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*Full Length Research Paper*

# *Mitragyna speciosa* Korth standardized methanol extract induced short-term potentiation of CA1 subfield in rat hippocampal slices

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***Mitragyna speciosa* Korth or ketum or kratom has long been used by local people in Thailand and Malaysia to treat various types of diseases and to boost energy. There is lack of information available on the effect of *M. speciosa* Korth in learning and memory function, therefore this study was conducted to understand its effect using a cellular model (hippocampus). The objective of this study was to delineate the effect of *M. speciosa* Korth standardized methanol extract (MS), we used extracellular recording in rat hippocampal slices *in vitro*. Acute hippocampal slices were prepared from 4 weeks-old male Sprague dawley rats. Field excitatory post-synaptic potentials (fEPSP) were investigated after the application of test materials in concentrations of 0.0001, 0.001, 0.005, 0.01, 0.05 and 0.1% dissolved in 0.1% DMSO. The 50% inhibitory concentration ($IC_{50}$) of test material was then calculated. Superfusion of MS (all concentrations) produced irreversible fEPSP amplitude reduction with an $IC_{50}$ of 0.008%. The same concentration of MS (0.008%) prevented the induction of long-term potentiation (LTP) and induced only short-term potentiation (STP) in CA1 neurons. In the CA1 region of the hippocampus, reduced concentration-dependently glutamatergic transmission and blocked LTP at the $IC_{50}$.**

**Key words:** *Mitragyna speciosa* Korth standardized methanol extraction (MS), extracellular recording, field excitatory post-synaptic potential (fEPSP), short-term potentiation (STP).

## INTRODUCTION

*Mitragyna speciosa* Korth or ketum has long been used by locals in Thailand and Malaysia to treat various types of diseases. According to Burkill (1966), ketum leaves have antipyretic, antidiarrheal, analgesic, and local anaesthetic properties. Ketum is also believed to have energy boosting effects (Shellard, 1974; Harvala and Hinou, 1988; Jansen and Prast, 1988) and mitragynine (a major alkaloid of *M. speciosa* Korth) has opioid like properties which can lead to addiction (Matsumoto, 1996a, b). Cumulative studies have been conducted to find the pharmacological potential of *M. speciosa* Korth (Kumarnsit et al., 2006, 2007; Shaik et al., 2009; De Moreasa et al., 2009; Aziz et al., 2010; Chittrakarn et al., 2010; Parthasarathy et al., 2010; Taufik et al., 2010), toxicology (Saidin and Gooderham, 2007; Saidin et al., 2008; Harizal et al., 2010) and the behavioural effects (Reanmongkol et al., 2007; Farah et al., 2010). However, only one study emphasized cognitive function (learning and memory). A study conducted by Apryani et al. (2010) suggested that chronic administration of mitragynine can alter the cognitive behavioural function (working memory) in mice by using object location tasks and motor activity in open-field test. There is a lack of information about the effect of *M. speciosa* Korth in learning and memory function, therefore this study attempted to understand its acute effect using an *in vitro* slice preparation (hippocampus). This investigation is crucial to find the potential of *M. speciosa* Korth standardized methanol

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extract (MS) in improving learning and memory. Neuronal potentiation involves several stages depending on calcium influx into the neuron. Long-term potentiation (LTP) is a long-lasting enhancement of synaptic strength which lasts from hours to days and is triggered by high frequency stimulation (HFS) of a critical number of afferent fibers (Castro-Alamancos and Connors, 1996; Bramham, 2010). Synaptic plasticity is fundamental in hypothesizing how the brain forms memories through synaptic changes recorded as LTP, although it does not constitute a memory (Rudy, 2008). The electrophysiological finding is very important to clarify the learning and memory formation associated with *M. speciosa* Korth.

The objective of this study was to delineate the effect of *M. speciosa* Korth standardized methanol extract (MS), we used extracellular recording in rat hippocampal slices *in vitro*.

## METHODOLOGY

### Plant material

The *M. speciosa* Korth leaves were collected from Jengka, Pahang, Malaysia. The plant was harvested and identified by Forest Research Institute Malaysia (FRIM) and conserved at USM herbarium with the voucher number USM 11074. These leaves were thoroughly washed with distilled water to remove dirt. The wet leaves were weighed and then dried in an oven at 50°C for 12 h. The dried leaves were grounded into a fine powder by a mill machine and the powder was weighed.

### Methanol crude extract preparation

One hundred grams of the powdered leaves were exhaustively soxhlet extracted in absolute methanol (MeOH) using an Ace Soxhlet Extractor 6730 and condenser 6740 (Quick fit, England) for 4 h at 60°C. The dark green extract was then concentrated under reduced pressure at 40°C using a rotary evaporator. The extract was further concentrated by allowing it to stand overnight in an oven at 30°C to remove any 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 gas chromatography-mass spectrometry (GC-MS). The dried extract was sealed in a bottle and stored in the refrigerator at 4°C until further used.

### Standardize extraction

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

### Animals and Brain Slice

Four weeks-old male Sprague dawley rats 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.

The rats were anesthetized with chloroform (Sigma Aldrich) and decapitated. Brain slices were prepared during the light-phase. The brains were rapidly removed and placed in an ice-cold artificial cerebrospinal fluid (aCSF). Transversal slices of the hippocampus (400 µm thick) were prepared using a microtome (Microm, Germany). After incubation in a holding chamber with aCSF (22 - 25 °C) for at least 60 min, the slices were placed in the recording chamber and superfused with oxygenated aCSF at a flow rate of 1.5 ml/min (Sajikumar *et al.,* 2009).

### Drug preparation

In the field excitatory post-synaptic potential (fEPSP) experiment, six concentrations of MS were used; 0.00004, 0.0004, 0.002, 0.004, 0.02 and 0.04 g were dissolved in dimethylsulfoxide (DMSO, Sigma Aldrich, Steinheim, Germany). The final concentration of MS were 0.0001, 0.001, 0.005, 0.01, 0.05 and 0.1% while DMSO was 0.1% in the 40 ml of artificial cerebrospinal fluid (aCSF). 0.0083% of MS showed 50% inhibitory concentration ($IC_{50}$) in fEPSP experiment (Figure 7) thus, the concentration of 0.0083% was chosen for the neuron potentiation experiment and therefore, 0.0033 g of MS was dissolved in DMSO. The final concentration of MS was 0.0083% while DMSO was 0.1% in the 40 ml of aCSF. As a control, an aCSF solution containing 0.1% DMSO alone was used. In both experiments (fEPSP and neuron potentiation experiment), 0.1% of DMSO was used as a dilution solvent since the MS was insoluble in aCSF. 0.1% DMSO did not affect synaptic transmission and LTP induction (Huber et al., 1995; Sajikumar et al., 2009).

### Extracellular recording of fEPSP

Acute hippocampal slices were prepared from 4-weeks-old male Sprague dawley rats. Under deep anaesthesia with chloroform (Sigma Aldrich), the rats were decapitated, and the hippocampal formation was rapidly dissected and placed in ice-cold oxygenated (95% $O_2$/ 5% $CO_2$) aCSF containing (in mM): 124 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.2 $KH_2PO_4$, 26 $NaHCO_3$, and 10 glucose. The pH was adjusted to 7.4. Transverse slices (400 µm thick) were cut with a microtome (Microm, Germany) in oxygenated ice-cold aCSF. Slices were incubated in the same aCSF at 27±1°C for at least 2 h prior to electrophysiological recording. Extracellular recordings were made using glass microelectrodes (2 to 3 MΩ) filled with bath solution (aCSF). fEPSPs were recorded using a discontinuous voltage clamp amplifier (SEC 10, NPI electronics, Tamm, Germany) in bridge mode. fEPSPs were evoked by test stimuli (0.066 Hz, 4 to 5 V, 20 µs) delivered via a bipolar tungsten electrode insulated to the tip (tip diameter 5 µm). In the dendritic region of CA1 (Figure 1), fEPSPs were evoked by stimulating the Schaffer collateral-commissural pathway (Sccp). The stimulus intensities were adjusted in a manner to produce responses of about 50% of the maximum amplitude. Various concentrations of *M. speciosa* Korth standardized methanol extract (0.0001, 0.001, 0.005, 0.01, 0.05 and 0.1%) were applied via the super-fusion system with oxygenated aCSF at a flow rate of 1.5 ml/min, respectively. In all experiments, baseline synaptic transmission was monitored for 20 min. All the measurements were conducted by using CellWorks Lite software (NPI electronics, Tamm, Germany).

### Inducing LTP

LTP in the CA1 region was induced by tetanizing the Schaffer test collateral with high frequency stimulation (HFS; 100 pulses/100 Hz).

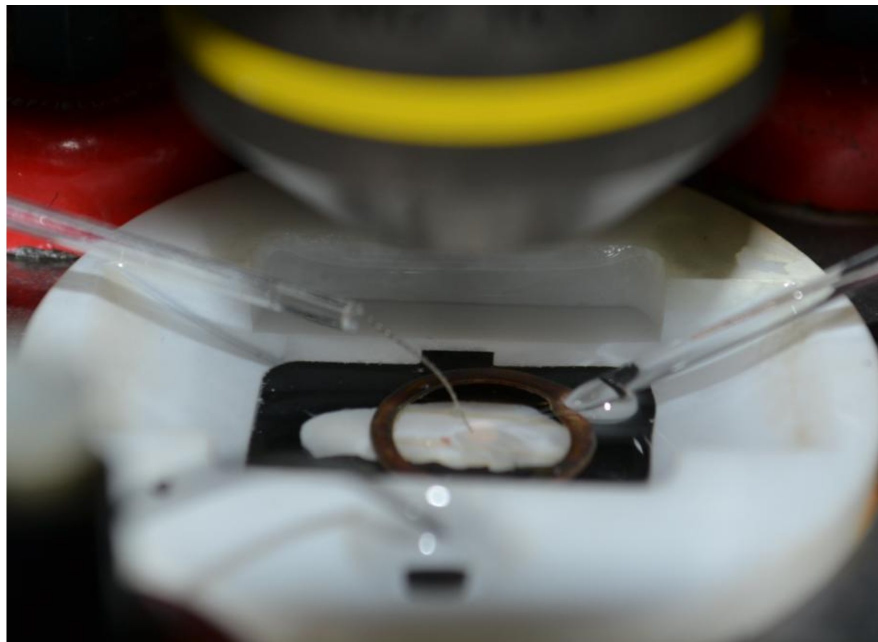

**Figure 1.** Setup of the stimulus electrode and the recording electrode on hippocampal slice during fEPSP experiment.

In all experiments, baseline synaptic transmission with a stimulus was monitored for 20 min before HFS was delivered. The slope of the EPSP was measured between 20% and 80% of the peak amplitude using glass microelectrodes (2-3 MΩ) filled with aCSF (Azad *et al*., 2004). 0.008% of MS + 0.1% DMSO and 0.1% DMSO alone (control group) were applied via the super-fusion system with oxygenated ACSF at a flow rate of 1.5 ml/min, respectively. All the measurements were conducted by using CellWorks Lite software (NPI electronics, Tamm, Germany). The data were pooled by normalizing with respect to the mean values obtained.

#### Statistical analysis

$IC_{50}$ value was estimated by fitting the dose–response curve to the logistic equation and Hill coefficient ($n_H$) was standardized as 1 using Graph- Pad Prism v5 (GraphPad Software). All data points showed the mean and standard error means (S.E.M).

## RESULTS

### Plant extraction (detection of MS using gas chromatography-mass spectrometer)

More than 25 peaks were detected after running the crude extract sample for 40 min in gas chromatography-mass spectrometry (GC-MS) (Figure 2). The suspected mitragynine peak was noted at a retention time of 29.43 min and 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.

### The effects of MS on field EPSPs

Figure 3 shows the effect of MS (0.0001% MS + 0.1% DMSO) on field EPSPs in the hippocampal CA1 area in rats. A concentration of 0.0001% MS + 0.1% DMSO reduced the fEPSP amplitude. In order to determine the recovery from the inhibitory effect of 0.0001% MS + 0.1% DMSO, the peak amplitude of fEPSP was measured during 20 min of washout period (aCSF + 0.1% DMSO) however, no recovery effect was detected (mean from last 10 min was 0.75 ± 0.003 mV).

At a concentration of 0.001% MS + 0.1% DMSO, fEPSP amplitude further decreased 20% more than the previous concentration of 0.0001% MS at 20 min (Figure 4). No recovery effect was observed during the 20 min of washout period. The mean from last 10 min was calculated as 0.56 ± 0.005 mV. Nevertheless, after washout no recovery was detected.

MS (0.005%) reduced fEPSP amplitudes over a range of stimulus levels. Initially, the decrease was observed until approximately 70 min, followed by another subsequent reduction of the amplitude after 70 min (Figure 5). No reversible effect was observed (last 10 min of the mean ± SEM, was 0.55 ± 0.005 mV). Figure 6 shows the inhibitory action of MS (0.01%) on the fEPSP by reducing the fEPSP amplitude. Again, no recovery effect was detected during washout. The mean was 0.18 ± 0.004 mV from the last 10 min. MS (0.05%) evoked a decrease of fEPSP in the CA1 dendritic region (Figure 7). The amplitude of the peak was measured during 20 min of washout. The last 10 min showed a mean of 0.16 ±

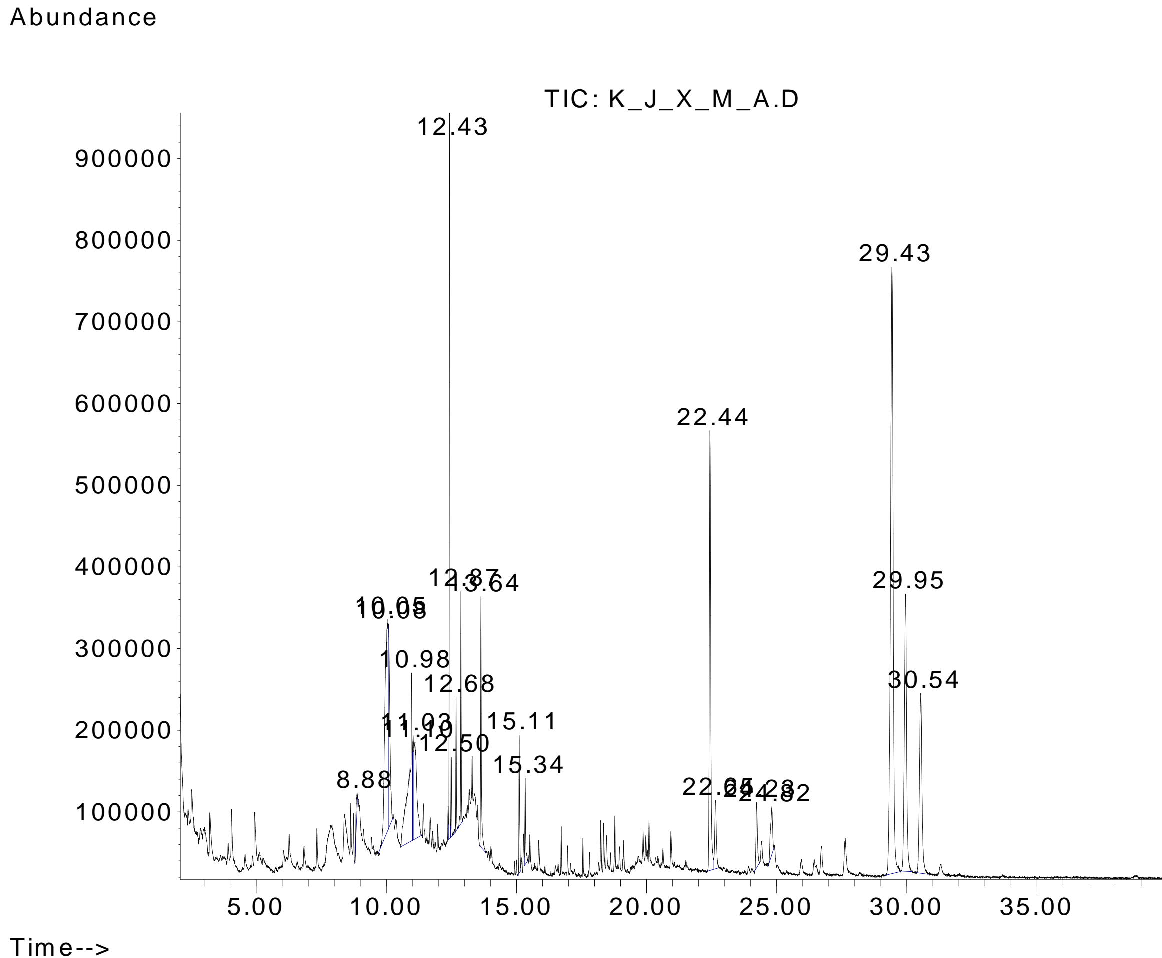


**Figure 2.** Chromatogram of *Mitragyna speciosa* Korth standardized methanol extract showing more than 25 peaks of the chemical substance.

0.007 mV. The fEPSP amplitude was irreversible for approximately 40 min (Figure 8). The recovery from the inhibitory effect exerted by 0.1% MS was measured during washout. Mean of the last 10 minutes was 0.09 ± 0.002 mV.

## Inhibitory concentration 50% ($IC_{50}$)

The values plotted were taken at the same time point (120 min) of all concentrations in fEPSP experiment. According to the dose response curve, 0.008% of MS showed 50% inhibitory effect (Figure 9) with $n_H$ = 1. This concentration was used for the LTP experiments.

## Long-term potentiation

In this experiment, the control group was dissolved in 0.1% DMSO. When using a final concentration of 0.1% DMSO, it was shown not to affect the synaptic transmission (Table 10).

## DISCUSSION

Extracellular post-synaptic potential (EPSP) experiments with various concentrations of MS have to be conducted to get 50% inhibitory concentration (IC50). Since no previous study has been conducted on MS using this approach, therefore there is no reference on exact concentration to be used in this experiment. The $IC_{50}$ concentration is vital to be conducted in order to choose the precise concentration for long term potentiation (LTP) experiment. Six different concentrations of MS (0.0001%, 0.001%, 0.005%, 0.01%, 0.05% and 0.1%) were chosen to cover a wide range of concentration, from lowest until the highest concentration.

Our experiments demonstrate that, in rat hippocampal slices, superfusion of MS resulted in a long-lasting reduction of neurotransmission at CA3-CA1. Normalized fEPSP response from all groups of MS showed an

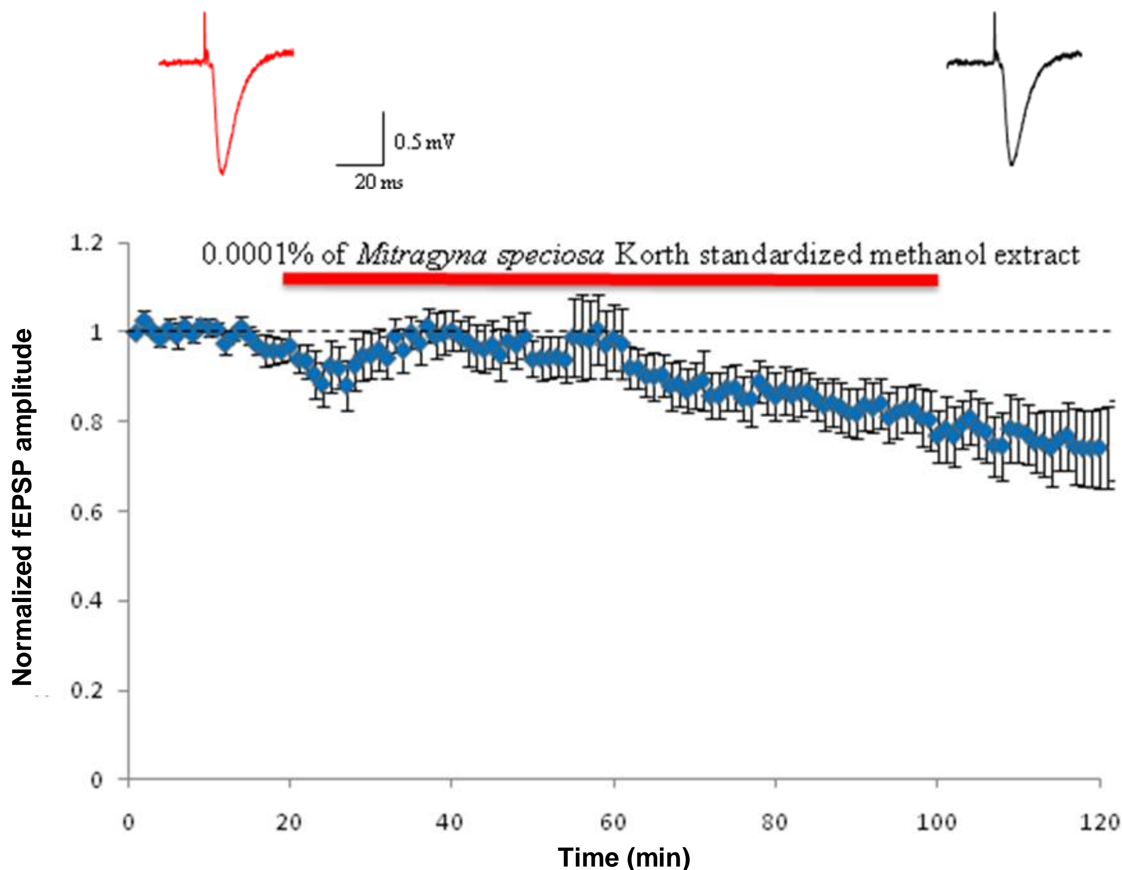


**Figure 3.** Effect of the *Mitragyna speciosa* Korth standardized methanol extract. Stable baseline responses were recorded in hippocampal CA1 slices for 20 min (0.1% DMSO + aCSF). The points represent means ±SEM, (n = 6).

irreversible decrease of the fEPSP amplitude. The drug-induced inhibition of fEPSP was irreversible during washout of at least 20 min. The extract affects fEPSP bidirectional with an excitatory effect at the beginning of superfusion and finally a concentration-dependent inhibition. This trend was because there are many compounds in MS which have various effects (Matsumoto et al., 2004; Takayama, 2004). Since a lot of compounds are present, we cannot differentiate which compound is producing this effect. From the trend we can also postulate that the response showed a continuously decreasing pattern due to the irreversible action of the compounds available. The two highest concentrations (0.05 and 0.1%) of MS showed a consistently decreasing pattern from the beginning. The highest concentration (0.1%) of MS showed drastically decreasing responses, implying that the high efficacy of the irreversible biocompound is producing this response.

With one episode of HFS, LTP which lasted at least two hours were detected in control group (DMSO), as shown by the previous findings of Gozlan et al. (1994), Diana et al. (2003), Chen et al. (2007) and Huang and Kandel (2007). The present finding of the control group (0.1% DMSO) has resulted in LTP. In the present study, the result for the treated group with 0.008% of MS dissolved in 0.1% DMSO showed inhibition of LTP induced, inducing STP. The result showed rapid decay approximately 20 min after HFS which is comparable to the characteristic of STP, lasting from 5 to 20 min (Bliss and Collingride, 1993). The major factor controlling the time course of synaptic potentiation lies on the degree and magnitude of NMDA receptor-mediated with the postsynaptic Calcium ($Ca^{2+}$) increase (Malenka, 1991). This is in accordance with the findings by Hannay et al. (1993) stating that STP is NMDA receptor dependent. Xie et al. (1996) have showed that STP should be both NMDA and AMPA receptor mediated in the dentate gyrus but with a different peak magnitude. STP of AMPA receptor has a peak magnitude of ~30 s after a HFS whereas the NMDA-STP peak was immediately after HFS (Xie et al., 1996). The result for this experiment showed a peak as soon as the onset of HFS was exerted, agreeing with the NMDA-STP.

In this experiment, the initiation of STP was clearly

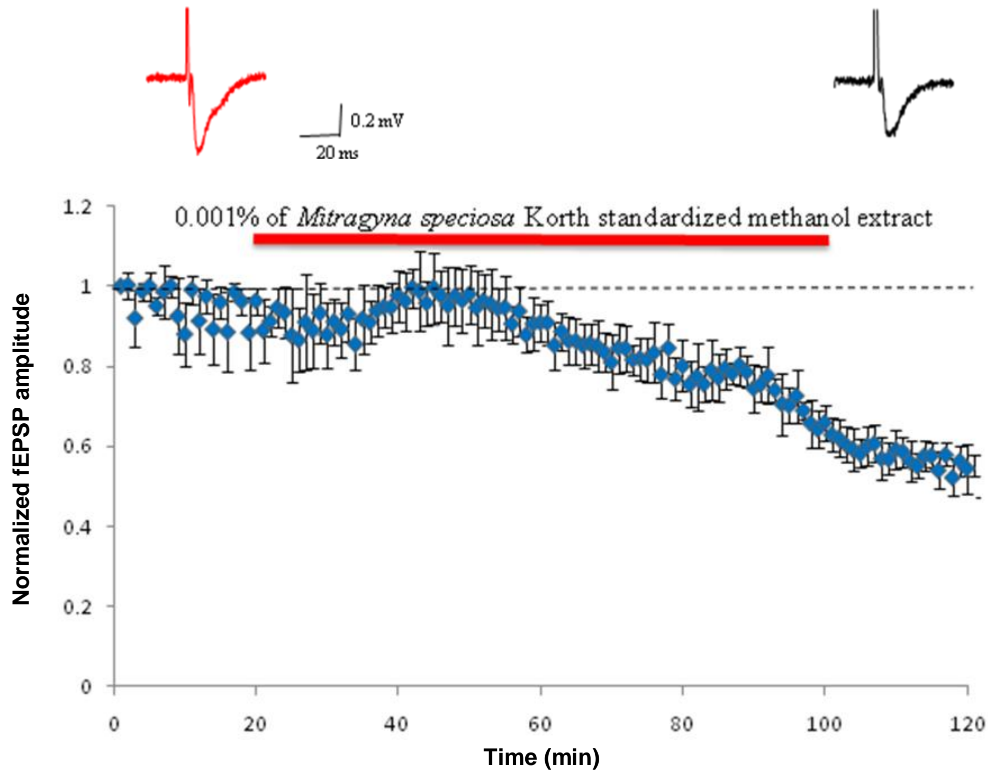


**Figure 4.** Effect of *Mitragyna speciosa* Korth standardized methanol extract (0.001%) in the hippocampal area CA1 of slices. The points represent means ±SEM, (n = 6).

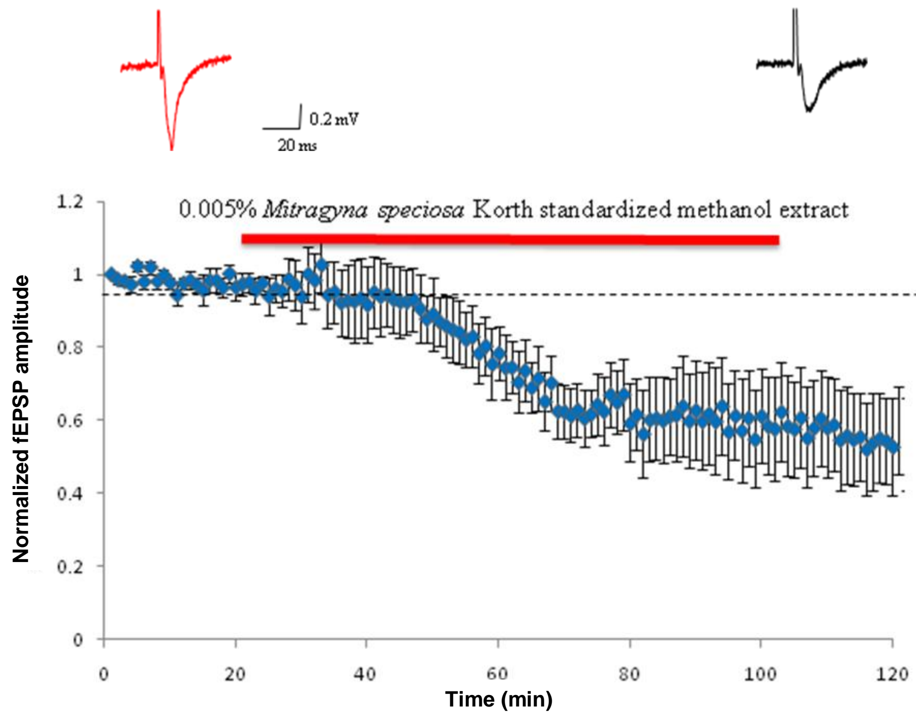


**Figure 5.** Effect of the *Mitragyna speciosa* Korth standardized methanol extract (0.005%) with stable baseline responses recorded in hippocampal area CA1 of slices for 20 min (0.1% DMSO + aCSF). The points represent means ±SEM, (n = 6).

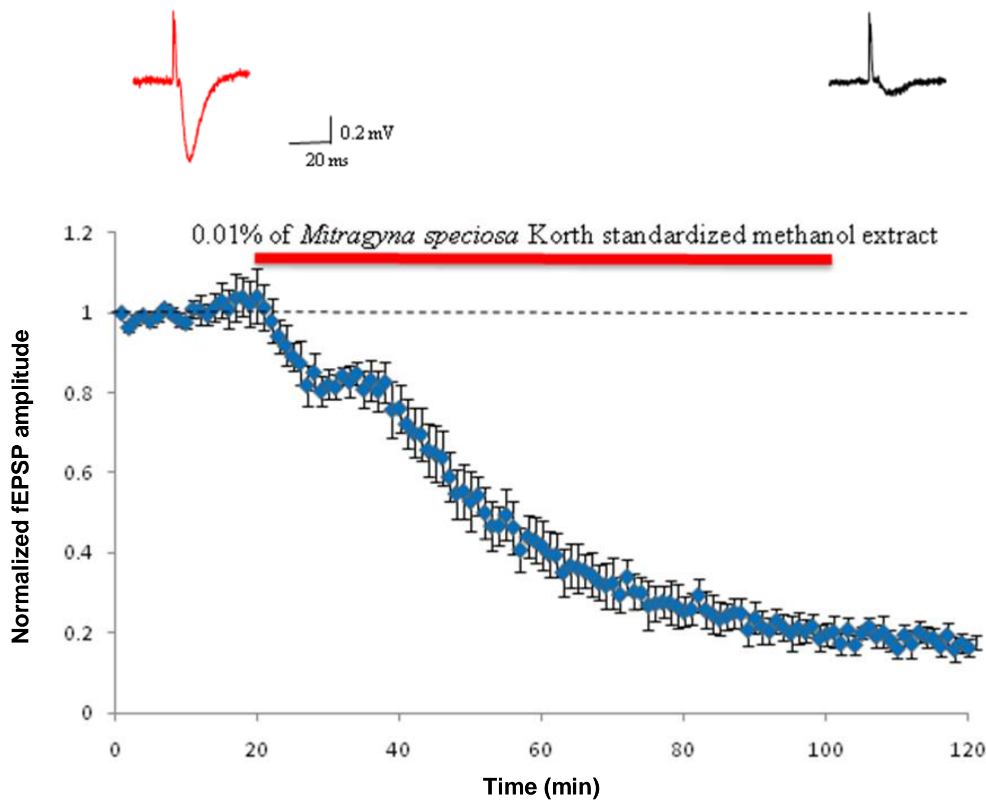


**Figure 6.** Effect of the *Mitragyna speciosa* Korth standardized methanol extract (0.01%) on the amplitude of fEPSP in hippocampal area CA1 of slices. Data are plotted as means ± SEM (n = 6).

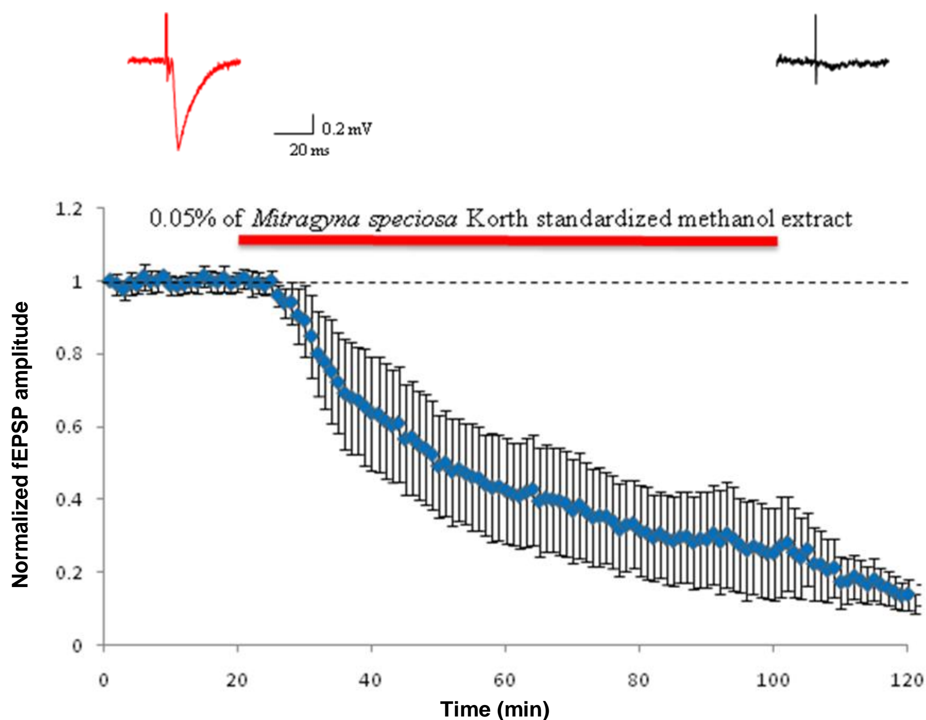


**Figure 7.** Effect of the *Mitragyna speciosa* Korth standardized methanol extract. Stable baseline responses were recorded in hippocampal CA1 slices for 20 min (0.1% DMSO + aCSF). The points represent means ±SEM, (n = 6).

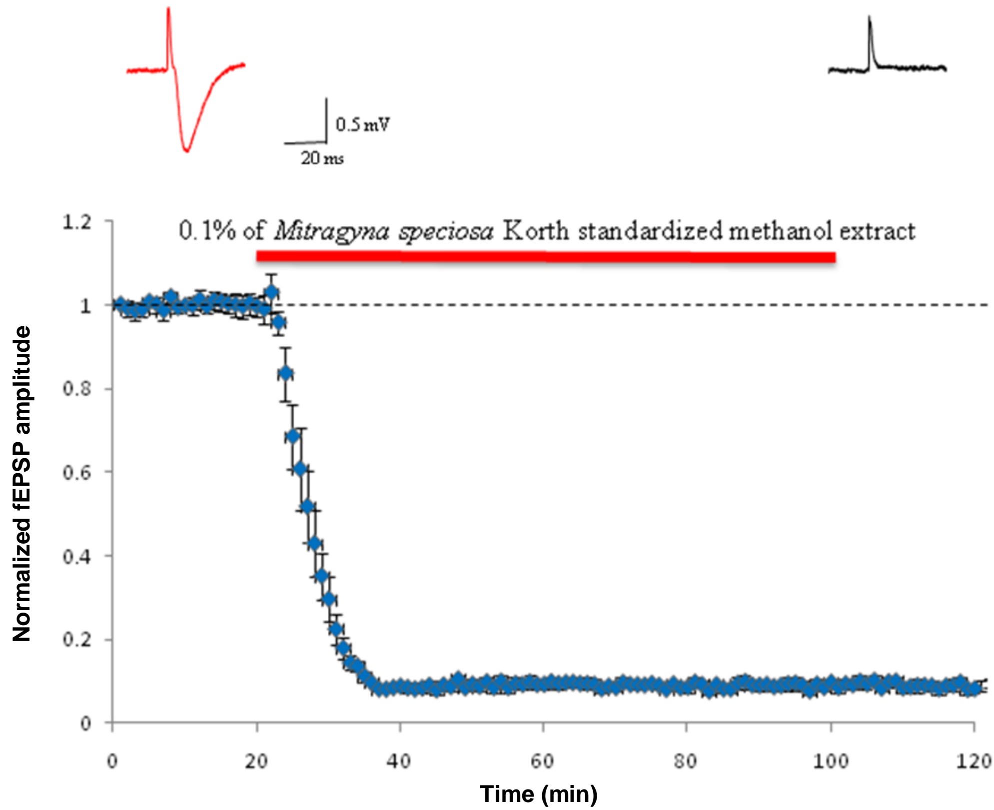


**Figure 8.** Effect of the *Mitragyna speciosa* Korth standardized methanol extract. Stable baseline responses were recorded in hippocampal CA1 slices for 20 min (0.1% DMSO + aCSF). The points represent means ±SEM, (n = 6).

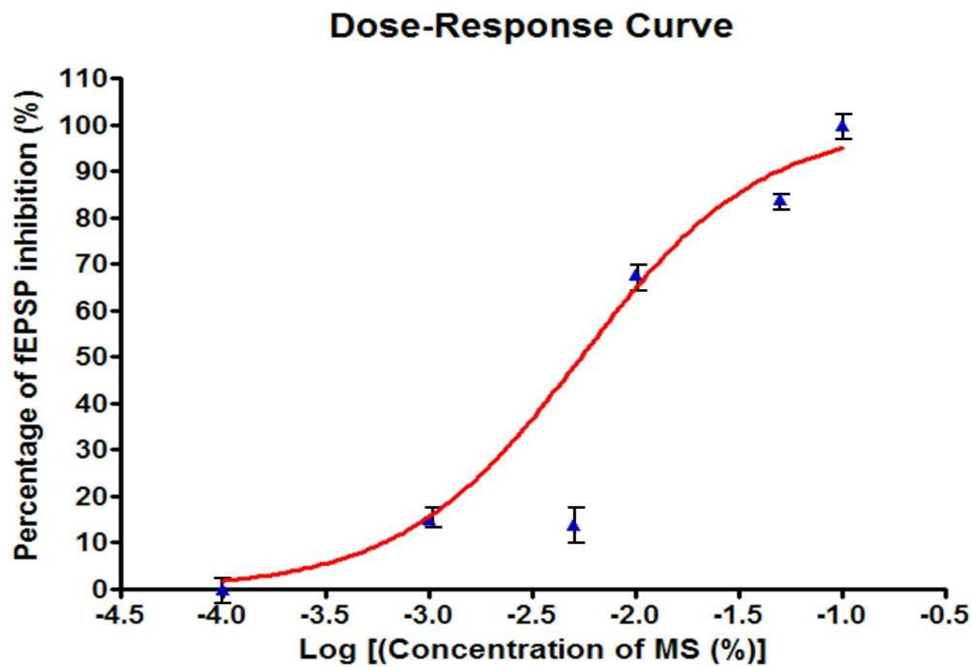


**Figure 9.** Dose response curve of fEPSP inhibitory effect plotted from 6 different concentrations of *Mitragyna speciosa* Korth standardized methanol extract.

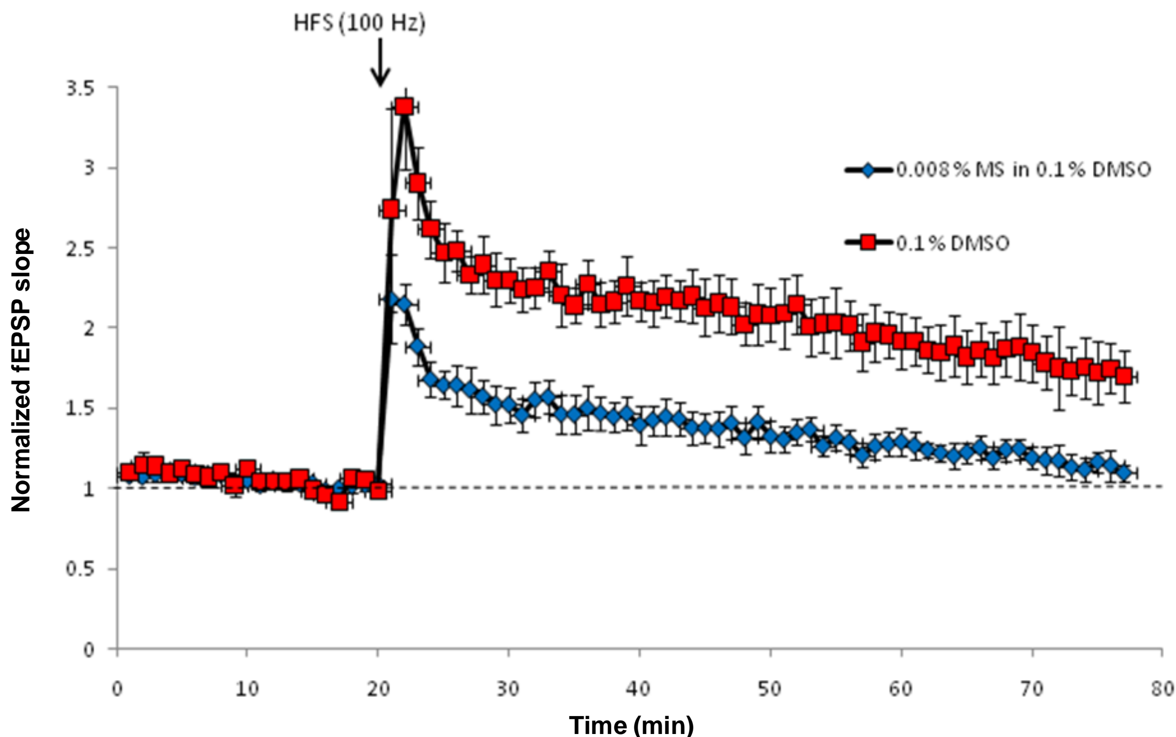


**Figure 10.** Stable baseline responses were recorded in hippocampal area CA1 of slices from both groups for 20 min. LTP-inducing HFS consisted of one train of HFS (100 pulses at 100 Hz). Perfusion with vehicle (0.1% DMSO) resulted in stable LTP (mean from last 10 min, 1.78 ± 0.2). The treated group with 0.008% has shown inhibition of LTP, a short-term potentiation (STP) (mean for the last 10 min, 1.17 ± 0.1). The initial slope of the EPSP has been normalized to the average baseline value during the perfusion. The points represent the means, and the error bars represent the SEM, (n = 6).

seen for the first few seconds of HFS and decayed rapidly to an asymptotic level. This is supported by Xie et al. (1996) showing STP with immediate visible response and fast decaying to a steady level. The time of decay and length of STP increase with the magnitude of initial potential. The biochemical involvement in STP is the inhibition of protein kinase activity induces STP (Malenka, 1991). The second messenger kinases are Ca2+ calmodulin (CAM), protein kinase C (PKC), CAMP-dependent protein kinase and tyrosine kinase are responsible to reduce LTP expression (Xie *et al*., 1996). STP are postsynaptic processes which involve relatively small or brief rises in extracellular and intracellular $Ca^{2+}$ (Hannay et al., 1993). Initiation of STP is mainly triggered by three types of $Ca^{2+}$ channels, the N, P/Q or L-type channel. Inhibition of STP is contributed by application of specific blocker for all these channels at the same time (Quinlan et al., 2008). One of the biocompounds in *M. speciosa* Korth, mitragynine was also found to block T- and L-type $Ca^{2+}$ channel currents and reduce $KCl^-$ induce $Ca^{2+}$ influx in neuroblastoma cells (Matsumoto et al., 2005). Therefore, from this study it might be hypothesized that MS extract inhibit the influx of $Ca^{2+}$.

Mitragynine is associated with opioid receptors, namely µ and δ-opioid receptor (Yamamoto et al., 1999). Opiates have been shown to impair memory functions upon chronic administration (Apryani et al., 2010). The study by Kalivas et al. (2001) has also shown that µ opioid can impair memory functions. When the induction of LTP is inhibited, memory functions are impaired (Apryani et al., 2010). Thus, in this study, mitragynine acted to impair memory processes by inhibiting the induction of LTP.

## Conclusion

In conclusion, MS reduced fEPSP in the CA1 region concentration-dependently and the $IC_{50}$ of MS (0.008%) blocked LTP. The discovery of which specific biocompound of MS is responsible for the mechanism might be an important subject for future research.

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