

Behavioural and Electrophysiological Evidence of Impaired Learning and Memory in Male Sprague Dawley Rats following Subchronic Exposure to Standardised Methanolic Extract of *Mitragyna Speciosa* Korth

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Abstract

Background: *Mitragyna speciosa* (MS) or ketum is primarily found in Southeast Asia, particularly in northern Malaysia and Thailand. The medicinal value of this plant has attracted significant attention from both herbal medicine practitioners and scientists worldwide. Despite having illegal consumption status, the plant merits study. We conducted a series of experiments to test our hypothesis that ketum impairs both learning and memory in rats.

Methods: Ketum leaves were extracted using methanol and standardised for the amount of its pure compound, mitragynine. Rats were divided into groups for a passive avoidance task and long-term potentiation (LTP) extracellular recording. In the extracellular recording condition, rats were grouped into control, MS100 (100 mg/kg of ketum extract), MS200 (200 mg/kg of ketum extract), and MS500 (500 mg/kg of ketum extract) groups. An additional group that received morphine was included in the passive avoidance task (10 mg/kg), and there were six animals per group. Rats received daily treatments orally for 28 days for both experiments.

Result: Using a passive avoidance task, our data revealed that the rats' memory significantly increased with increasing doses of MS compared to the morphine-treated group. Our findings from LTP recordings showed that LTP was fully blocked by the higher doses of MS.

Conclusion: We speculate on the possibility that additional factors were involved in the passive avoidance task because it was an in vivo animal study, while the LTP experiment solely involved brain slices.

Keywords: Subchronic exposure, Methanolic extract, *Mitragyna speciosa*

Introduction

Ketum, which is known scientifically as *Mitragyna speciosa* Korth, has been studied for its potentially positive and negative effects on humans. Ketum may contain mitragynine as a pure compound at up to 66.2% (1) of the total alkaloid content, depending on the region. Takayama (2) calculated that mitragynine

comprises approximately 12% of the total alkaloid content in Malaysian species. Ketum has an opiate-like effect and a coca-like stimulant ability that prevents tiredness and increases tolerance for working under high temperature conditions. This plant also acts as a substitute for opium and for weaning addicts off morphine. Pharmacological and phytochemical studies of mitragynine have been conducted to investigate its unique medicinal properties (3–7). Apyani (8) reported

the effects of mitragynine on learning and memory. Using object location tasks and motor activity in an open field test, their results suggest that chronically administered mitragynine could alter cognitive behavioural function, specifically working memory in mice. However, in the present study, we focused on the behavioural and electrophysiological aspects of learning and memory. An experiment in our laboratory has demonstrated that acute administration of mitragynine can induce short-term potentiation in the CA1 region of rat hippocampus (9). As an extension of this study, here we were interested in studying the in vivo and in vitro effects of a crude standardised methanolic extract of *Mitragyna Speciosa* (MS). Long-term potentiation (LTP) is a crucial mechanism of learning and memory. LTP results from coincident activity of pre- and post-synaptic elements, which cause a facilitation of chemical transmission that lasts for hours in vitro, and persists for weeks or months in vivo (10,11). These results led us to hypothesize that MS compounds, especially mitragynine, might impair the activity of ion channels in neuronal cells, particular those in the dendrites. Thus, the aim of this study was to investigate the effects of subchronic MS exposure on LTP in rat hippocampal slices and on the cognitive function of rats in passive avoidance conditioning.

Materials and Methods

Mitragyna Speciosa Korth Methanolic Extraction and Standardisation

The leaves of the plant were collected and thoroughly washed with distilled water to remove dirt. The wet leaves were weighed and then dried in an oven at 50 °C for 12 hours. During this time, the leaves were periodically turned over to provide uniform drying. The dried leaves were ground to fine powder by a mill machine and the powder was weighed. Then, 100 g of the powder was exhaustively Soxhlet extracted in absolute methanol (100% v/v MeOH) using an extractor and condenser (Ace Soxhlet Extractor 6730, Condenser 6740, QuickFit, Staffordshire, United Kingdom) for 4 hours at 60 °C. Next, the extract was concentrated under reduced pressure at 40 °C using a rotary evaporator. Then, it was further concentrated by allowing it to stand overnight in an oven at 30 °C to remove any trace of methanol. The final product yielded 20 g of a green extract, which was then screened for the presence of the alkaloid mitragynine using gas chromatography mass spectrum (GC-MS). The extract produced was standardized with reference to the amount

of mitragynine content using a validated GC-MS method. Dried extract was stored at 4 °C until further use.

Animals

Male Sprague-Dawley rats that weighed 70–80 g of age four weeks were obtained from the breeding colony of Animal Research and Service Centre (ARASC) Universiti Sains Malaysia (USM). They were housed at a density of two rats per cage and maintained at a constant temperature on a standard 12 h:12 h light/dark cycle with lights on at 7 am. Food and water were available ad libitum. The experiments were conducted according to the ethical norms approved by the Animal Ethics, USM.

Administration

There were four groups of animals involved in the behavioural tests, which were control, MS100 (100 mg/kg), MS200 (200 mg/kg), and MS500 (500 mg/kg). An additional behavioural study involved a group that was treated with morphine (10 mg/kg). The extracts were administered to each rat via an oral gavage. The subchronic administration of these drugs were given daily at approximately 10.00 am for 28 days.

Brain Slice Preparation

Transverse hippocampal slices (350 µm thick) were prepared as described by Biessels (12) using a vibrating microtome (HM650V, Microm International, Walldorf, Germany). The slices were maintained in a submerged recording chamber and perfused (1–2 mL/min) with artificial cerebrospinal fluid (aCSF) of the following composition in mmol/l: 124 of NaCl; 3.3 of KCl; 1.2 of KH₂PO₄; 1.3 of MgSO₄; 10.0 of glucose; 20.0 of NaHCO₃; and 2.5 of CaCl₂ and gassed with 95% O₂ and 5% CO₂. All experiments were carried out at room temperature. The slices were allowed to recover for at least 60 minutes incubation after preparation.

LTP Recording

Field excitatory post-synaptic potentials (fEPSPs) were recorded in the stratum radiatum of the Schaffer collateral pathway with glass micro-electrodes filled with the incubation medium (aCSF) placed at the CA1 region of the hippocampus. Bipolar stainless steel stimulation electrodes, insulated except at the tip, were placed on the afferent fibres of the stratum radiatum of the CA3 region of the hippocampus. Before each experiment began, the stimulus intensity to elicit threshold and maximum fEPSPs were

determined. Next, a stimulus-response relation was determined and the stimulus intensity was adjusted to evoke fEPSPs of half maximum amplitude using an amplifier (NPI Electronic, Tamm, Germany) and kept constant thereafter. Stimulation frequency was 0.05 Hz. The first 10 minutes of every recording served as a baseline value. High frequency stimulation (HFS) of 100-Hz tetanisation was given after the recorded baseline was stable. Next, the response obtained was recorded for approximately 1 hour. The average slope of the fEPSP at baseline was set at 100%, and changes in the slope were expressed as a change from baseline. Responses were recorded using CellWorks version 5.1.1 (NPI Electronic, Tamm, Germany).

Behavioural Assessment: Passive Avoidance Conditioning

A passive avoidance task was adapted from Khajehpour (13) with a slight modification. A total of six rats were used in the passive avoidance (PA) experiment. One day after the last administration day (day 28), each rat underwent an acquisition test in a shuttle box (PACS-30, Columbus Instruments, Ohio, USA), and the protocol was set in the software (PASC Shuttle Box V3.13, Columbus Instruments, Ohio, USA). The protocol was as follows: a 5 seconds exploration duration (the time a rat spent in a light compartment before the guillotine door opened), a 2 minutes maximum trial duration, 10 mA of the conditioned stimulus (CS) light intensity, 1.0 mA of the unconditioned stimulus (US) grid intensity, and a 1.5 seconds US grid duration. The US was given immediately after the rat entered the dark compartment. The time the rat took to enter the dark compartment was recorded. The experiment was terminated when the rat completed the 2 minutes maximum trial duration. For the retention test, which was conducted 48 hours after the acquisition test, the same rat was placed in the light compartment, and 5 seconds later, the guillotine door opened for 2 minutes without giving an electrical shock at the dark compartment. The time the rat took to enter the dark compartment was recorded (step through latency).

Results

*GC-MS Analysis of Standardized Methanolic *Mitragyna speciosa* Extract*

The spectrum of the unknown components was matched with the spectrum of the known components stored in the NIST library. The GC-MS analysis of the active compounds of the

standardised methanolic *Mitragyna speciosa* extract (MS) showed there were 17 peaks of compounds detected in the extract. The main alkaloid mitragynine was detected at peak 15 with a retention time (RT) of 19.51 (Figure 1). Mitragynine accounted for 12.27% of the total alkaloid and was the most abundant alkaloid in the plant extract.

Behavioural Studies

The number of trials in the acquisition phase for all groups was only 1 (result not shown), which indicates that all animals learned the passive avoidance (PA) task. There were no significant differences recorded for all experimental groups in comparison to the control group for this phase. Thus, MS in all doses has no effect on PA acquisition. In the retention phase, there were significant differences for all groups compared to the morphine group. These findings demonstrated that MS in all doses does not impair the PA task, as shown by morphine, and might increase the memory of the animals in the higher doses (Figure 2). Furthermore, the paired t test between the acquisition phase and retention phase for each group showed statistically significant differences, with the exception of the morphine group.

LTP Recording After Subchronic Exposure to MS

The LTP data from the brain slices of the subchronically exposed rats were obtained from the 1 hour recording, which consists of 10 of minutes baseline responses and 50 minutes of LTP responses that occurred post-high frequency stimulation. Figure 3 shows the responses obtained after the HFS for groups MS100, MS200, and MS500 when compared to control. The first

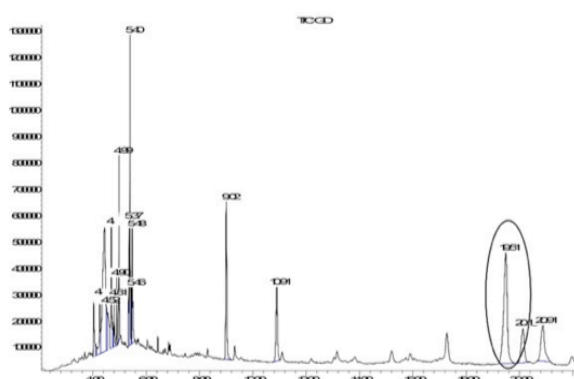


Figure 1: Chromatogram of crude standardized methanolic *Mitragyna speciosa* extract. Note the elliptical area covers the peak of mitragynine.

10 minutes shows the stable baseline responses recorded at the CA1 region of the hippocampus with 0.05 Hz of stimulation. High frequency stimulations consisting of 100 Hz tetani were given immediately after the baseline recording. Note that LTP was induced in the control group. Recordings from the MS100 group shows that LTP was lower compared to the control, and prolonged up to 40 minutes. No LTP events were recorded for both MS200 and MS500. The points represent the means and the error bars represent the SEM. There was an N=6 for the brain slices in each experimental group. The arrows indicate the point of high frequency stimulation.

Discussion

The present series of experiments assessed the role of oral subchronic (28 days) doses of standardised methanolic extract of *Mitragyna speciosa* Korth (MS) on the acquisition and retention of a passive avoidance task, and on the induction and maintenance of long-term potentiation at the CA1 region of the rat hippocampus. The results indicate that subchronic administration of MS at higher doses did not impair memory, as assessed in behavioural studies, but inhibited EPSP-LTP induction in the CA1 region of the hippocampus.

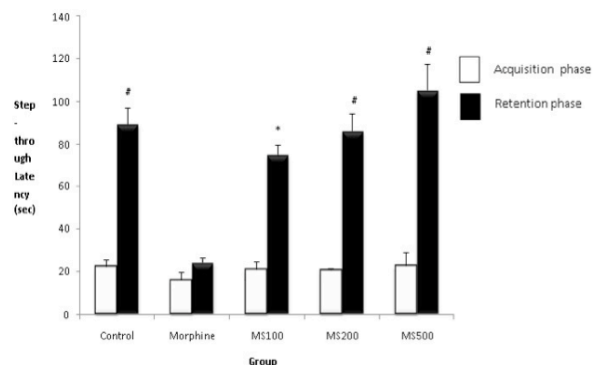


Figure 2: The effects of different doses of MS and morphine as a positive control. The dark bars represent step-through latency during the acquisition phase while the white bars represent the retention phase, which was recorded 48 hours later. The error bars represent \pm SEM. * $P < 0.01$ and # $P < 0.001$ for the comparison of all groups with the morphine group by one-way ANOVA. $N = 6$ for each experimental group.

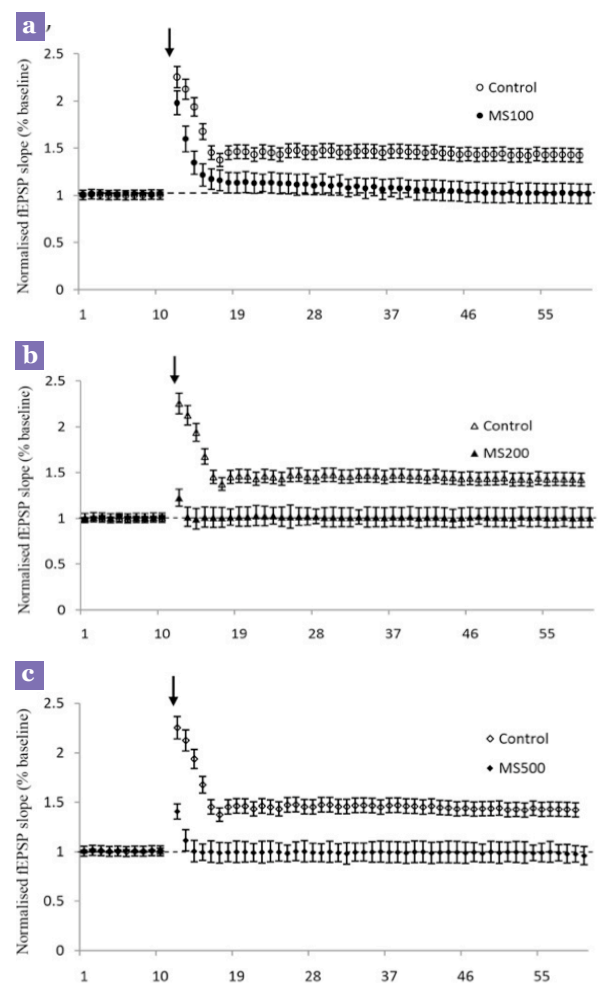


Figure 3: The effects of subchronically-exposed MS to rats' brain slices. The first 10 minutes shows the stable baseline responses recorded at the CA1 region of the hippocampus with 0.05 Hz of stimulation. High frequency stimulations consisting of 100 Hz tetani were given right after the baseline recording. Note that LTP has been induced in the control group. (a) Recording from the group MS100 shows that LTP was lower compared to control and prolonged up to 40 minutes. (b, c) No LTPs were recorded for both MS200 and MS500. The points represent the means, and the error bars represent the SEM. $N = 6$ brain slices for each experimental group. Arrows indicate the point of high frequency stimulation.

Behavioural Studies

Our experiments showed that subchronic oral administration of 100, 200, and 500 mg/kg of MS have no effect on learning in a PA task because all groups learned the task. This result is consistent with an acute study reported by Senik (14). In addition, in the retention phase, the experimental groups and the control group had a significantly higher step-through latency compared to the morphine group. Among all of the groups, only the MS500 group had a higher step-through latency than the control group, suggesting that MS at higher doses increases memory consolidation. A paired t test analysis of the acquisition and retention phase showed that all groups except for the morphine group differed significantly in step-through latency, suggesting that animals learned and became conditioned to the tasks in the shuttle box experiment.

An acute study conducted by Senik (14) for the retention phase showed no significant differences among treatment groups. However, the present study contradicted that acute study because the animals in our study memorised the punishment, while the memory in the animals from the acute study was impaired. Furthermore, mitragynine after chronic treatment impaired cognitive function in mice, i.e., working memory (8). We speculate that improvement in memory consolidation in the MS-treated groups (especially at higher doses) in the present study was due to a combination of the period of exposure and the various compounds present in the extract.

Memory impairment was observed in mice during a step-down passive task with substantial residual effects that lasted as long as 24 hours after the pre-training administration of morphine (15). On the other hand, 30% of the animals in the morphine-addicted group learned passive avoidance slower than the control and sham group (15). This result is consistent with the present study in which morphine-treated animals learned more slowly than other groups.

Electrophysiological Assessments

The electrophysiological results show that subchronic oral administration of MS (200 and 500 mg/kg) inhibited LTP induction following 100 Hz of tetanus at the Schaffer collateral pathway of the hippocampal CA1 region.

LTP is defined as an activity-dependent enduring increase in synaptic activity that is thought to be a cellular correlate of learning and memory. The activation of N-methyl-D-aspartate (NMDA) receptors in the hippocampus is involved in the induction of LTP of excitatory

synaptic transmission. MS is believed to affect the propagation of EPSP, disrupting integration by blocking receptors. LTP depends crucially on glutamatergic receptor activity, especially NMDA and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (17). The connection between synapses is impaired due to the blockage of the receptors and will thus prevent LTP from being induced (18). Several alkaloids, such as mitragynine (19,20), rhynchophylline (1), and corynoxine A (21), have been reported to act as calcium channel blockers. In our study, we speculate that the actions of these alkaloids contributed to the blockage of LTP at the Schaffer collateral pathway of the CA1 region of the hippocampus.

MS acts as a psychoactive drug similar to opium. Chronic exposure of rats to morphine or heroin induces severe drug tolerance and dependence, and markedly reduces the capacity of hippocampal CA1 LTP during the period of drug withdrawal (22). Eisch (23) reported that long-term administration of heroin or morphine reduced neurogenesis in the adult rat hippocampus.

Our passive avoidance task data are not supported by the findings from our electrophysiological assessments. The blocking of hippocampal LTP supposedly reduces the memory consolidation in the shuttle box experiment. Different parts and systems in the brain mediate distinct forms of memory (24). The hippocampal and parahippocampal areas of the cerebral cortex constitute a system that supports declarative or cognitive memory (25). Other forms of non-declarative memory or memories underlying other learned behaviours do not crucially rely on the hippocampus and other structures of the medial temporal lobe, but instead rely on the amygdala, striatum, and cerebellum (26). In the present study, electrophysiological assessments were performed on brain slices (in vitro) that possessed limited neuronal connection, which might contribute to the differences in the findings from the behavioural performance in the passive avoidance task. We also speculate that hormones play a role in mechanisms of learning and memory in the in vivo condition. Korz and Frey (27) found that LTP consolidation and memory retrieval were dependent on β -adrenergic, dopaminergic, and mineralocorticoid receptor activities.

Conclusion

In conclusion, MS affects in vivo and in vitro learning and memory differently. Inhibition

of LTP induction after subchronic exposure to MS was observed in this study, suggesting that MS might act on specific receptors in the brain, particularly receptors in the hippocampus. Furthermore, MS improves memory consolidation to have a positive effect on learning and memory in a passive avoidance task. The standardized extract of *Mitragyna speciosa* may have different effects in vivo compared to the effects in vitro, especially with respect to the functions of the brain. Additional studies of mitragynine should be performed to further understand the specific medicinal properties of the plant.

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Conflicts of Interests

None.

Authors' Contributions

Conception and design, critical revision of the article for important intellectual content, final approval of the article, provision of study materials or patients, obtaining of funding: SMM, JMA
Analysis and interpretation of the data, statistical expertise: MUL
Administrative, technical, or logistic support, drafting of the article: SMM, JMA, MUL

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