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Pre-treatment of Diclofenac Prevents Memory Impairment After *Mitragyna speciosa* (Korth.) Havil. Administration: Role of the Arachidonic Acid Cascade

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Abstract: *Mitragyna speciosa* (MS) has been reported to cause memory impairment, and early studies suggest the involvement of the arachidonic acid cascade in cognitive disorders. This study was conducted to investigate the potential of diclofenac pre-treatment to prevent memory impairment caused by MS. Male Wistar rats were divided into five groups: vehicle group (CMC Na 0.5%; p.o), mitragynine ethanolic extract (MSEE) groups (50 and 100 mg/kg BW; p.o), and the MSEE groups pretreated with diclofenac sodium (5 mg/kg BW; i.p). The treatments were given for two weeks. The memory performances were evaluated using MWM and Y-maze tests. The changes in IL-1β level, profiles of the microglia, and degenerated neurons in the hippocampal CA1 were observed. The administration of MSEE was demonstrated to impair memory in behavioral tests, accompanied by an increase in IL-1β levels in the hippocampus compared to the vehicle group ($P<0.05$). Histological examination revealed increased numbers of microglia and degenerated neurons after MSEE. Pre-treatment of diclofenac significantly improved ($P<0.05$) the memory performance as well as IL-1β levels and ameliorated the histological presentation. The results of the present study indicate the essential of the arachidonic acid cascade in MS-induced memory impairment.

Keywords: Mitragyna speciosa (Korth. (Havil.); memory impairment; Morris water maze (MWM); Y-maze; diclofenac; arachidonic acid; IL-1β; CA1.

1. Introduction

Mytragina speciosa (MS) is part of the Rubiaceae family and is widely found in Southeast Asia, including Indonesia [1]. Currently, MS has been commonly used in the United States and Europe as an antidepressant, anxiolytic, and opioid substitute [2-4]. The current research indicates that kratom leaves contain a greater number of the 40 compounds, with four having been identified as exhibiting pharmacological activity: mitragynine, 7-hydroxymitragynine (7-HMG), speciociliatine, and corynantheidine [5].

MS has been reported to result in significant cognitive deficits and emotional disturbances in animals following acute or chronic administration [6-9]. Recent research has indicated that mitragynine has been associated with spatial/place learning deficits, accompanied by impaired synaptic transmission and long-term potentiation (LTP) in the CA1 of the rat hippocampus and electroencephalogram (EEG) deficits [9, 10].

The mechanisms underlying the adverse effects of MS administration on cognition remain incompletely understood. Testing of metabolomic parameters identified several pathways that may be involved in the effects of MS exposure, including the arachidonic acid pathway [11]. IL-1β induction was involved in the arachidonic acid cascade pathway [12]. Repeated morphine injection increased IL-1β production [13]. Increased levels of IL-1β and increased expression of arachidonic acid cascade enzymes have been implicated in the initiation of nerve damage [14]. Arachidonic acid has also been linked to neuroplasticity, signal transduction, and cognitive impairment [15]. Diclofenac, a COX inhibitor, has been demonstrated to prevent memory deficit in stress rats [16]. The present study investigated the potential of diclofenac pre-treatment to prevent memory impairment caused by MS.

2. Materials and Methods

2.1. MSEE preparation.

The Powder simplisia of Mitragyna specioa (Korth.(Havil.) was obtained from Pontianak, Kalimantan, Indonesia, and was confirmed from The Herbarium of Jatinangor, Taxonomical Laboratory, Padjajaran University, with certificate number 36/HB/09/2023. The powder simplisia was extracted using the soxhlet apparatus method, and 96% ethanol was used as the solvent.

2.2. Animals subject.

The subjects utilized in this research were male Wistar rats aged 2-3 months and weighing 180-240 grams as obtained from CV. Kencana, Bandung, Indonesia. This research has received approval from The Ethics Committee for Animal Research of the Institute of Technology Bandung, based on the Certificate of Ethical Approval with the certificate number KEP/I/2023/VIII/H190723CA/EKTA.

2.3. Protocols and experimental design.

The subjects were acclimated for seven days prior to the commencement of the experiment, with the experimental conditions set at room temperature of 25 ± 2℃, and a 12-hour dark-to-light cycle. *Ad libitum* access to food and drink was provided. The experimental animals were divided into 5 groups as follows: group 1 was administered with vehicle (0.5% CMC Na; p.o), group 2 received MSEE at 50 mg/KgBW/Day (p.o), while group 3 was given MSEE at 100 mg/KgBW/Day (p.o). Groups 4 and 5 received diclofenac (Novell, Indonesia) pre-treatment at 5 mg/kg, 30 mins prior to the two doses of MSEE. The experimental timeline is described in Figure 1.

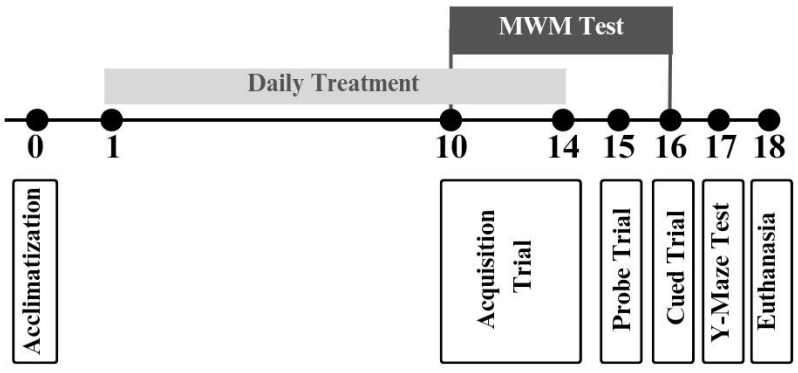

Figure 1. Experimental timeline (Day).

2.4. Behavioral memory evaluation.

2.4.1. Morris water maze test.

The Morris Water Maze (MWM) was used to ascertain spatial learning and memory [17]. The following methods, as described by Uygur and Arslan [18] with several modifications, were used. A circular pool with a height of 60 cm and a diameter of 135 cm was used in this study. The pool is divided into four quadrants, designated as ((North-East (NE), North-West (NW), Southeast (SE), and South-West (SW)). A platform with a height of 18 cm and a diameter of 13 cm is utilized and positioned in the NE quadrant in the same location throughout the test period. The pool is filled with water at a temperature of 25 ± 2°C and mixed with non-toxic material (milk) to a height of 1.5-2.5 cm above the water in order to render it invisible. The pool contains several fixed-visual cues, such as a calender, a light source, a door, and two observers placed above the pool to observe behavior during the MWM test.

In the acquisition trial, rats were trained four times daily to find the invisible platform. The trials commenced from one of the randomly selected quadrants, except for the quadrant in which the platform was located. The time taken by the mouse to reach the platform was recorded. Following each exercise, the rats were dried using a dry towel. Subsequently, following the completion of the test, the rats were returned to their cages.

Following the training session, a probe trial was conducted by removing the platform and allowing the rat to swim for 60 seconds. The percentage of time and frequency when the rat was in the platform quadrant was recorded. One day after the probe trial was completed, a cued trial test was carried out to assess the deficit learning or memory that was not caused by the weakness of sensory, motor, or motivational weaknesses[19]. The test is conducted by elevating a platform above the surface of the water and affixing a flag to the surface of the platform. The latency to the platform was recorded.

2.4.2. Y-maze test.

The Y-maze was used to assess spatial working memory, utilizing spontaneous alternation parameters [20]. On the day of testing, the rats were placed in the experimental room 30 minutes prior to the Y-Maze test. Each rat was placed in a Y maze (without any form of training, reward, or punishment) at the end of one arm and allowed to explore the arm for 8 minutes. The Y-Maze is constructed with a length of 50 cm, a width of 10 cm, and a height of 10 cm with an angle of 120°. At the end of each test, the maze was cleaned with 70% ethanol solution and dried with paper towels to remove any residual odors from the apparatus [21]. The number of alternations and arm entries were recorded. The percentage of alternations was calculated as follows: (Number of alternations)/(Total number of arm entries-2) x100. This study quantified the time spent grooming (time spent licking feet, rubbing and scratching various body parts). An increase in grooming behavior can be interpreted as an indication of an elevated stress response to the novel environment [22].

2.4. Measurement of hippocampus IL-1β.

The ELISA kit (BT-Lab) was employed to ascertain the concentration of IL-1β in the hippocampus. Following euthanasia, the hippocampus was washed using ice-cold phosphate-buffered saline (PBS) with a pH of 7.4. The hippocampus was then weighed and homogenized in PBS at a organ weight (g) ratio to PBS volume. The PBS (ml) volume was calculated as 1:9

and homogenized on ice using a glass homogenizer. Subsequently, the homogenates were centrifuged for 15 minutes at 4°C and 12,000 rpm to obtain the supernatant, which was then stored in a freezer at a temperature of -80°C. The results of the ELISA kit analysis are read using an ELISA reader (Technan) at an absorption wavelength of 450 nm.

2.6. Histological examination.

One sample of the hippocampal tissues of each group was isolated, rinsed in saline solution, and then collected in 10% buffered formalin pH 7.4. Following this, the tissues were prepared for the paraffin-embedding technique. Sections of 5 µm thickness were obtained from the paraffin block. The sections were then stained with hematoxylin and eosin (H&E). A pathologist read the histological evaluation results, and each group was blinded to avoid bias in the reading. The readings were carried out in the CA1 hippocampus area under a microscope at 400 times magnification. Degenerated neurons were identified as shrunken cells with pyknotic and hyperchromatic nuclei. Microglia are small cells with elongated nuclei and minimal cytoplasm [23, 24].

2.7. Statistical analyses.

Statistical analysis was carried out using GraphPad Prism 8.4 software. The normality was evaluated with the Saphiro-Wilk test. The latency of escape was analyzed with a mix-model two-way ANOVA, followed by an LSD test for multiple comparisons. The other data was analyzed using one-way ANOVA and the *post hoc* LSD test for multiple comparisons. Differences between groups were considered significant if $p<0.05$.

3. Results and Discussion

3.1. Diclofenac prevents spatial learning and memory impairment in MSEE administration.

The spatial learning capacity is represented by the latency time needed to find the platform. The process required the rats to remember the correct location [25]. The mean latency of escape of MS on spatial memory performance was recorded and presented in Figure 2.

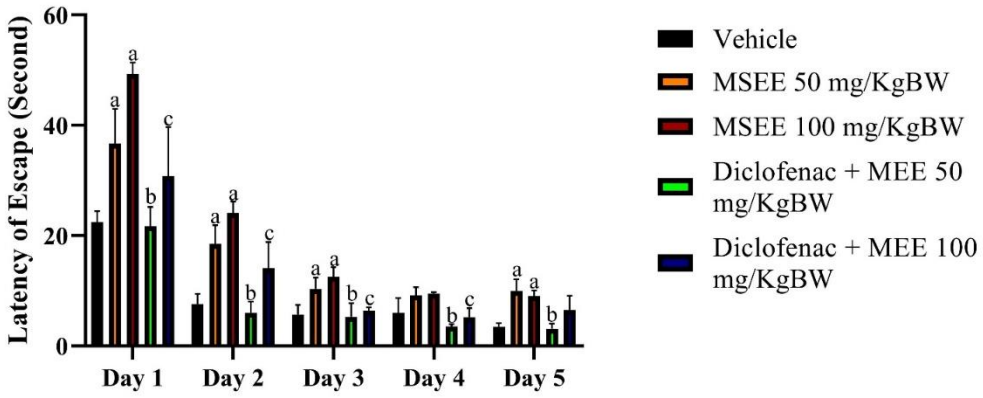

Figure 2. Evaluation of MSEE on spatial memory uses the MWM Test ($^{a,b,c}p<0.05$ vs vehicle, MSEE 50 mg/kg, and MSEE 100 mg/kg, respectively).

The memory deficit is interpreted through an increase in the time spent finding platforms and a decrease in the time spent in the target quadrant [26]. During the five days of the learning process (Figure 2), the group that received MSEE experienced a learning deficit

when compared to the normal group ($p< 0.05$). Previous studies have demonstrated that mitragynine impairs behavioral performance and causes deficits in learning and memory function [6-9] and reduction in reference memory in the water maze task [11]. Chronic use of MS has been associated with impaired long-term potentiation (LTP), which can weaken long-term memory consolidation [27, 28]. Furthermore, the methanol MS extract has been demonstrated to prevent the formation of long-term potentiation (LTP) in short-term potentiation, resulting in a reduction in excitatory post-synaptic potentials (EPSPs) [28].

The involvement of the arachidonic acid cascade pathway was evaluated in this study by administering diclofenac sodium (which has a known role as a COX inhibitor) prior to MSEE administration. The latency time for test animals to reach the platform was compared between those given only MSEE and those given diclofenac before MSEE administration at the same dose. The statistical analysis results indicated a statistically significant difference ($p< 0.05$) between the two groups, with the diclofenac-treated group exhibiting a lower latency time. A reduction in escape latency has been demonstrated to result in a memory improvement [29]. This is consistent with the findings of Zul Aznal [11], which indicate that one of the pathways involved in the influence of memory when given mitragynine is the arachidonic acid cascade pathway. Arachidonic acid can be metabolized by three different enzyme pathways, namely cyclooxygenase (COX), lipooxygenase (LOX), and cytochrome p450 (CYP) enzymes, to produce a variety of biologically active fatty acid mediators [30]. Drug-induced changes in arachidonic acid are associated with neuroplasticity and signal transduction [14, 15].

Twenty-four hours after the final acquisition trial, a probe trial (without the administration of the extract) was conducted. The test animal was allowed to explore the pool for 60 seconds, starting from the same starting point. The probe trial was conducted to determine whether the animals had retained the memory of the location or formed a spatial reference memory. The observations were made by measuring the time and frequency spent in each of the quadrants. The results of the test are presented in Figure 3A and Figure 4.

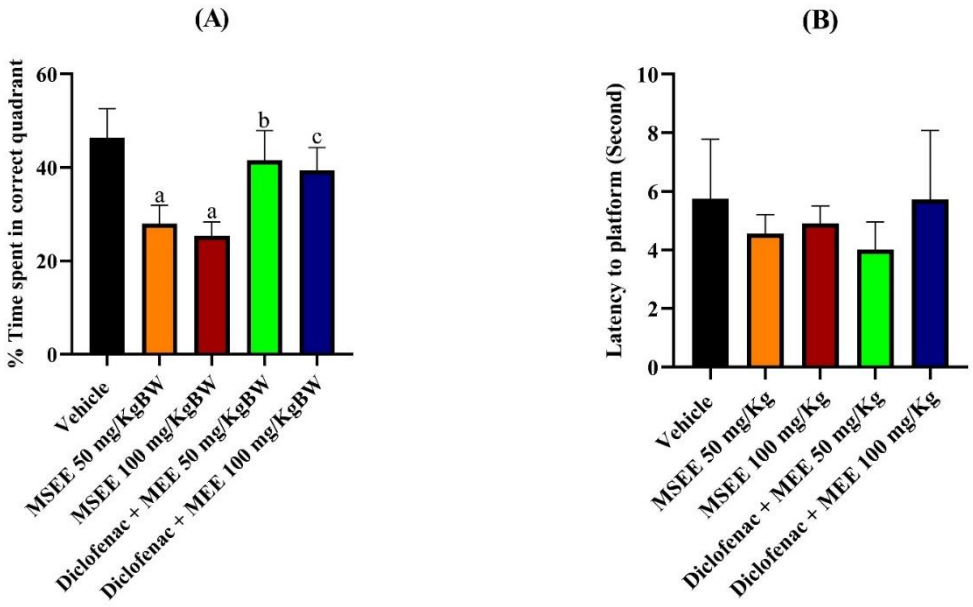

Figure 3. The profile of spatial memory performance in MWM. (A) probe trial test to evaluate spatial memory; (B) cued trial test to measure the increase in latency due to memory impairment not caused by deficit sensor, motor, or motivational. ($^{a,b,c}p<0.05$ vs vehicle, MSEE 50 mg/kg, and MSEE 100 mg/kg, respectively).

The occurrence of a spatial learning process can be observed in the reduced time spent reaching the platform and the increased time spent in the target quadrant [26]. The results of the probe trial evaluation indicated that the repeated administration of MSEE 50 mg/KgBW (27,98±3,9) and 100 mg/KgBW (25,41±2,8) resulted in a reduction in the percent of time spent in the target quadrant compared to the vehicle group (46,32±6,2) ($p<0.05$) in a one-way ANOVA analysis. The group administered the 100 mg/kg dose exhibited the most pronounced spatial reduction, as evidenced by the results of time and frequency in the target quadrant compared to other doses. This suggests that MSEE administration may be associated with spatial memory deficits. In the group that received diclofenac pre-treatment on MSEE 50 mg/KgBW (41,53±6,3) and 100 mg/KgBW (39,44±4,8), it was demonstrated that this had a preventive effect, as evidenced by the longer time of test animals in the probe trial evaluation ($p < 0.05$) compared to the group that received MSEE at the same dose.

Cued trials (platform visible and additional flag present) were conducted the day after probe trials. The performance of all groups demonstrated no significant differences in latencies compared to the carrier group ($p>0.05$; Figure 3B). The research findings indicated that the spatial memory deficit was not attributable to the subject's sensory, motor, or motivation [18,19].

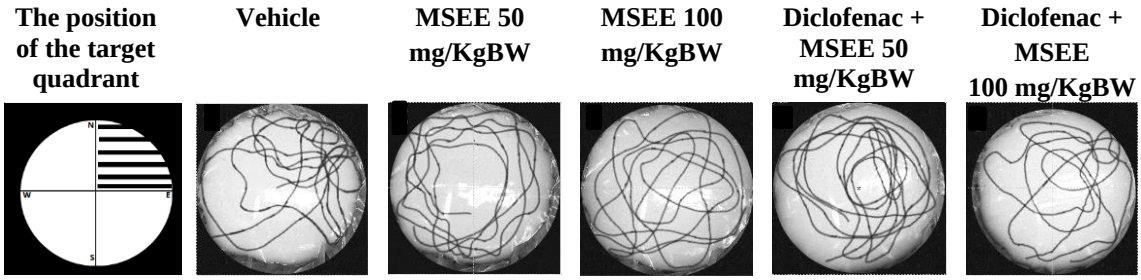

Figure 4. The MWM track during the probe trial.

3.2. Diclofenac discourages spatial working memory following MSEE administration.

The Y-maze is a relatively simple method that has been widely used to evaluate spatial working memory. Spontaneous alternation behavior is based on the animals' natural curiosity to explore new environments. A high percentage of spontaneous alternation indicates good working memory [21]. The results of measuring the percent of alternation are presented in the following Figure 5.

The image depicts a statistically significant ($P < 0.05$; Figure 5A) reduction in the percentage of alternation following the administration of KEE 50 (46.68 ± 6.3) and KEE 100 (29.66 ± 5.4) in comparison to the vehicle group (77.85 ± 8.1). This result aligns with other studies that MS have reported a working memory deficit using different procedures [6]. In accordance with the spatial memory test, the group that had previously received diclofenac exhibited a greater number of spontaneous turns in the Y-maze test than the group that had received KEE in the same dose ($p < 0.05$; Figure 5A; Figure 6). In addition, grooming behavior was assessed (Figure 5B). Grooming has been considered an index of behavioral adaptation to situations of stress, pain, and inflammation [31]. This increase in behavior reflects an increased stress response to the new environment [22]. This study found that the group given MSEE experienced a stress response to the new environment, as evidenced by the increase in grooming time compared to the vehicle group ($p<0.05$).

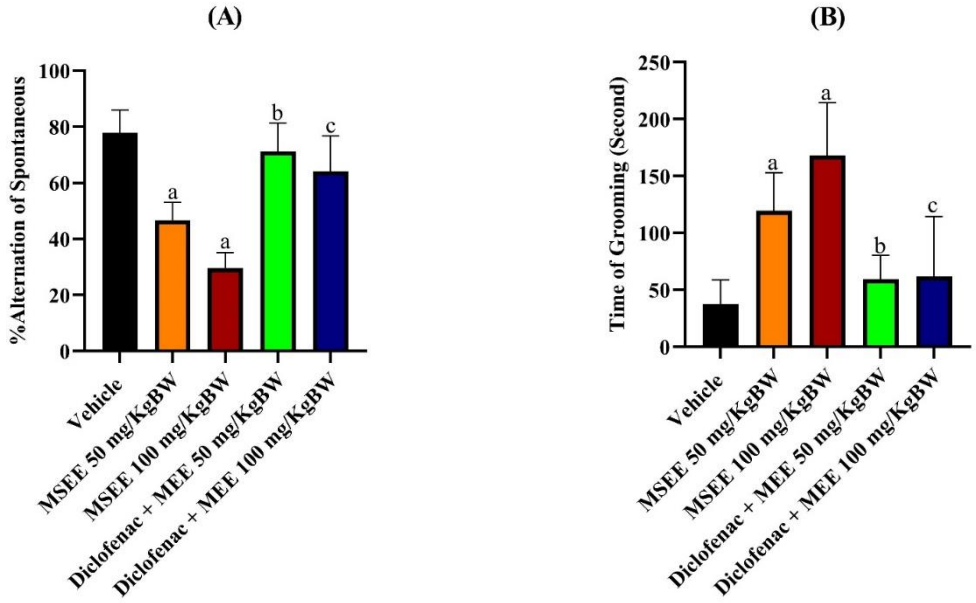

Figure 5. Evaluation of spatial working memory uses Y-Maze. **(A)** alternation of spontaneous to evaluated of spatial working memory; **(B)** Grooming during the Y-Maze test to indicate stress in a novel environment. ($^{a,b,c}p<0.05$ vs vehicle, MSEE 50 mg/kg, and MSEE 100 mg/kg, respectively)

This is consistent with the observed increase in IL-1β levels in the group MSEE-treated group. This is similar to a report by Song, who reported that the administration of IL-1 increased grooming scores [32]. Diclofenac sodium is a potent cyclooxygenase inhibitor and has been shown to reduce the release of arachidonic acid [16]. Furthermore, diclofenac has been reported to prevent stress-induced repetitive memory deficits in mice.

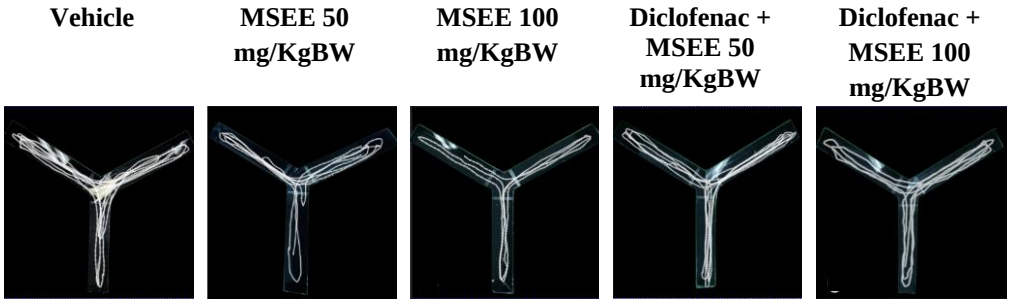

Figure 6. The track of arm entries during the Y-Maze test.

3.3. Diclofenac attenuated IL-1β hippocampus following MSEE administration.

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine with receptors distributed widely throughout the brain, with the highest concentration observed in the hippocampus [33]. The results of measuring IL-1β are presented in Figure 7.

The results of the evaluation of IL-1β levels in the vehicle group indicate an average IL-1β level of 2,146 ± 0.24. In the group that received MSEE at doses of 50 and 100, there was an increase in IL-1β levels dose-dependence with an average of 3,160 ± 0.19 and 3,718 ± 0.89. The results obtained from the one-way ANOVA analysis indicated that the group that received MSEE was significantly different ($p<0.05$) from the vehicle group. According to Barbosa [34], chronic administration of opioids also upregulates IL-1β in the brain periphery.

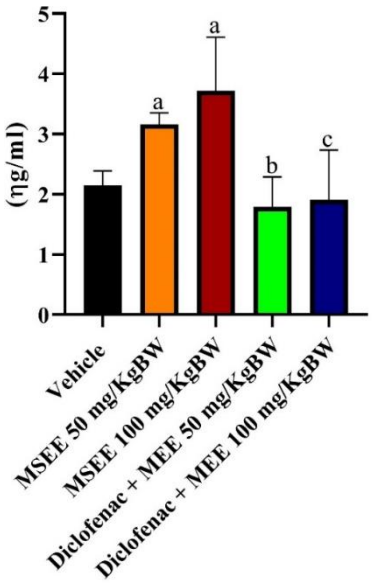

Figure 7. Effect of MSEE on the levels of IL-1 β in the hippocampus. ($^{a,b,c}p<0.05$ vs vehicle, MSEE 50 mg/kg, and MSEE 100 mg/kg, respectively).

The administration of high levels of IL-1β (in the pathological range) to rat hippocampal slices reduced LTP in area CA 1 (Bear, 2016). Furthermore, IL-1β has been shown to increase JNK activation and p38, decreasing LTP [35]. Mitragynine has been demonstrated to induce short-term potentiation in rat hippocampal CA1 [29]. Furthermore, investigations of a standardized MS extract and mitragynine in hippocampal CA1 sections demonstrated a significant reduction in fEPSPs (field excitatory post-synaptic potentials) and the blocking of LTP [28, 29, 9]. This study provides additional evidence that the reduction in fEPSPs may be attributed to an increase in IL-1β.

It has been postulated that memory impairment due to mitragynine results from arachidonic acid metabolism [11]. Furthermore, cytokines induce the production of COX-2 (an enzyme that catalyzes the production of prostaglandins). This is supported by several other pieces of evidence involving the arachidonic cascade and IL-1β in memory. The bilateral injection of IL-1β in the rat hippocampus has increased cyclooxygenase-2 (COX-2) levels and activated the arachidonic enzyme, releasing PGE2 in the hippocampus. Furthermore, PGE2 injection has been shown to cause a greater number of working memory errors than the control group [12].

Diclofenac has been reported to be a more selective inhibitor of COX-2 than COX-1. The chemical structure of diclofenac is similar to that of meclofenamic acid, an NSAID belonging to the fenamate group [35]. The fenamate group has been demonstrated to be more effective in inhibiting the release of IL-1β compared to other NSAID groups, such as celecoxib and ibuprofen. Western blot analysis has demonstrated that the fenamate specifically blocks caspase-1-dependent IL-1β processing without significant effects on cell death [36]. This study demonstrated that the administration of diclofenac prior to administering 50 and 100 doses of MSEE resulted in a significant reduction in IL-1β levels, with a mean decrease of 1,791 ± 0.49 and 1,909 ± 0.82, respectively. The results of the one-way ANOVA analysis indicate a statistically significant difference ($p < 0.05$; Figure 7) between the group that received only MSEE at the same dose and the group that received both MSEE and diclofenac.

3.4. Diclofenac decreases the profile of degenerated neurons and microglia in the CA 1 hippocampus.

The hippocampus is an organ that plays an important role in the process of learning and memory. It is comprised of three Cornu Ammonis, namely the CA1, CA2, and CA3, and the dentate gyrus. CA1 is the primary site of Cornu Ammonis and the final site of memory processing [37]. In this study, the histological profile of the number of glial cells and the percentage of neuronal damage was observed histologically in the hippocampal CA1 area, as presented in Figure 8.

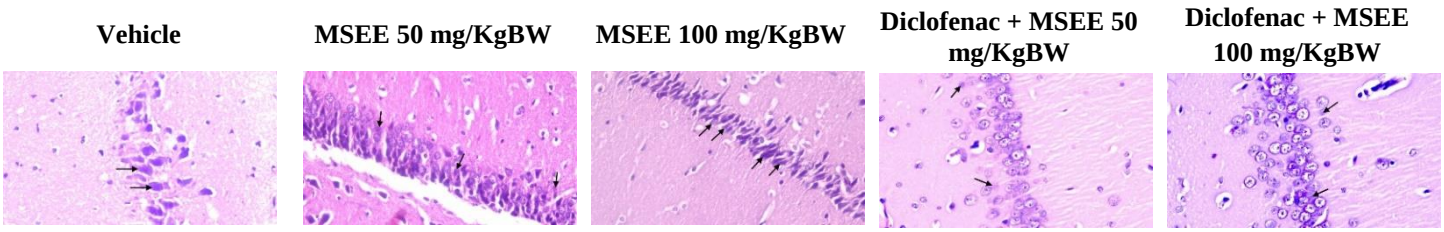

Figure 8. Histological presentation of hippocampal CA1.

The higher the dose of MSEE administered, the higher the percentage of degenerated neurons (Figure 9A). This finding is consistent with research indicating that IL-1β causes neuronal cell death in the hippocampus and indirectly causes neuronal cell death caused by several other factors [38]. This is in contrast to the group that received diclofenac pre-treatment, which did not increase the percentage of degenerated neurons. The bilateral injection of IL-1β in the rat hippocampus has increased cyclooxygenase-2 (COX-2) levels and activated the arachidonic enzyme, releasing PGE2 in the hippocampus [12]. Diclofenac has been reported to be more selective for COX-2 and has been shown to inhibit COX-2, especially in neurons [16, 36].

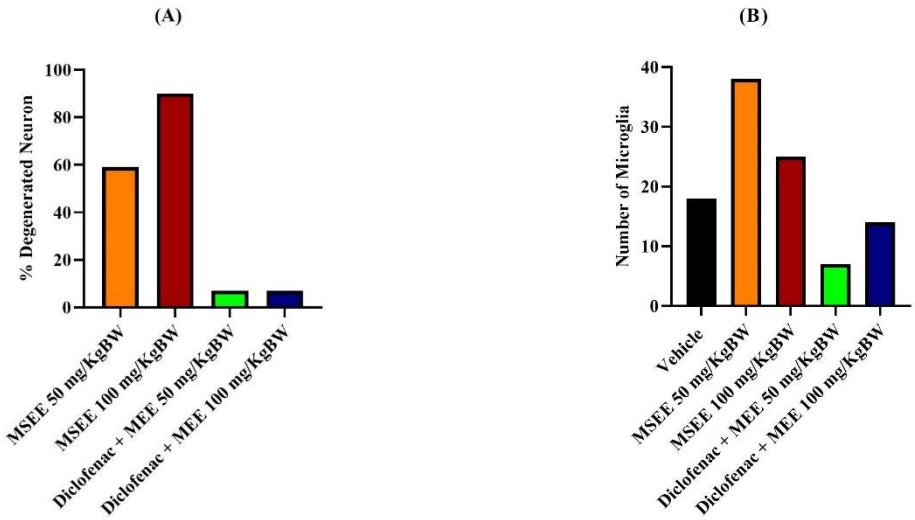

Figure 9. The Histological profile of hippocampal CA1. **(A)** Percent of the degenerated neuron; **(B)** Number of microglia.

Microglia are the brain's primary immune cells. It is postulated that activated microglia are the primary cellular source of inflammatory cytokines such as IL-1β, IL-6, and TNFα. In this study, there was an increase in the number of microglia following MSEE administration. Several studies demonstrate that opioid addiction causes the activation of microglia in the central nervous system [39]. The group that received diclofenac prior to MSEE administration exhibited a lower number of microglia than those who received only MSEE (Figure 9B). This

is due to the structural similarity between diclofenac and the fenamate family [36]. This is consistent with reports that mefenamic acid can reduce neuroinflammation in transgenic AD mice, including microglia activation and IL-1β release [40].

In general, animals that have undergone hippocampal lesions perform poorly in the Y-maze and MWM tests [41]. These findings are consistent with the results of behavioral memory performances on the MWM and Y-maze tests.

4. Conclusions

Results of the present study demonstrate that MSEE induces a deficit in spatial and working memory, accompanied by an increase in brain IL-1β. Diclofenac pre-treatment prior to MSEE improved the memory deficit and prevented IL-1β elevation. The data suggests the involvement of the arachidonic acid cascade in kratom-induced memory impairment.

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Conflicts of Interest

The authors declare no conflict of interest

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