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Full Length Research Paper

Effect of acute administration of *Mitragyna speciosa* Korth. standardized methanol extract in animal model of learning and memory

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***Mitragyna speciosa* Korth. or ketum is classified under medicinal plants, used by locals in Thailand and Malaysia to treat various types of diseases. Due to lack of information present on the effect of *M. speciosa* in learning and memory function, therefore this study is conducted to understand its effect on cognitive functions (learning and long-term memory). The aim of this study is to evaluate cognitive functions of rats after acute exposure of *M. speciosa* standardized methanol extract using avoidance tasks. Sprague-Dawley rats were treated with three different concentrations of *M. speciosa* standardized methanol extract (100, 500 and 1000 mg/kg). Morphine was given to positive control group and carboxyl-methyl-cellulose (CMC) and piracetam were given to negative control group. Learning and memory were evaluated using one-way passive avoidance test and two-way active avoidance test. This study showed significant improvement in learning acquisition in the *M. speciosa* standardized methanol extract treated group compared to controls, however no benefit was observed on memory consolidation, in both passive and active avoidance tests. In conclusion, acute administration of *M. speciosa* standardized methanol extract facilitated learning, but there was no benefit on long-term memory consolidation.**

Key words: *Mitragyna speciosa* standardized methanol extract (MS), one-way passive avoidance test, two-way active avoidance test, memory impairment.

INTRODUCTION

Mitragyna speciosa Korth. or its common name ketum belongs to the family Rubiaceae and also sometimes known as the Naucleae tribe from the subfamily Naucleoideae. Genus mitragyna can usually be found in swamps and valleys in tropical and subtropical Asia and Africa. Other than Southeast Asian countries such as Thailand, Laos, Cambodia and Malaysia (Burkill, 1966; Jansen and Prast, 1988; Hinou and Harvala, 1988), *M. speciosa* may also be found in the east such as Borneo, Philippines and New Guinea (Burkill, 1966) in the East and West Africa as well as India (Harvala and Hinou, 1988). Genus *Mitragyna* is a large trunk tree and some species may even reach to a height of 100 feet. Though, usually *M. speciosa* only grows 50 feet and is recognized by its globular shape flower with 120 dark yellow florets, shiny green ovate-acuminate leaves arranged opposite to one another and paired decusatively, having a fruit-like capsule with flat seeds (Shellard, 1974). *M. speciosa* is a type of tropical evergreen and non-seasonal plant. This species grows heavily in damp areas rich with humus and is sensitive towards drought and extreme coldness. During extremely cold temperature, the leaves of *M. speciosa* will fall down and become infertile and consequently die (Macko et al., 1972). Genus Mitragyna was previously named by Korthals due to its stigma shape similar to a mitra bishop (Shellard, 1974).

M. speciosa is one of the species classified under

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medicinal plants. It has long been used by locals in Thailand and Malaysia to treat various types of diseases. According to Burkill (1966), *M. speciosa* leaves have antipyretic, antidiarrheal, analgesic, and local anesthetic properties. Besides that, *M. speciosa* is also believed to have energy boosting effects (Shellard, 1974; Harvala and Hinou, 1988; Jansen and Prast, 1988) and mitragynine (major alkaloid of *M. speciosa*) has opioid like properties which can lead to addiction (Matsumoto, 1996a, b). The high dose of the extract (1000 mg/kg) has also been revealed to induce acute severe hepatotoxicity and mild nephrotoxicity (Harizal et al., 2010).

Most studies have focused on pure mitragynine, for its antinociceptive actions on the central nervous system mediated by opioid receptor and numerous studies are conducted on *M. speciosa* because of its similar effect to morphine (Matsumoto et al., 1996a, b; Watanabe et al., 1997; Thongpradichote et al., 1998; Idid et al., 1999; Yamamoto et al., 1999; Matsumoto, 2006a, b). Lack of investigation has been conducted on the effect of *M. speciosa* standardized methanol extract on the aspect of learning and memory processes. A study conducted by Apryani et al. (2010) suggested that chronic administration of mitragynine can altered the cognitive behavioural function (working memory) in mice by using object location task and the motor activity in open-field test. Due to lack of information present on the effect of *M. speciosa* in learning and memory function, therefore this study is conducted to understand its effect on cognitive functions (learning and long-term memory). Avoidance task as a measure to assess cognitive behavioural function has been associated with memory consolidation studies since it is relatively fast to acquire within seconds and is long-lasting, and it occurs in CA1 (hippocampus region) where long-term potentiation (LTP) has been studied (Izquierdo et al., 2008).

The present study investigated the acute administration of *M. speciosa* standardized methanol extract to the cognitive behavioural function (learning and long-term memory) in rats through one-way passive avoidance test and two-way active avoidance task.

MATERIALS AND METHODS

Plant material

The *M. speciosa* leaves were collected from Jengka, Pahang, Malaysia. The plant was harvested and identified by Forest Research Institute Malaysia (FRIM). These leaves were thoroughly washed with distilled water to remove dirt. The wet leaves were weighed and then dried in an oven at 50ºC for 12 h. The dried leaves were ground into a fine powder by a mill machine and the powder was weighed.

Methanol crude extract preparation

One hundred grams of the powdered leaves was exhaustively soxhlet extracted in absolute methanol (MeOH) using an Ace Soxhlet Extractor 6730 and condenser 6740 (Quick fit, England) for 4 h at 60ºC. The dark green extract was then concentrated under reduced pressure at 40ºC using rotary evaporator. The extract was further concentrated by allowing it to stand overnight in an oven at 30ºC to remove trace of methanol solvent. The final product yielded 20.0 g of a green solid methanol extract which were then screened for the presence of the alkaloid mitragynine using GCMS. The dried extract was sealed in a bottle and stored in the refrigerator at 4ºC until further used.

Gas chromatography-mass spectrometer (GC/MS)

Analysis was conducted using Agilent Gas Chromatography 6890N (Agilent, Atlanta) and Agilent Mass Spectrometer 5973I with HP-5MS column (diameter 0.25 mm, length 30 m, film thickness 0.25 um) and helium gas as the carrier. The inlet temperature of gas chromatography was set to 280ºC with splitless mode. One microliter of sample was injected into the GC/MS system. Gas flow was set to 1.2 mL/min. The initial oven temperature was 70ºC, increased to 280 at 30ºC and the final temperature was maintained for 18 min. MSD Transfer Line Heater was set to 285ºC. The target ion for mass spectrometer was 186, 199, 200, 214, 215, 269, 383, 397, 398 and 399 Da. Automatic peak detection and mass spectrum was performed for a total runtime of 29 min. The suspected mitragynine peak was identified using the peaks of *M. speciosa* spectrum by matching it with the NIST 02 Library and confirmed by matching the retention time with the mitragynine standard (external standard) provided by the Institute of Medical Research Malaysia (IMR). Compounds were quantified using total ion current (TIC) peak area, and converted to compound mass using qualitative calibration curves of external standards.

The amount of mitragynine in the *M. speciosa* standardized methanol extract (MS) was determined by the calibration curve, constructed by using five standard concentrations of mitragynine: 8, 16, 32, 64 and 128 ng/mL. The calibration curve was constructed by plotting the ratio of the abundance of mitragynine from 5000 ng/mL MS to that of the standard (y) against the concentrations of mitragynine (x). The linearity of the curve was evaluated by linear regression analysis.

Standardize extraction

The *M. speciosa* leaves extraction was standardized in reference to the amount of mitragynine content using validated GCMS method. Dried extract were stored at 4ºC and its stability was monitored for 12 months.

Animals

Adult male albino rats (Sprague Dawley) weighing 150 to 200 g were obtained from the breeding colony of the Laboratory Animal Research Unit (LARU), Universiti Sains Malaysia (USM). They were housed one rat per cage and maintained at a constant temperature on a standard 12:12 light/dark cycle with light on at 7 am. Food and water were available *ad libitum* in the normal acrylic cage. The experiments were conducted according to the ethical norms approved by Universiti Sains Malaysia, Kubang Kerian Health Campus Animal Ethics Committee guideline for animal care. Six groups of rats were used in this study based on different treatments (100 mg/kg MS, 500 mg/kg MS, 1000 mg/kg MS, vehicle (CMC), 430 mg/kg morphine (positive control, 500 mg/kg piracetam (negative control); ten rats per group were tested.

Extract and drug administration

The dried methanol extract was insoluble in saline solution; hence it

was re-suspended using 1% CMC as a vehicle. The suspension was then stored at 4℃ until further used (Amresh et al ., 2007). The MS with various concentrations (100, 500 and 1000 mg/kg body weight), morphine with toxic concentrations (430 mg/kg body weight) and 500 mg/kg piracetam were homogenously mixed together with CMC and administrated orally by using gavage needle to six groups of animals, respectively. The sixth group was taken as a negative control and given vehicle (1% CMC in distilled water). The maximum volume of CMC administered was not greater than 2 mL/100 g body weight. Animals were fasted prior to conducting the experiment (only food but not water was withheld overnight). Following the 24 h of fasting, the animals were weighed and the test substance administered. After the substance has been administered, food may be withheld for further 3 to 4 h.

One-way passive avoidance response

A two compartment step–through passive avoidance (PA) apparatus was used as has been reported previously (Jafari-Sabet, 2006). In acquisition trial, the animals were placed in the illuminated compartment and 5 s later a guillotine door was raised. After entering the dark compartment, the guillotine door was closed and immediately a 50 Hz, 1 mA constant current shock was applied for 1.5 s. Training was terminated when the rat remained in the light compartment for 120 consecutive seconds. The number of trials (entries into the dark chamber) was recorded (Motamedi et al., 2003).

The rat was then removed from the apparatus and immediately given an oral dose of drug (morphine, piracetam and MS or CMC, respectively). Twenty-four hours after training, a retention test was performed to determine long-term memory. Each animal was placed in the light compartment for 10 s, the door was opened, and the latency for entering into the shock compartment (as described in the training session) was measured. The test session ended when the animal entered the shock compartment or remained in the light compartment for 600 s (criterion for retention). During these sessions, no electric shock was applied (Jafari-Sabet, 2006). The latency of entering the dark compartment (step-through latency = STL) was recorded (Motamedi et al., 2003). The retention test (Day 2) was performed 24 h after the acquisition test (Day 1). Learning was considered if there was a significant increase in the latency on Day 2 compared to Day 1 (Carrie et. al., 2007). In this study, ten rats per group were tested. All responses were analyzed by two blinded reviewers.

Two-way active avoidance response

The apparatus consisted of a shuttle box with two compartments (90 × 2 × 18) separated by a wall and equipped with an electrifiable grid floor (3 mm in diameter, 10 mm apart). The two compartments were connected by a guillotine door. In this study, a lamp (4w) in the shuttle box was used as the conditioned stimulus (CS) and presented for 10 s until the rat crossed to the opposite compartment. If the rat did not cross to the opposite compartment, then it would receive a foot shock (0.5 mA constant current for maximum 5 s). When the rat crossed to the opposite compartment during the conditioned stimulus, an avoidance response was recorded.

All animals which showed positive response were selected to avoid bias. The selected animals were given an oral injection of drug (morphine, piracetam and MS or CMC, respectively). The animals were trained in two consecutive days; each day 50 trials in the shuttle box apparatus, and their memory retention were tested one week later using 20 trails. Active avoidance learning was performed daily at 9 to 11 am (Motamedi et al., 2003). Difference between training session and the test session 1 week later (number of trials) was the indicator for learning process. In this study, ten rats per group were tested. All responses were analyzed by two blinded reviewers.

Statistical analysis

Kruskal Wallis test was done to see any significant changes between *M. speciosa* standardized extract groups, morphine, piracetam and CMC group. Differences occurring at a *p*-value of 0.05 or less were considered significant. If there was any significant differences among groups, a Mann-Whitney test were used instead. All data points showed the mean of ten rats and standard error means (S.E.M).

RESULTS

Plant extraction (detection of mitragynine by using gas chromatography-mass spectrometer)

More than 25 peaks were detected after running the crude extract sample for 40 min in GC-MS (Figure 1). The suspected mitragynine peak was noted at a retention time of 29.43 min. The suspected mitragynine peak was identified using the peaks MS spectrum by matching it with the NIST 02 Library and confirmed by matching the retention time with the mitragynine standard provided by the Institute for Medical Research (IMR) Malaysia.

Passive avoidance response

In this test, the result showed that learning was increased significantly in rats treated with piracetam and MS 1000 mg/kg in the retention test compared to acquisition trial, $p > 0.05$. However, there were no learning processes detected in other groups of study (Figure 2). The step-through latency of rat after treated with all doses of MS showed impairment in memory consolidation (Figure 3). The same effect can also be seen in rat treated with morphine. However, rats from piracetam group showed significant increase in memory consolidation, $p > 0.05$.

Active avoidance response

In this study, the initial result showed that in 2-way active avoidance task, treated group (1000 mg/kg MS) were significantly increased in the first day of training. In the second day of training only piracetam, 500 mg/kg MS and 1000 mg/kg MS treated group showed good performance in learning acquisition (Figure 4). The other two groups (vehicle, morphine and 100 mg/kg MS group) do not escape or avoid the foot shock in the consecutive days of training. Figure 5 showed there is no significant difference of mean number of trials between groups after one week post training. This result indicated that no information was retrieved for memory consolidation of rats in all group of study.

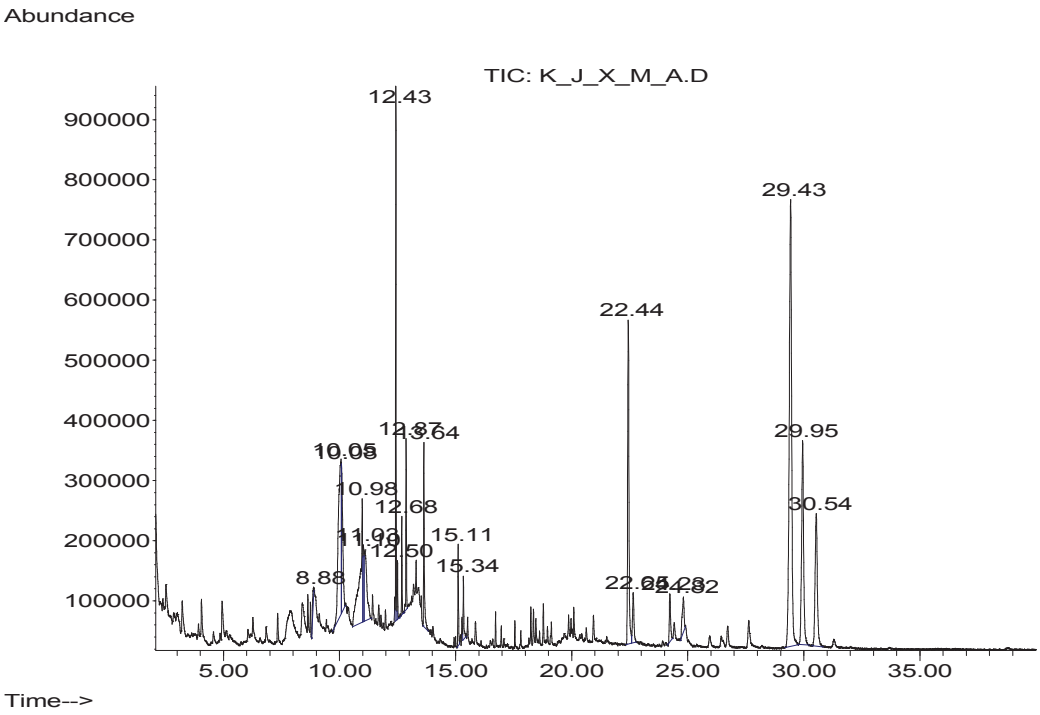

Figure 1. Chromatogram of *Mitragyna speciosa* Korth. standardized methanol extract showing more than 25 peaks of chemical substance.

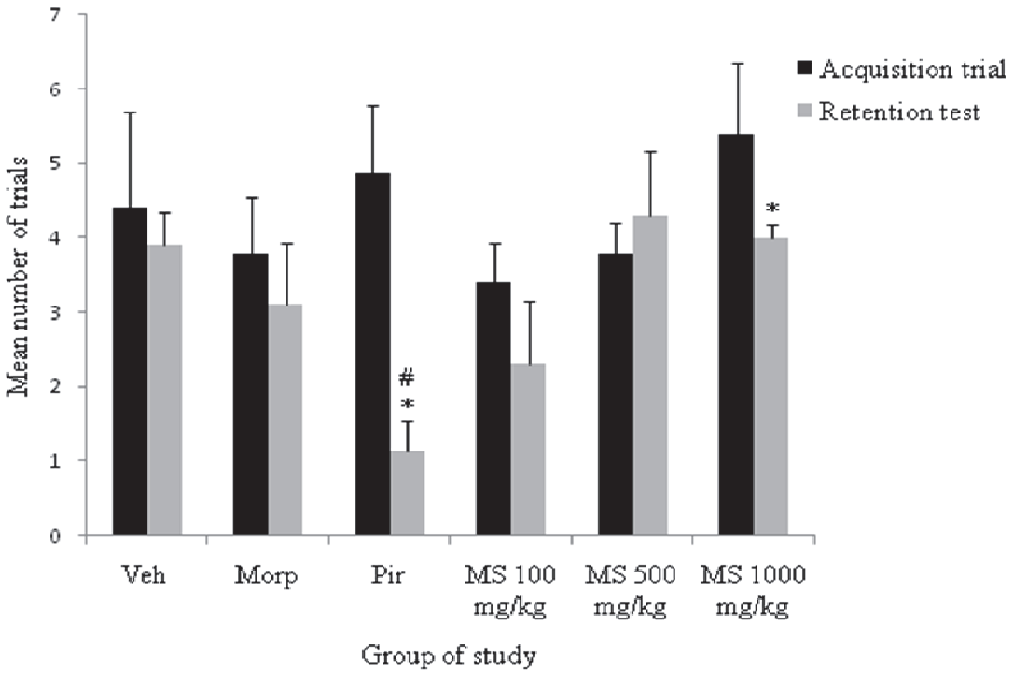

Figure 2. Histogram showed number of trial in rat's passive avoidance response in all groups of animal model. Each data represent the value of n = 10 for each group. * Statistically significant difference compared to the acquisition trial $p < 0.05$. # Statistically significant difference compared to the negative control groups (Veh) $p < 0.05$. Abbreviation, Veh = negative control (CMC), Morp = positive control (430 mg/kg of morphine), Pir = negative control (500 mg/kg of piracetam) and MS = *Mitragyna speciosa* Korth. standardized methanol extract.

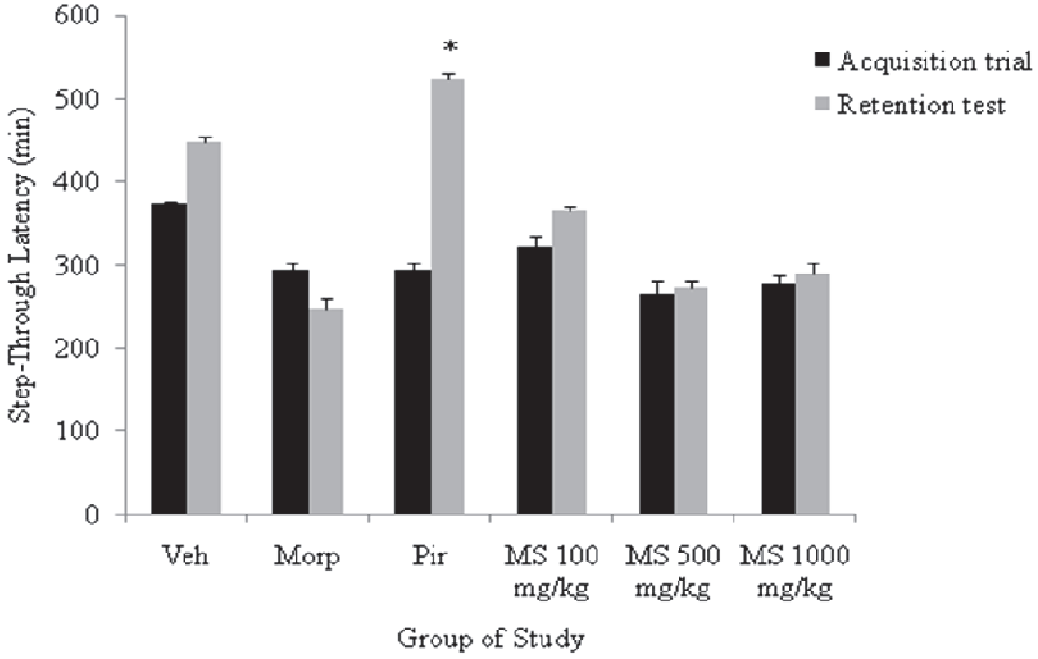

Figure 3. Histogram of step-through latency in rat's passive avoidance response in all groups of animal model. Each data represent the value of n = 10 for each group. * Statistically significant difference compared to the Veh and Morp, $p > 0.05$. Abbreviation, Veh = negative control (CMC), Morp = positive control (430 mg/kg of morphine), Pir = negative control (500 mg/kg of piracetam) and MS = *Mitragyna speciosa* Korth. standardized methanol extract.

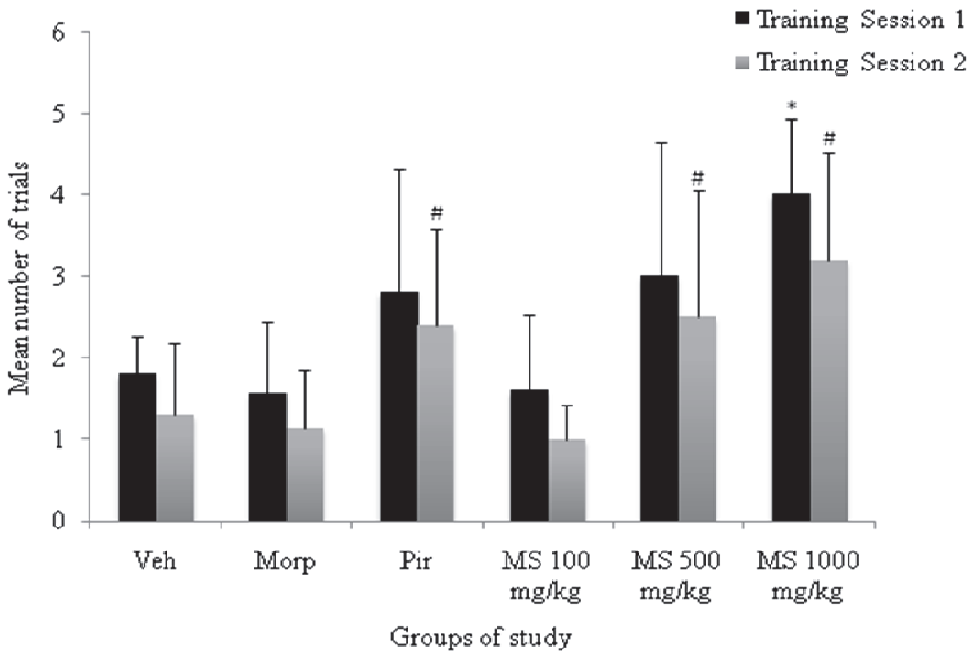

Figure 4. Comparison of active avoidance in two consecutive days training between groups. Each data represent the value of n = 10 for each group. * Statistically different from positive control (Morp) and negative control groups (Pir and Veh), $p < 0.05$. # Statistically different from Morp and Veh treated group, $p < 0.05$. Abbreviation, Veh = negative control (CMC), Morp = positive control (430 mg/kg of morphine), Pir = negative control (500 mg/kg of piracetam) and MS = *Mitragyna speciosa* Korth standardized methanol extract.

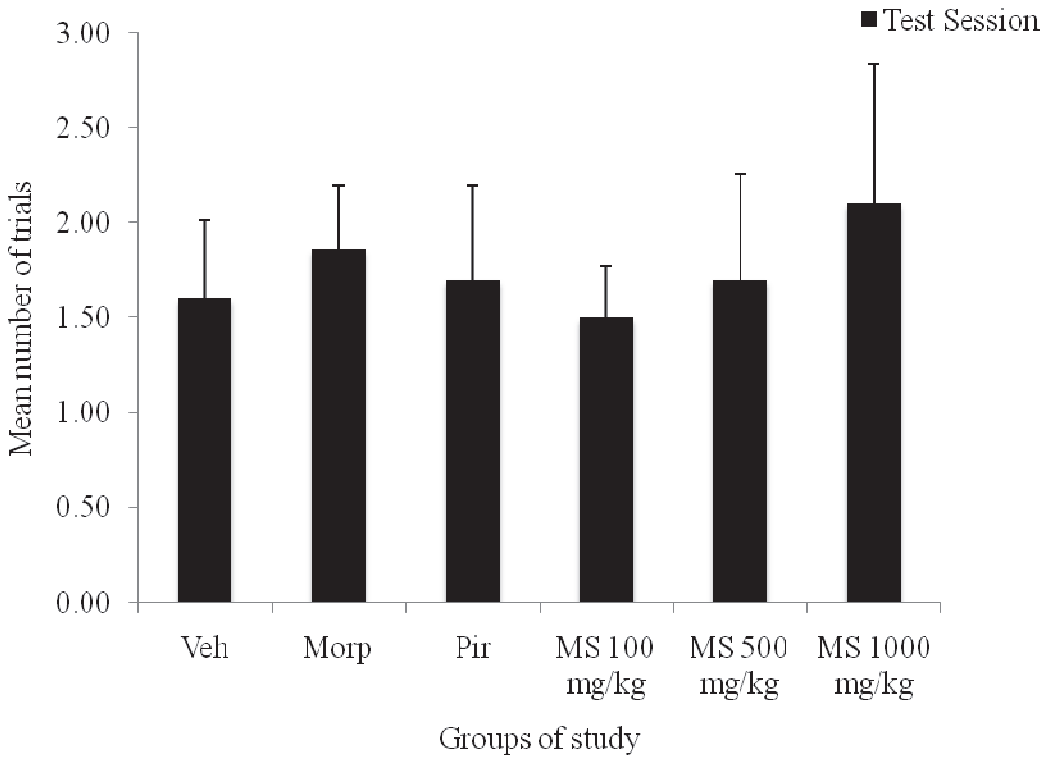

Figure 5. Number of trials of active avoidance response between groups after one week post training. Each data represent the value of n = 10 for each group. This data showed there is no statistical difference from negative and positive control, $p > 0.05$. Abbreviation, Veh = negative control (CMC), Morp = positive control (430 mg/kg of morphine), Pir = negative control (500 mg/kg of piracetam) and MS = *Mitragyna speciosa* Korth. standardized methanol extract.

DISCUSSION

According to present study, the finding indicated that MS (1000 mg/kg) could be regarded as a learning and memory stimulant agent in terms of its facilitator effect on retention of acquired learning. On the contrary, it had minimal effect on the acquisition of passive avoidance learning (Figure 2). Previous studies have shown that pre-training administration of morphine impaired memory consolidation that was partially restored by pretest administration of the same opioid (Jafari et al., 2006). In this study, the effect of MS (1000 mg/kg) on memory is different from that of morphine. Morphine is known to be a competitive opioid agonist. In a study conducted by Motamedi et al. (2003), rats treated with chronic administration of morphine learned the passive avoidance task slower than the control group. The result in this study is in agreement with the report by Jafari-Sabet et al. (2005) demonstrating an impairment of passive avoidance when administered with morphine. As for piracetam, it clearly enhanced passive avoidance learning in rats. Song et al. (1997) reported the ability of piracetam in reversing the deficit in passive avoidance learning in thymectomized rats.

The present result of this study suggests that, rats treated with MS have the ability to avoid the test environment when it previously received a noxious stimulus. The effective dose of MS is found to be 1000 mg/kg. Rat's ability in learning and retaining information about the environment and the situation which it have been exposed to previously during the training period was increased, but the memory consolidation during the test session one week later was impaired. Working memory impairment by mitragynine (major alkaloids of *M. speciosa*) was also described by Apryani et al. (2010).Active avoidance test are also commonly used to examine various memory functions in rat. This test observes the ability of the rats to make a proactive response to escape a chamber where foot shock was previously administered, to the next chamber. A study by Aguilar et al. (1998) has shown the impairing effects of morphine on active avoidance task. This is in accordance with this study showing a significant effect of morphine in rats. Piracetam influences positively on active avoidance learning in this study. This is parallel with findings by Sansone and Oliverio (2002) demonstrating facilitation of active avoidance task when tested animals were administered with piracetam. Our results show that in 2-

way active avoidance task, treated group- 500 mg/kg MS, 1000 mg/kg MS and piracetam group showed good performance. The other groups of study did not escape or avoid the foot shock in the consecutive days of training. This suggests that MS can act as a stimulant in high dosage- 500 and 1000 mg/kg. Thus, at this dose rat can increase its ability in learning about the environment and the situation which it have been exposed to previously during the training period.

Since *M. speciosa* has properties similar to opiate family (Matsumoto et al., 1996a, b), a lot of researches have been done to evaluate and to prove the potential of this plant. Opiate agonists and antagonists may influence memory consolidation in laboratory animals. Reports have suggested that the effective treatments for memory processes depend on interactions among different systems, which is in accordance with the complexity of brain systems (Costa and Xavier, 2007). Previous researches have demonstrated the interaction of opiate with various neurotransmitter and hormones in learning and memory consolidation (Castellano et al., 1996). This leads to behavioural and neurochemical correlations that provide unique information on the biological systems underlying behaviour. Neurotransmitters responsible in modulating memory consolidation are acetylcholine, dopamine, norepinephrine, epinephrine, serotonine, NMDA and AMPA (Hsieh et al., 1998; Zhu and Barr, 2004; Jafari et al., 2006; Jafari-Sabet, 2006). Thus, present significant finding in one-way passive avoidance and two-way active avoidance test raise the possibility of one or more neurotransmitters being facilitated by *M. speciosa*.

Conclusion

In conclusion, acute administration of *M. speciosa* standardized methanol extract facilitated learning, but there was no benefit on long-term memory consolidation. In order to get a clear understanding of learning and memory in animal treated with *M. speciosa* extract, various types of animal behaviour test should be considered. Object recognition test, Y-maze test and T-maze test should be included in future studies to evaluate which pathway is involved in learning and memory after being treated with *M. speciosa* extract.

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