



Original Article

Possible mechanisms of antinociception of methanol extract of *Melastoma malabathricum* leavesErman Shah Jaios^a, Suzana Abdul Rahman^b, Siew Mooi Ching^c, Arifah Abdul Kadir^d, Mohd. Nasir Mohd. Desa^{a,e}, Zainul Amirudin Zakaria^{a,e,f,*}^a Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor, Malaysia^b Department of Biomedical Sciences, Kulliyah of Allied Health Sciences, International Islamic University Malaysia, Pahang, Malaysia^c Department of Family Medicine, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor, Malaysia^d Department of Veterinary Pre-Clinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor, Malaysia^e Halal Product Research Institute, Universiti Putra Malaysia, Selangor, Malaysia^f Integrative Pharmacogenomics Institute, Universiti Teknologi MARA, Selangor, Malaysia

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ABSTRACT

Melastoma malabathricum L., Melastomaceae, has been traditionally used to relieve diverse pain-related ailments. The objectives of the present study were to determine the antinociceptive activity of methanol extract of *M. malabathricum* leaves and to elucidate the possible mechanisms of antinociception involved using various rats' models. The extract (100, 250, and 500 mg/kg) was administered orally 60 min prior to subjection to the respective test. The *in vivo* acetic acid-induced abdominal constriction, formalin-induced paw licking, and hot plate tests were used as the models of nociception to evaluate the extract antinociceptive activity. Further studies were carried out to determine the role of opioid and vanilloid receptors, glutamate system and nitric oxide/cyclic guanosine phosphate (NO/cGMP) pathway in modulating the extract antinociceptive activity. From the results obtained, *M. malabathricum* exhibited significant ($p < 0.05$) antinociceptive activity in all the chemical- and thermal-induced nociception models. Naloxone (5 mg/kg), a non-selective opioid antagonist, failed to significantly affect the antinociceptive activity of MEMM when assessed using the abdominal constriction-, hot plate- and formalin-induced paw licking-test. *M. malabathricum* also significantly ($p < 0.05$) reversed the nociceptive response in capsaicin- and glutamate-induced paw licking test. Furthermore, only L-arginine (a nitric oxide precursor) alone, but not, N^G-nitro-L-arginine methyl esters (L-NAME; an inhibitor of NO synthase), methylene blue (MB; an inhibitor of cGMP), or their combination thereof, significantly ($p < 0.05$) block the antinociceptive activity of *M. malabathricum*. In conclusion, *M. malabathricum* exerted a non-opioid antinociceptive activity at the central and peripheral levels partly via the inhibition of vanilloid receptors and glutamatergic system, and activation of the NO-mediated/cGMP-independent pathway.

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Introduction

Some of the most common ailments that affected millions of people worldwide are pain and inflammation (Raghav et al., 2006; Rang et al., 2011). Therefore, researches in the fields of pain and inflammation, particularly on finding appropriate drugs to treat them, have tremendously increased these past few years. This claim is suggested based on the rapid developments in the field of synthetic and medicinal chemistry that saw increase in reports on newly synthesized drugs including those for the treatment of pain and inflammation. However, many of synthetic drugs are

withdrawn lately after their introduction into the market because of the adverse side effects associated with their prolonged use. For example, chronic use of morphine, a currently prescribed analgesic drug, has been associated with the development of tolerance and dependence. Therefore, alternative agents with less or, possibly, no unwanted side effects are required and plant-based natural products are and have been the important sources of those alternative agents (Verpoorte, 1998). Natural products from plants have long been recognized as the important sources of therapeutically effective medicines (Cragg et al., 2003).

Melastoma malabathricum L. (family Melastomaceae) is a flowering plant native to the Southeast Asian region including Malaysia. It is the sole species in the genus *Melastoma* and has been classified as a weed, and can be found to grow extensively in the wasteland areas. Commonly called the "Straits *Rhododendron*" and locally

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known to the Malay as “Senduduk”, *M. malabathricum* has been widely used in the Malay as well as others traditional medicines (Rajenderan, 2010). According to several reports, the leaves, in particular, have been applied either pounded, grounded or as decoction, by several tribes and population to treat ailments such as stomach ulcers, dysentery and diarrhoea, those associated with pain (*i.e.*, toothache and stomachache), to accelerate wound healing, for post-natal care and prevention of scars from small pox infection, and, postpartum remedy (Grosvenor et al., 1995; Ong and Nordiana, 1990; Sharma et al., 2001; Sulaiman et al., 2004; Roosita et al., 2008). Scientifically, the leaves of *M. malabathricum* have been reported to exert no acute toxicity and (Sunilson et al., 2009), antibacterial (Grosvenor et al., 1995; Wiart et al., 2004), antiviral (Grosvenor et al., 1995), antioxidant (Nazlina et al., 2008), cytotoxic (Nazlina et al., 2008), anticoagulant (Manicam et al., 2010), antiulcer (Hussain et al., 2008), antidiarrheal (Sunilson et al., 2009), anti-inflammatory (Susanti et al., 2008; Zakaria et al., 2008), antinociceptive (Sulaiman et al., 2004; Zakaria et al., 2008) and antipyretic (Zakaria et al., 2008) activities. Phytochemical screening of the *M. malabathricum* leaves demonstrated the presence of flavonoids, triterpenes, tannins, saponins, steroids (Zakaria et al., 2008; Simanjuntak, 2008; Faravani, 2009), alkaloids (Zakaria et al., 2008), glycosides and phenolics (Simanjuntak, 2008; Faravani, 2009). However, the phytochemical analysis of methanol extract of *M. malabathricum* leaves (MEMM) revealed only the presence of flavonoids, glycosides, phenolics, triterpenes, tannins, saponins and steroids but no alkaloids. The previous antinociceptive activity of *M. malabathricum* leaves, in particular, has been investigated using the aqueous and ethanol extract of the leaves with attempt only made to determine the role of opioid receptors reported. In the present study, the methanol extract of *M. malabathricum* leaves (MEMM) was used to further study the antinociceptive activity of *M. malabathricum* and the mechanisms of action involved. The mechanisms of action to be studied include the role of opioid and vanilloid receptors, glutamate system and nitric oxide/cyclic guanosine phosphate (NO/cGMP) pathway.

Materials and methodology

Plant collection and preparation of MEMM

The leaves of *Melastoma malabathricum* L. was collected between June and July 2012 around the Universiti Putra Malaysia (UPM), Malaysia and certified by a botanist, Dr. Shamsul Khamis, from the Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. A voucher specimen (ACP0017) has been earlier deposited at the Herbarium of the Laboratory of Natural Products, IBS, UPM, Malaysia. The procedure for preparation of MEMM was carried out as described in detail by Zakaria et al. (2008).

Drugs and chemicals

The following reagents and drugs were used: methanol (Fischer Scientific, UK), DMSO, formalin, acetic acid, morphine, acetylsalicylic acid (ASA), naloxone, capsaicin, glutamate, L-arginine, N^G-nitro-L-arginine methyl esters (L-NAME) and methylene blue (MB) (Sigma, USA). The drugs were prepared by dissolving them in distilled water. The MEMM was dissolved in vehicle (10% DMSO) immediately before used. All solutions were administered in the volume of 10 ml/kg.

Animals

Male Sprague Dawley (SD) rats (180–200 g; 8–10 weeks old) and male ICR mice (25–30 g; 5–7 weeks old), purchased from Che Nur

Supplier, Selangor, Malaysia, were transferred to the Animal Holding Unit, International Islamic University Malaysia (IIUM), Pahang, Malaysia and allowed to acclimatize for one week prior to the experimentation. The animals were cared and handled according to the procedures described in detail by Mohd. Sani et al. (2012). The study protocol of the present study was approved by the Animal Ethics Committee of International Islamic University Malaysia [IIUM / IACUC Approval / 2016/ (9) (58)] and were performed in accordance with the Integrated Centre for Research of Animal Care and Use (ICRACU) guidelines.

Acute toxicity study of MEMM

Acute toxicity studies were carried out according to the “Guideline for Testing of Chemicals – Acute Oral Toxicity – Fixed Dose Procedure (OECD No. 423)” (OECD, 2002). Rats were fasted overnight prior to the administration of test solutions. The treated group obtained a single dose of 5000 mg/kg MEMM while one group each obtained either vehicle or distilled water (10 ml/kg) by gavage. Then, the animals were monitored separately at least once during the first 30 min after dosing, occasionally during the first 24 h and daily thereafter for 14 days. Food and water were provided *ad libitum*. The mortality, body weight and behavioural screening were recorded daily for 14 days after treatment. The rats that survived were euthanized and macroscopic analysis and the weight of vital organs were recorded. These organs were fixed in 10% formalin for histological assessment.

Antinociceptive activity

Acetic acid-induced abdominal constriction test

The acetic-acid-induced abdominal constriction test was carried out according to the method described in detail by Collier et al. (1968) but with slight modifications. The mice ($n=6$) were pre-treated orally (*p.o.*) with 10% DMSO (negative control), 100 mg/kg ASA (positive control), or MEMM (100, 250, and 500 mg/kg) prior to assessment using the abdominal constriction test. Sixty minutes after the respective test solution administration, the mice were injected via intraperitoneal (*i.p.*) route with phlogistic agent (0.6% acetic acid). The animals were immediately placed individually into glass cage and 5 min were allowed to elapse. The abdominal constriction resulting from the injection of acetic acid consists of a contraction of the abdominal together with a stretching of at least one hind limb. The number of abdominal constrictions produced in these animals was counted cumulatively for 25 min. Antinociceptive activity, indicated by the reduction in the mean of the number of abdominal constrictions in the test groups compared to the control group, was calculated as the percentage inhibition of abdominal constrictions (percentage of inhibitory level) using the following formula: $[\text{mean of (control - test group)}/\text{control group} \times 100\%]$.

Hot plate test

The hot plate test was carried out according to the method described by Hunskaar et al. (1986) but with slight modifications. The mice ($n=6$) were pre-treated (*p.o.*) with 10% DMSO (negative control), 5 mg/kg morphine (positive control), or MEMM (100, 250, and 500 mg/kg) prior to assessment using the hot plate test. The temperature of the metal surface (Ugo Basile 7280) was set at 50 ± 0.2 °C. Sixty minutes after the respective test solution administration, the mice were placed on the heated metal surface and the latency to a discomfort reaction (licking paws or jumping) was recorded. The cut-off time of 20 s was chosen to avoid tissue injury. Latency was record before and 60, 90, 120, 150, 180, 210 min following the oral administration of treatments. The prolongation of latency times compared with the values of the controls was used for statistical comparison.

Formalin-induced paw licking test

The formalin induced paw licking test (also known as formalin test) was carried out as described by [Hunnskaar and Hole \(1987\)](#) but with slight modifications. Rats ($n=6$) were administered *p.o.* with 10% DMSO (negative control), 5 mg/kg morphine or 100 mg/kg ASA (both act as the positive controls), or MEMM (100, 250, and 500 mg/kg) prior to assessment using the formalin test. Pain was induced by injecting 50 μ l of 5% formalin in the sub-plantar region of the right hind paw 60 min after the test solutions administration. Immediately after the phlogistic agent administration, the rats were individually placed in a transparent glass cage observation chamber. The amount of time that the animal spent licking the injected paw, considered as an indicator of pain, was recorded for the duration of 30 min in two phases, known as the early (0–5 min) and late (15–30 min) phases.

Mechanisms of antinociceptive activity of MEMM

Capsaicin-induced paw licking test

The capsaicin-induced paw licking test was used to investigate the role of vanilloid receptors in the modulation of MEMM antinociceptive action and the procedure adopted has been described in detail by [Sakurada et al. \(1992\)](#) but with slight modifications. Rats were pre-treated orally with 10% DMSO or MEMM (100, 250, and 500 mg/kg) 60 min prior to assessment using the respective test. Sixty min after the administration of test solutions capsaicin was injected (1.6 μ g/paw, 20 μ l) into the intraplantar (*i.pl.*) region of the rat's right hind paw. Immediately after the phlogistic agent administration, the rats were individually placed in a transparent glass cage observation chamber and observed individually for 5 min after the capsaicin injection. The amount of time the animals spent licking the injected paw was recorded with a chronometer and was considered as an indication of nociception.

Glutamate-induced paw licking test

The glutamate-induced paw licking test was used to investigate the role of glutamatergic system in the modulation of MEMM antinociceptive action and the procedure adopted has been described by [Luiz et al. \(2007\)](#) with slight modifications. Rats were pre-treated orally with 10% DMSO or MEMM (100, 250, and 500 mg/kg) 60 min prior to assessment using the respective test. A volume of 20 μ l of glutamate (10 μ mol/paw, in normal saline) was injected via *i.pl.* route in the right hind paw of rats 60 min following the test solutions administration. Immediately after the phlogistic agent administration, the rats were individually placed in a transparent glass cage observation chamber and observed individually from 0 to 15 min after the glutamate injection. The amount of time the animals spent licking or biting the injected paw was recorded with a chronometer and was considered as an indication of nociception.

Involvement of opioid receptor

To determine the role of opioid receptors in the modulation of MEMM antinociceptive activity, six groups of animals ($n=6$) were pre-treated (*i.p.*) with a non-selective opioid antagonist, naloxone (5 mg/kg; *i.p.*) for 15 min followed by the oral administration of the most effective MEMM dose (500 mg/kg) or 10% DMSO. Sixty minutes later, the animals were assessed using the acetic acid-induced abdominal constriction test, hot plate test or formalin test, respectively ([Mohd. Sani et al., 2012](#)).

Involvement of nitric oxide/cyclic-guanosine monophosphate (NO/cGMP) pathway

To determine the role of nitric oxide/cyclic-guanosine monophosphate (NO/cGMP) pathway in the modulation of MEMM antinociceptive activity, the method described in detail

by [Mohd. Sani et al. \(2012\)](#) was adopted with slight modifications. In this study, the mice ($n=6$) were pre-treated with 20 mg/kg L-arginine, L-NAME, MB, or their respective combination (L-arginine with L-NAME or L-arginine with MB) followed 5 min later by pre-treatment with 10% DMSO or MEMM (500 mg/kg), respectively. Sixty minutes later, the animals were assessed using the abdominal constriction test.

HPLC and GCMS analysis of MEMM

The HPLC analysis of MEMM has been carried out previously and the detailed method was published by [Kamisan et al. \(2014\)](#). From the analysis, flavonoids were generally detected based on the UV-vis spectra wavelength while quercitrin was specifically detected based on the comparison of chromatogram obtained for MEMM against those of several pure flavonoids. In addition to the HPLC finding, GCMS analysis was also performed on MEMM.

GC-MS analysis of MEMM was performed using Agilent 7890A (Agilent Technologies) coupled with MSD quadrupole detector 5975C (Agilent Technologies). Separation of analytes by gas chromatography was carried out using the Hewlett Packard HP-5MS silica capillary column (30 m \times 0.25 mm \times 0.25 mm). For GC-MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1 ml/min and an injection volume of 1 μ l was employed (split ratio of 1:10); injector temperature 250 °C; ion-source temperature 280 °C. The oven temperature was programmed from 100 °C (isothermal for 2 min), with an increase of 10 °C/min to 200 °C, then 12 °C/min to 280 °C, ending with a 17 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. Total GC running time was 35.50 min. The relative % amount of each component was calculated by comparing its average peak area to the total areas. The software adopted to handle mass spectra and chromatograms was a Turbomass. For the identification of compounds, interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

Results

Acute toxicity effect of MEMM

All animals in the treatment and control groups demonstrated an increase in body weight at weeks 1 and 2 in comparison to day 0. Neither alteration in the behavioural pattern nor mortality was observed throughout the duration of experimentation. In addition, no changes in food and water intake, and behaviour were detected among the animals. The vital organs showed no significant changes in their respective relative weight while the respective microscopic analysis demonstrated no signs of toxicity (data not shown). Moreover, the single oral administration of 5000 mg/kg MEMM did not generate any sign of toxicity in the treated animals after 14 days. Based on these observations, the extract was suggested to possess an LD₅₀ that is greater than 2000 mg/kg body weight.

Acetic acid-induced abdominal constriction test

The MEMM, at all tested doses, demonstrated a significant ($p<0.001$) and dose-dependent antinociceptive activity when assessed using the abdominal constriction test ([Fig. 1](#)) with the percentage of analgesia ranging between 33 and 62%. The 250 mg/kg MEMM produced an antinociceptive activity that was of equal

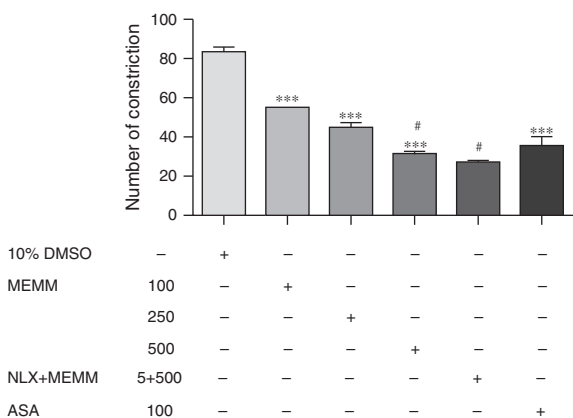


Fig. 1. Antinociceptive activity of MEMM assessed by the acetic acid-induced abdominal constriction test in mice. Acetic acid administered by intraperitoneally 60 min before pre-treated with DMSO as vehicle (control), acetylsalicylic acid (ASA), or MEMM (100, 250 and 500 mg/kg). All treatments administered via oral route. ***Data differed significantly ($p < 0.001$) when compared to 10% DMSO-treated group. #Data was not significant when compared together.

effectiveness when compared to the positive control (100 mg/kg ASA).

Hot plate test

Table 1 shows the antinociceptive profile of orally-administered MEMM as assessed using the hot plate test. The MEMM, at the dose of 250 mg/kg, exhibited significant ($p < 0.001$) activity at the interval time of 60, 90 and 120 min while the 500 mg/kg MEMM exerted significant ($p < 0.001$) antinociception until the end of experiment. Co-administration with naloxone (5 mg/kg; *i.p.*) failed to inhibit the 500 mg/kg MEMM antinociceptive activity. Overall, the 5 mg/kg morphine demonstrated the most effective effect when compared to the MEMM at all doses used.

Formalin-induced paw licking test

MEMM demonstrated a significant ($p < 0.001$) antinociceptive activity in a dose-dependent manner in both phases of the formalin-induced paw licking test as shown in Fig. 2A and B, respectively. In comparison to MEMM, 5 mg/kg morphine also attenuated both phases while 100 mg/kg ASA only reduced the nociception in the late phase. Overall, morphine was effective than the ASA and MEMM in both phases of the formalin test.

Capsaicin-induced paw licking test

The antinociceptive profile of MEMM assessed using capsaicin-induced paw licking test is shown in Fig. 3. All doses of MEMM demonstrated a significant ($p < 0.05$) and dose-dependent

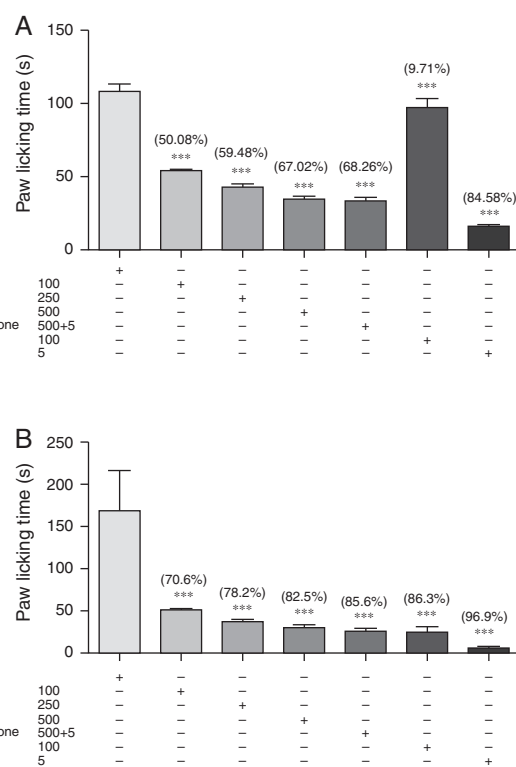


Fig. 2. Antinociceptive activity of MEMM assessed using the formalin-induced paw licking test. (A) Effect of MEMM recorded at the early phase (0–5 min), and; (B) effect of MEMM recorded at the late phase (15–30 min). Each column represents the mean \pm SEM of 6 rats. The rats were pre-treated with vehicle (10% DMSO) as control, MEMM (100, 250, and 500 mg/kg, *p.o.*), acetylsalicylic acid (ASA, *p.o.*), or morphine (5 mg/kg, *p.o.*), 60 min before *i.p.* injection of formalin. The asterisks denote the significance levels as compared to control, *** $p < 0.001$ by one-way ANOVA followed by Dunnett's post hoc test. ***Data differed significantly ($p < 0.05$) when compared to the 10% DMSO-treated group.

antinociception against capsaicin-induced nociception with the percentage of analgesia ranging between 29% and 64%.

Glutamate-induced paw licking test

Fig. 4 shows the antinociceptive profile of MEMM assessed using the glutamate-induced paw licking test. All doses of MEMM also exerted a significant ($p < 0.05$) and dose-dependent antinociception against glutamate-induced nociception with the percentage of analgesia ranging from 17% to 72%.

Involvement of opioid receptors

The effects of non-selective opioid antagonist (naloxone, 5 mg/kg) on MEMM antinociceptive activity was evaluated using

Table 1
Antinociceptive activity of MEMM assessed by the hot plate test in mice.

Treatment	Dose (mg/kg)	Latency of discomfort(s) at respective time interval (min)						
		0 min	60 min	90 min	120 min	150 min	180 min	210 min
10% DMSO	-	6.97 \pm 0.22	6.97 \pm 0.22	6.90 \pm 0.23	6.15 \pm 0.15	6.92 \pm 0.23	6.88 \pm 0.29	6.35 \pm 0.17
Morphine	5	5.77 \pm 0.15	17.37 \pm 1.03 ^a	18.25 \pm 0.74 ^a	16.52 \pm 1.22 ^a	13.67 \pm 1.43 ^a	11.22 \pm 1.11 ^a	10.48 \pm 0.58 ^a
MEMM	100	6.84 \pm 0.33	10.19 \pm 0.91 ^a	10.56 \pm 1.68 ^a	6.70 \pm 0.32	6.73 \pm 0.37	7.77 \pm 0.33	7.38 \pm 0.46
	250	6.84 \pm 0.23	12.38 \pm 1.00 ^a	11.34 \pm 0.89 ^a	11.80 \pm 1.37 ^a	9.10 \pm 0.61 ^a	9.39 \pm 1.00 ^a	8.74 \pm 0.37 ^a
	500	6.75 \pm 0.30	12.51 \pm 1.38 ^a	14.1 \pm 0.67 ^a	15.12 \pm 1.00 ^a	13.14 \pm 1.10 ^a	10.28 \pm 0.20 ^a	9.70 \pm 0.130 ^a
Naloxone	5	6.38 \pm 0.27	6.43 \pm 0.41	5.98 \pm 0.46	6.1 \pm 0.21	5.93 \pm 0.68	6.13 \pm 0.58	5.67 \pm 0.54
Naloxone + MEMM	5 + 500	6.97 \pm 0.16	13.55 \pm 2.06 ^a	14.15 \pm 1.86 ^a	12.68 \pm 1.63 ^a	11.90 \pm 0.36 ^a	10.62 \pm 0.36 ^a	9.83 \pm 0.31 ^a

^a Data differed significantly ($p < 0.05$) when compared against the control (10% DMSO-treated) group.

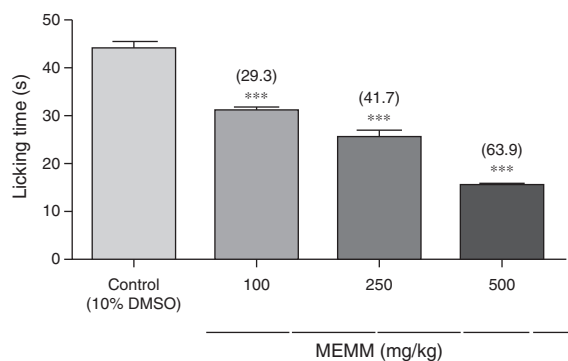


Fig. 3. Antinociceptive activity of MEMM assessed using the capsaicin-induced paw licking test in rats. Each column represents the mean \pm SEM of 6 rats. The rats were pre-treated with vehicle (10% DMSO) as control or MEMM (100, 250 and 500 mg/kg, *p.o.*) 60 min before of capsaicin (1.6 μ g/paw, 20 μ l, *i.pl.*). The asterisks denote the significance levels as compared to control, *** p < 0.001 by one-way ANOVA followed by Dunnett's post hoc test.

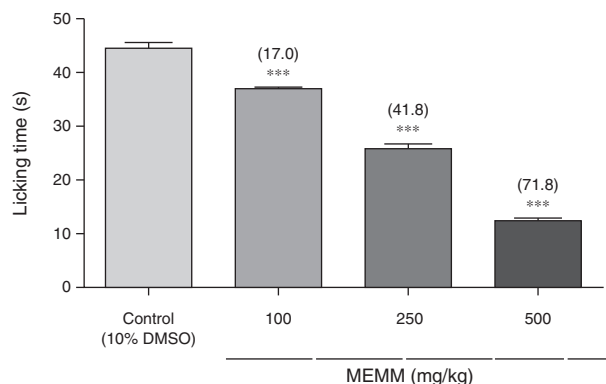


Fig. 4. Antinociceptive activity of MEMM assessed using the glutamate-induced paw licking test in rats. Each column represents the mean \pm SEM of 6 rats. The rats were pre-treated with vehicle (10% DMSO) as control or MEMM (100, 250 and 500 mg/kg, *p.o.*) 60 min before of glutamate (10 μ mol/paw, 20 μ l, *i.pl.*). The asterisks denote the significance levels as compared to control, *** p < 0.001 by one-way ANOVA followed by Dunnett's post hoc test.

the abdominal constriction test, hot plate test, and formalin test and are shown in Fig. 1, Table 1, and Fig. 2A and B, respectively. Naloxone did not significantly (p < 0.05) reversed the antinociceptive activity of 500 mg/kg MEMM when assessed using the abdominal constriction test, hot plate test and formalin test.

Involvement of NO/cGMP pathway

Fig. 5A shows the effect of L-arginine, L-NAME, or their combination on antinociceptive activity of 500 mg/kg MEMM assessed using the abdominal constriction test. Pre-treatment of rats with L-arginine alone did not affect the acetic acid-induced nociception but significantly (p < 0.05) reversed the antinociception of 500 mg/kg MEMM. On the other hand, pre-treatment of rats with L-NAME alone exerted significant (p < 0.05) antinociceptive activity but failed to enhance the extract's antinociceptive activity. Moreover, pretreatment with both L-arginine and L-NAME also failed to significantly change the extract's antinociception. Therefore, only the presence of NO donor (L-arginine) significantly affects MEMM antinociceptive activity.

Fig. 5B shows the effect of L-arginine, MB or their combination on antinociceptive activity of 500 mg/kg MEMM assessed using the abdominal constriction test. Pre-treatment of rats with MB alone produce a significant (p < 0.05) antinociceptive effect, which is not affected by early pre-treatment with L-arginine. Moreover, MB alone or in combination with L-arginine did not help to increase the antinociceptive activity of MEMM. Thus, inhibitor of cGMP pathway did not affect the extract's antinociception.

GCMS analysis of MEMM

A total of 30 peaks were identified from MEMM with the major compounds constituted of 9-octadecenamide (48%), 3-methyl quinoline (7.8%), propanoic acid (5.8%), methyl- β -D-galactopyranoside (6.8%), methyl- α -D-glucopyranoside (3.9%), hexadecanoic acid, methyl ester (3.4%), hexadecanoic acid (2.9%), hexadecanamide (2.9%), furfural (2.3%), pyrogallol (2.3%), myristamide (1.8%), 9,12,15-octadecatrienoic acid, methyl ester (2.1%), 2-methyl-l-mannomethylpyranoside (1.7%), γ -conicein (1.6%),

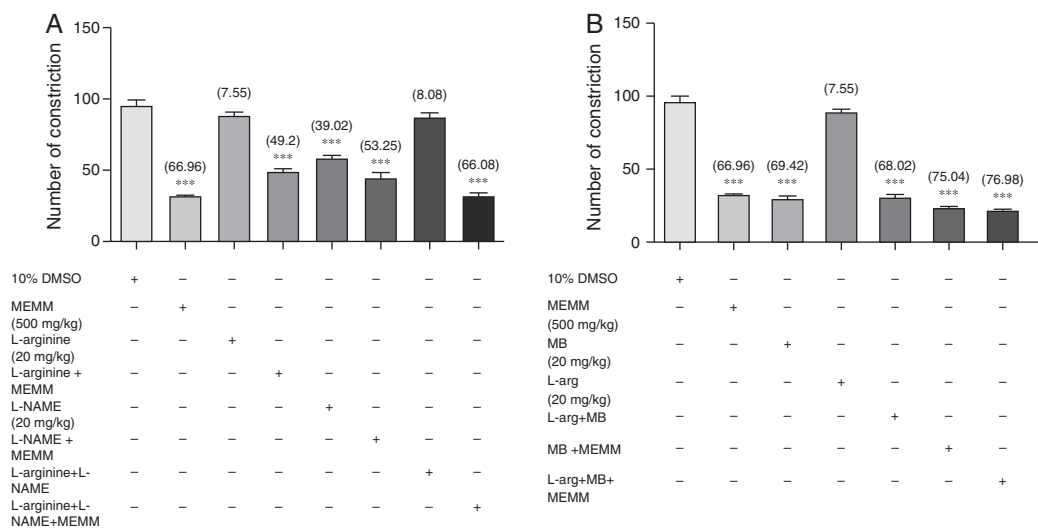


Fig. 5. Involvement of NO/cGMP pathway in the modulation of antinociceptive activity of MEMM. (A) Effect of L-arginine, L-NAME and their combination on antinociceptive activity of MEMM assessed using the abdominal constriction test. (B) Effect of L-arginine methylene blue (MB) and their combination on antinociceptive activity of MEMM assessed using the abdominal constriction test. The asterisks denote the significance levels as compared to control, *** p < 0.001 by one-way ANOVA followed by Dunnett's post hoc test.

dodecanedioic acid, dimethyl ester (1.6%) and 9-octadecenoic acid (1.3%).

Discussion

The management of pain using currently available analgesics have been over shadowed by various adverse effects. Morphine, which has been the drug of choice for the treatment of pain, has been known to cause dependence and tolerance upon its prolong use (Rang et al., 2011). In an attempt to contribute towards finding new analgesic drug with low or, possibly, no adverse effects, the present study was conducted to determine the antinociceptive potential of MEMM using various chemical- and thermal-induced models of nociception in laboratory animals. Although there have been three reports on the antinociceptive activity of *M. malabathricum* leaves, the three papers reported the use of ethanol, aqueous and chloroform extracts as their source of antinociceptive study and that those extracts were administered systemically, which are either by the intraperitoneal or subcutaneous routes (Sulaiman et al., 2004; Zakaria et al., 2006; 2008). In contrast to those reports, the present study used methanol extract that was administered orally into the rats. Although the use of ethanol extract almost resembles the current used of methanol extract, the former administration via the intraperitoneal route did not represent the traditional way of consuming the extract. Moreover, the aqueous and chloroform extracts of *M. malabathricum* have been proven to exert antinociceptive activity and, therefore, justify the use of intermediate solvent like methanol to extract out both the antinociceptive-bearing polar and non-polar bioactive compounds from *M. malabathricum*.

In the present study, MEMM was found to exert antinociceptive activity at both the peripheral and central levels with the non-opioid-mediated activity seen at both levels. The non-opioid mechanism is suggested based on our findings that naloxone failed to inhibit the extract's activity when assessed using all nociceptive assays. Interestingly, the antinociceptive activity of MEMM is also suggested to involve inhibition of the vanilloid receptors and glutamatergic system as MEMM attenuated both the capsaicin- and glutamate-induced models of nociception. Moreover, MEMM is also suggested to exert antinociceptive activity via the mechanisms of action that did not involve activation of the NO/cGMP pathway as pre-challenging the extract with L-NAME or MB also failed to inhibit MEMM antinociceptive activity. The finding that only L-arginine, but not L-NAME or MB, significantly reversed the antinociceptive activity of MEMM suggested that the presence of NO affected the extract via a pathway that was independent of cGMP action (Cui et al., 2005).

Several other mechanisms of action could be proposed based on the results obtained. Pain sensation can be produced by various types of stimuli (i.e., mechanical, thermal and chemical), hence the existence of mechanosensitive, thermosensitive and chemosensitive pain receptors are suggested (Tandon et al., 2003). The extract ability to inhibit at least two of the stimuli, which is represented by the respective acetic acid-induced abdominal constriction and formalin-induced paw licking tests, and the hot-plate test, is an indicative of its ability to inhibit the peripheral and central nociceptive mechanisms. Moreover, MEMM also attenuated both phases of the formalin test and, taking these things together, it is plausible to suggest that MEMM possesses the characteristic of centrally acting analgesics. However, since MEMM did not work on the opioid receptors at the peripheral and central levels, it might be a right candidate for the development of analgesic to replace morphine.

Several mechanisms of antinociception could also be suggested based on the nociceptive models applied in the present study. The acetic acid-induced abdominal constriction test is a typical model for assessment of peripheral antinociceptive activity

of new or potential analgesic agents. Other than being a sensitive model (Collier et al., 1968; Bentley et al., 1981; Mohd. Sani et al., 2012), this assay represents the stimulation of peripheral nociceptive mechanism via the acetic acid-induced release of several pro-inflammatory or nociceptive-endogenous mediators (i.e., bradykinin, serotonin, histamine, substance P or prostaglandins (PGE₂ and PGF_{2α})) (Deraedt et al., 1980; Ribeiro et al., 2000; Tandon et al., 2003), which caused subsequent activation of peripheral nociceptive neurons within the peritoneal cavity. In addition, the nociceptive response induced by acetic acid depends on the production of nitric oxide (NO) (Larson et al., 2000). Moreover, the acetic acid-induced nociception observed using the abdominal constriction test was attenuated by both the peripherally- and centrally-mediated analgesics. Therefore, our present results imply that MEMM may exerted in part a peripherally-mediated antinociceptive activity, which may to a certain extent resulted from the inhibition of the synthesis or action of some of the pro-inflammatory mediators mentioned above. In addition, the extract might also cause decrease in the production of NO and/or cytokines, thereby interfering with the mechanisms of signal transduction in the primary afferent nociceptors.

Nevertheless, the abdominal constriction test is not a specific test as certain type of non-analgesics like muscle relaxants can also give false positive results, which could lead to misinterpretation of the results (Le Bars et al., 2001; Mohd. Sani et al., 2012). Therefore, additional assessment of MEMM's antinociceptive potential using other models of nociception need to be conducted. To confirm the possible mechanisms of antinociception involved, the MEMM was further subjected to the hot-plate and formalin-induced paw licking tests. These tests are considered as the more specific tests for determining the involvement of the central and/or peripheral levels of antinociception (Mohd. Sani et al., 2012).

The hot plate test, which measured thermal-induced nociception at the supra-spinal and spinal levels, is a nociceptive model suitable for assessing the potential of any compounds/extracts to exert antinociceptive activity at central level. It has the advantage of being selective and sensitive only to the centrally-, but not peripherally-acting analgesics (Hosseinzadeh and Younesi, 2002; Giglio et al., 2006). The ability of MEMM to prolong the latency to feeling discomfort indicates the extract potential to inhibit the thermal-induced nociception and, therefore, suggested the involvement of centrally-mediated antinociception. Taking into account the ability of MEMM to attenuate nociceptive stimuli when assessed using the abdominal constriction and hot plate tests, it is plausible to suggest that MEMM exerts peripheral and central antinociceptive activity and, hence, demonstrated the characteristic of strong analgesics.

The ability of MEMM to exhibit antinociceptive activity at the peripheral and central levels was further confirmed by findings made using the formalin-induced paw licking test (Tjolsen et al., 1992; Mohd. Sani et al., 2012). Other than that, this model of nociception can also provide information on the effect of compounds/extracts towards the inflammatory- and non-inflammatory-mediated pain (Hunnskaar and Hole, 1987; Mohd. Sani et al., 2012). These advantages of using formalin test could be attributed to its characteristic distinct biphasic nociceptive response resulting from the intraplantar injection of phlogistic agent (formalin) (Hunnskaar and Hole, 1987; Tjolsen et al., 1992). The two distinct phases known as the first/early phase and the second/late phase occur between 0–5 min and 15–30 min following the phlogistic agent administration, respectively (Shibata et al., 1989). The early phase corresponds to the neurogenic and non-inflammatory-mediated nociceptive response, and represents the centrally-acting nociception. This neurogenic pain is described as an intensely painful process resulting from direct formalin activation of transient receptor potential A1 (TRPA1) cation channels

located at the sensory C-fibres that reflect centrally-mediated pain (McNamara et al., 2007). The late phase, on the other hand, corresponds to the inflammatory-mediated nociceptive response, and represents the peripherally-acting nociception. This inflammatory-induced nociception is due to the action of various inflammation mediators released due to damage to the cells resulting from formalin injection (Parada et al., 2001). It was reported that substance P and bradykinin act as mediators in the first phase response, while histamine, serotonin, prostaglandin and bradykinin are involved in the nociceptive response of the second phase. It is well established that, centrally acting drugs (i.e., opioids) can inhibit both phases; however, peripherally acting drugs (i.e., NSAID) only inhibit the second phase (Tjolsen et al., 1992; Mohd. Sani et al., 2012). In the present study, MEMM attenuated both phases of nociception further confirming the extract's centrally-acting effect.

In the early part of the discussion, MEMM is suggested to probably possess a characteristic of strong opioid analgesics based on the extract ability to inhibit the chemically- and thermally-induced nociceptive stimuli and to attenuate the nociceptive response in the two phases of formalin test. However, pre-treatment with naloxone did not affect the antinociceptive activity of MEMM against all models of nociception. This finding contradicted previous report made by Sulaiman et al. (2004), who demonstrated the involvement of opioid system in the modulation of antinociceptive activity of ethanol extract of *M. malabathricum* (EEMM). This discrepancy could be due, particularly, to the different route of administration of the respective extract. The effect of route of administration can be seen in report by Matsumoto et al. (2004), wherein morphine was reported to exert weak antinociceptive efficacy when given via the oral route in comparison to the subcutaneous route. In this case, EEMM was given intraperitoneally in comparison to MEMM, which was given orally. The bioactive compounds in MEMM might have lost or decreased in its opioid action due to metabolism processes in the liver following the extract's oral administration in comparison to EEMM, which was not metabolized by liver following its intraperitoneal administration and directly transfer to the site of action. The processes might have destroyed some of the antinociceptive compounds, possibly those acting at the opioid receptors. To further support the contradiction mentioned above, a report by Rebolledo et al. (2012) could be used to explain the discrepancy in opioid receptors role on the antinociception of MEMM and EEMM. According to Rebolledo et al. (2012), the polar compounds were easily digested than the non-polar compounds and this could be used to explain the inability of naloxone to block the extract antinociception when administered orally, but not systemically.

Further tests were also conducted to examine the involvement of MEMM in the modulation of nociceptive transmission via the vanilloid receptors, glutamatergic system and NO/cGMP pathway. To determine the role of vanilloid receptors in the modulation of antinociceptive activity of MEMM, the capsaicin-induced paw licking test were carried out in rats. Capsaicin, the pungent substance from chilli peppers, has been repeatedly used in pain research for its ability to induce both hyperalgesia and analgesia, depending on the concentration and route of application (Numazaki and Tominaga, 2004). Capsaicin has the ability to activate C- or A δ -fibres in afferent neurons through stimulation of TRPV1 receptors thus allowing the influx of Ca $_2^+$ and Na $^+$ leading to neurogenic pain. In addition, TRPV1 receptors are considered to be integrators of noxious chemical and physical stimuli that can be activated by capsaicin, heat and low pH (Numazaki and Tominaga, 2004). Previous studies have also shown that capsaicin induces the release of neurokinins, neuropeptides, excitatory amino acids (glutamate and aspartate), nitric oxide (NO) and pro-inflammatory peripheral mediators, besides promoting the transmission of nociceptive information to the spinal cord and the activation of vanilloid receptors (Caterina and Julius, 2001). The vanilloid receptors also can

be sensitized or activated by several inflammatory mediators (i.e., bradykinin, nitric oxide and prostaglandin) and studies have shown that activation of these receptors causes a sharp increase in inflammatory mediator levels (Cortright and Szallasi, 2004; Numazaki and Tominaga, 2004). In the present study, MEMM reversed the capsaicin-induced nociception in a dose-dependent manner indicating that the extract was also effective in attenuating nociceptive transmission modulated via the vanilloid receptors as well as interfering with the release/action of those inflammatory mediators. The latter ability might explain the extract potential to inhibit the abdominal constriction test and the second phase of the formalin test, which are related to the inflammatory-mediated nociception.

In an attempt to assess the ability of MEMM to interfere with the glutamate-mediated nociceptive transmission, the glutamate-induced paw licking test was performed. Glutamate, one of the important excitatory amino acids, is most wide spread in the central nervous system (CNS) (Neugebauer, 2002). It acts as a major excitatory neurotransmitter where they participate in a great number of physiological and pathological states. Glutamate induces nociceptive transmission at the peripheral, spinal and supra-spinal sites via different types of glutamate receptors (i.e., AMPA, Kainate and NMDA receptors) (Neugebauer, 2001a,b). Additionally, the nociceptive response induced by glutamate is greatly mediated by both activation of *N*-methyl-D-aspartate (NMDA) and α -amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (non-NMDA) receptors, as well as releasing of NO or some NO-derived substances (Beirith et al., 2002). The release of NO will trigger the synthesis of pro-inflammatory mediators such as cytokines, which help to enhance the inflammatory reaction (Beirith et al., 1998, 2002). Moreover, there were reports that the activation of glutamate receptors contribute to the maintenance of peripheral nociceptive process associated with inflammation (as seen in the late phase of formalin test) (Neugebauer, 2002), while the presence of glutamate receptors antagonist inhibited the inflammatory (late phase), but not neurogenic phase of the formalin test (Bhave et al., 2001). In the present study, oral administration of MEMM exerted a dose-dependent inhibition of the glutamate-induced nociceptive response. Therefore, it is strongly suggested that the MEMM antinociceptive activity against the glutamate-induced nociception occurs through the interaction of respective extract with any glutamate receptors in the glutamatergic system or by interfering with the NO production.

To investigate the role of NO/cGMP pathway in the modulation of MEMM antinociceptive activity, the extract were pre-challenged against L-arginine (acts as a NO donor), L-NAME (acts as an inhibitor of NO synthase) and MB (acts as an inhibitor of cGMP pathway). NO, a biological molecule found inside and between cells, is a major player in physiological functions such as the impulse transmission in the central and peripheral nervous systems (Garthwaite and Boulton, 1995). It has been reported that NO is involve in the mechanism of nociception at the supraspinal and peripheral sites by acting as a pro-nociceptive or an antinociceptive agent depending on the doses presence (Ferreira et al., 1991; Machelska et al., 1997). From the results obtained, only L-arginine significantly reversed MEMM's antinociception suggesting that the presence of NO reduced but did not inhibit the extract's antinociceptive potential. Hence, it is plausible to suggest that MEMM works via the NO-mediated/cGMP-independent pathway. It has been well acknowledged that NO exerts various biological roles that are mediated in a cGMP-independent manner. For example, NO has been shown to interact directly and indirectly with various inhibitory neurotransmitters such as GABA, glycine, opioid, and muscarinic receptor mechanisms (Ichinose et al., 1998; Cui et al., 2005).

Several classes of bioactive compounds have been detected in MEMM, namely flavonoids, tannins, saponins, triterpenes. Recent study using the HPLC analysis has also reported the presence

of quercitrin in the extract (Kamisan et al., 2014). The ability of quercitrin to inhibit the pro-inflammatory mediators, especially cytokines, engaged in pain modulation has been reported (Comalada et al., 2005; Gadotti et al., 2005), thus, suggested the compound to partly responsible for the observed antinociceptive activity of MEMM. In the present study, GCMS analysis was performed on MEMM and approximately 53% of the constituents detected were fatty acid amide, namely 9-octadecenamide, hexadecanamide and myristamide. The present of fatty acid amide might contribute to the observed antinociceptive activity of MEMM based on previous reports that several fatty acid amides demonstrated antinociceptive activity (Dray and Dickenson, 1991; Déciga-Campos et al., 2007; Barrière et al., 2013). Derivatives of 9-octadecenamide (Dray and Dickenson, 1991) and hexadecanamide (Déciga-Campos et al., 2007), in particular, have been proven to exert antinociceptive activity and, hence, are believed to contribute to MEMM's antinociceptive activity. Nevertheless, further studies are needed to refine and validate these early findings.

Conclusion

In conclusion, MEMM exerted a non-opioid antinociceptive activity at the peripheral and central levels via mechanisms involving modulation of the vanilloid receptors, glutamatergic system, and NO-mediated/cGMP-independent pathway. Moreover, the antinociceptive activity of MEMM might be attributed to the presence of flavonoid-based bioactive compounds, including quercitrin. The ability to exert a non-opioid antinociceptive activity at the peripheral and central level suggests that the extract could be a good candidate for the development of new analgesic drug that is lack of dependence/tolerance effects seen with morphine.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Authors' contribution

ESJ carried out the experiments and drafted the manuscript. SAR and AKA participated in the experimental design, and helped to draft the manuscript. SMC and MND involved in the statistical analysis and manuscript preparation. ZAZ conceived of the study, participated in its design and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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