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Full Length Research Paper

The effects on motor behaviour and short-term memory tasks in mice following an acute administration of *Mitragyna speciosa* alkaloid extract and mitragynine

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***Mitragyna speciosa* Korth. leaves have been traditionally used by Malay and Thai natives for its opium-like effect and coca-like stimulant ability to enhance tolerance for hard work. Mitragynine is the major indole alkaloid in this plant. In the present study, we investigated the effects of the alkaloid extract of *M. speciosa* leaves and its major alkaloid mitragynine on exploratory behaviour, short-term memory and motor coordination in mice using Y-maze and rota-rod tests, respectively. Male Swiss albino mice were orally administrated with 20, 40 and 80 mg/kg alkaloid extract of *M. speciosa* leaves or mitragynine. Acute oral administration of the alkaloid extract or mitragynine significantly increased the total number of arm entries, rearing frequency and decreased grooming and immobility time in Y-maze test when compared to control group ($P<0.05$). However, the alkaloid extract or mitragynine did not affect short-term memory or motor coordination. These findings suggest that acute administration of *M. speciosa* alkaloid extract or mitragynine may enhance exploratory behaviour due to the decrease in stress to a novel environment.**

Key words: *Mitragyna speciosa*, mitragynine, motor behaviour, short-term memory, Y-maze, rota-rod.

INTRODUCTION

Medicinal plants have been used for centuries to prevent and cure different ailments (Thomson, 2010). According to the World Health Organization, more than 80% of the population in developing countries resort to medicinal plants for their primary health care remedies (Soetan and Aiyelaagbe, 2009). *Mitragyna speciosa* Korth. (Rubiaceae) is an indigenous evergreen tree found in Southeast Asian countries particularly in Malaysia and Thailand. The leaves of this plant are known as "Biak-Biak" or "Ketum" in Malaysia and "Kratom" in Thailand. *M. speciosa* leaves have been traditionally used by Malay and Thai natives for its opium and coca-like effects to enhance tolerance for hard work under the hot sun (Grewal, 1932; Suwanlert, 1975). In addition, the leaves were used to relieve pain, reduce coughing, treat diarrhoea, and substitute morphine in treatment of addicts (Watanabe et al., 1997; Vicknasingam et al., 2010). Over 25 alkaloids have been isolated from the mature leaves of *M. speciosa*. Mitragynine is the most abundant alkaloid accounting for approximately 66 and 12% of the total alkaloid extract obtained from the Thai and Malaysian *M. speciosa* leaves, respectively (Takayama et al., 1998; Chittrakarn et al., 2008). Earlier studies have reported the role of supraspinal opioid receptors and both descending and serotonergic systems in the antinociceptive effect of mitragynine (Matsumoto et al., 1996a,b). Moreover, mitragynine has been shown to inhibit the electrically stimulated contraction of guinea-pig ileum and mouse vas deferens through the activation of μ- and δ–opioid receptors, respectively (Watanabe et al., 1997; Yamamoto et al., 1999).

Recent findings include central inhibition of gastric acid

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Abbreviations: **GC/MS,** Gas chromatography/mass spectrometry; **EI,** electron impact; **SIM,** selected ion monitoring; **USM,** universiti sains Malaysia; **i.p,** intraperitoneally; **MOSTI,** Ministry of Science, Technology and Innovation; **IPS,** Institute of Postgraduate Studies.

secretion by mitragynine through opioid receptors stimulation (Tsuchiya et al., 2002) and radioligand binding assay that revealed mitragynine binding to opioid receptors, with the highest affinity to κ- followed by μ- and δ–opioid receptor subtypes (Boyer et al., 2008; Taufik Hidayat et al., 2010). Animal studies on the effects of *M. speciosa* extracts and mitragynine on open field locomotion have been previously reported (Reanmongkol et al., 2007; Moklas et al., 2008). These studies were focused on locomotor activity rather than exploratory behaviour following drug administration. A recent study by Apryani and colleagues (2010) examined the chronic effect of mitragynine on working memory processes in mice. However, the acute effects of *M. speciosa* alkaloid extract or mitragynine on short-term memory is unknown. A recent *in-vitro* study has reported the muscle relaxant effects of the methanolic extract of *M. speciosa* leaves and mitragynine (Chittrakarn et al., 2010). However, these effects have not yet been evaluated using whole-animal model (*in-vivo*). Therefore, the present study was designed to investigate the acute effects of different doses of *M. speciosa* alkaloid extract and mitragynine on exploratory behaviour, short-term memory and motor coordination in mice.

MATERIALS AND METHODS

Plant material

Fresh *M. speciosa* leaves were collected from Kedah, Malaysia. Taxonomic identification was confirmed by the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, where voucher specimen was deposited with the number, USM11074. The leaves were cleaned and oven dried at 40ºC for three days, then ground to fine powder. Powdered leaves (500 g) were extracted with methanol using soxhlet apparatus for three days. The mixture was filtered before evaporating to dryness under reduced pressure at 35°C with a Buchi R110 Rotavapor (Buchi Labortechnik AG, Flawil, Switzerland) to give the crude methanolic extract. The extraction and evaporation procedures were repeated three times. One part of the methanolic extract was mixed with 10% acetic acid for 24 h, filtrated and washed with hexane. The acidic layer was then basified with ammonia solution to pH9 and extracted with chloroform. The combined chloroform extract was evaporated using a rotary evaporator to yield 0.6% (w/w) of crude alkaloid mixture. Isolation of mitragynine from the crude alkaloid extract of *M. speciosa* leaves was performed as described by Ponglux et al. (1994) with a slight modification.

Standardization of *M. speciosa* alkaloid extract

The quantitative determination of mitragynine in *M. speciosa* alkaloid extract was achieved by gas chromatography/ mass spectrometry (GC/MS) system. An Agilent 6890N gas chromatograph coupled to an Agilent 5973 mass spectrometry detector was used in the electron impact (EI) mode with 70 eV ionization energy. Chromatographic separation was performed with HP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm). The inlet temperature was 280°C and the column oven temperature was set at 70°C and increased linearly to 280°C at a rate of 30°C/min, then held for 17 min. One microlitre of the diluted extract was automatically injected in the splitless mode. Helium was used as a carrier gas with a flow rate of 1.0 ml/min. *M. speciosa* alkaloid extract was injected at concentrations ranging from 128 to 1024 ng/μl. Quantification was performed in a selected ion monitoring (SIM) mode and the calibration curve for mitragynine was generated using linear regression analysis.

Animals

Male Swiss albino mice (weighing 20 to 30 g) were obtained from the breeding colony of the Animal House facility, Universiti Sains Malaysia (USM). They were housed in groups of 5 per cage, for a minimum of 5 days prior to behavioural testing, with free access to the standard commercial food pellets and water *ad libitum*. Animals were housed in a temperature controlled room at 25±2°C and maintained on a reversed light/dark cycle. Behavioural testing was performed during the dark phase of the light cycle (07:00 to 19:00). The experimental protocols for care and use of laboratory animals described in the present study were guided and approved by the Animal Ethics Committee, [USM/2010/56 (186)].

Drugs and chemicals

Diazepam ampoules (10 mg/2 ml; Hospira, USA), *d*-amphetamine sulphate (Lipomed, Arlesheim, Switzerland) and Scopolamine hydrobromide (Acros Organics, USA) were used as reference drugs. Tween 20 (ChemPur, Germany) was used as a vehicle.

Drugs administration

Alkaloid extract of *M. speciosa* leaves and mitragynine were freshly dissolved in 20% (v/v) tween 20 and administered orally (p.o.) using an intragastric gavage needle. Diazepam, amphetamine and scopolamine were dissolved in sterile saline (0.9% NaCl) and administered intraperitoneally (i.p.). Mitragynine was administered 60 min before testing. Vehicle, alkaloid extract, diazepam and scopolamine were administered 30 min before testing whereas amphetamine was administered 15 min prior to testing (Hahn et al., 1986; Heo et al., 2009). In the present study, the optimal doses and time of administration of the alkaloid extract and mitragynine were determined in a preliminary study conducted in our laboratory. All administrations were performed in a dose volume of 10 ml/kg body weight. Each mouse was tested only once. Behavioural testing and data collection were performed by an investigator blinded to the identity of the treatment groups.

Y-maze test

Y-maze test is based on the innate curiosity of rodents to explore novel environments (Luszczki et al., 2005). It has been effectively used to assess exploratory behaviours, learning and memory function in rodents (Kokkinidis et al., 1976; Hahn et al., 1986; Jing et al., 2008; Kwon et al., 2009). In the present study, Y-maze apparatus consisted of three identical arms (33 × 11 × 12 cm each) in which the arms are symmetrically separated at 120°. The maze floors and walls were constructed from stainless steel. Animals were randomly divided into nine groups (each, $n = 9$): control (20% tween 20); *M. speciosa* alkaloid extract (20, 40 and 80 mg/kg); mitragynine (20, 40 and 80 mg/kg); amphetamine (10 mg/kg) and scopolamine (1 mg/kg). Non-habituated mice were gently placed at the end of one arm, and were allowed to freely explore the Y-maze during 8 min. The following behavioural parameters: (I) the number of arm visits; (II) sequence of arm visits; (III) rearing frequency (number of times the animal stood on its hind legs with t he front

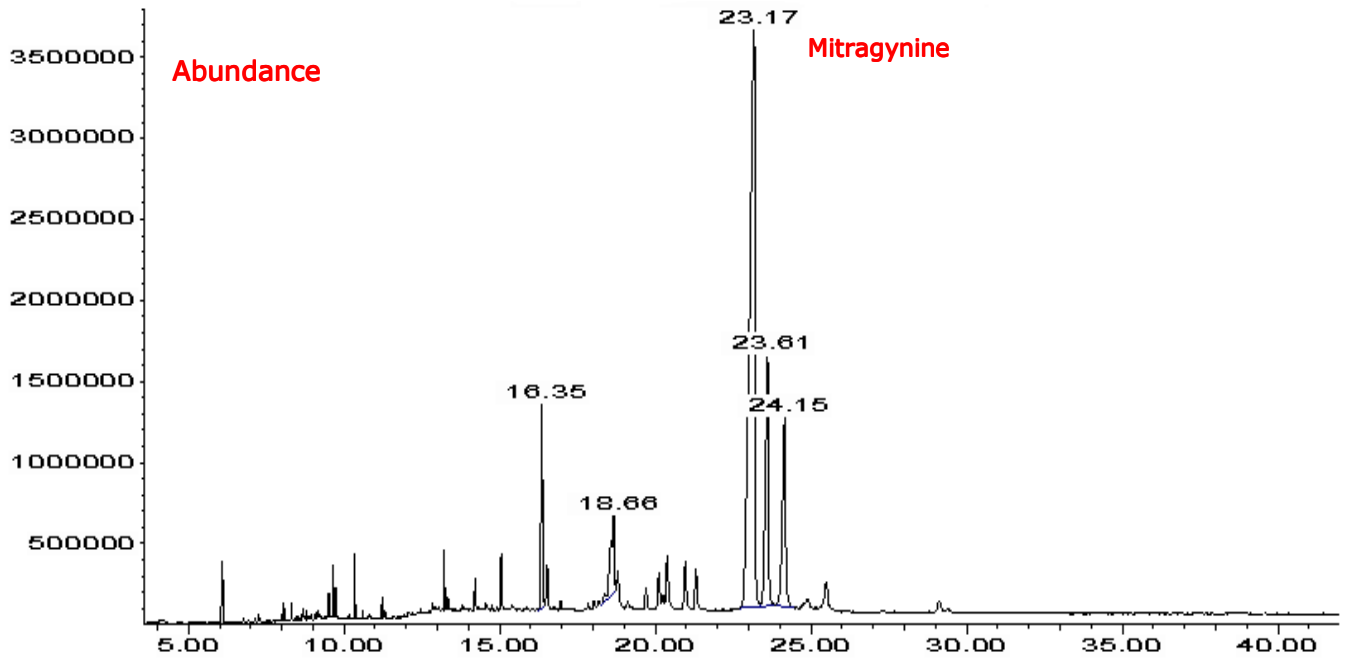

Figure 1. GC/MS chromatogram of *M. speciosa* alkaloid extract. The retention time of mitragynine is at 23.17 min.

limbs either against the wall or feely in the air); (IV) grooming time (time spent in paws licking, face cleaning, rubbing and scratching various parts of the body) and (V) immobility duration (total seconds of lack of movements) were recorded for each mouse using video camera (Sony, DCR-SX44E). The maze arms were cleaned with 70% ethanol between tasks to remove residual odours. An actual alternation was defined as entries into all three arms consecutively (that is, ABC, CAB or BCA but not BAB). The percentage alternation for each mouse was determined as the ratio of actual to possible alternations (defined as the total number of arm entries minus 2), multiplied by 100 as shown by the following equation: % Alternation = [(Number of alternations) / (Total arm entries-2)] x 100 (Kim et al., 2007; Heo et al., 2009). The total number of arm entries was considered to reflect spontaneous locomotor activity.

Motor coordination test

The effect of the *M. speciosa* alkaloid extract and mitragynine on motor coordination was determined via the use of the Roto-Rod (IITC Life Science, CA, USA). Fresh mice were placed on a horizontal rod (32 mm diameter) rotating at a steady speed of 12 revolutions per min. The mice capable of remaining on the rod for 3 min or more, in two successive trails were selected and grouped into eight groups (each, $n = 9$): control (20% tween 20); *M. speciosa* alkaloid extract (20, 40 and 80 mg/kg); mitragynine (20, 40 and 80 mg/kg) and diazepam (5 mg/kg). The test consisted of placing mice 30 min post treatment and at intervals of 30 min for a total of 180 min. The time in sec for the animal to fall off within the cut off time of 180 s was automatically recorded through sensory platforms located below the rotating rod (Fujimori and Cobb, 1965; Felipe et al., 2008).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.0 software, using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. Results were expressed as mean ± S.E.M. *P*-values < 0.05 were considered statistically significant.

RESULTS

Standardization of *M. speciosa* alkaloid extract

GC/MS results demonstrated relatively high abundance of mitragynine in the alkaloid extract of *M. speciosa* leaves (Figure 1). Quantitative analysis revealed the presence of (13.7%) mitragynine in the standardized extract.

Y-maze test

Acute administration of *M. speciosa* alkaloid extract or mitragynine increased spontaneous locomotor activity in mice. Significant increase in the total number of arm entries was observed in mice orally treated with 20, 40 and 80 mg/kg alkaloid extract or mitragynine when compared to control group ($P<0.05$). However, the total number of arm visits following acute administration of *M. speciosa* alkaloid extract or mitragynine was significantly lower than that of amphetamine group ($P<0.05$) (Figure 2a). Moreover, alkaloid extract of *M. speciosa* leaves or mitragynine increased novelty-induced rearing behaviour in mice. Compared to control group, mice treated with 20, 40 and 80 mg/kg alkaloid extract or mitragynine

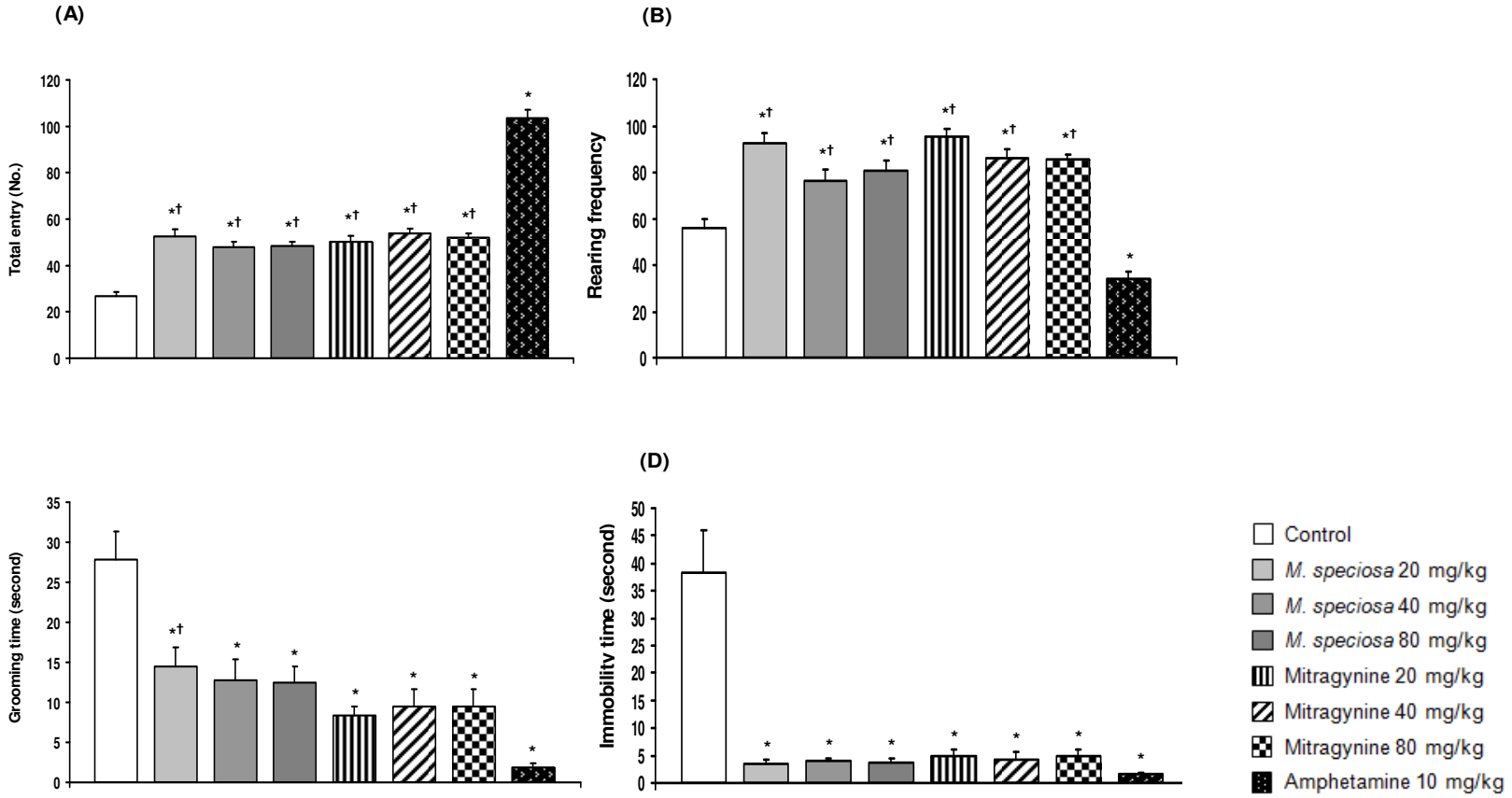

Figure 2. Effects of *Mitragyna speciosa* alkaloid extract, mitragynine and amphetamine on spontaneous motor behaviour in Y-maze test. Results are expressed as mean ± SEM of the total number of arm entries (A), rearing frequency (B), grooming time (C) or immobility time (D). Comparisons were made by using one way ANOVA followed by a post hoc Bonferroni test. * $P<0.05$ when compared to the control group. † $P<0.05$ when compared to amphetamine group.

demonstrated significant increase in rearing frequency ($P<0.05$). However, i.p. administration of 10 mg/kg amphetamine significantly decreased rearing behaviour in Y-maze test ($P<0.05$). In addition, mice treated with *M. speciosa* extract or mitragynine exhibited a significantly higher frequency of rearing compared with the amphetamine group ($P<0.05$) (Figure 2b). Self-grooming and immobility time were significantly decreased in mice treated with alkaloid extract, mitragynine or amphetamine, compared to the control group ($P<0.05$). In addition, the decrease in grooming time in mice treated with 10 mg/kg amphetamine was significantly lower than that of mice treated with 20 mg/kg alkaloid extract ($P<0.05$) (Figure 2c and d). In the assessment of the effect on short-term memory, the percentage of spontaneous alternation was evaluated. Similar to vehicle treated group, acute administration of *M. speciosa* alkaloid extract or mitragynine did not affect the innate tendency of mice to alternate arm choices in Y-maze test (Figure 3). However, i.p. administration of 1 mg/kg scopolamine significantly

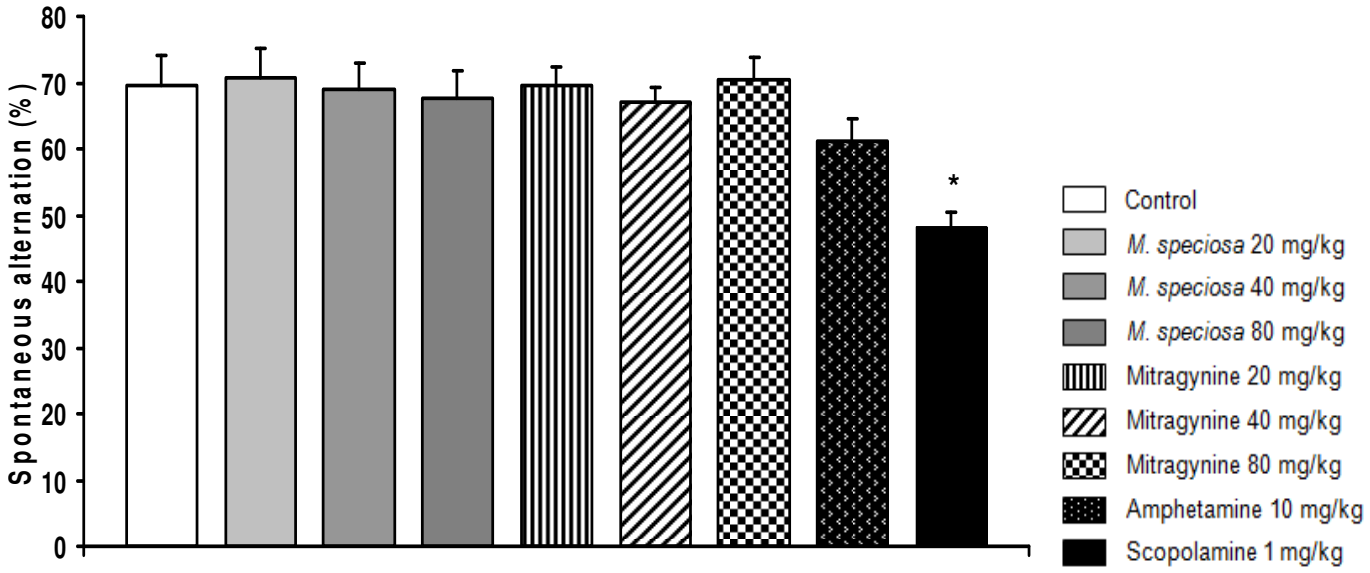

Figure 3. Effects of *Mitragyna speciosa* alkaloid extract, mitragynine, amphetamine and scopolamine on spontaneous alternation behavior in Y-maze test. Results are expressed as mean ± SEM. Comparisons were made by using one way ANOVA followed by a post hoc Bonferroni test. * $P<0.05$ when compared to the control group.

reduced alternation behaviour (48.23±2.24) as compared to control group (69.71±4.32) ($P<0.05$). Similarly, mice treated with amphetamine (10 mg/kg, i.p.) demonstrated slight, but not significant, decrease in the percentage of alternation when compared to control group.

Motor coordination test

Alkaloid extract of *M. speciosa* leaves and mitragynine did not affect muscle coordination in the rota-rod test. Mice orally treated with 20, 40 and 80 mg/kg alkaloid extract or mitragynine were able to maintain their balance on the rotating rod during the 3 min testing sessions. On the other hand, motor coordination was significantly impaired after i.p. injection of 5 mg/kg diazepam to mice. However, the muscle relaxant effect of diazepam was gradually recovered within 120 min following the administration diazepam (Table 1).

DISCUSSION

Our findings demonstrated that acute administration of *M. speciosa* alkaloid extract or mitragynine in Swiss albino mice significantly increased horizontal (locomotion) and vertical (rearing) components of exploratory behaviour in a novel Y-maze environment. On the other hand, the duration of immobility and self-grooming appeared significantly decreased. In addition, our findings demonstrated that administration of the alkaloid extract or mitragynine had no significant effect on the alternation scores or motor coordination. The findings of the exploratory behaviour in the present study supported previous studies that showed stimulation of opioid receptors within the mesolimbic and nigrostriatal dopamine systems increase locomotion and rearing behaviour in rodents (Iwamoto and Way, 1977; Walter and Kusehinsky, 1989; Ford et al., 2006). In addition, mitragynine has been shown to possess the opioid receptors agonistic properties (Watanabe et al., 1997; Yamamoto et al., 1999; Tsuchiya et al., 2002). Therefore, it seems likely that alkaloid extract of *M. speciosa* leaves and mitragynine may enhance locomotion and exploratory behaviour through their action on opioid receptors in the mesolimbic and nigrostriatal dopamine systems. Self-grooming, other than to clean and care of body suface, has been considered as an index of behavioural adaptation to stressful situations (Ladurelle et al., 1998). Exposure to the mild stress of a novel environment induces grooming behaviour in rodents (Devine et al., 2001; Liu et al., 2010).

It has been reported that stimulation of μ-opioid receptors in the hippocampus reduces stress to novel environment through the inhibition of hippocampal serotonin release (File et al., 1993; Passarelli and Costa, 1989; Bechtholt et al., 2007). Thus, our findings on self-grooming suggest the possibility that *M. speciosa* leaves extract and mitragynine may reduce stress in a novel environment through their action on opioid receptors in areas of the brain that modulate responses to stress. Future studies are necessary to elucidate the involvement of opioidergic system of the amygdala and hippocampus in stress-mitigating effects of *M. speciosa*

Table 1. Effects of *M. speciosa* alkaloid extract (MS), mitragynine (MG) and diazepam (DZP) on motor coordination in mice using rota-rod test. Results are expressed as mean ± SEM. Comparisons were made by using one way ANOVA followed by a post hoc Bonferroni test. * $P<0.05$ when compared to the control group. † $P<0.05$ when compared to treated groups.

Treatment	Time (second)					
	30	60	90	120	150	180
Control (10 ml/kg)	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0
MS (20 mg/kg)	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0
MS (40 mg/kg)	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0
MS (80 mg/kg)	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0
MG (20 mg/kg)	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0
MG (40 mg/kg)	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0
MG (80 mg/kg)	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0
DZP (5 mg/kg)	8.3±0.4*†	25.6±1.9*†	149.4±5.7*†	180.0±0.0	180.0±0.0	180.0±0.0

alkaloid extract and mitragynine. Spontaneous alternation behaviour is regarded as a measure of short-term memory in rodents (Hritcu et al., 2007; Heo et al., 2009). A mouse must remember the least recently visited arm in order to alternate the arm choice (Hooper et al., 1996; Lee et al., 2010). Our results demonstrated that acute oral administration *M. speciosa* leaves extract or mitragynine did not affect short-term memory processes in mice. However, works on the long-term administration of *M. speciosa* extract or mitragynine and their effects on spontaneous alternation behaviour in Y-maze test are currently underway in our laboratory. Interestingly, Apryani and colleagues (2010) demonstrated that chronic administration of mitragynine impaired working memory processes in mice. On motor behaviours, we performed rota-rod test to evaluate whether *M. speciosa* extract or mitragynine affect motor coordination in mice. Acute oral administration of *M. speciosa* alkaloid extract or mitragynine did not significantly alter the motor coordination in the rota-rod test.

These results suggest that mitragynine, at the doses employed in this study did not produce any adverse effects on motor coordination which is an important safety criterion for new psychoactive compounds (WHO, 2004). Even though, mitragynine had shown muscle relaxant effect *in-vitro*, our data unable to affirm the findings of Chittrakarn et al. (2010) from the whole-animal model (*in-vivo*).

In the present study, differences between the behavioural effects observed following acute administration of *M. speciosa* extract or mitragynine were not statistically significant. These findings suggest that minor constituents of *M. speciosa* extract exhibit more potent opioid agonistic effects than mitragynine. Similarly, previous studies reported more potent antinociceptive effect following acute administration of *M. speciosa* extract when compared to mitragynine (Watanabe et al., 1992, 1999). It has been well documented that increased novelty exploration is an intrinsic manifestation of the reduced stress in a novel environment (Gil et al., 1999; Strekalova et al., 2004). Our data demonstrated that acute administration of *M. speciosa* leaves extract or mitragynine increased locomotion, rearing behaviour and reduced grooming time in a novel Y-maze environment. Together, these results suggest that *M. speciosa* alkaloid extract and its major alkaloid mitragynine may enhance exploratory behaviour by mitigating stress to a novel environment. Mechanisms underlying these effects may involve, to a certain extent, activation of opioid receptors in the central nervous system which mediate other neural processes in motor behaviours in response to stress. Future researches should be conducted to determine the exact mechanisms, aside opioid receptors that underlie these behavioural alterations.

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