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ORIGINAL ARTICLE

Methanolic Extract of *Mitragyna speciosa* Korth Leaf Exhibits Place Preference Only at Higher Doses in Mice

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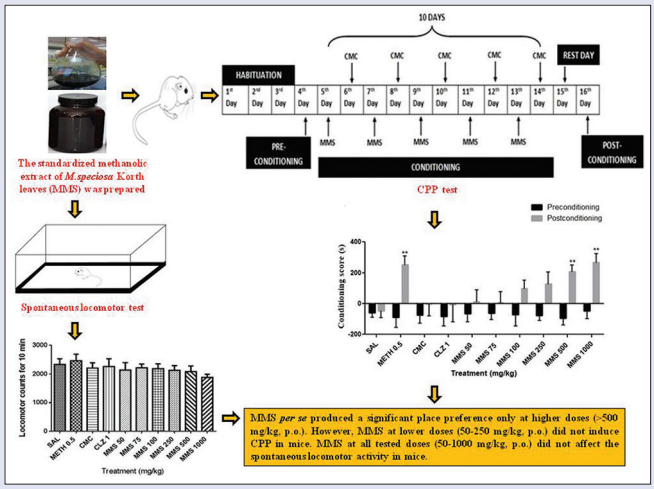
ABSTRACT

Background: *Mitragyna speciosa* Korth possesses a wide range of therapeutic benefits, despite having abuse liability. **Objectives:** The purpose of this research was to investigate the reinforcing properties of *M. speciosa* Korth leaf extract obtained via methanol extraction using mouse conditioned place preference (CPP) test. **Materials and Methods:** In CPP study, following baseline preference test (preconditioning score), the mice were subjected to conditioning trials at varying doses of methanolic extract of *M. speciosa* (MMS, 50, 75, 100, 250, 500, and 1000 mg/kg, p.o.) or reference drugs methamphetamine (0.5 mg/kg, intraperitoneally [i.p.]) and clozapine (1 mg/kg, p.o.) or vehicle controls (1% w/v sodium carboxy methyl cellulose [10 mL/kg, p.o.] and saline [10 mL/kg, i.p.]) followed by a preference test performed under drug-free state (postconditioning score). In addition, the effect of all tested drugs on the spontaneous locomotor activity was assessed. **Results:** The CPP study results revealed that MMS *per se* produced a significant place preference only at higher doses (>500 mg/kg, p.o.). Nevertheless, MMS at lower doses (50–250 mg/kg, p.o.) did not induce CPP in mice. In addition, MMS at all tested doses (50–1000 mg/kg, p.o.) did not affect the spontaneous locomotor activity in mice. **Conclusion:** MMS *per se* exhibits reinforcing properties at only an increased dose of >500 mg/kg, and therefore, it is best to administer at lower doses (<250 mg/kg) for the potential therapeutic benefits in preclinical studies.

Key words: Clozapine, conditioned place preference, drugs abuse, methamphetamine, *Mitragyna speciosa*

SUMMARY

- The methanolic extract of *Mitragyna speciosa* (MMS) leaf showed a place preference in conditioned place preference test only at higher doses (>500 mg/kg) in mice, which was comparable to the reference drug methamphetamine (0.5 mg/kg). However, MMS did not display place preference at lower doses (<250 mg/kg) in mice. The study results suggest that MMS could have addictive potential at higher doses, and it should be used only at lower doses (<250 mg/kg) for any therapeutic investigations in preclinical research.



Abbreviations Used: MMS: Methanolic extract of *Mitragyna speciosa*; METH: methamphetamine; CLZ: clozapine; CMC: carboxy methyl cellulose; SAL: saline; CPP: conditioned place preference.

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INTRODUCTION

The application of cannabis, coca, and opium poppy in hospitals and medical surgeries was dated back to few centuries ago. Morphine obtained from the opium poppy drips in hospitals served as a potent pain killer; cocaine obtained from *Erythroxylon coca* plant served as an anesthetic agent in certain medical surgeries; and cannabinoids obtained from the cannabis plant are used for the treatment of chemotherapy-induced nausea and vomiting and epileptic seizures.[1-4] Although these plants were proved to be beneficial in all aspects mentioned above, the addictive and fatality potentials of these plants are undeniable. Appropriate use of these drugs in terms of dose, dosage forms, and duration of the treatment makes them beneficial rather than harmful. *Mitragyna speciosa* is one such plant that possesses vast therapeutic benefits with addictive potential. Identifying the nonaddictive dose of *M. speciosa* leaf extract is crucial in novel drug discovery for various ailments.

M. speciosa is a tropical tree mostly found in Thailand and Northern Malaysia, which has been used conventional for therapeutic and recreational purposes. *M. speciosa* is known as "Kratom, Thang, Thom, and Kakuam" in Thailand and "Ketum or Biak-biak" in Malaysia, and it has been reported for many pharmacological activities such as analgesic, antidiarrheal, antipyretic, local anesthetic, and antipsychotic activities.[5,6] Boyer *et al.* reported that *M. speciosa* acts as an opioid agonist and exhibits a strong analgesic (pain-relieving) effect, which is often used in place of powerful prescription of opioids.[7] Besides, it is evident that

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M. speciosa has conventionally been used for the treatment of muscle ache, fatigue, pain management, cough, high blood pressure, diarrhea along with functioning as opioid substitute to treat opioid addiction.[8-10] A total of 25 alkaloids were isolated and characterized from *M. speciosa* leaves since the 1960s, and the main bioactive compounds were found to be mitragynine, paynanthenine, speciociliatine, speciogynine, and 7-hydroxymitragynine. Among all the active alkaloids present in *M. speciosa*, mitragynine is considered to be the predominant alkaloid which is accountable for the various pharmacological activities of the plant.[10,11] Mitragynine has been reported for antidepressant-like[12,13] and antinociceptive effects mediated through the supraspinal opioid receptors and descending noradrenergic and serotonergic systems in animal models.[14]

The usage of *M. speciosa* is not being limited to Southeast Asia but has been spread into European countries and the United States.[15,16] In 2014, a study conducted in the USA by Sufka *et al.* using methanol extract of *M. speciosa* leaf (MMS, 50, 100, 300 mg/kg, intraperitoneally [i.p.]) and mitragynine (5, 10, and 30 mg/kg, i.p.) revealed that MMS leaf had insignificant effect on place preference compared to vehicle control in rats, whereas mitragynine significantly induced place preference in rats and these results postulated that the presence of other psychoactive constituents or lower concentration of mitragynine in the plant extract that have antagonistic properties on mitragynine's rewarding effects.[17] According to Hassan *et al*., the content of alkaloid in *M. speciosa* leaf varies by season and geographical region.[10] Besides, new types of alkaloids namely corynantheidaline, mitragynaline, corynantheidalinic acid, and mitragynalinic acid were identified in the Malaysian *M. speciosa* leaf.[18] Therefore, the rewarding effect of the Malaysian variety of *M. speciosa* leaf could be differed from the plant obtained from the other origin as reported earlier by Sufka *et al.* The present study aimed at investigating the reinforcing properties of the Malaysian variety of this plant using a mouse conditioned place preference (CPP) test. This familiar animal model, CPP was utilized to evaluate the addictive/aversive properties of psychoactive substances.

MATERIALS AND METHODS

Animals

Swiss albino male mice (7–8 weeks old) with an approximate body mass of 25–30 g were used. The experimental animals were provided unlimited access for standard laboratory food pellets and water and housed in individually ventilated cages in a group of four animals. Standard animal laboratory conditions (room temperature: 22°C ± 1°C; relative humidity: 45%–55%; 12 h light/dark cycle [lights on/off at 7.00/19.00 h]) were maintained. The animals were brought to experimental room 1 week before experimentation to adjust with the laboratory environment. Humane care and environmental enrichment were provided to reduce the animal discomfort. The Institutional Animal Care and Use Committee (IACUC) of Faculty of Medicine, University of Malaya, appraised and approved (IACUC Ethics No. 2016-190908/PHAR/R/VP) the animal study protocol in accordance to the "Guide for the Care and Use of Laboratory Animals" by the National Research Council of the National Academies of the USA.[19]

Drugs and chemicals

M. speciosa Korth leaves methanolic extract (standardized) and clozapine (CLZ, served negative control in this study) were produced as suspensions utilizing sodium carboxy methly cellulose (CMC) solution (1% w/v) and administered by oral gavage (p.o). As a positive control, methamphetamine hydrochloride (Sigma Aldrich, St. Louis, MO, USA) solution was injected i.p. which was prepared in sterile normal saline (SAL). The vehicle control groups received CMC/SAL. The experimental animals received a constant volume (1 mL/100 g body weight) of drugs prepared freshly before the experiment session. The present MMS doses were chosen based on the therapeutic and lethal doses (4.90 g/kg in mice) of MMS published in the literature.[8] Similarly, in another study, the standardized MMS (1.6%w/w of mitragynine) at 100, 500, and 1000 mg/kg, p.o resulted no mortality during 14-day observation period in male Sprague–Dawley rats.[20] Furthermore, numerous central nervous system (CNS) implications of MMS has been reported at 50–1000 mg/kg.[5,8,21-23] The methamphetamine (METH) dose was selected based on the published literature and standardized in our laboratory.[24,25]

Plant collection and identification

The *M. speciosa* leaves were obtained from Alor Setar Kedah, Malaysia, in December from a tree (about 12 m height). The authentication of the leaf was carried out by Dr. Sugumaran Manickam (Rimba Ilmu, Institute of Biological Science, University of Malaya). For future reference, a voucher specimen bearing a number (KUL 47980) was deposited.

M. speciosa leaves extract

Standardized *M. speciosa* Korth leaves methanolic extract (presence of mitragynine at 4.4% w/w) prepared for the previous study[5] was used in the present study. Briefly, using methanol as an extractant, *M. speciosa* Korth leaves were subjected to cold extraction with sonication to obtain the plant extract which was later phytochemically characterized and reported.[5] For further studies, the obtained extract MMS was placed in an amber screw-capped container and preserved at 4°C. On the basis of previous published reports, the MMS doses were employed for the present CPP study.[5,17]

Apparatus

CPP test was carried out using three-compartment rectangular box, which was fabricated using a Plexiglas measuring 45 (L) cm × 15 (W) cm × 15 (H) cm. Two removable Plexiglas partitions were inserted to divide the box as two equal-sized large compartments (20 cm × 15 cm × 15 cm) with a small middle gray zone (5 cm × 15 cm × 15) separating both compartments. Both large compartments were distinguish by different visual and tactile cues. The compartment on one side had white walls with vertical black stripes and a white wire mesh on the floor served as a white compartment. While the compartment on the other side had black wall with horizontal white stripes and a polished black floor served as a black compartment. A visible line is drawn on the floor of all three compartments of CPP box. Twenty-seven squares with a measurement of 5 cm × 5 cm were drawn on the floor. A transparent Plexiglas lid was attached to this apparatus to observe the animal behavior through a webcam (Logitech HD) mounted above the CPP apparatus connected to a computer. This specific apparatus was earlier used to demonstrate a successful establishment of ethanol and METH-induced CPPs in mice in our laboratory.[24,26,27]

Effect of methanolic extract of *Mitragyna speciosa per se* on conditioned place preference in mice

Conditioned place preference procedure and experimental design

The experiment was performed as described previously.[24-26] Habituation, preconditioning, conditioning, and postconditioning are the four distinct phases in CPP methodology. Figure 1 illustrates the experimental design of the study. All tests were carried out over the same time period daily.

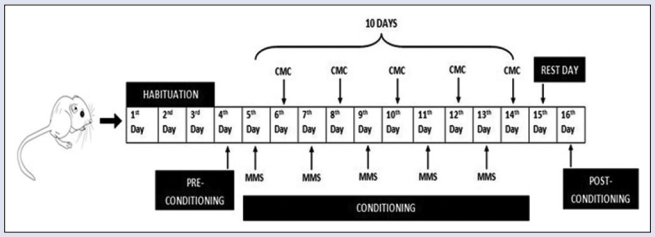

Figure 1: Experimental design and timeline

Habituation and preconditioning

Naïve mice were acclimatized in CPP box for 3 days by placing each mouse in the middle compartment (gray) for 5 min before allowing the animals to access both compartments freely for another 15 min by lifting both removable partitions. On day 4 (preconditioning day), the experiment was conducted in a similar pattern as habituation except the recording of preconditioning baseline (the times spent of mice in each compartment) as described previously.[26] In addition, MMS-induced locomotor activity/sensitization was assessed concurrently in this study. The locomotion of each mouse was assessed by counting the number of lines traversed by the animal on the CPP floor during preconditioning and postconditioning tests.[25] The animals were immediately transferred to the respective home cage at the end of each trial. Conditioning scores were calculated based on the following formula: conditioning score = time spent (white compartment) − time spent (black compartment). Mice which had indicated high inclination (>66% conditioning score) or a high aversion (<33% conditioning score) were exempted from the study.

Conditioning

The conditioning sessions were initiated a day after preconditioning test for 10 days (day 5–14). During conditioning, the mice received a varying doses of MMS (50, 75, 100, 250, 500, and 1000 mg/kg, p.o.) or CLZ (1 mg/kg, p.o.) or METH (0.5 mg/kg, i.p.) and confined immediately in the white compartment for 60 min (for drugs administered orally) and 30 min (for drugs administered i.p.). However, on alternate days, MMS/CLZ-treated mice received CMC (1% w/v; 1 mL/100 g, p.o.); METH-treated group received SAL (1 mL/100 g, i.p.) and confined in the black compartment for 60 and 30 min, respectively. On the other hand, the vehicle control group received CMC (1% w/v; 1 mL/100 g bw, p.o.) and the SAL control group was injected with SAL (1 mL/100 g bw, i.p.) on both compartments (both white and black compartments) during the entire 10-day conditioning session (both odd and even days). Upon completion of conditioning session, the animals were sent back to their corresponding home cages.

Postconditioning

The postconditioning test was performed after 48 h of last conditioning. The mice were not administered with MMS, METH, CLZ, CMC, or SAL on postconditioning test day (day 16). The drug-free mice were kept for a period of 5 min in the middle gray compartment, and later, the partitions were lifted to ensure that the mice could access both compartments without restriction for 15 min. The halting time in each compartment and the total number of lines traversed (to assess locomotor sensitization) by the animal were recorded. Later, a trained personnel with no knowledge of the treatment design analyzed the data. Conditioning scores were calculated as mentioned earlier in preconditioning.

Effect of methanolic extract of *Mitragyna speciosa* treatment on locomotor activity in mice

An actimeter procured from Orchid's Scientific, Nasik, India (model: ACT01), was used to measure the spontaneous locomotor activity in mice. The actimeter possess 32-infrared sensors with a transparent square Plexiglas arena (50 cm × 50 cm). Mice were habituated to the test chamber for 3 days once daily for 10 min, to avoid initial exploratory behavior when exposed to a new environment for the first time. No significant change in locomotor activity was observed on the 3rd day between the groups (data not shown). The animals were segregated into 10 groups ($N = 8$) and treated with SAL (1 mL/100 g, i.p.); METH (0.5 mg/kg, i.p.); CMC (1 mL/100 g, p.o.); CLZ (1 mg/kg, p.o.); and MMS at varying doses (50, 75, 100, 250, 500, and 1000 mg/kg, p.o., respectively). After 60 min and 30 min of oral or i.p. drug administration, respectively, the treated animals were positioned at center of actimeter and the spontaneous locomotor activity was measured for a period of 10 min. The data are projected as the cumulative light beam interruption (locomotive count by 10 min). In between tests, the test arena was sanitized with 20% v/v ethanol.

Statistical analysis

The data are expressed as means ± standard error of the mean. Effects of MMS on CPP score and locomotor count were analyzed using repeated-measure ANOVA with *post hoc* Sidak's multiple comparison test and one-way ANOVA with *post hoc* Dunnett's multiple comparison test. For data analysis, GraphPad Prism version 5 (GraphPad Software, San Diego, California, USA) statistical software was utilized. A statistically significant value was set at $P < 0.05$.

RESULTS

Effect of methanolic extract of *Mitragyna speciosa per se* on conditioned place preference in mice

The repeated-measures ANOVA results indicated a significant effect of trial ($F(1, 140) = 35.00$; $P < 0.0001$) and treatment × trial interaction ($F(9, 140) = 1.927$; $P = 0.0527$) and a nonsignificant effect of treatment ($F(9, 140) = 1.711$; $P = 0.0918$). The *post hoc* Sidak's multiple comparisons test (preconditioning versus postconditioning) revealed that MMS at higher doses (500 and 1000 mg/kg, p.o.) and positive control METH (0.5 mg/kg, i.p.) significantly ($P < 0.01$) induced place preference in CPP test in mice. However, MMS at lower doses (50, 75, 100, and 250 mg/kg, p.o.) and CLZ (1 mg/kg, p.o.) did not significantly induce CPP as seen with vehicle-treated (CMC 1% w/v) and SAL-treated control groups, which implies that MMS *per se* at lower doses (50–250 mg/kg, p.o.) and CLZ (1 mg/kg, p.o.) did not induce CPP in mice [Figure 2a]. In addition, it has been documented that the locomotor sensitization due to repeated administration of drugs in CPP test was failed to achieve (treatment [$F(9, 70) = 1.131$; $P = 0.3533$; trial [$F(1, 70) = 2.549$; $P = 0.1149$]; and treatment × trial interaction [$F(9, 70) = 0.7379$; $P = 0.6729$]). In general, the drugs of abuse have a potential to induce behavioral sensitization (increase in locomotor activity) in rodents, and this phenomenon is thought to share neural mechanisms with drug craving in humans. In this study, METH nonsignificantly increases the locomotor activity. However, all tested doses of MMS and CLZ failed to show any behavioral (locomotor) sensitization in mice [Figure 2b].

Effect of methanolic extract of *Mitragyna speciosa* treatment on locomotor activity in mice

Based on one-way ANOVA results ($F(9, 70) = 0.6134$; $P = 0.7816$), all tested doses of MMS (50–1000 mg/kg, p.o.), METH (0.5 mg/kg, i.p.), and

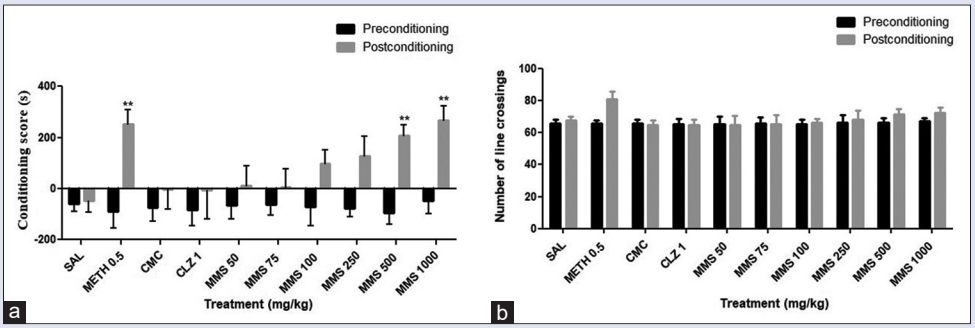

Figure 2: The effect of MMS *per se* on place preference in mice. (a) The data represent the differences between the times spent in the compartment associated with drugs (MMS/METH/CLZ) and vehicles (CMC/SAL). The negative values represent a preference for the black compartment and vice versa. (b) The number of line crossings in CPP box. Each bar represents the mean ± SEM (n = 8). Statistical significance was observed at $^{**}P < 0.01$ when compared between pre- and post-conditioning scores. MMS: Methanolic extract of *Mitragyna speciosa*; METH: Methamphetamine; CLZ: Clozapine; CMC: Carboxy methyl cellulose; SAL: Saline; CPP: Conditioned place preference; SEM: Standard error of the mean

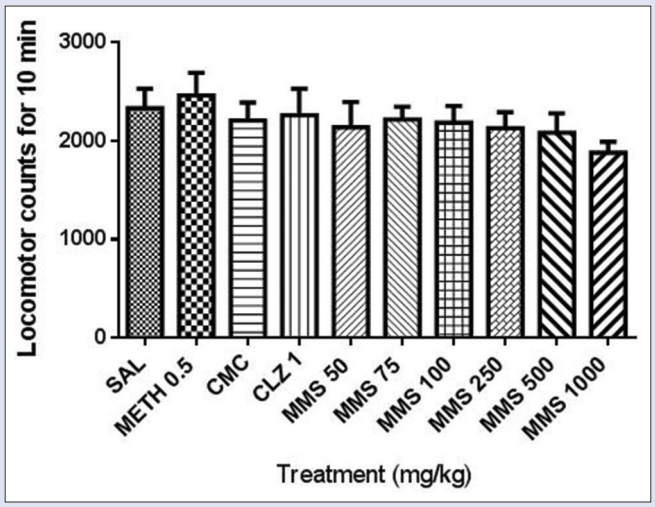

Figure 3: Effect of the acute treatment of MMS (50–1000 mg/kg, p.o.), METH (0.5 mg/kg, intraperitoneally), and CLZ (1 mg/kg, p.o.) on the spontaneous locomotor activity in mice. Values are expressed as mean ± SEM (n = 8). The statistical differences between the treatment groups (MMS and METH) and vehicle control groups (CMC and SAL) were analyzed using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test. MMS: Methanolic extract of *Mitragyna speciosa*; METH: Methamphetamine; CLZ: Clozapine; CMC: Carboxy methyl cellulose; SAL: Saline; CPP: Conditioned place preference; SEM: Standard error of the mean

CLZ (1 mg/kg, p.o.) did not affect the spontaneous locomotor activity of mice as shown in Figure 3.

DISCUSSION

For the research on psychoactive botanicals for their potential therapeutic benefits, it is necessary to characterize the putative addictive liabilities and motor impairment of botanicals in animal models. This component of research is important to ensure that the therapeutic efficacy of the botanicals outweigh their side effects. In this study, MMS's effect *per se* on place preference test and locomotor activity was evaluated.

From the data obtained, like a vehicle control group (CMC), the treatment groups CLZ (1 mg/kg) and MMS (50–250 mg/kg) did not alter place preference in mice. This result validated the initial findings of Sufka *et al.*,[17] which showed that MMS did not induce a significant CPP at lower doses (50, 100, and 300 mg/kg). In our earlier study, the neuromodulatory effect of MMS on dopaminergic system was tested at a range of doses from lower to higher (50–500 mg/kg). From the data obtained, an inverted bell-shaped dose–response was observed and this study highlighted that the antidopaminergic property of MMS was seen only at lower doses.[5]. Besides that, numerous *in vivo* studies have been conducted using *M. speciosa* on CNS activities at doses between 50 and 1000 mg/kg.[8,17,21,22,28] Therefore, it has been postulated that the addictive properties of MMS could be lost at lower doses due to predominant antidopaminergic activity at these doses. Coincidently, many therapeutic effects of *M. speciosa* leaf extract were reported at lower doses.[5,12,13,29]

On the other hand, MMS produced a significant place preference at higher doses (500 and 1000 mg/kg) in the mouse CPP test, which was comparable to the result obtained for METH (0.5 mg/kg) treated group, which infers that MMS can exhibit rewarding effect only at higher doses. The prime bioactive phytoconstituent of *M. speciosa*, mitragynine has been studied for its interaction with several receptors, including opioid, dopaminergic, serotonergic, and adrenergic receptors that are mainly contributing to various CNS pharmacological effect of this plant. A competitive binding assay showed that mitragynine interact with three types of opioid receptors at varying affinities (high affinity to low affinity: μ, κ, and δ opioid receptors).[7,8,14,30-33] It has been well established that μ-opioid receptors are involved in mood-enhancing and euphoria effects by activating dopaminergic reward pathways.[34] Therefore, the rewarding effect of MMS at higher doses might be mediated by the interaction of mitragynine with μ-opioid receptors. Moreover, MMS at higher doses (>100 mg/kg) was found to facilitate the dopaminergic neurotransmission as demonstrated in earlier publication.[5] Therefore, the abusive liability of MMS at higher doses could be stem from its facilitatory effect on μ-opioid receptors and dopaminergic system. Further receptor–ligand-binding assays using MMS at varying doses are essential to validate the exact MMS's mechanism of action for its addictive properties at higher doses.

It is well known that a repeated administration of addictive drugs enhances the motor activity, a phenomenon called behavioral (locomotor) sensitization.[35] Interestingly, in this study, though MMS at higher doses produced a significant place preference, it did not affect the locomotor activity (behavioral sensitization in CPP test) of the mice. Based on the literature, it has been hypothesized that mitragynine, the major phytoconstituent of MMS, might attribute to the rewarding effect of MMS. In a study conducted by Mohammad

Yusoff *et al.*, mitragynine *per se* was reported to produce a significant place preference at higher doses (>10 mg/kg). However, mitragynine at all tested doses (1, 5, 10, or 30 mg/kg) did not alter the locomotor activity in the CPP test.[36] Besides, this reported study revealed that mitragynine exhibited CPP without causing locomotor sensitization or conditioned locomotor effect, and these rewarding properties of mitragynine might be the key factor responsible for the development of addiction following *M. speciosa* consumption.[33] On the other hand, the reference drug METH (0.5 mg/kg) treated group also did not increase locomotor activity during the CPP test. This result corroborates with previous findings, in which the data demonstrated that METH significantly induced hyperlocomotor activity only at 1 and 2 mg/kg but not at a dose of METH (0.5 mg/kg)[37] when given repeatedly daily for 7 consecutive days. In another study, METH at lower doses (0.01 and 0.03 mg/kg) exhibited hypolocomotion whereas METH at moderate doses (0.3, 1, and 2.5 mg/kg) produced hyperlocomotion and METH at higher doses (5 and 10 mg/kg) significantly induced stereotypy in mice.[38]

Generally, CPP test is accompanied with a test for motor activity to eliminate the potential influence on motor activity by the tested compounds that can affect the CPP results. In the present study, MMS at all tested doses (50–1000 mg/kg) did not alter the spontaneous locomotor activity which implies that MMS is devoid of any motor disturbances. This result is consistent with previous findings, in which the spontaneous locomotive activity in mice was not affected by the administration of the MMS (50–200 mg/kg) and its alkaloidal fraction (5–20 mg/kg).[8] Hence, the outcome of the current CPP study is unlikely due to changes in the motor activity.

CONCLUSION

This study shed some lights on the putative abuse liabilities of MMS is seen only at higher doses (>500 mg/kg) and suggests to use MMS at lower doses (<250 mg/kg) for the potential therapeutic outcome.

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Conflicts of interest

There are no conflicts of interest.

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