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Original Article

Antinociceptive effect of semi-purified petroleum ether partition of *Muntingia calabura* leaves

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ABSTRACT

Muntingia calabura L., Muntingiaceae, is a medicinal plant for various pain-related diseases. The aims of the present study were to determine the antinociceptive profile and to elucidate the possible mechanisms of antinociception of petroleum ether partition obtained from crude methanol extract of *M. calabura* leaves using various animal models. The antinociceptive profile of petroleum ether fraction (given oral; 100, 250 and 500 mg/kg) was established using the *in vivo* chemicals (acetic acid-induced abdominal constriction and formalin-induced paw licking test) and thermal (hot plate test) models of nociception. The role of glutamate, TRPV1 receptor, bradykinin, protein kinase C, potassium channels, and various opioid and non-opioid receptors in modulating the partition's antinociceptive activity was also determined. The results obtained demonstrated that petroleum ether partition exerted significant ($p < 0.05$) antinociception in all the chemicals-, thermal-, capsaicin-, glutamate-, bradykinin, and phorbol 12-myristate 13-acetate (PMA)-induced nociception models. The antinociceptive activity was reversed following pretreatment with opioid antagonists (*i.e.* naloxone, β-funaltrexamine, naltrindole and nor-binaltorphimine), and the non-opioid receptor antagonists (*i.e.* pindolol (a β-adrenoceptor), haloperidol (a non-selective dopaminergic), atropine (a non-selective cholinergic receptor), caffeine (a non-selective adenosinergic receptor), and yohimbine (an α2-noradrenergic)). In addition, pretreatment with L-arginine (a nitric oxide (NO) donor), N^G-nitro-L-arginine methyl esters (L-NAME; an inhibitor of NO synthase (NOS)), methylene blue (MB; an inhibitor of cyclic-guanosine monophosphate (cGMP) pathway), or their combination failed to inhibit petroleum ether partition's antinociception. In conclusion, petroleum ether partition exerts antinociceptive activity at the peripheral and central levels via the modulation of, partly, the opioid (*i.e.* μ, κ and δ) and several non-opioids (*i.e.* β-adrenergic, dopaminergic, cholinergic, adenosinergic, and α2-noradrenergic) receptors, glutamatergic, TRPV1 receptors, PKC and K^+ channels systems, but not L-arg/NO/cGMP pathway.

Introduction

Various neurotransmitters and receptor systems take part in the modulation of pain processes in the central nervous system (CNS) and peripheral nervous system (PNS), such as the vanilloid (Cui et al., 2006), opioid (Craig and Sorkin, 2011), glutamate (Osikowicz et al., 2013), protein kinase C (PKC; Velázquez et al., 2007), potassium ion (K^+) channels (Ocana et al., 2004), and nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathways (Schmidtko et al., 2009). Despite the rapid advancements in the development of various treatments for pain, the clinical efficacy and tolerability of conventional analgesics can be overshadowed by its unwanted adverse effects. Some patients have taken alternative approach to treat pain, including complementary alternative medicine (CAM) in order to avoid these side effects (Astin, 1998). The use of natural product as alternative therapies is an increasingly popular method to treat such discomfort, either alone or as a complement to conventional medical approaches (Wirth et al., 2005). Holt and Chandra (2002) categorized natural products as herbs, herbal concoctions, traditional Chinese medicines, dietary supplements and alternative medicines. The research in natural product based on its ethnopharmacological knowledge has

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provided substantial contributions to drug innovation through the discovery of novel chemical structure and/or mechanism of actions (Rates, 2001). *Muntingia calabura* L., Muntingiaceae, the sole species in the genus, has been traditionally used in the Southeast Asia and tropical America (Kaneda et al., 1991; Nshimo et al., 1993) to treat headaches and gastric ulcer, and as an emmenogogue, antidyspeptic, antispasmodic, diaphoretic, tranquillizer and tonic (Kaneda et al., 1991; Perez-Arbelaez, 1975). Various medicinal properties have been scientifically reported from *M. calabura* leaves, including anti-tumor (Kaneda et al., 1991; Su et al., 2003), hypotensive (Shih et al., 2006), antibacterial (Zakaria et al., 2006a,b; Zakaria et al., 2010; Sufian et al., 2013; Isnarianti et al., 2013), antiplatelet aggregation (Chen et al., 2007), anti-inflammatory, antipyretic and antinociceptive (Zakaria et al., 2007a,b), anti-myocardial infarction (Nivethetha et al., 2009), antihypertensive (Shih, 2009), antioxidant (Siddiqua et al., 2010; Zakaria et al., 2011; Aruna Sindhe et al., 2013) cytotoxic (Chen et al., 2005; Sufian et al., 2013), antiproliferative (Zakaria et al., 2011), antihyperglycemic (Aruna Sindhe et al., 2013), antiulcer (Balan et al., 2014; Zakaria et al., 2014a,b), and hepatoprotective (Mahmood et al., 2014) activities. Other parts of *M. calabura* have also been reported to exert insecticidal (Neto Bandeira et al., 2013), anti-inflammatory (Preethi et al., 2012; Gomathi et al., 2013), antioxidant (Preethi et al., 2010; Gomathi et al., 2013) activities. With regards to the antinociceptive studies, the methanol extract of *M. calabura* has been reported to show antinociceptive activity (Mohd Sani et al., 2012), which was later shown to involve activation of the non-selective opioid (*i.e.* μ-, δ- and κ-opioid) and non-opioid (*i.e.* adenosinergic, α2-noradrenergic, and β-adrenergic) receptors, modulation of the ATP-sensitive K^+ channel, and inhibition of bradikinin and protein kinase C actions (Zakaria et al., 2014a,b). Moreover, the authors also suggested that the observed antinociceptive activities and its mechanism of actions were resulted from the synergistic effect of the bioactive compounds, flavonoids, saponins, tannins and steroids, possessed by the plant extracts. Recent laboratory research proved that the crude methanol extract of *M. calabura* (MEMC) leaves possesses good therapeutic effect in reducing nociceptive response (Mohd Sani et al., 2012; Zakaria et al., 2014a,b) and further study by Mohamad Yusof et al. (2013) leads to the isolation of four flavonoid-based antinociceptive-bearing bioactive compounds, of which one is a new compound called calaburone (8-hydroxy-6-methoxyflavone) and three were known compounds, namely 5-hydroxy-3,7,8-trimethoxyflavone,3,7-dimethoxy-5-hydroflavone and 2′,4′-dihydroxy-3′-methoxychalcone. In this study, we further evaluate the possible antinociceptive activity and its mechanism of action of the petroleum ether partition extracted from MEMC.

Materials and methods

Plant collection

The leaves of *Muntingia calabura* L., Muntingiaceae, were collected from its natural habitat in Shah Alam, Selangor, Malaysia. Plant identification has been made earlier (Balan et al., 2014) and a voucher specimen (SK 964/04) has been deposited at the Herbarium of Institute of Biosciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

Preparation of methanol extract of *M. calabura* (MEMC) and its petroleum ether fraction (PEMC)

The method for preparation of MEMC was described in detail by Balan et al. (2014). The obtained MEMC was then extracted subsequently with petroleum ether using the method described in detail by Mohamad Yusof et al. (2013). MEMC (2 g) dissolved in a solvent with a ratio of 1:7:2 which consist of MeOH (100 ml), petroleum ether (700 ml) and distilled water (200 ml). The mixture was rigorously shaken using a separatory funnel and then left to separate. The supernatant was collected and filtered while the residue was soaked again with petroleum ether until the supernatant become colorless. The collected petroleum ether supernatant was also subjected to rotary evaporator at 40 °C under reduced pressure to obtain a concentrated extract of petroleum ether of *M. calabura* (PEMC).

Experimental animals

Male ICR mice (25–30 g) and Sprague Dawley rat (150–180 g) were obtained from a private supplier (Chenur Supplier, Selangor, Malaysia) and acclimatized for three days in the Animal Holding Unit, Faculty of Medicine and Health Sciences, UPM. Details on the handled and cared of these animals have been described by Balan et al. (2014) and was in compliance with current UPM guidelines for the care of laboratory animals (Ethical approval no.: UPM/FPSK/PADS/BR-UUH/00404) and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983). All experiments ($n = 6$) were conducted between 09:00 and 16:00 h to minimize the effect of environmental changes.

Drugs and chemicals

Capsaicin, glutamate, naloxone, β-funaltrexamine, naltrindole, nor-binaltorphimine, apamin, charybdotoxin, tetraethylammonium, atropine, haloperidol, pindolol, yohimbine, caffeine, glibenclamide were purchased from Sigma–Aldrich (USA) while bradykinin was purchased from Tocris Bioscience, UK. They were prepared at the desired dose by dissolving them in distilled water (dH_2O). Acetyl salicylic acid (ASA), morphine and capsezepine (Sigma–Aldrich, USA) were dissolved in 10% DMSO while phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich, USA) was dissolved in PBS solution. Acetic acid, petroleum ether, methanol and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (U.K.). All drugs and PEMC were given in a 10 ml/kg basis.

Antinociceptive analysis

Acetic acid-induced abdominal constriction test

The acetic acid-induced abdominal constriction was carried out with slight modifications according to the method described in detail by Mohd Sani et al. (2012). The mice were pretreated orally with 10% DMSO (vehicle, negative control), 100 mg/kg ASA (positive control) or PEMC (100, 250 and 500 mg/kg) and 60 min later subjected to the abdominal constriction test.

Hot plate test

The hot-plate test, used to assess the central antinociceptive activity of PEMC, was performed according to the method described in detail by Mohd Sani et al. (2012). The mice were pretreated orally with vehicle (10% DMSO, negative control), 5 mg/kg morphine (positive control) or PEMC (100, 250 and 500 mg/kg) 60 min prior to subjection to the hot plate test.

Formalin test

The formalin test was carried out as described in detail in previous research by Mohd Sani et al. (2012). Rats were administered orally with the test solutions (*i.e.* 10% DMSO (negative control), 100 mg/kg ASA (peripherally acting positive control), 5 mg/kg morphine (centrally acting positive control) or PEMC (100, 250 and 500 mg/kg)) 60 min prior to the formalin injection. The nociceptive response described as the early phase and the late phase was recorded at the respective 0–5 min and 15–30 min following the formalin administration.

Determination of the motor coordination and sedative effects of PEMC

Effect of PEMC on motor coordination

Rota-rod test

A rota-rod tread mill device (Ugo Basil, Italy) was employed to assess the effect of PEMC on motor coordination (Melo et al., 2011) with slight modifications. In the beginning, mice capable of remaining on the rota-rod apparatus longer than 210 s (9 rpm) were selected 12 and 24 h prior to the test. Thirty minutes after the administration of either 10% DMSO (negative control), diazepam (2.5 mg/kg, *i.p.*) or PECM (100, 250 and 500 mg/kg, *p.o.*), each mouse was tested on the rota-rod apparatus at 0.5, 1 and 2 h post-treatment. The time (s) the mice were able to remain on top of the bar was recorded for up to 210 s. Decrease in fall off time is suggestive of depression of the central nervous system (CNS).

Sedative effect of PEMC

Sodium pentobarbital-induced sleeping time test

The sedative effect of PEMC in combination with sodium pentobarbital (SP) was evaluated (Martínez-Vázquez et al., 2012). For this purpose, a total of 30 mice were divided into five groups ($n = 6$) and orally administered with either 10% DMSO (negative control), diazepam (2.5 mg/kg, *i.p.*) or PECM (100, 250 and 500 mg/kg, *p.o.*) 30 min before the intraperitoneal administration of sodium pentobarbital (42 mg/kg, *i.p.*). Each mouse was placed on a warm table and cautiously monitored for the onset of uncoordinated movements subsequent to the sedative phase of the test. Loss of the righting reflex related to the hypnosis phase and the duration of sleep were also observed. The effects were recorded as follows: time passed between the administrations of pentobarbital until loss of righting reflex was recorded as of the onset of sleep, whilst the time from the loss to recovery was considered as the duration of sleep.

Investigation on the possible mechanisms of antinociception of PEMC

Capsaicin-induced paw licking test

Antinociceptive action of PEMC on vanilloid receptors (TRPV1) were investigated using the procedure described by Goncalves et al. (2005) with slight modifications. Rats were pretreated orally with vehicle, capsazepine (0.17 mmol/kg) or PEMC (100, 250 and 500 mg/kg) 60 min before capsaicin injection (1.6 μg/paw, 50 μl) into the i.pl. region of the rat's right hind paw. The induced rat individually observed in a transparent glass chamber for 5 min, and the amount of time the animals spent licking the injected paw was recorded with a chronometer.

Glutamate-induced paw licking test

A slight modification from the procedure described by Beirith et al. (2002) was used to study the role of glutamatergic system in the modulation of PEMC antinociceptive action. Rats were pretreated orally with vehicle, ASA (100 mg/kg) or PEMC (100, 250 and 500 mg/kg) 60 min prior to glutamate injection. A volume of 50 μl of glutamate (10 μmol/paw) was injected into the i.pl. region on the right hind paw of each rat. Immediately after the phlogistic agent administration, the induced rat were placed in a transparent glass cage observation chamber and individually observed from 0 to 15 min. The amount of time the animals spent licking or biting the injected paw was recorded with a chronometer.

Involvement of protein kinase C

The experiment was conducted based on the previously described method by Savegnago et al. (2007). A volume of 50 μl of PMA (a protein kinase C activator) solution (0.05 μg/paw) was injected into the ventral surface of the right hind paw of the rat 60 min after the oral administration of vehicle, ASA (100 mg/kg) or PEMC (100, 250 and 500 mg/kg). The animals were observed individually from minute 15 to 45 following PMA injection and the amount of time the rat spent licking the injected paw was recorded using a chronometer.

Bradykinin-induced nociception

Based on the method previously described by Ferreira et al. (2004), bradykinin (10 nmol/paw in 50 μl) was injected into the intraplantar surface of the right hind paw of each rat 60 min after the oral administration of vehicle, ASA (100 mg/kg) or PEMC (100, 250 and 500 mg/kg). The bradykinin-induced rat was observed individually for 10 min, and the amount of time they spent licking the injected paw was recorded.

Involvement of L-arginine/nitric oxide/cyclic guanosine monophosphate pathway

To determine the role of nitric oxide/cyclic-guanosine monophosphate (NO/cGMP) pathway in the modulation of PEMC antinociceptive activity the method described in detail by Zakaria et al. (2006a,b) was adopted. Mice were pretreated 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 pretreatment with vehicle or PEMC (500 mg/kg), respectively. Sixty minutes after the administration of test solutions, the mice were subjected to the abdominal constriction test.

Involvement of potassium channels

To determine the effect of PEMC-induced antinociception on K^+ channels, a method previously described by Alves and Duarte (2002) was used. Rats were pre-treated with glibenclamide (an ATP sensitive K^+ channel inhibitor; 10 mg/kg, *i.p.*), apamin (small conductance Ca^{2+}-activated K^+ channels, 0.04 mg/kg, *i.p.*), charybdotoxin (an inhibitor of large conductance Ca^{2+}-activated K^+ channels, 0.02 mg/kg, *i.p.*) and tetraethylammonium chloride (a non-selective voltage dependant K^+ channel inhibitor, 4 mg/kg, *i.p.*) 15 min before oral administration of either vehicle or PEMC (500 mg/kg). Sixty minutes later, pain was induced using 0.6% acetic acid. The number of writhing will be recorded for 25 min, 5 min following acetic acid injection.

Effect of various receptor antagonists on PEMC-induced antinociception

The detail method described by Zakaria et al. (2014a,b) was adopted to study the effect of various receptor antagonist on the antinociceptive activity of PEMC. Groups of animal were

pre-treated with caffeine (3 mg/kg, *i.p.*), atropine (10 mg/kg, *i.p.*), haloperidol (20 mg/kg, *i.p.*), pindolol (1 mg/kg. *i.p.*), or yohimbine (0.15 mg/kg, *i.p*) 15 min before the oral administration of vehicle or PEMC (500 mg/kg). Sixty minutes later after the administration of PEMC or vehicle, the mice were subjected to the abdominal constriction test.

Analysis of opioid receptor subtypes

Evaluation on the involvement of opioid receptor subtype was done using the abdominal constriction test which is similar with previously described. The preparation of the opioid antagonists' doses and timing of administration were based on previously conducted studies by Choi et al. (2003), Reeta et al. (2006) and Zakaria et al. (2014a,b). The μ opioid antagonist, β-funaltraxamine (β-FNA; 10 mg/kg, *i.p.*), δ opioid receptor antagonist, naltrindole (NALT; 1 mg/kg. *i.p.*) or κ opioid receptor antagonist, nor-binaltorphimine (nor-BNI; 1 mg/kg, *i.p.*) were administered 90 min, 15 min and 30 min respectively, before oral administration of 500 mg/kg of PEMC. The nociceptive stimulus was injected 60 min after PEMC administration.

HPLC profile of PEMC at various wavelengths

The HPLC profile of PEMC was established according to the methods described in detail for MEMC by Mohd Sani et al. (2012). In addition, PEMC was also spiked with a list of flavonoid-based compounds, namely pinostrobin, hesperetin, flavanone, 4′,5,7-trihydroxy flavanone, 2,4,4′-trihydroxy chalcone, quercitrin, dihydroquercitin, fisetin, quercetin, rutin, quercitrin, naringenin, silibinin, and genistein, to determine their presence in the extract. The HPLC analysis was carried out in the Laboratory of Phytomedicine, Medicinal Plants Division, Forest Research Institute of Malaysia (FRIM), Kepong, Malaysia.

Statistical analyses

The results are presented as mean ± standard error of mean (SEM). The one-way ANOVA test with Dunnett's multiple comparison and two-way ANOVA with Bonferroni post hoc test were used to analyze and compare the data. $p < 0.05$ was set as the limit of significance.

Result

Antinociceptive profile of PEMC

Fig. 1 shows that orally administered PEMC produced a significant ($p < 0.001$) and dose-dependent antinociceptive effect in mice subjected to the acetic acid-induced abdominal constriction test. The percentage analgesia produced ranged between 26 and 70%, while the positive control treatment (100 mg/kg ASA, *p.o.*) produced an analgesic effect of 59.31%.

Fig. 2 shows the centrally and peripherally mediated antinociceptive profile of orally administered PEMC as indicated by the formalin test. The extract exerted significant ($p < 0.05$) and dose-dependent antinociceptive activity in both the early and late phases of the assay. The antinociceptive activity, in both phases, was lower than that of 5 mg/kg morphine.

The central antinociceptive profile of PEMC, performed using hot plate test, a thermal-induced nociception model was shown in Fig. 3. PEMC exerted significant ($p < 0.001$) antinociceptive activity only by the 500 mg/kg which is the highest dose tested. However, the antinociception induced by PEMC was again lower than that induced by 5 mg/kg morphine administered via *i.p.* injection.

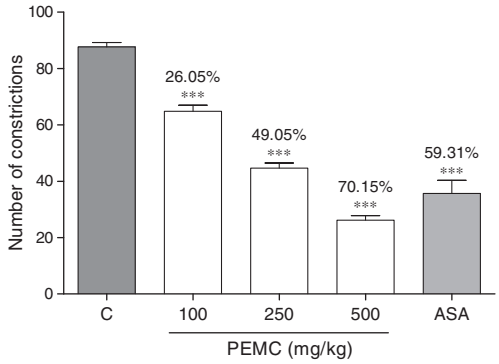

Fig. 1. Number of abdominal constrictions in mice during the acetic acid-induced abdominal constriction test after treatment with PEMC, acetylsalicylic acid (ASA; positive control), or vehicle ([C] negative control). Acetic acid was administered intraperitoneally 60 min before oral administration of treatment. $n = 6$ in each group. Numbers above the bars indicate the percentage of analgesia. ***$p < 0.001$ as compared to negative control treatment by one-way analysis of variance followed by Dunnett's post hoc test.

Motor coordination and sedative effects of PEMC

Effect of PEMC on motor coordination

In the rota rod test, all doses of PECM did not impair motor coordination at 0.5, 1 and 2 h post-administration in contrast to 2.5 mg/kg diazepam, which caused a significant ($p < 0.05$) decrease in time that the animals stayed on the rota-rod apparatus compared to the control group (Table 1).

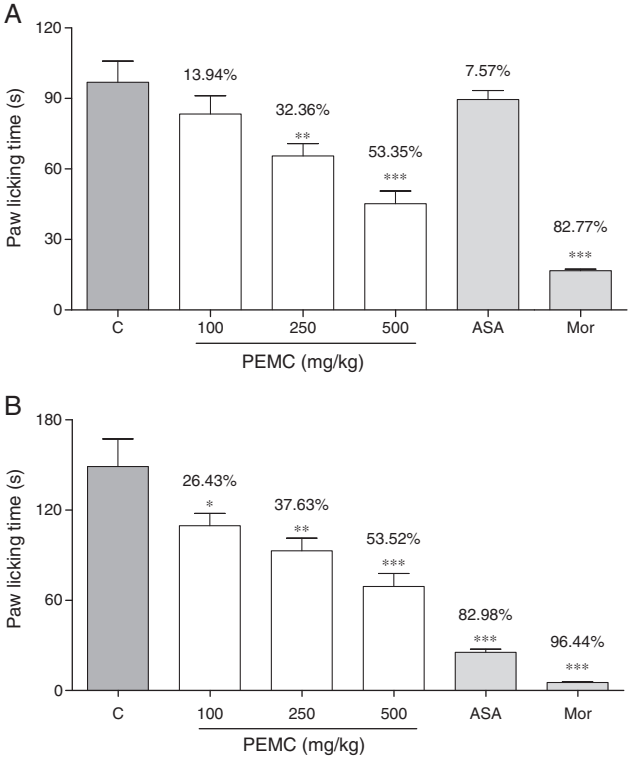

Fig. 2. Effect of PEMC in formalin-induced paw licking test. Data represents the mean ± SEM of six rats. The rats were pretreated with vehicle (10% DMSO), PEMC (100, 250, and 500 mg/kg, *p.o.*), acetylsalicylic acid (ASA, *p.o.*), or morphine (5 mg/kg, *p.o.*), 60 min before i.pl. injection of formalin. 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.

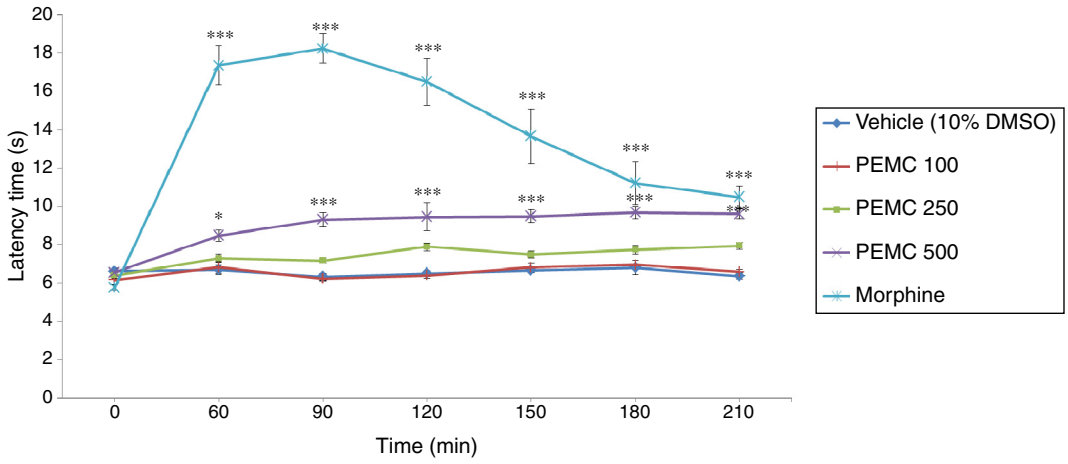

Fig. 3. Antinociceptive profile of PEMC assessed using the hot-plate test in mice. *$p < 0.05$ when compared with the control group at same the respective interval. Data are the mean ± SEM; $n = 6$ mice per group.

Table 1
Effect of PEMC on motor coordination assessed by the rota-rod test.

Treatment	Dose (mg/kg)	Time (s) on Rota-rod/210 sa		
		30 min	60 min	90 min
10% DMSO	–	210.0 ± 0.0	207.18 ± 2.11	210.0 ± 0.0
Diazepam	3	41.15 ± 6.32[a]	28.18 ± 7.35[a]	56.92 ± 7.43[a]
PEMC	100	205.49 ± 4.26	204.75 ± 3.64	210.0 ± 0.0
	250	202.35 ± 6.14	207.03 ± 2.08	206.47 ± 2.52
	500	208.08 ± 1.12	205.87 ± 3.02	210.00 ± 0.00

Data are the mean ± SEM; $n = 6$ mice per group. The rats were pretreated with 10% DMSO (negative control), 2.5 mg/kg diazepam (i.p.) or PEMC (100, 250, and 500 mg/kg, p.o.) 30 min before subjection to the test.

[a] Significantly differed at $P < 0.05$ when compared to the control group as assessed by the one-way ANOVA followed by Dunnett's post hoc test.

Table 2
Effect of PEMC on mice sleeping activity assessed using the sodium pentobarbital-induced sleeping test.

Treatment	Dose (mg/kg)	Onset of sleep (min)	Duration of sleep (min)
10% DMSO	–	3.48 ± 0.41	41.12 ± 0.33
Diazepam	1	1.72 ± 0.28[a]	94.33 ± 0.66[a]
PEMC	100	3.08 ± 0.17	68.66 ± 0.47[a]
	250	3.26 ± 0.39	71.66 ± 0.49[a]
	500	2.98 ± 0.32	88.83 ± 0.55[a]

Data are the mean ± SEM; $n = 6$ mice per group.

[a] Significantly differed at $P < 0.05$ when compared to the control group at the same respective interval as assessed by the one-way ANOVA followed by Dunnett's post hoc test.

Sodium pentobarbital-induced sleeping time test

Administration of PEMC did not cause changes in neither the latency sedation nor the sleeping time induced by sodium pentobarbital suggesting that PEMC did not exert sedative effects at any of the doses tested (Table 2).

Role of TRPV1, glutamatergic, B_2 receptors and PKC pathway

The ability of PEMC to inhibit the vanilloid receptor-induced nociceptive transmission is shown in Fig. 4. The 250 and 500 mg/kg PEMC significantly ($p < 0.001$) attenuated the capsaicin-induced paw licking test in a dose-dependent manner with the percentage of analgesia recorded ranging between 30 and 64%, respectively. The inhibition percentage of 63.52% produced by 500 mg/kg PEMC was higher than the positive control capsazepine (59.72%).

Fig. 5 shows the antinociceptive profile of PEMC against glutamate-induced paw licking test. PEMC significantly ($p < 0.001$) attenuated the nociceptive effect assessed using the

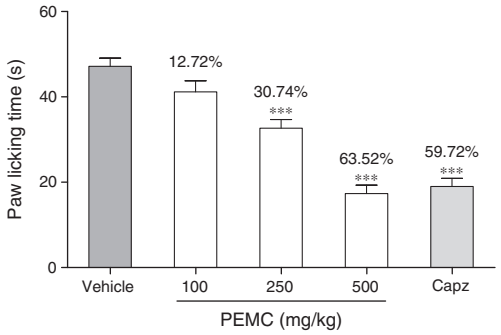

Fig. 4. Effect of PEMC on capsaicin-induced paw licking test in rats. Each column represents the mean ± SEM of six rats. The rats were pretreated with vehicle (control, 10% DMSO) or PEMC (100, 250, and 500 mg/kg, *p.o.*) 60 min before injection of capsaicin (1.6 μg/paw, 50 μl, i.pl.). The asterisks denote the significance levels as compared to control, ***$p < 0.001$ by one-way ANOVA followed by Dunnett's post hoc test.

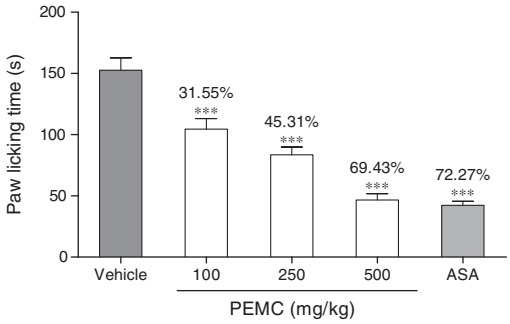

Fig. 5. Effect of PEMC on glutamate-induced paw licking test in rats. Each column represents the mean ± SEM of six rats. The rats were pretreated with vehicle (control, 10% DMSO) or PEMC (100, 250, and 500 mg/kg, *p.o.*) 60 min before injection of glutamate (10 μmol/paw, 50 μl, i.pl.). The asterisks denote the significance levels as compared to control, ***$p < 0.001$ by one-way ANOVA followed by Dunnett's post hoc test.

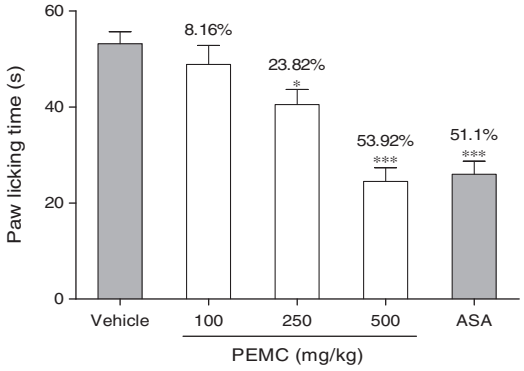

Fig. 7. The antinociceptive effect of PEMC against bradykinin-induced paw licking. Each column represents the mean ± S.E.M. of six rats. Vehicle (negative control; 10% DMSO, *p.o.*), PEMC (100, 250 and 500 mg/kg, *p.o.*) and acetylsalicylic acid (ASA: 100 mg/kg, *p.o.*). ***$p < 0.001$ when compared to control group.

glutamate-induced paw licking test in a dose-dependent manner. The percentage of analgesia obtained from the administration of 100, 250 and 500 mg/kg PEMC ranging between 32 and 69%.

Fig. 6 shows that the oral administration of 250 and 500 mg/kg PEMC produced significant ($p < 0.001$) inhibition of PMA-induced nociception in rat. Interestingly, the 500 mg/kg PEMC exerted slightly better antinociceptive activity compared to 100 mg/kg ASA indicated by the calculated percentage of analgesia (66.38% and 55.36% respectively).

As seen in Fig. 7, PEMC given orally exhibits significant inhibition in a dose-dependent manner on the nociception caused by intra-plantar injection of bradykinin in rat. The maximal inhibition observed was 53.92% for dose 500 mg/kg. Similar inhibitory effect was observed for 100 mg/kg aspirin.

Evaluation of L-arginine/NO/cGMP pathway

Fig. 8a and b show the role of the abdominal constriction tests incorporating L-arginine, L-NAME and MB to explore the role of the L-arginine/NO/cGMP pathway in the modulation of PEMC-induced antinociceptive activity. In Fig. 8a, pretreatment with 20 mg/kg L-arginine did not produce any significant effect when compared to the negative control. Meanwhile, pre-treatment with 20 mg/kg L-NAME alone exerted a significant antinociceptive effect. However, the positive effect was diminished when L-NAME given in combination with L-arginine. In the groups treated with PEMC, pretreatment with L-NAME did not affect the extract antinociceptive activity, whereas pretreatment with L-arginine significantly reduced, but

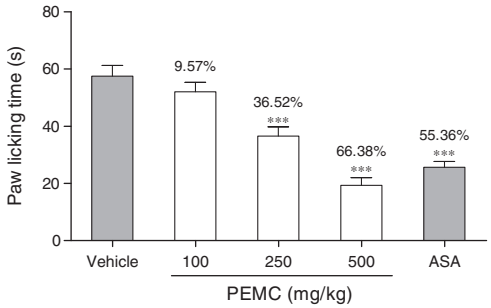

Fig. 6. Effect of PEMC on nociception induced by phorbol 12-myristate 13-acetate (PMA) on rats. Each column represents the mean ± SEM time spent on paw-licking behavior of six animals. Animals were administered with vehicle (negative control), PEMC (100, 250, and 500 mg/kg, *p.o.*) or acetylsalicylic acid (ASA; 100 mg/kg, *p.o.*) 60 min before injection of PMA (50 ml solution containing 0.05 mg PMA/paw) in the right hind paw. Numbers above bars indicate percentage of analgesia. ***$p < .001$ as compared to the control group by one-way analysis of variance followed by Dunnett's post hoc test.

did not completely inhibit, the antinociceptive effects of PEMC. Pretreatment with a combination of L-arginine and L-NAME also failed to affect the intensity of PEMC antinociception.

Pretreatment with 20 mg/kg MB alone (Fig. 8b) exerted significant ($p < 0.05$) antinociceptive activity and co-treatment with 20 mg/kg L-arginine failed to reverse MB antinociception. Pre-treatment with MB significantly ($p < 0.05$) enhanced PEMC antinociception while co-treatment with MB and L-arginine failed to affect the antinociceptive intensity of PEMC.

Involvement of potassium channels, non-opioid and opioid receptors on PEMC-induced antinociception

Fig. 9 shows the involvement of potassium channels in the modulation of PEMC-induced antinociceptive activity. Pretreatment with glibenclamide (10 mg/kg, *i.p.*), Apamin (0.04 mg/kg, *i.p.*), charybdotoxin (0.02 mg/kg, *i.p.*) and tetraethylammonium chloride (4 mg/kg, *i.p.*), significantly ($p < 0.01$) reversed the antinociceptive activity of PEMC (500 mg/kg) when assessed using acetic acid-induced abdominal writhing test.

The effect of antinociceptive activity of PEMC was significantly ($p < 0.05$) reversed following intraperitoneal administration of several receptor antagonists (Fig. 10) namely caffeine (3 mg/kg), atropine (10 mg/kg), yohimbine (0.15 mg/kg), haloperidol (0.2 mg/kg), pindolol (0.2 mg/kg), or naloxone, naltrindole, nor-binaltorphimine and β-funaltrexamine (Fig. 11).

HPLC profile of PEMC

The HPLC analysis of PEMC was measured at various different wavelengths (*e.g.* 210, 254, 280, 300, 330 and 366 nm and the best separation was obtained at 300 nm (Fig. 12a). Four major peaks were separated at these wavelengths, which were labeled as P1 (R_T 13.772 min), P2 (R_T 20.171 min), P3 (R_T 21.592 min), and P4 (R_T 28.144 min). Further analysis demonstrated that these four peaks showed λ_{max} values in the region of 211.9–272.0, 234.3–308.8, 240.1–270.8, 241.3–292.2 nm, respectively (Fig. 12b), suggesting, in part, the presence of flavonoid-based compounds. Comparison between chromatogram of PEMC and chromatogram of the standard compounds demonstrated the presence of pinostrobin and flavanone (Fig. 12c).

Discussion

The present study was the continuation of previous studies by Mohd Sani et al. (2012) and Zakaria et al. (2014a,b) on antinociceptive and mechanism of actions profile for methanolic crude extract

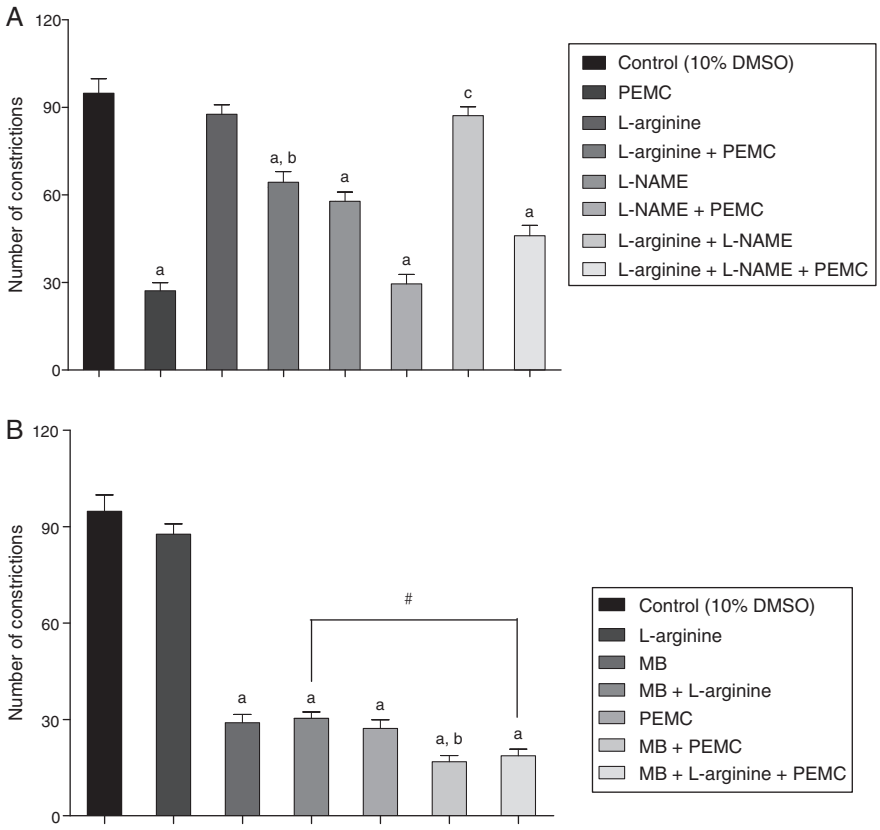

Fig. 8. (A) Effects of L-arginine, L-NAME, and their combination on PEMC antinociception as assessed by acetic acid-induced abdominal constriction test. The letters represent the significance levels between compared groups; [a]$p < 0.001$ when compared with control group; [b]$p < 0.001$ when compared with PEMC-treated group; [c]$p < 0.001$ when compared with L-NAME-treated group, by one-way ANOVA followed by Dunnett's post hoc test. (B) Effects of L-arginine, methylene blue, and their combination on PEMC antinociception as assessed by acetic acid-induced abdominal constriction test. The letters and symbol above the columns represent significance levels between compared groups; [a]$p < 0.001$ when compared with control group, [b]$p < 0.05$ when compared with MB-treated group, [#]$p < 0.05$ when compared between selected group.

of *M. calabura* (MEMC). Recent laboratory study conducted focusing on the antinociceptive potential of partition extracted from the MEMC, where the crude extract partitioned using three solvent with different polarity namely petroleum ether, ethyl acetate and aqueous using the method described above. Using acetic acid-induced abdominal writhing test to screen for antinociceptive activity, petroleum ether partition produced significantly better activity (data not shown). Hence, the PEMC selected to be further tested for its antinociceptive properties as well as the mechanism of actions.

Our data shown the PEMC attenuated the nociceptive response induced by chemical and thermal stimuli, suggesting that the compound exerts peripheral and central antinociceptive activities. Its ability to influence both peripheral and central nociception was further confirmed by our observation of its antinociceptive effects in both phases of the formalin test. Overall, these observations suggest that the antinociceptive activity of PEMC resembles that of opioid analgesics, such as morphine. Moreover, the ability of PEMC to attenuate nociception in both the abdominal constriction and hot plate tests suggests that the compound is effective

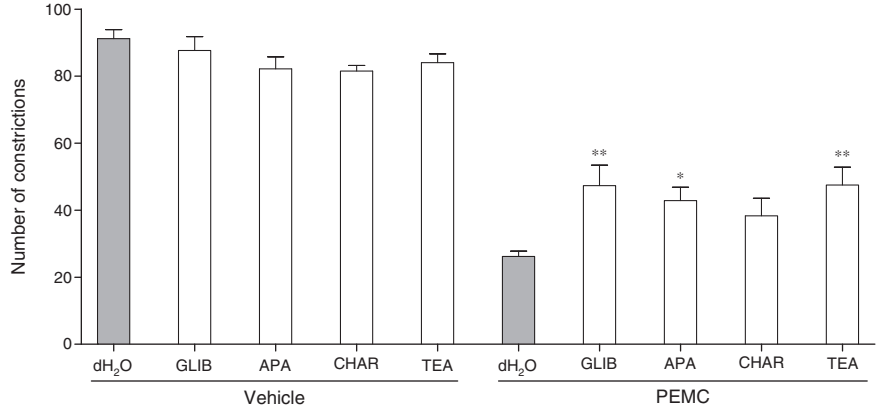

Fig. 9. Effect of PEMC on nociception in the acetic acid-induced abdominal constriction test in mice following pretreatment with potassium channels inhibitors. Each column represents the mean ± SEM number of constrictions observed in six animals. Vehicle (10% DMSO, *p.o.*), PEMC (500 mg/kg, *p.o.*), glibenclamide (GLIB: 10 mg/kg, *i.p.*), apamin (APA: 0.04 mg/kg, *i.p.*), tetraethylammonium chloride (TEA: 0.01 mg/kg, *i.p.*) The asterisks, $^{*}p < 0.05$ and $^{**}p < 0.01$, denote the significance levels as compared to their respective control group. Comparisons between groups were made by one-way analysis of variance followed by Dunnett's post hoc test.

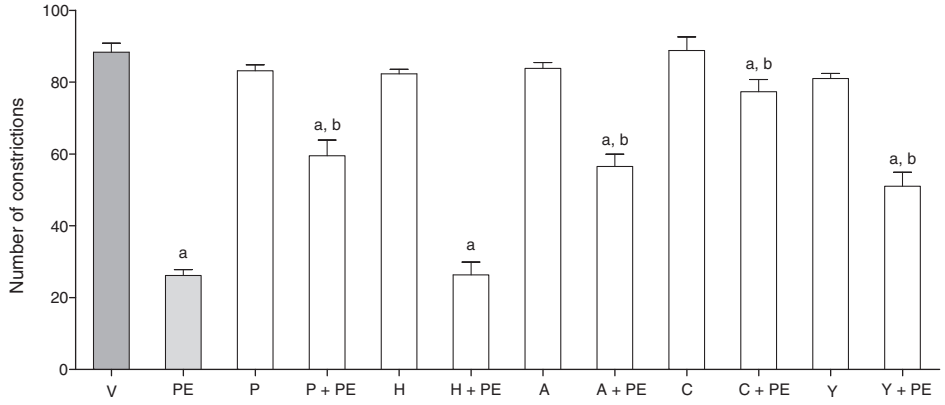

Fig. 10. The involvement of several non-opioid receptor antagonists in PEMC-induced antinociception against acetic acid-induced abdominal writhing test in mice. Vehicle (V: 10% DMSO, *p.o.*), PEMC (PE: 500 mg/kg, *p.o.*), atropine (A: 10 mg/kg, *i.p.*) haloperidol (H: 20 mg/kg, *i.p.*), pindolol (P: 1 mg/kg, *i.p.*), caffeine (C: 3 mg/kg, *i.p.*), yohimbine (Y: 0.15 mg/kg, *i.p.*). [a] $p < 0.001$ significantly different when compared to vehicle-treated group; [b] $p < 0.001$ significantly different when compared to PEMC-treated group.

with both inflammatory-induced and non-inflammatory-induced nociception, respectively (Zakaria et al., 2007a,b), which also supported by our data from the formalin-induced paw licking test where both phases, the early (neurogenic) and late (inflammatory) phase, attenuated by PEMC treatment.

The acetic acid-induced abdominal constriction test, described as a typical model for inflammatory pain, has long been widely used as a tool to screen for analgesic or anti-inflammatory properties of new agents (Collier et al., 1968; Vongtau et al., 2004). The acetic acid-induced abdominal constriction test reflects the inflammatory-mediated nociceptive response (Mohd Sani et al., 2012) where the injected acetic acid into the peritoneal cavity increases the level of cyclooxygenase (COX) and lipooxygenase (LOX) in the peritoneal fluid and indirectly leads to the release of endogenous mediators such as prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, serotonin, histamine, cytokines, and eicosanoids. These endogenous mediators, in turn, excite the peripheral nociceptive neurons within the peritoneal cavity that are sensitive to non-steroidal anti-inflammatory drugs (NSAID) (Ikeda et al., 2001). The increased levels of PGE_2 due to prolong irritation of peritoneal cavity lead to enhanced capillary permeability (Deraedt et al., 1980; Vogel and Vogel, 1997) as well as the release of glutamate and substance P from peripheral afferent fiber (Millan, 1999), indicating the stimulation of peripheral nociceptive mechanisms. The ability of PEMC to inhibit acetic acid-induced nociception suggests that the compound attenuates the levels of peripheral COX and LOX, which indirectly decreases PGE_2 synthesis and obstructs the transduction of pain in primary afferent nociceptors. However, the acetic acid-induced abdominal construction test is not specific, thus further studies using other nociceptive models, namely the hot plate and formalin tests are required to determine whether the antinociceptive effects of PEMC are centrally or peripherally mediated.

The hot plate test, predominantly a spinal reflex test that involves activation of the supraspinal nociceptive processing, has been applied in various researches to show that only the analgesic acting centrally, but not peripherally, have an effect on this test (Chen et al., 1995; Le Bars et al., 2001; Hosseinzadeh and Younesi, 2002; Giglio et al., 2006). The ability of PEMC to inhibit both the early and late phases of the formalin test in the present study confirmed the extract's ability to exert centrally and peripherally mediated antinociceptive effects (Mohd Sani et al., 2012; Mohamad Yusof et al., 2013). The early phase, classified as a neurogenic pain, is an acute reaction, lasted for 5 min which immediately occur after the injection of formalin where it directly activate the nociceptors. The late phase, appears between 15 and 30 min following formalin injection, is classified as an inflammatory pain as it is caused by inflammatory mediators released such as histamine, serotonin, bradykinin and PGE_2 (Verma et al., 2005). Our results in the present study indicate that PEMC possesses mild centrally mediated antinociceptive characteristics as seen in both the hot plate test and early phase of formalin test.

Over the past decade, tremendous research being devoted in developing the best medication for pain reliever, and extensive studies has been perform to explore their precise mechanism of action in order to understand and limit any unwanted side effect. However, the neuronal basis of pain transmission and modulation

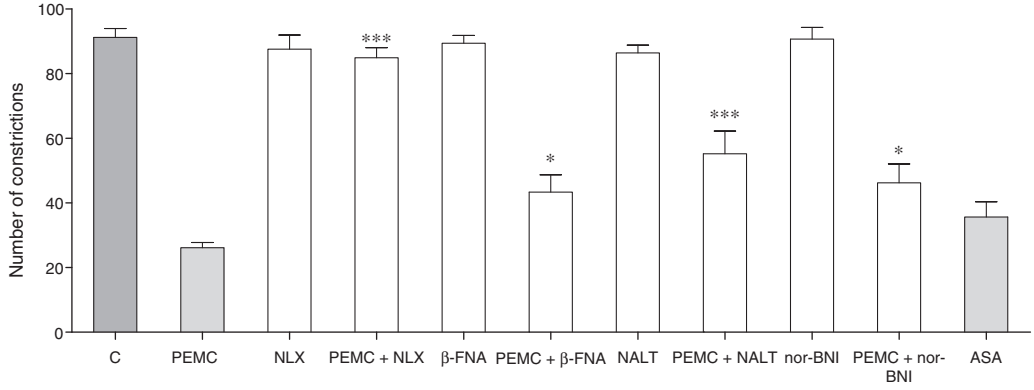

Fig. 11. Analysis of opioid receptor subtypes involvement in PEMC-induced antinociception against acetic acid-induced writhing test in mice. Control (C: 10% DMSO, *p.o.*), PEMC (500 mg/kg, *p.o.*), naloxone (NLX: 5 mg/kg, *i.p.*), naltrindole (NALT: 1 mg/kg, *i.p.*), β-funaltrexamine (β-FNA: 10 mg/kg, *i.p.*), nor-binaltorphimine (nor-BNI: 1 mg/kg, *i.p.*) and acetylsalicylic acid (ASA: 100 mg/kg, *p.o.*). $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared to the group treated only with PEMC.

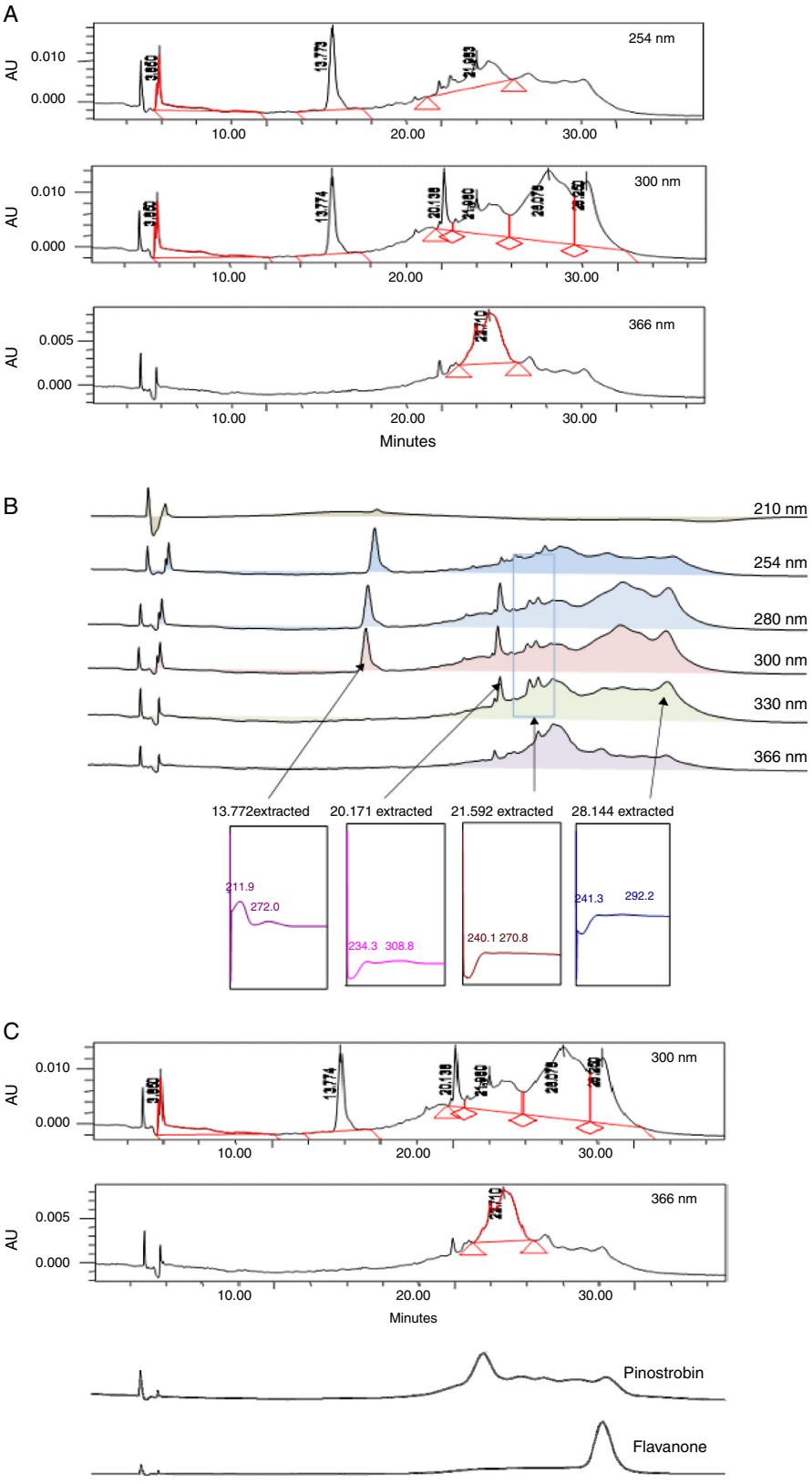

Fig. 12. (A) The HPLC profile of PEMC at different wavelengths (*i.e.* 254, 300 and 366 nm). Several major peaks were detected at the retention time (R_T) of 13.77, 20.14, 21.98, 26.08 and 29.25 s at the wavelength of 330 nm. (B) The HPLC profile of PEMC at different wavelengths (*i.e.* 210, 254, 280, 300. 330 and 366 nm) supported by the UV spectra analysis of PEMC. The UV spectra demonstrated the presence of four major peaks labeled as P1 (R_T 13.772 min), P2 (R_T 20.171 min), P3 (R_T 21.592 min), and P4 (R_T 28.144 min), which were observed at their respective λ_{max} at the region of 211.9–272.0, 234.3–308.8, 240.1–270.8, 241.3–292.2 nm, respectively, suggesting, in part the presence of flavonoid-based compounds. (C) Comparison of the HPLC profile of PEMC against several pure flavonoids at the wavelength of 366 nm. Only comparison with pinostrobin and flavanone demonstrated matching HPLC profile to that of the extract.

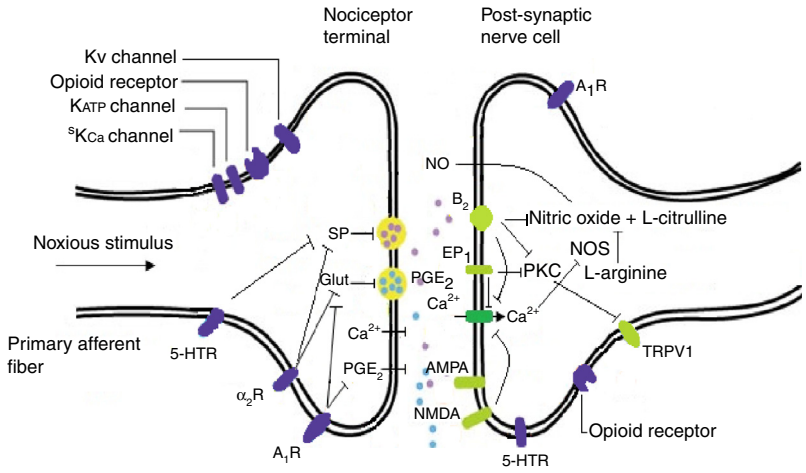

Fig. 13. Schematic diagram of the proposed mechanisms of antinociceptive action demonstrated by PEMC. PEMC exerts antinociceptive activity at peripheral and central levels via modulation of several receptors and neurotransmitters systems. Ca^{2+}: calcium ion; cGMP: cyclic guanosine monophosphate; GC: guanylate cyclase; GTP: guanosine triphosphate; K_v: voltage-gated potassium channel; K_{ATP}: ATP-sensitive potassium channel; $^{S}K_{Ca}$: small conductance calcium-activated potassium channel; 5-HTR: serotonergic receptor; α_2R: α_2-adrenergic receptor; A_1R: adenosine receptor; Glut: glutamate; PGE_2: prostaglandin E_2; EP_1: prostaglandin receptor; B_2: bradykinin receptor; NMDA: *N*-methyl-D-aspartate; PKC: protein kinase C enzyme; NOS: nitric oxide synthase; NO: nitric oxide; TRPV1: transient receptor potential cation channel subfamily V member 1.

is still not completely understood. Numerous chemicals and factors such as opioids, substance P, glutamate, nitric oxide, serotonin, and other inflammatory mediators, has been documented to engaged in the activation of the PNS and CNS nociceptive pathways (Mohd Sani et al., 2014). The existence description of these modulatory mechanisms such as the role of vanilloid receptors, glutamatergic system, opioid and non-opioid receptors, nitric oxide, PKC as well as K^+ channels pathways, have benefited and provide guidance for researchers in the development of new analgesic candidates (Rodella et al., 2010).

Our previous study (Mohd Sani et al., 2012; Zakaria et al., 2014a,b) using the crude methanol extract of *M. calabura* (MEMC) revealed the participation of most the modulatory mechanisms. In the present study, we employed the same approach to explore the possible mechanisms of action responsible for the observed antinociceptive activity of PEMC. Based on our findings, PEMC; (1) attenuate the nociception induced by capsaicin indicating possible involvement of vanilloid receptors, (2) inhibit glutamate-induced nociception which in turn inhibit the release of NO and NO-related substance (Beirith et al., 2002), (3) significantly inhibit PMA-induced nociception suggesting its involvement in PKC-activated phosphorylation of vanilloid receptor pathway (Vellani et al., 2001), (4) inhibit bradykinin-induced nociception showing its ability to block B_2 receptor from being activated, (5) affected by L-arginine, but not L-NAME and MB, suggesting PEMC antinociception did not involve modulation via the L-arginine/NO/cGMP pathway, (6) involved in the activation of ATP-sensitive K^+ channels, small conductance Ca^{2+}-activated K^+ channels and non-selective voltage-dependant K^+ channels, but not large conductance Ca^{2+}-activated K^+ channels, (7) play significant role in antinociception modulated by adenosinergic, α_2-noradrenergic, cholinergic and β-adrenergic receptors, (8) involved in the activation of opioid receptor subtypes as PEMC antinociceptive effect was significantly reduced by the pretreatment of μ, δ and κ opioid receptor subtype inhibitors.

Our findings, apart from the possible participation of cholinergic receptor and inability of PEMC to activate large conductance Ca^{2+}-activated K^+ channels, were in line with our results obtained from the crude methanol extract of *M. calabura*. The involvement of cholinergic receptor seen in PEMC-induced antinociceptive might probably due to higher purity of the expressed bioactive compounds extracted from the crude extract.

Earlier studies implied that the depression of CNS and the effect of nonspecific muscle relaxation can diminish the response of motor coordination and, accordingly, may nullify the antinociceptive results (Quintans-Júnior et al., 2010). Interestingly, the present findings demonstrated that PEMC-treated (100, 250 and 500 mg/kg, orally) mice exerted no performance alterations in the rota-rod- and pentobarbital-induced sleeping-test indicating that PEMC did not interfere in the mice anxiety behavior or in the motor function, when compared to the control group. In contrast, diazepam induced anxiety reduction demonstrated by an increase in the number of visits and time spent in the open arms and by an increase in the total number of entries. Overall, these findings suggested that there is no alteration in motor coordination among the treated animals, therefore, eliminating a nonspecific muscle relaxation as well as sedative effects of PEMC at the doses used. These results suggest that the antinociceptive effect of PEMC is not related to sedative effect or to alteration in motor function (de Vasconcelos et al., 2011).

The phytochemical screening of PEMC was in accordance with the phytoconstituents found in the crude extract (MEMC) as previously reported by Zakaria et al. (2014a,b). Phytochemical constituents of PEMC include flavonoids, triterpenes, tannins and steroids, and some of these compounds have been reported to exert antinociceptive activity (Starec et al., 1988; Beirith et al., 1999; Karumi et al., 2003; Musa et al., 2009). Moreover, flavonoids like pinostrobin have been identified from the leaves of *M. calabura* (Su et al., 2003; Chen et al., 2005) and also detected in PEMC via the qualitative HPLC method. Despite various reports on the pharmacological potential of pinostrobin, none of those reports were related to the antinociceptive activity. However, pinostrobin has been reported to exert anti-inflammatory activity (Tuchinda et al., 2002; Patel and Bhutani, 2014) and might, therefore, supported the PEMC antinociceptive activity. Moreover, recently published report by Mohamad Yusof et al. (2013) revealed that the antinociceptive activity of PEMC assessed using only the formalin test could be due to the presence of several flavonoid-based bioactive compounds, namely 5-hydroxy-3,7,8-trimethoxyflavone, 3,7-dimethoxy-5-hydroyflavone, 2′,4′-dihydroxy-3′-methoxychalcone, and calaburone. These compounds have been shown to exhibit antinociceptive activity when given separately and, therefore, they are suggested to act synergistically to exert the antinociceptive effect.

Further analysis of the identified peaks based on the recorded UV spectra revealed that the four peaks falls between the λ_{max} of 210 and 310 nm suggesting the presence of flavonoid-based bioactive compounds as reported by Aznam et al. (2012). There are five major subgroups of flavonoids namely flavones, flavonols, flavanonols, flavanones and dihydroflavonols (Tsimogiannis et al., 2007). The presence of flavanones-based bioactive compounds, in particular, can be suggested based on the recorded UV–Vis spectra obtained for each of the peak (Aznam et al., 2012). According to Aznam et al. (2012), several flavonoids that have methoxyl and hydroxyl groups, detected at the UV spectra that range between 210 and 290, have been isolated from fractions of methanol extract of *Kaempferia rotunda*. Recent studies by Mohamad Yusof et al. (2013) tend to support the presence of flavanones or methoxyl- and hydroxyl-contained flavonoids in PEMC as the authors have successfully isolated several antinociceptive-bearing flavanones, namely 5-hydroxy-3,7,8-trimethoxyflavone, 3,7-dimethoxy-5-hydroyflavone, 2′,4′-dihydroxy-3′-methoxychalcone, and 8-hydroxy-6-methoxyflavone (calaburone).

It is reasonable to conclude from our findings in the present study that the antinociceptive properties of PEMC occur via interaction of a variety of physiological pathways within the PNS and CNS. PEMC appears to act as an agonist for potassium channels, opioid, cholinergic, adenosinergic, adrenergic, and serotonergic receptors, while at the same time inhibiting the PKC, vanilloid receptors and glutamatergic system. Fig. 13 represents a model of the mechanisms of PEMC's antinociceptive action based on these findings. These activities could be due to the synergistic effect of flavonoids, triterpenes, tannins and steroids. The knowledge gained from such studies in the present and future will be useful for the design or discovery of new chemical entities that exhibit similar antinociceptive activity.

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 no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contribution

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

Conflicts of interest

The authors declare no conflicts of interest.

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