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Research Article

Antinociceptive Activity of *Melicope ptelefolia* Ethanolic Extract in Experimental Animals

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Melicope ptelefolia is a medicinal herb commonly used in Malaysia to treat fever, pain, wounds, and itches. The present study was conducted to evaluate the antinociceptive activity of the *Melicope ptelefolia* ethanolic extract (MPEE) using animal models of nociception. The antinociceptive activity of the extract was assessed using acetic acid-induced abdominal writhing, hot-plate, and formalin-induced paw licking tests. Oral administration of MPEE produced significant dose-dependent antinociceptive effects when tested in mice and rats using acetic acid-induced abdominal constriction test and on the second phase of the formalin-induced paw licking test, respectively. It was also demonstrated that MPEE had no effect on the response latency time to the heat stimulus in the thermal model of the hot-plate test. In addition, the antinociception produced by MPEE was not blocked by naloxone. Furthermore, oral administration of MPEE did not produce any effect in motor performance of the rota-rod test and in acute toxicity study no abnormal behaviors as well as mortality were observed up to a dose level of the extract of 5 g/kg. These results indicated that MPEE at all doses investigated which did not produce any sedative and toxic effects exerted pronounce antinociceptive activity that acts peripherally in experimental animals.

1. Introduction

Melicope ptelefolia Champ Ex. Benth (Rutaceae), locally known as "tenggek burung," is one of the most common medicinal herbs that are widely distributed in many areas of Peninsular Malaysia and also in several other Asian countries [1]. Apart from being one of the most popular traditional fresh vegetables among the Malays of Malaysian community, different parts of *M. ptelefolia* has been used traditionally for centuries as natural remedy for fever, emmenagogue, stomach ache, and rheumatism as well as treatment of wounds and itches [2, 3]. In addition there have been many other usages of the herb, for example, to prevent premature ejaculation, as an aphrodisiac, and for its blood pressure lowering effects. However, many of these so-called usages are not substantiated by any written documents. Pharmacologically, extracts of the plant are reported to possess antimicrobial and cytotoxic properties, as well as being rich in antioxidants [4, 5]. Previous phytochemical studies on the plant revealed the presence of 2,2-dimethyl-2H-1-benzopyrans, benzopyrans dimmers, and bisisoquinoline alkaloids as major constituents [6–8]. The Malaysian variety, on the other hand, have reported to contain O-geranylcoumaric acid, furoquinoline alkaloids, and several polyprenylated acetophenones including 2,4,6-trihydroxygernylacetophenone (tHGA) [9, 10].

Although the *M. ptelefolia* appears to have some traditional use for pain relief, there is no substantial pharmacological report on the possible antinociceptive effect of this plant, to date. In addition, *M. ptelefolia* has also been demonstrated to attenuate nitric oxide (NO) in lipopolysaccharide-induced RAW264.7 murine macrophages, which has also been associated with the development of pain [4]. Therefore, the present study was conducted to evaluate the antinociceptive effect of the *M. ptelefolia* ethanolic extract (MPEE) using chemical and thermal models of nociception in mice and rats.

2. Materials and Methods

2.1. Plant Material and Sample Preparation. The fresh leaves of *M. ptelefolia* were collected from Serdang area in the state of Selangor, Malaysia in March 2008. The plant material was identified and authenticated by a resident botanist through comparison with herbarium specimens of *M. ptelefolia* (SK153/02) kept at the Mini Herbarium, Institute of Bioscience, Universiti Putra Malaysia (UPM). The freshly collected leaves of *M. ptelefolia* were air-dried under shade for 48 hours. The dried leaves were then grounded to a fine mesh and then extracted via sonication (30-minute session repeated 5 times) with 95% ethanol as a solvent to sample ratio of 5 : 1 (v/v). The extracts obtained after each 30-minute session were pooled and processed to complete dryness via rotary evaporation and lyophilization to yield a greenish black colored ethanolic extract of *M. ptelefolia* (MPEE).

2.2. Experimental Animals. Experiments were carried out using adult male ICR mice (20–30 g) and adult male Sprague-Dawley rats (200–250 g), maintained at 22 ± 2°C under a 12-hour light/12-hour dark cycle and with access to food and water *ad libitum*. The animals were first acclimatized for at least 7 days before experiment and were only used once throughout the experiments. All the experiments were conducted in accordance with the ethical guidelines on animal experimentation [11], approved by the Animal Care Unit Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Animals were fasted 12 hours prior to experiment.

2.3. Drugs and Chemicals. The following drugs and chemicals were used: morphine hydrochloride, acetylsalicylic acid (ASA), naloxone hydrochloride, formaldehyde, and acetic acid (Sigma Chemical Co.). All drugs and MPEE were dissolved and diluted in saline (0.9% NaCl) just before use. Oral or intraperitoneal administration of the drugs and MPEE were given at all times in a volume of 10 ml/kg of animal's body weight. Respective controls received only saline as vehicle and had no effects per se on the nociceptive responses.

2.4. Assessments of the Antinociceptive Activity of M. ptelefolia Ethanolic Extract (MPEE)

2.4.1. Acetic Acid-Induced Abdominal Constriction Test. The acetic acid-induced abdominal constriction test was conducted according to a previously described method [12], with slight modifications. Mice were divided into 5 different groups ($n = 6$) and treated with oral administration (p.o.) of MPEE (30, 100, and 300 mg/kg), acetylsalicylic acid (ASA, 100 mg/kg), or similar volume of vehicle (0.9% NaCl, 10 ml/kg). The number of abdominal constrictions, consisting constriction of abdominal part together with full stretching of both hind limbs, following intraperitoneal administration of 0.6% acetic acid (10 ml/kg) was counted and recorded over a period of 30 min, starting 5 min after acetic acid injection.

2.4.2. Hot-Plate Test. The hot-plate test was conducted according to the method that was described previously in [13]. This test measures the time that elapses before the test mice show hind paw licking/shaking and jumping as an indication of pain in response to the applied heat. The hot plate (Ugo Basile, model-7280) was maintained at 52.0 ± 0.2°C, and animals were placed into a Perspex cylinder on the heated stage. Response latency was measured by recording the time between placement and the described responses. MPEE (30, 100, and 300 mg/kg, p.o.), morphine (5 mg/kg, i.p.), or vehicle (10 ml/kg, p.o.) were administered 60 min before the experiment. Response to heat for every treated mouse was observed before and at 0-, 30-, 60-, 90-, 120-, 180-, and 210-minute intervals following the treatment. The cut-off time was 20 s in order to minimize skin injury.

2.4.3. Formalin-Induced Paw Licking Test. The test was conducted as previously described [14]. Briefly, 60 min after p.o. administration of the MPEE (30, 100, and 300 mg/kg), ASA (100 mg/kg), vehicle (10 ml/kg) or intraperitoneal administration (i.p.) of morphine (5 mg/kg), 50 μL of 2.5% formaldehyde (v/v in distilled water) was injected subcutaneously into the plantar surface of the left hind paw of the rats. The duration of nociceptive behavioral responses to including biting, licking, and scratching of the injected paw were noted and recorded up to 30 min. The first 5 min was considered as the first phase (neurogenic phase) and the period of 15–30 min as the second phase (inflammatory phase) of the nociceptive response.

2.4.4. Involvement of Opioid Receptors. In order to investigate the involvement of the opioidergic system in MPEE-induced antinociception, separate groups of rats ($n = 6$) were pretreated with nonselective opioid receptor antagonist, naloxone (5 mg/kg, i.p), which was injected 10 min before p.o. administration of MPEE (300 mg/kg) and morphine (5 mg/kg, i.p.), and tested using the formalin-induced paw licking test.

2.5. Motor Performance Assessment. The test was conducted as previously described in [15]. Briefly, mice were selected 24 h prior to the test by selecting only those that were able to remain successfully on the revolving bar (14 rpm) of the rota-rod apparatus (Ugo Basile, Model 7600) for two consecutive periods of 60 s. The selected mice were then divided into 5 groups ($n = 6$) and were treated with MPEE (30, 100, and 300 mg/kg, p.o.) and vehicle or diazepam

(4 mg/kg, i.p.). Motor performance was evaluated at 30, 60, and 120 min following treatments, and the amount of time of permanence(s) on the revolving bar during a 60-second period was recorded.

2.6. Preliminary Acute Toxicity Assessment. The method described by Lorke was employed [16]. In brief, mice were separated into four groups of 6 mice each. They were fasted overnight and then were orally administered with the MPEE at the doses of 300, 1000, and 5000 mg/kg, while the control group only received the vehicle. The mice were observed for any abnormal behavior such as sedation, respiratory distress, motor impairment, and hyperexcitability for 3 h. Furthermore, the incidence of mortality for each group was recorded up to 24 h after administration. Food and water were provided *ad libitum*.

2.7. Statistical Analysis. The data obtained was statistically analyzed using one-way ANOVA. This was followed by Dunnett's or Tukey's post hoc tests when the ANOVA produced significant results. All data were expressed as the mean ± S.E.M of 6 animals per group. The tests were performed using GraphPad Software ver 5.01 (GraphPad Software Inc., San Diego, CA). Differences are considered significant when $P < .05$.

3. Results

3.1. Antinociceptive Activity of the M. ptelefolia Ethanolic Extract (MPPE)

3.1.1. Acetic Acid-Induced Abdominal Constriction Test. The effect of MPEE on writhing response in mice is depicted in Figure 1. Oral administration (p.o.) of MPEE at the doses of 30, 100, and 300 mg/kg caused significant inhibition ($P < .05$) on the writhing response induced by acetic acid with an apparent dose dependency. The percentage of inhibition produced by MPEE at 30, 100, and 300 mg/kg was 63.3, 73.3, and 95.3% as compared to control, respectively. Such effects were also observed in mice pre-treated by ASA (60.5%, $P < .05$).

3.1.2. Hot-Plate Test. As presented in Table 1, MPEE (30, 100, and 300 mg/kg) did not exerted any significant changes in the response latency against the thermal stimulus-induced nociception of the hot-plate test as compared to the control. Morphine (5 mg/kg, i.p.) significantly increased the latency response on the hot plate.

3.1.3. Formalin-Induced Paw Licking Test. Figure 2 demonstrated that p.o. administration of the MPEE at the doses of 30, 100, and 300 mg/kg caused significant inhibition of the pain response only on the second phase (inflammatory phase, Figure 2(b)) but not the first phase (neurogenic phase, Figure 2(a)) of the formalin-induced paw licking test with inhibition of 20.4, 60.1, and 64.5%, respectively. Similarly, ASA (100 mg/kg, p.o.) caused significant inhibition (66.0%) of the second phase but not the first phase. In contrast,

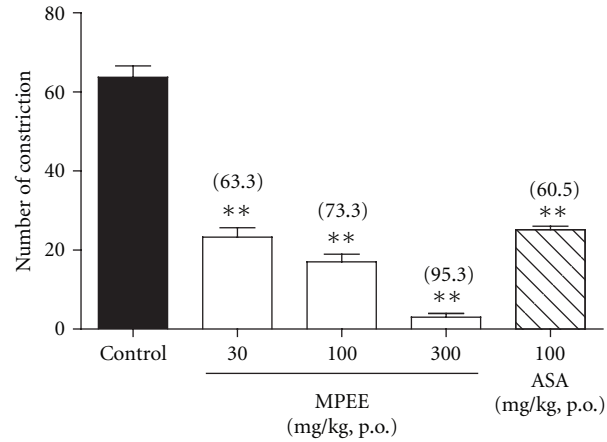

Figure 1: Effect of *M. ptelefolia* ethanolic extract (MPEE) on acetic acid-induced abdominal constriction in mice. Each column represents the mean ± S.E.M. of 6 mice. $^{**}P < .01$ compared to the control group (Dunnett's test). Values in parentheses are percentage of inhibition.

morphine produced marked inhibition of both first and second phases of the formalin-induced paw licking test.

3.1.4. Involvement of Opioid Receptors. The results presented in Figure 2 show that pretreatment of mice with naloxone (5 mg/kg, i.p.), given 10 min beforehand, did not reverse the MPEE-induced antinociception (300 mg/kg, p.o.). However, the antinociception produced by morphine was significantly reversed.

3.2. Motor Performance Assessment. Table 2 demonstrated that MPEE (30, 100, and 300 mg/kg, p.o.) did not cause any significant alterations in the motor performance of the mice. In contrast, diazepam (4 mg/kg) significantly reduced the time of permanence on the rota-rod.

3.3. Preliminary Acute Toxicity Assessment. Administration of MPEE (300, 1000, and 5000 mg/kg, p.o.) did not produce any noticeable effect on behaviour or mortality in treated animals during observation period.

4. Discussion

The potential antinociceptive activity of the ethanolic extract of *M. ptelefolia* leaves (MPEE) was investigated using acetic acid-induced abdominal constriction test, hot-plate test, and formalin-induced paw licking test. The acetic acid-induced abdominal constriction test and the hot-plate test have been reported to be useful methods to investigate peripheral and central antinociceptive activity, respectively [17, 18], while the formalin-induced paw licking test is a valuable method to detect antinociceptive activity both peripherally and centrally [19–21].

In the present study, it was demonstrated that the MPEE significantly inhibited the acetic acid-induced abdominal constriction in mice. The positive control group treated with

Table 1: The effect of the *M. ptelefolia* ethanolic extract (MPEE) on the hot-plate test in mice. Results are expressed as the mean ± S.E.M. in seconds of 6 mice. $^{**}P < .01$ compared to the control group (Dunnet's test).

Treatment	Dose (mg/kg; p.o.)	Interval following treatment (min)						
		0	30	60	90	120	180	210
		Latency time (s)						
Control (0.9% NaCl)		7.9 ± 1.3	6.9 ± 1.3	7.8 ± 1.35	8.8 ± 1.3	8.3 ± 0.9	8.8 ± 1.8	9.1 ± 1.4
MPEE	30	6.8 ± 0.8	6.9 ± 0.3	7.0 ± 1.0	7.5 ± 1.1	7.2 ± 0.7	8.1 ± 1.5	8.3 ± 2.0
	100	7.5 ± 1.6	8.0 ± 1.3	8.1 ± 1.3	9.2 ± 0.8	10.2 ± 2.1	10.4 ± 2.8	11.1 ± 2.4
	300	7.4 ± 2.4	8.8 ± 1.6	8.3 ± 1.8	10.8 ± 1.5	9.7 ± 2.1	9.9 ± 2.5	8.5 ± 1.8
Morphine	5	8.0 ± 1.1	15.9 ± 1.2**	17.8 ± 2.7**	14.3 ± 2.2**	12.6 ± 3.4**	10.9 ± 4.0	10.9 ± 1.2

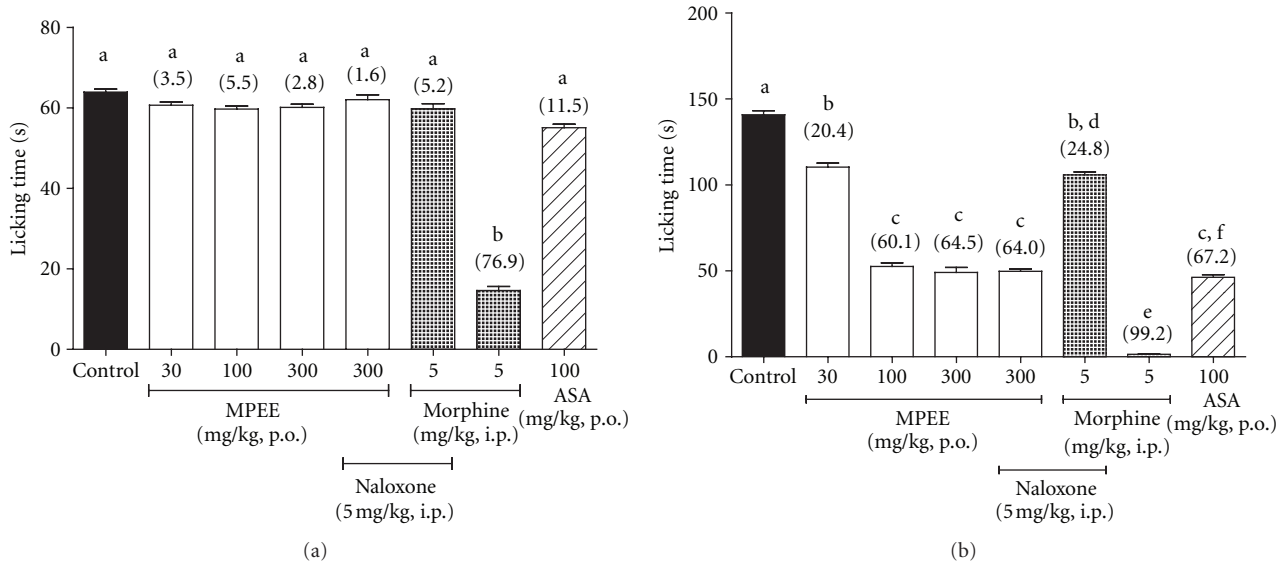

Figure 2: Effect of *M. ptelefolia* ethanolic extract (MPEE) in formalin-induced paw licking test (early phase (a) and late phase (b)) in rats. Each column represents the mean ± S.E.M. of 6 mice. The rats were pre-treated with vehicle (control), MPEE, morphine, or acetylsalicylic acid (ASA), 30 min before i.pl injection of formalin. Statistical analysis was determined by one-way ANOVA followed by Tukey's test with values of similar superscript letters not statistically different from each other ($P < .05$). Values in parentheses are percentage of inhibition.

ASA (100 mg/kg) also manifested significant reduction in the number of writhes. Acetic acid acts indirectly by inducing the release of endogenous mediators, such as prostaglandins, (PGs) as well as increase in lipooxygenase (LOX) production in the peritoneal that eventually stimulate local peritoneal nociceptors [17, 22]. In addition, it have been shown elsewhere that centrally and peripherally acting drugs such as morphine and aspirin are able to inhibit the inflammatory pain induced by acetic acid [23, 24]. Therefore, the present results of the acetic acid-induced abdominal constriction test strongly suggest that the mechanism of MPEE may be linked partly to inhibition of LOX and/or cyclooxygenase (COX) in peripheral tissues, thereby reducing PG synthesis and interfering with the mechanism of transduction in primary afferent nociceptors.

MPEE was also subjected to the formalin-induced paw licking test, a test model which is sensitive to various classes of analgesic drugs [25], and characterized by the first phase (neurogenic), which is evoked by direct formalin stimulation of the sensorial C-fibers followed by substance P release [26], and the second phase (inflammatory) mainly due to a subsequent inflammation reaction in the peripheral tissue mediated by the release of various inflammatory mediators has been associated with the increased level of PG, induction of COX and release of nitric oxide (NO) [18, 27]. The biphasic nature of pain response in this test, which reflects different pathological processes, can be used to elucidate the possible mechanism involved in analgesia [20]. Centrally acting drugs, such as opioids, inhibit both phases of pain, while peripheral-acting drugs such as acetylsalicylic acid, that inhibit COX activity, only inhibit the second phase [25, 26, 28]. Our results showed that p.o. administration of MPEE inhibited significantly only the second phase of formalin-induced paw licking test, suggesting an involvement of its active analgesic principles at the peripheral levels, which may be due to inhibition of COX and consequent prostaglandin synthesis. In addition, recent *in vitro* studies have shown that *M. ptelefolia* methanolic extract and its compound kokusaginine attenuated NO in lipopolysaccharide induced RAW264.7 murine peritoneal macrophages [9, 29]. For this

Table 2: The effect of the *M. ptelefolia* ethanolic extract (MPEE) on the rota-rod test in mice. Results are expressed as the mean ± S.E.M. in seconds of 6 mice. $^{**}P < .01$ compared to the control group (Dunnet's test).

Treatment	Dose (mg/kg)	Interval following treatment (min)		
		30	60	120
		Time of permanence (s)/60 s		
Control (0.9% NaCl)		60	60	59.3 ± 0.3
MPEE (p.o.)	30	60	59.8 ± 0.9	58.9 ± 0.5
	100	59.8 ± 0.4	59.8 ± 0.4	59.6 ± 0.2
	300	59.2 ± 0.5	57.9 ± 0.6	58.6 ± 0.6
Diazepam (i.p.)	4	37.6 ± 8.9**	32.5 ± 2.2**	39.6 ± 1.7**

reason, the peripheral antinociceptive activity showed by the MPEE in the present study can be explained, at least in part, by the presence of this compound and other constituents, not excluding the possibility of synergism between other constituents present in the MPEE that perhaps inhibited COX as well as release of NO.

Additionally, the peripheral analgesic effect of the MPEE in the present study is strongly supported by the results obtained from the hot-plate test which is a preferential method to screen centrally acting opiate analgesic drugs [19]. It was demonstrated that p.o. administration of the MPEE had no effect on the response latency time to the heat stimulus (Table 1). As expected, morphine (centrally acting drug) significantly increased the latency time to the nociceptive response compared with control group. It is also interesting to note that pretreatment with a nonselective opioid receptor antagonist, naloxone, failed to reverse the antinociceptive effect of MPEE in the second phase of the formalin-induced paw licking test, compared to extract and morphine alone (Figure 2). These results strongly suggest that opioid system and central antinociception effects were not involved in the MPEE-induced antinociception. Another interesting finding in the present study demonstrated that treatment of MPEE was also largely devoid of significant effects on the motor coordination of mice in the rota-rod test, therefore eliminating a nonspecific muscle relaxation and sedative effects in MPEE-induced antinociception, and the preliminary acute toxicity test obtained showed no occurrence of death and abnormal behavior, even at the highest dose of MPEE (5 g/kg), indicating that it may have a reasonable safety margin with regard to acute toxicity.

In conclusion, the results of the present study provide convincing evidences that MPEE possesses a significant peripheral antinociceptive effect that is probably mediated by inhibiting the production of inflammatory mediators. The precise mechanism underlying the antinociceptive action of MPEE has yet to be determined and currently under investigation, but it is unlikely to be associated with an interaction associated with opioid receptors. These findings support, at least in part, the use of the plant in traditional medicine for the treatment of some painful and inflammatory conditions and also established the presence of biologically active principles whose activities may need further investigation.

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