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# DREAM and C-fos Proteins Expression after Treatment with Malaysian *Mitragyna speciosa.*

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**ABSTRACT**

The herb plant *Mitragyna speciosa* (MS) is indigenous in swampy region of Southeast Asia and its traditionally used as a medicine for various illnesses. Recent researches suggested that MS has analgesic effect and it has sedative and stimulant effects depended on given dose. The study aimed to investigate the histopathological changes and expression of both C-fos and DREAM that could be caused bychloroform-methanolic extract of MS. Dawley's Sprague rats were orally given the extract at three doses (10, 30, 100 mg/kg) for three weeks and restraint for two hours in restrainer device. The histopthological effects were assessed microscopically, and the C-fos and DREAM expression were evaluated via immunoblot. For the histopathological examination there were no significant changes among (testes, and brain), but kidney showed hyperemia at 30 and 100 mg/kg of nonstress and glomerulus shrunked at 30 mg/kg of stress rats, and for liver there was only slight hyperemia at 30 of stress and 100 mg/kg of nonstress groups. The immunoblot results for proteins expression showed that; C-fos was expressed in prefrontal (PFC) was significantly increased ($p<0.05$) at dose 10 mg/kg in both stressed and non-stressed, while it decreased in hippocampus at 100 mg/kg in stressed, and did not show changes in non-stressed rats. DREAM expressed decrease in PFC at 10 mg/kg in stressed animals, while in hippocampus DREAM expression level was raised significantly at 100 mg/kg in both stressed and non-stressed groups. Thus study showed that MS does not cause histopathological changes, but c-Fos and DREAM showed different expression in targeted areas.

**Keywords:** *Mitragyna speciosa*; mitragynine; histopathological; DREAM; C-fos

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## INTRODUCTION

*Mitragyna speciosa* (MS) is a tropical herb tree belongs to Rubiaceae family (coffee family), it grows in swampy territory in the tropical and sub tropical regions of Africa and Asia. Some of MS grow to height of 30 meters. Leaves are the used part from tree, they grow to over 18 cm long and 10 cm wide with an ovateacuminate shape and tapered ends [21], [12]. MS trees called among native citizen as "ketum" in Malaysia and "kratom" in thaland and also named Kakuam, Ithang, and Thom. In Thailand There are two varieties of MS, classification came based on leaf's vein, one with red veins in the leaf, the second with green veins. The red veined leaves are supposed to have the stronger biological activities [5]. Because of MS medical properties, it traditionally used as a treatment for diarrhea and as a substitute in case of opium addiction [22]. (Chuakul, 1995) reported that, traditionally MS often used to treat diarrhea or intestinal infection by amoeba and protozoa [5]. Additionally MS used to elevate pain from cutting wound, treating diabetes, and hypertension [24]. Also native may use MS for it is euphoric effects [1]. MS acts as a stimulant at low dose and a sedative at higher dose [3]. It has been reported as central nervous system stimulant rather than a depressant. (Chittrakarn, 2008). MS leaves are a reach with alkaloids. Shellard in (1974) named twenty two alkaloids. Mitragynine is the dominant alkaloid in MS (66% of total crude extract) [23], this mitragynine effectively reduce the tolerance due to the chronic administration of morphine when it combines with morphine [10]. The two alkaloids in krom mitragynine and 7-hydroxymitragynine (13 times higher potency than morphine, and about 46 times more potent than mitragynine based on animal studies) act as agonists to supraspinal mu and delta-opioid receptors and produce effects similar to morphine [16]. Expression of opioid receptors is higher on the rats' brain compared to other tissues. Mitragynine bound to those three opioid receptors (μ, δ, and κ) but have the higher affinity towards κ opioid receptors [14].

DREAM (Downstream Regulatory Element Antagonist Modulator), of the neuronal calcium sensor (NCS) family [26]. DREAM represents the first known Ca2+-binding protein to function as a DNA-binding transcriptional regulator [8]. DREAM represses endogenous opioid production [7]. DREAM binds to a downstream response element (DRE) on the prodynorphin gene, and repressed dynorphin transcription [9]. DREAM alters apoptosis in neural cells by binding hexokinase I, and reducing mitochondrial hexokinase I localization [7].

C-FOS belongs to the Fos family [17], it is (a product of an immediately early gene *c-fos*) is a transcription factor thought to play a role in neuronal adaptations and brain plasticity [15]. C-Fos protein expression has been used as a marker of neuronal activation [25].

This study was designed to investigate the subchronic histopathological effects of Malaysian MS chloroform-methanol extract. Also the study was designed to evaluate the expression of c-Fos and DREAM proteins in both hippocampus and prefrontal cortex after the administration of MS in subjected to stress and nonstress rats.

## MATERIALS AND METHODS

### Mitragyna speciosa crude extract

Green MS leaves was cleaned, washed with distilled water and left to dry in room temperature, and then dried in the oven at 35°C over night. After leave dry, grinned in blender to obtain powder form. Organic solvent extraction method was used to extract the powdered MS leaves as a described by Houghton and Ikram [27] with some modification. MS powder mixed with 100% methanol in ration 1:3 and soaked for three days. In third day the mixture was filtered , the solvent was poured into bottle and the Soult was mixed with methanol in same ratio and soaked for other three days, soaked and filtered processes were repeated for three times.

The filtered solution was evaporator using rotated evaporated, with water path at temperature 45°C. Continued with evaporator till got the brownish – crystal powder. The powder dissolved into 300 ml of chloroform then transferred into separate funnel, and 400 ml of distilled water was added into the funnel. The mixture was mixed well inside the funnel. Fix funnel into its stand, later two layer will formed, lower layer run out and discarded, add more distilled water to suspension and shake again , wait till layer formed again and discard the lower layer. The procedure was repeated three times. Finally the upper layer which contained chloroform was collected. Chloroform suspension was dried via rotator evaporator to yield the chloroform-methanolic extract of MS, and used to prepare the alkaloid three doses, 10, 30, 100 mg/kg.

### Experimental animals

In this study, 70 adult male Sprague Dawley rats with average weight 250-300 g each will be used. Rats were obtained from the Laboratory Animal Unit, Faculty of Medicine and Health Science, University Putra Malaysia (UPM). Animals were confirmed free from any diseases or abnormalities that could affect the experiments. For familiarization before proceed with any treatment, the rats were housed in cages for at least one week under controlled temperature (25 ± 2), with dark/light environmental cycle of 12 hours. The animals were provided with food (pellet) once a day, and tap

water *ad libitum.* The cages' beddings were changed once a week to maintain rats' cleanness. The experimental rats randomly divided into three groups: one control group (normal saline), which contained 10 rats, and two treated groups (one subjected to stress while the other did not subjected to stress). Each treated group (with and without stress) was divided into 3 sub-groups, 10 rats per group, and each group was treated with a three doses of MS (10, 30, 100 mg/kg).

**Extraction administration**

The chloroform-methanolic extract of MS with various three concentrations (10, 30. and 100 mg/kg) were administered orally by using oral gavages through force feeding into the rats. First the oral gavages was used to feel the trachea and esophagus before feeding and it can be differentiated by the disc forming trachea would have a bumpy movement when passing the gavages. Pharmacological treatment was performed for three weeks.

The experiment was carried out on 70 rats (males) which were divided into three groups. Group (I) was served as a control group which consists of 10 rats was given orally the drug carrier "sterile saline" and the other two groups were Group II (with stress) and Group III (without stress). For the each Group (II) and (III), three different concentrations of MS were forced oral feed at 10, 30 and 100 mg/kg respectively and each was consist of 10 rats equally.

The group (III), rats after feeds MS doses was daily subjected to the immobilization stress for two hours.

**Immobilization stress**

All group (III) rats were assigned to restrain stress for two hours daily after three extract doses were given. Rats were removed from their cage and individually placed into 25 cm × 6 cm (L×D) plexiglas tubes, which length was adjusted with a piston, so that the animal was unable to move.

**Summary of specimens Histology Procedure**

Liver, kidney, testes, and brain were collected and placed in 10% formalin. Organs were grossed in about 3 mm thick, and then grossed samples were processed via tissue processor and embedded in paraffin wax. Paraffin blocks were sliced by using a microtome to produce 4 µm slices and later stained with Hemotoxylin and Eosin (H&E), and Nissl staining was used for staining brain slices.

**Western blot analysis**

In order to release protein from tissue, small tissue sample weighted and placed in labeled eppendorf tube, protease inhibitor tablet solved in sucrose lyses buffer and added to sample. Samples were homogenized with a hand held homogenizer (Polytron PT1600E) on ice for 5 seconds.

Homogenized sample was centrifuged at -4°C in speed of 10000 rpm for 10 minutes. As a result of high speed centrifuging, samples were separated to supernatant and pellet. Supernatant was collected as a cytosolic fraction, while pellet re-centrifuged after 200 µl of sucrose lyses buffer was added. After centrifuge time up, supernatant and pellet were formed. Supernatant was discarded, and 100 µl of lyses buffer was added to pellet to get nuclear fraction.
The protein concentration was measured using Bovine Serum Albumin (BSA) and brad ford reagent protein assay. Test samples and standards were run on 12% SDS PAGE, and then immuno-blotted with primary antibody (anti-DREAM sc 1:500) and (anti-C-fos ab 1:500) and with goat anti rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000 Serotec). The membrane was developed using enhanced laminate for western HRP substrate for chemiluminescente. The developed membrane was viewed using gel documentation (Vilberlourmat chemi smart 3020 wl/26 mx). The optical density of specific bands were measured with Mac Bi photonic ImageJ. The blot was reprobed with β-actin antibody (anti- ß-actin ab 1:10000) to ensure equal protein loading.

**Data analysis**

DREAM and c-Fos expression levels are presented as a percentage and compared to control groups as 100%. Differences among different doses were analyzed by one way ANOVA followed by post-hoc Tukey's multiple comparison tests that was used to compare inter-group results. Significance was presented as $p<0.05$.

**Histopathologic study result**

Examination of following organs kidney, liver, testes, and brain for the Group 2 (treated with stress) and Group 3 (treated without stress) showed normal appearance of their size , shape, and color, at the three dosages 10, 30 and 100 mg/kg ofchloroform- methanolic extraction of MS, by comparing with the control.

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Histopathological investigation for all groups treated show no change in brain and testes tissues, while some histopathological changes presented in liver and kidney. For kidney there was slight glomerulus shrunken in 30 mg/kg stress group, and slight hyperemia in 30 and 100 mg/kg of nonstress groups when compared with control group. As showed in Figures 1, 2 and 3. Liver Histopathology estimate, there were Slight and moderate hyperemia was observed for groups of 100 mg/kg without stressed and 30 mg/kg with stressed. As showed in Figures 4 and 5.

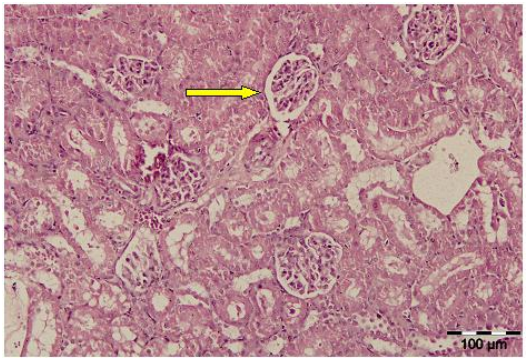


**Figure 1: The slide for rat kidney from group treated with 30 mg/kg of MS and subjected to stress, which was observed under magnification 20x with 0.1 mm measurement scale. Slide shows normal histological structures, yellow arrow represent slight glomerulus shrunken.**

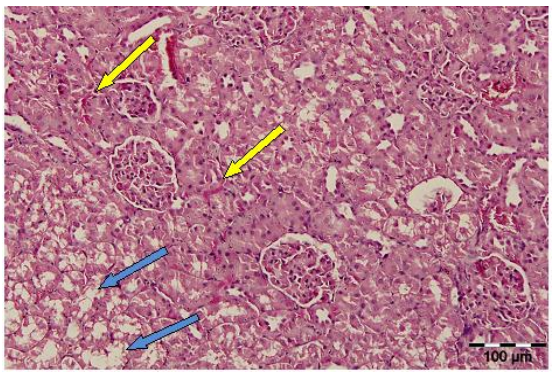


**Figure 2: The slide for rat kidney from group treated with 30 mg/kg of MS and did not subject to stress, which was observed under magnification 20x with 0.1 mm measurement scale. Slide shows normal histological structures, with vacoulation (blue arrows) and slight hyperemia (yellow arrows).**

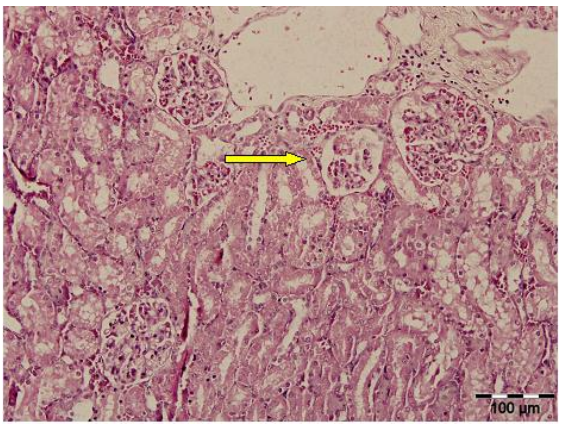


**Figure 3: The slide for rat kidney from group treated with 100 mg/kg of MS did not subjected to stress, which was observed under magnification 20x with 0.1 mm measurement scale. Slide shows normal histological structures, with slight glomerulus shrunken (yellow arrow).**

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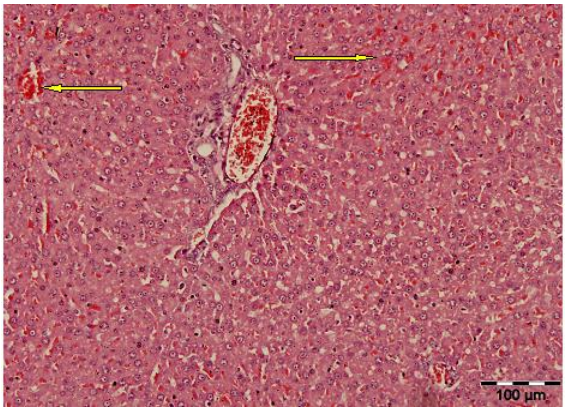



**Figure 4: Light micrograph of rat's liver taken from rat was given 10 mg/kg of MS and did not subject to stress. Specimen slide was stained with H&E stain, and it was observed under 20x power with 0.1 mm measurement scale. Slide shows normal feature and components of liver with moderate hyperemia (yellow arrow).**

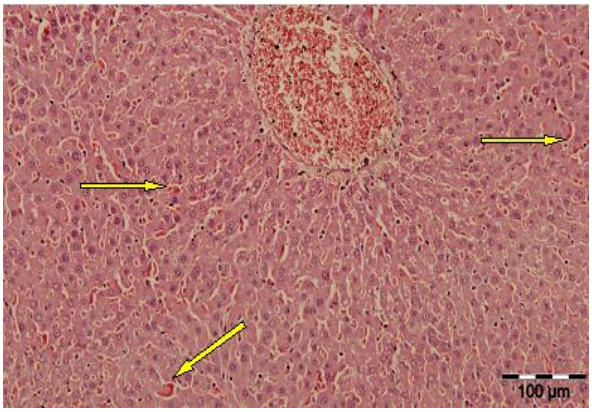
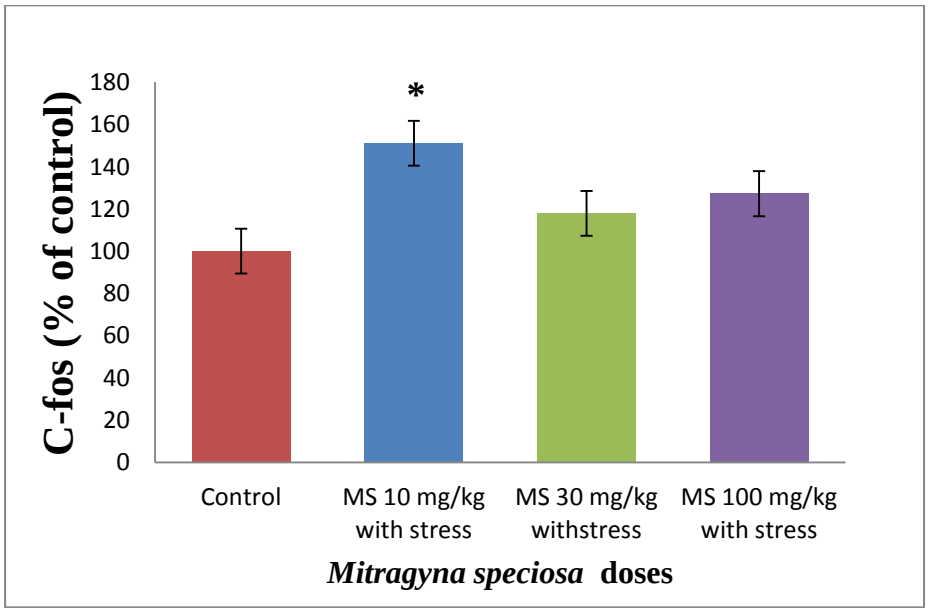



**Figure 5: Light micrograph of rat's liver treated with 100 mg/kg of MS, did not subject to stress. Slide was been stained with H&E stain, and was observed under 20x power with 0.1 mm measurement scale. Slide shows normal feature and components of liver with slight hyperemia (yellow arrow).**

**c-Fos expression**




**Graph 1: c-Fos expression in prefrontal cortex in rats treated with MS and subject to stress**

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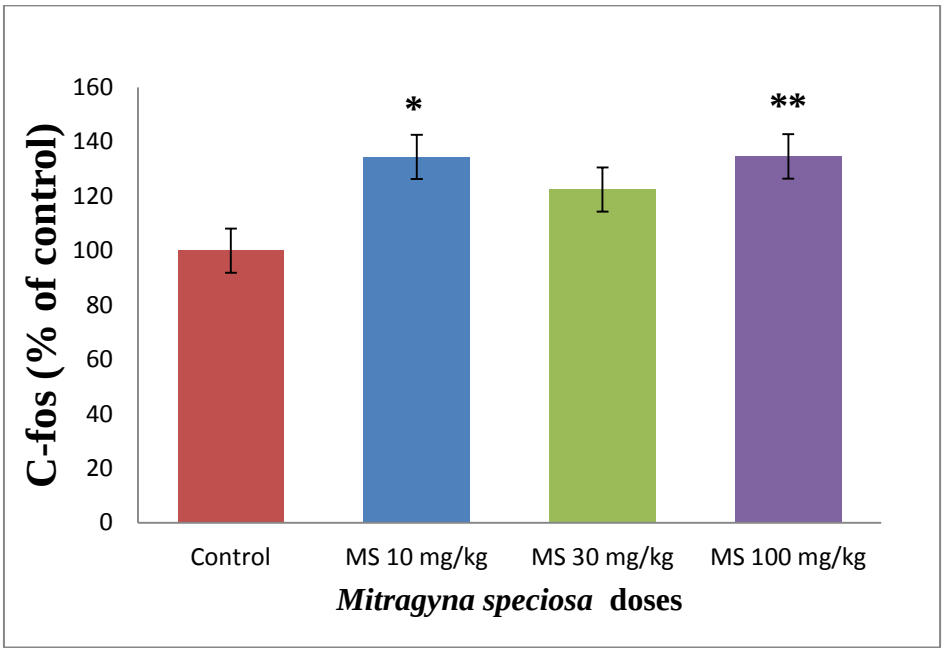
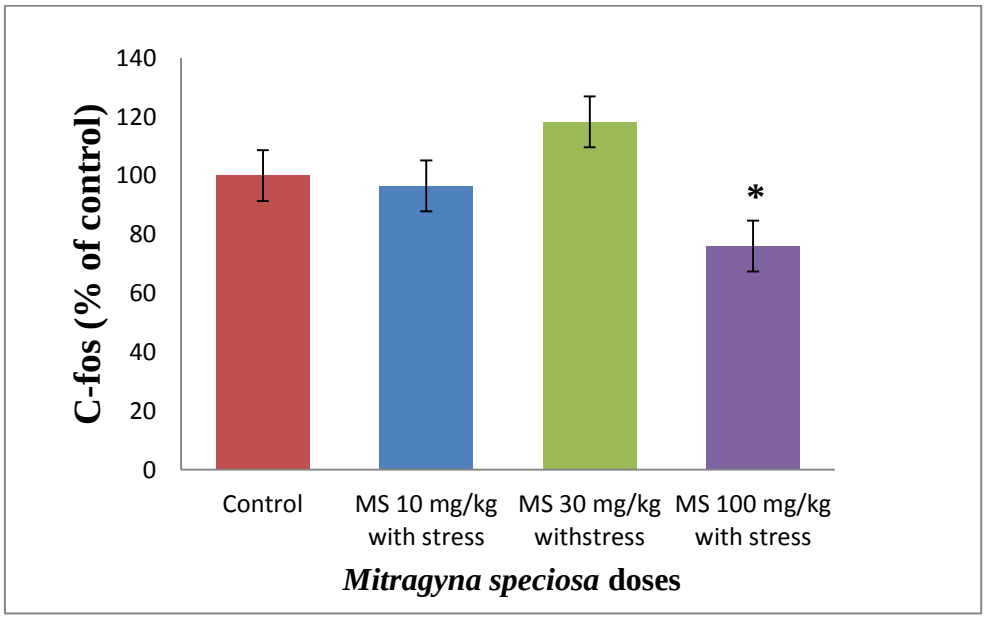
Western blot were used to measure the effect of MS on c-Fos expression in prefrontal cortex. The graph (1) shows control group that was given normal saline, and other three groups which were received three different doses of MS and subjected to stress (10, 30, and 100 mg/kg). The data (quantification of bands from western blots and normalized by ß-Actin) is expressed as percentage of c-Fos value and compared to control. The significance '*' was determined for 10 mg/kg restraint animals group with $p < 0.05$, compared with control group. Meanwhile the expression in groups of 30, and 100 mg/kg were low and showed no significance when compared to the control groups. While, c-Fos expression in prefrontal cortex (graph 2) shows control group that was given normal saline, and other three groups which were given three different doses of MS and did not subjected to stress. Statistically the significante expression presented with doses 10 and 100 mg/kg with $p < 0.05$. Meanwhile, 30 mg/kg showed less significance differences when compared to the control groups.

The graph (3) shows c-Fos expression in hippocampus in Restrained groups in three doses of MS (10, 30, and 100mg/kg). Only 100 mg/kg had expressed when compared with control group with $p < 0.05$. While, non-stress treated (graph 4) groups did not show statistically significant protein expression.




**Graph 2: c-Fos expression in prefrontal cortex in rats treated with MS and nonstress**




**Graph 3: c-Fos expression in hippocampus in rats treated with MS and subject to stress**

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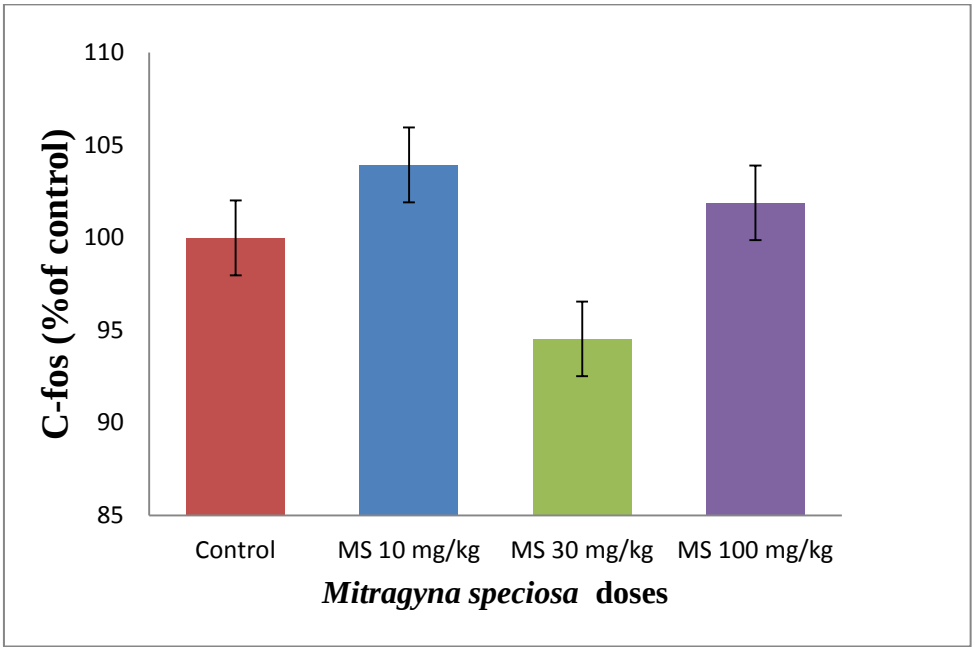
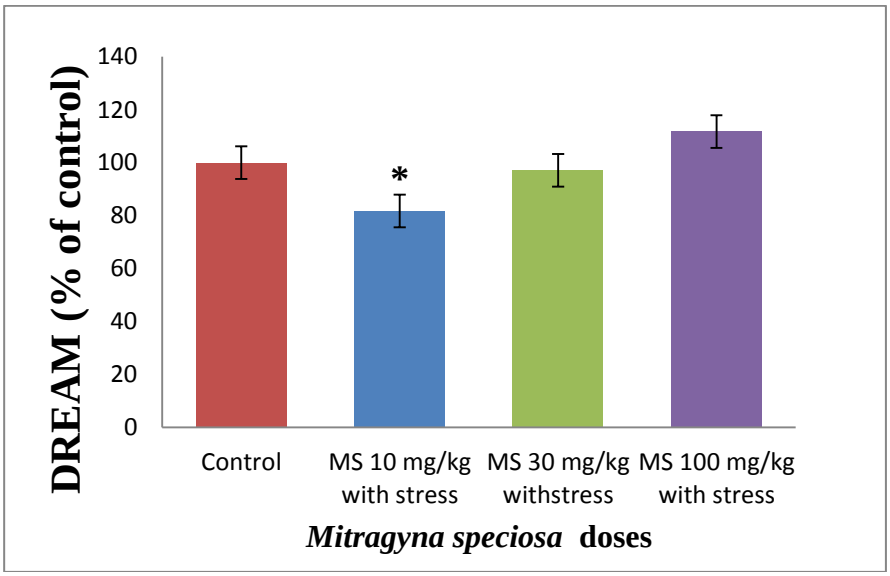



**Graph 4: c-Fos expression in hippocampus in rats treated with MS and nonstress.**

**DREAM expression**

The expression of DREAM in prefrontal cortex in rats subjected to restraint stress and treated with MS doses (10, 30, and 100 mg/kg) as shown in graph (5). At dose 10 mg/kg DREAM was highly expressed with $p < 0.05$. Meanwhile the level of expression in groups given 30, and 100 mg/kg did not present any statistically significant when compared to the control. In other non restrained groups DREAM expression in prefrontal cortex. The protein expression occurs only at dose 100 mg/kg as shown in graph (6).

DREAM expression in hippocampus stress groups with MS doses (10, 30, and 100 mg/kg). DREAM statistically expressed in dose 100 mg/kg with $p < 0.05$, in compare with control group. Whereas in nonstress groups the significance was determined for dose 100 mg/kg with $p < 0.05$, compared with control group.




**Graph 5: The expression of DREAM in prefrontal cortex in rats treated with MS and subject to stress.**

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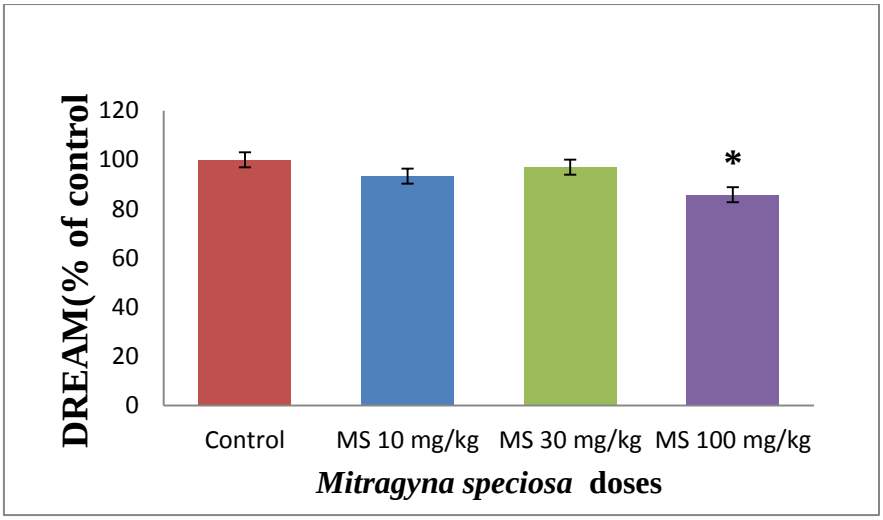
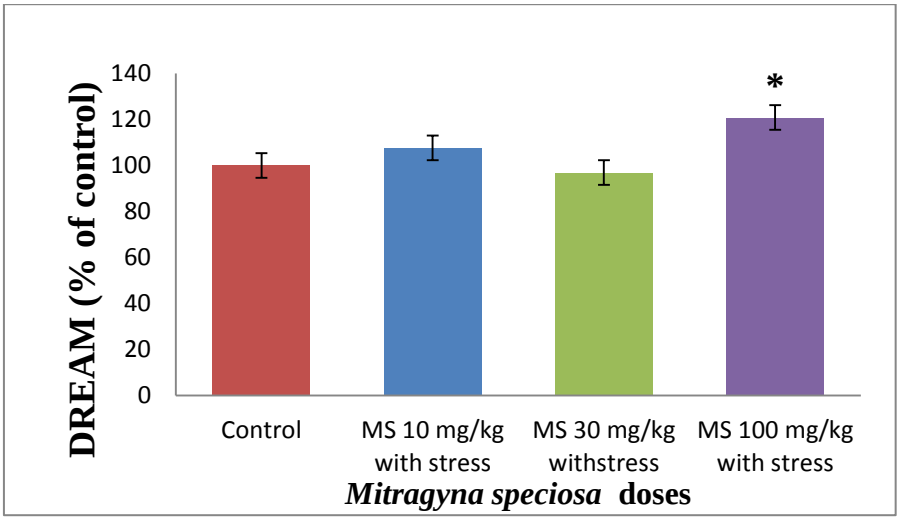
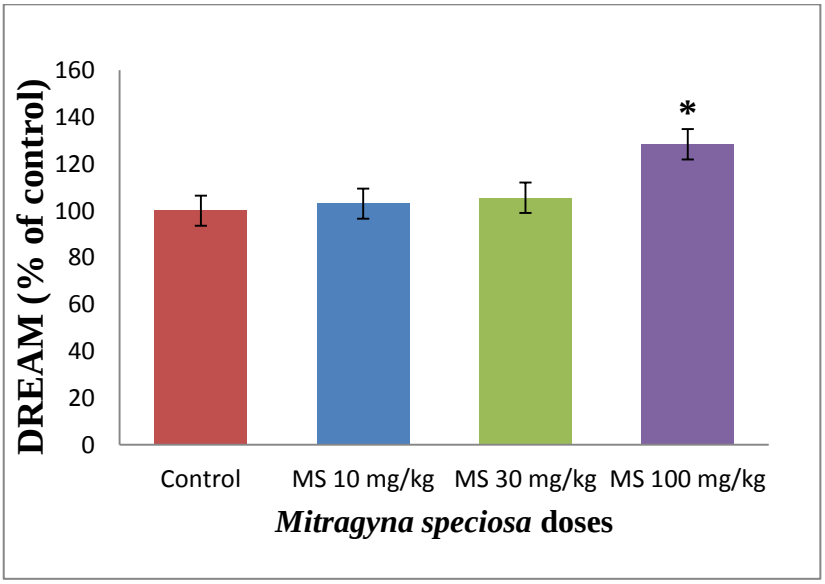



**Graph 6: The expression of DREAM in prefrontal cortex in rats treated with MS and nonstress.**




**Graph 7: The expression of DREAM in hippocampus in rats treated with MS and subject to stress.**




**Graph 8: The expression of DREAM in hippocampus in rats treated with MS and nonstress.**

## DISCUSSION

Few studies were carried out to investigate the effect of MS on histological structure of fatal organs, and its physiological effect on specific brain area. Kidney at 30 mg/kg of stress group presented slight shrunken and in 30 and 100 mg/kg of nonstress group there were slight hyperemia. Similar to report of Sabetghadam *et al.* (2013), mitragynine at 100 mg/kg showed swollen glomerulus capsule and red blood cells between lumens which may indicate early stage of mitragynine renal toxicity [19].

Hyperemic change was detected in liver microscopic slides at 30 mg/kg of stress and 100 mg/kg of nonstress groups. While liver severe damage was observed at 1000 mg/kg [13]. Also, Chronic administration of mitragynine induced hepatocytes hyper-trophy, dilation of sinusoids and hepatocytes hemorrhage [19].

MS was assumed to be physiologically active and has morphine like properties [2]. Therefore it was supposed to have the similar effect as morphine with stress did not induce any histopathological changes on liver, kidney, testes, and brain. Our histopatholgical findings of testes are well in agreement with the findings of study done by Cicero *et al.* in 2002 which was preformed with morphine.

However, current study histopatholodicaal results for brain were consistent with previous research findings that; MS at dose 1000 mg/kg did not induce histopathologic changes [13]. Whereas, mitragynine caused medulla base damaged and represented by local vacuolation and necrosis of neuronal cells. Similar changes were noted in the white matter of hippocampus [19].

MS has known to induce effect of 'cocaine-like' stimulation in small doses; while at high doses 'morphine-like' sedation and nausea are reported [3]. These effects of MS could explain the reduction in DREAM expression in prefrontal cortex at doses 10 mg/kg and 100 mg/kg in stressed and non-stressed rats respectively. As DREAM is well-known role as a transcriptional repressor [26]. Mitragynine caused local vacuolation and necrosis of neuronal cells in medullary base. A similar change in the white matter of hippocampus, and further accompanied by degenerative nerve cells damage [19]. DREAM was defined by their ability to alter apoptosis in neural cells by binding hexokinase I, and reducing mitochondrial hexokinase I localization [7]. Which could mean the increase of DREAM expression in hippocampus after extract administration and stress, may be taken as an early sign of hippocampus neuron damage.

Mapping of the expression of c-Fos used to map the brain regions that are influenced by antipsychotic drugs [18]. In current study MS increase the c-Fos expression in PFC, while c-Fos in hippocampus was significantly lower in restraint rats. However in non- stresse the c-Fos did not expresse at all doses. In rats morphine sulfate increased the number of Fos positive cells in limbic areas, indicating that these regions were more active during exposure to the drug-paired environment PFC was on of the most activated region [20]. c-Fos expression increased in various limbic and motor structures after acute exposure to drugs of abuse including cocaine, morphine, nicotine, and alcohol [25]. Exposure to stressors markedly increases neuronal and genomic activation within the mPFC, as reflected in the pronounced expression of the immediate early gene c-Fos [11]. Increase in c-Fos mRNA expression in prefrontal cortical regions immediately after stress session [4]. However in this study MS did not induce c-Fos expressions in hippocampus unless it was combined with stress, and this expression may come as a result of stress not MS.

## CONCLUSION

It was evident from the present study; the chloroform-methanolic extract of MS may have less potent effect on tissue than pure mitragynine and morphine. Western blot data shows different expression for c-Fos and DREAM protein in PFC and hippocampus, as C-fos expressed was increased in prefrontal but decreased in hippocampus while DREAM was induced in hippocampus, but reduced in prefrontal cortex. These findings may indicate that, MS has different effects in different brain areas. Further investigations need to be conducted with higher doses of the extract and a chronic study need to be conducted to investigate this herb effects among long term users. In addition, to get more data about its effect on the nervous system more areas of the CNS such as amygdala have to be involved in future studies.

## ACKNOWLEDGEMENTS

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## REFERENCES

[1] Ahmad K, and Aziz Z. J Ethnopharm 2012; 141: 446-450.
[2] Adkins JE, Boyer EW, and McCurdy CR. Curr Top Med Chem 2011; 11:1165-1175.

[3] Babu KM, McCurdy CR, and Boyer EW. Informa Healthcare 2008; 46:146–152.
[4] Bland ST, Schmid MJ, Der-Avakian A, Watkins LR, Spencer RL and Maier SF. Brain Res 2005; 1051: 90-99.
[5] Chittrakarn S, Sawangjaroen K, Prasettho S, Janchawee B, and Keawpradub N. J Ethnopharm 2008; 116:173-178.
[6] Cicero TJ, Davis LA, LaRegina MC, Meyer ER, and Schlegel MS. Pharmacol Biochem Behavior 2002; 72: 157 - 163.
[7] Craig TA, Ramachandran PL, 3rd HRB, Podratz JL, Windebank AJ and Kumar R. Biochem Biophys Res Comm 2013; 433: 508-512.
[8] Carrion AM, Link WA, Ledo F, Mellstrom B, and Naranjo JR. Nature 1999;398:80-4.
[9] Costigan M, and Woolf C. J Cell 2002; 108: 297-30.
[10] Fakurazi S, Abdul Rahman S, Hidayat MT, Ithnin H, Moklas MAM and Arulselvan P. Molecules 2013;18:666-681.
[11] Gratton A and Sullivan RM. Techniq Behav Neural Sci 2005; 15: 807-817.
[12] Hassana Z, Muzaimi M, Navaratnam V, Yusoff NHM, Suhaimi FW, Vadivelu R, Vicknasingam BK, Amato D, Hörsten S, Ismail NIW, Jayabalan N, Hazim AI, Mansor SM and Müller CP. Neurosci Biobehav Rev 2013; 37: 138–151.
[13] Harizal SN, Mansor SM, Hasnan J, Tharakan JK, and Abdullah J. J Ethnopharm 2010;131:404–409.
[14] Hidayat MT, Apryani E, Nabishah BM, Moklas MAA, Sharida F and Farhan MA. Adv Med Dental Sci 2010; 3: 65-70.
[15] Kaczmarek L. Acta Neurobiologiae Experimentalis 2000; 60:419–424.
[16] Matsumoto K, Horie S., Ishikawa H., Takayama H., Aimi N., Ponglux D. and Watanabe K. Life Sci 2004; 74: 2143-2155.
[17] Milde-Langosch K. European J Cancer 2005; 41: 2449-2461.
[18] Rajkumar R, See LKY, and Dawe GS. Brain Res 2013; 1508:34-43.
[19] Sabetghadam A, Ramanathan S, Sasidharan S, and Mansor SM. J Ethnopharm 2013;146: 815–823.
[20] Schroeder B, Holahan MR, Landry CF, and Kelley AE. Synapse 2000; 37:146-158.
[21] Shellard EJ. Bull Narcotics 1974; 26:41-55.
[22] Suwanlert S. Bull narcotics 1975; 21-27.
[23] Takayama H. Chem Pharm Bull 2004; 52: 916-928.
[24] Vicknasingama B, Narayananb S, Benga GT, Mansora SM. Int J Drug Policy 2010; 21: 283–28
[25] Wedzony K, Koros E, Czyrak A, Chocyk A, Czepiel K, Fijal K, Mackowiak M, Rogowski A, Kostowski W, and Bienkowski P. Arch Pharmacol 2003; 368: 331-341.
[26] Zhang Y, Li Y, Yang YR, Zhu HH, Han JS, and Wang Y. Neurochem Res 2007; 32:1592-1599.
[27] Houghton, PJ, and Ikram, MS. Phytochem. 1986; 25: 2910-2912.