in the inhibition of macrophage transformation into epithelioid cells following injury. This may account for the anti-inflammatory activities produced by MSM in both the acute and chronic models of inflammation employed. Even though the exact mechanism of action is unknown, the anti-inflammatory activity of *M. speciosa* may result from a combination of inhibition of pro-inflammatory mediator release and vascular permeability in addition to enhanced immunity, stimulation of tissue repair and healing processes.

Furthermore, phytochemical analysis of MSM has demonstrated the presence of alkaloids, saponins, flavonoids, tannins and sterols. The anti-inflammatory and/or antinociceptive actions of these compounds have been reported by many researchers. Moreover, the suppression of inducible nitric oxide synthase and cyclo-oxygenase-2 enzymes has been shown for alkaloids and flavonoids [28, 29]. Saponins have also been reported to have anti-inflammatory activities by inhibition of the enzymes inducible nitric oxide synthase, cyclo-oxygenase-2 and lipoxygenase [30]. Therefore, it seems that the anti-inflammatory and antinociceptive effects of MSM could also be attributed to the presence of alkaloids, saponins, flavonoids, tannins and sterols in the leaves of *M. speciosa*.

Conclusion

This study showed that MSM possesses antinociceptive and anti-inflammatory properties. However, further investigation is advocated to elucidate the active principle(s) and exact mechanism(s) of its action.

Acknowledgments

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