ORIGINAL ARTICLE DOI: https://doi.org/10.31577/ansr.2024.66.3.4

The effects of ethanol and aqueous extracts of *Moringa oleifera* leaves on learning, spatial memory and anxiety in male mice

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Submitted: 2024-10-29 Accepted: 2024-12-09 Published online: 2024-12-19

Key words: ***Moringa oleifera;* learning; memory; anxiety; Barnes Maze; Elevated Plus Maze**

Act Nerv Super Rediviva 2024; **66**(3): 125–134 ANSR66324A04

Abstract

OBJECTIVE: *Moringa oleifera* leaves are widely used in the treatment of a variety of disorders such as cognitive deficits. Several studies show that *M. oleifera* 's leaves, roots, and seeds all contain high levels of micronutrients, which enhance brain function. The objective of this study is to investigate and compare the effects of ethanolic and aqueous extracts of *M. oleifera* leaves on spatial memory and anxiety-like behavior at various doses.
METHOD: Thirty male mice were randomly divided into five groups (n = 6) and administrated those extracts via intraperitoneal injection for 30 consecutive days. Behavioral assays were performed using the Barnes maze and elevated plus maze tests.
RESULTS: No significant change was observed in the final body weight. Both aqueous and ethanol extracts lead to improved learning and memory by reducing errors. However ethanol extract at 200 mg/kg had a greater effect on improving learning and memory, and did not enhance anxiety compared to the other doses.
CONCLUSION: Ethanol extract of *M. oleifera* leaves in high dose has a more positive cognitive effect rather than aqueous extract of *M. oleifera* leaves.

Introduction

Moringa oleifera (Moringaceae) is a fast-growing tree native to the sub-Himalayan regions of India, Pakistan, Bangladesh, Afghanistan, and the southern part of Iran, where it is employed in traditional medicine (Bakre *et al.* 2013). Because of the high concentration of micro- and macronutrients in *M. oleifera* leaves, it is a widespread dietary source worldwide (Vargas-Sanchez *et al.* 2019). Its leaves contain several compounds studied in animal models, but the findings suggest that they positively impact some human ailments (Vargas-Sanchez *et al.* 2019). Vitamin A, minerals, essential amino acids, flavonoids, and isothiocyanates are all found in *M. oleifera* (Chahardehi & Lim 2022; Kou *et al.* 2018). Therefore, it is generally recognized for its therapeutic potential (Hodas *et al.* 2021). The scientific research sheds light on the utilization of *M. oleifera* with various aqueous, hydroalcoholic, alcoholic, and other organic solvent preparations of various parts of this plant for medicinal purposes (Dhakad *et al.* 2019). The LD_{50} achieved for acute toxicity studies administered orally was more than 6.4 g/kg (Bakre *et al.* 2013). On the other hand, a genotoxic impact has been recently observed at concentrations of *M. oleifera* leaves alcoholic extract higher than 3000 mg/kg (Okechukwu *et al.* 2013). Hence, *M. oleifera* extracts possess several nutraceutical or pharmacological properties (Zhou *et al.* 2018), including anti-inflammatory, antioxidant, anti-cancer, hepatoprotective, neuroprotective, hypoglycemic, and blood lipid-lowering properties (Kou *et al.* 2018). Recent experimental investigations have demonstrated the neuroprotective properties of *M. oleifera* against Alzheimer's, dementia, Parkinson's, stroke, and neurotoxicity-related symptoms. The extract of *M. oleifera* leaves is used to alleviate dementia and enhance spatial memory. It has been demonstrated that these extracts inhibit acetylcholine esterase (AChE) activity, consequently enhancing cholinergic function and cognition (Sutalangka *et al.* 2013). Many neuroprotective phytochemicals have been identified in *M. oleifera*, indicating that it has the potential to exert neuroprotective effects (Ghimire *et al.* 2021) in both *in vitro* and *in vivo* studies (Vargas-Sanchez *et al.* 2019). Notably, flavonoids, such as quercetin and rutin, and polyphenolic compounds present in the extract of *M. oleifera* are known to possess antioxidant and neuroprotective effects (Bhattacharya *et al.* 2018).

The plant *M. oleifera* has been utilized in various ways, as demonstrated in several publications (Hodas *et al.* 2021). For instance, in animal models, *M. oleifera* was discovered to protect rats against cerebral ischemia (Kirisattayakul *et al.* 2013) and oxidative DNA damage (Singh *et al.* 2009). In studies on rats with induced arthritis, ethanolic extracts of *M. oleifera* leaves were found to have antinociceptive and anti-inflammatory properties (Mahdi *et al.* 2018). Also, Zhou *et al.* (2018) discovered that the anti-amnesic property of *M. oleifera* seeds might be regulated through cholinergic activity, hippocampus neurogenesis, and the Akt/ERK1/2/CEB signaling pathways (Zhou *et al.* 2018). Treatment with a supplement diet from this plant mitigated the loss of spatial memory function via a considerable decrease in escape latency and a significant increase in the frequency of crossing when mice spent time in the platform quadrant in the Morris water maze (MWM) test (Onasanwo *et al.* 2021). In addition, Adebayo *et al.* (2021) used a mixture of *M. oleifera* with a rat's diet continuously divided into 1, 5, 10, and 20% for 12 weeks and observed that diets improve spatial memory in the MWM test and non-spatial memory in the object recognition test, reduce AChE activity, protect against oxidative stress, and prevent neuronal degeneration in the hippocampus as stained with Cresyl violet dye (Adebayo *et al.* 2021). In another study, 100, 200, and 400 mg/kg of hydroalcoholic extract of *M. oleifera* were investigated for neurodegeneration in a rat model of age-related dementia to assess memory, MDA level, neuron density, the activities of SOD, CAT, GSH-Px, and AChE in the hippocampus (Sutalangka *et al.* 2013). Although the extract enhanced spatial memory and lowered MDA levels and AChE activity, it also raised SOD and CAT activities in CA1, CA2, CA3, and the dentate gyrus in the hippocampus, according to the study's findings (Sutalangka *et al.* 2013).

This study was conducted in order to compare the differences in cognitive behaviors including learning, memory and anxiety levels caused by aqueous and ethanol extracts of *M. oleifera* leaves. In order to improve the quality of memory and learning assessment, Barnes' Maze was used that permits the rapid and precise evaluation of various elements of spatial learning and memory compared to other maze types (Gawel *et al.* 2019).

Materials and methods

Aqueous extract (AE) of Moringa oleifera

The fresh *M. oleifera* leaves, harvested in September 2023 from Hormozgan province, Iran. The plant materials were air-dried under the shade for two weeks and then grounded into powdered form. The aqueous extract was prepared by maceration 200 g of plant leaves powder in one liter of hot water (80°C) and for 2 hours. During maceration, hot water was incrementally added until the process was complete. The resulting mixture underwent filtration through Whatman No. 1 filter paper and concentration via a rotary evaporator at 45°C (Jadhav *et al.* 2022).

Ethanol extract (EE) of Moringa oleifera

The ethanol extract was prepared by maceration 200 g of plant leaves powder in one liter of alcoholic solvent (96% ethanol) for three consecutive days placed on a shaker at room temperature in the dark. The extraction process was carried out with the help of ultra-

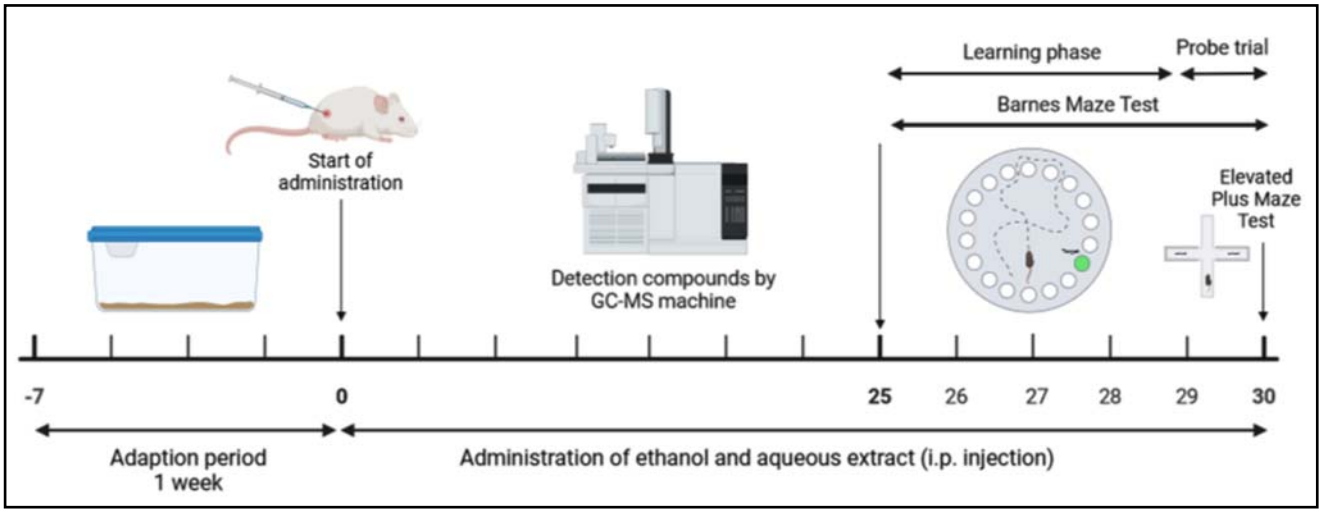

Fig. 1. A simplified experimental design procedure

sound (time 35 minutes, temperature 35 degrees Celsius and frequency 37 kHz). Afterward, the solution was smoothed with Whatman No. 1 filter paper for 10 minutes at a speed of 3500 rpm (1008g) in a centrifuge. Then, using a rotary evaporator, the resulting extract was obtained. Both extracts (aqueous and ethanol) was storage in dark tubes at 4°C in a refrigerator for future use (El-Shehawi *et al.* 2021)

Animals

A total of thirty male mice, aged 5 -6 weeks old with an average weight of 25-30 g, were randomly administrated to the ethanolic and water extract of *M. oleifera* groups with their control groups at the AJA University of Medical Sciences Experimental Animal Laboratory. We performed animal experiments following the U.K. Animals (Scientific Procedures) Act, 1986, and its associated guidelines, the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). For the experiment, five groups were considered.The animals were housed in 12:12 hour dark/light cycle, room temperature (24°C ± 2°C), 60% relative humidity and provided with sufficient food and water (Husseini *et al.* 2021; Sadeghi *et al.* 2024). Every attempt has been made to reduce the pain. The ethics number was registered by the Council of Laboratory Animals of AJA University of Medical Sciences, Tehran, Iran (Approval No. IR.AJAUMS.REC.1400.145 in April 2021).

Experimental design

All mice were randomly assigned to five groups of 6 animals after a week of acclimatization, each as follows:

1) Mice were kept in their cage with no administration (n = 6, Control).
2) Mice were treated with 5 mg/kg of aqueous extract of *M. oleifera* (n = 6, AE 5 mg/kg).
3) Mice were treated with 200 mg/kg of aqueous extract of *M. oleifera* (n = 6, AE 200 mg/kg).
4) Mice were treated with 5 mg/kg of ethanolic extract of *M. oleifera* (n = 6, EE 5 mg/kg).
5) Mice were treated with 200 mg/kg of ethanolic extract of *M. oleifera* (n = 6, EE 200 mg/kg)

A one-month experimental period did not result in any documented injuries or fatalities. The schematic design of the experiment is shown in Fig. 1.

Behavioral analysis

Barnes maze test

The Barnes maze is a spatial learning device where animals look for an escape box (Barnes 1979). The maze is a darkish circular platform (120 cm in diameter), elevated 90 cm from the floor, with 20 circular holes (10 cm in diameter) arranged at the platform's edge. A 10×10×15-cm escape box is linked to one of the holes (the target hole). There were visible signals on the walls 50 cm away from the equipment. The lights were turned on (420 lux) during the behavioral sessions to boost the escape incentive. All experiment activities were captured on video using a video camera mounted above the equipment and evaluated using video-tracking software (Borj Sanat, Tehran, Iran).

Barnez Maze

During the habituation period, the animals were permitted to openly explore the maze for 1 hour. The animals were subjected to four daily training sessions and one probe trial. The initial training session was conducted directly following the habituation period. Each session started with the animals being placed under the escape box in the middle of the maze for 90 seconds. The escape box was then raised, and the animal was set free to investigate the maze. Every trials lasted 90 seconds or until the animals entered the target chamber. Nevertheless, if the rats did not reach the target chamber after the trial, the researcher gently directed them toward it. The animals stayed

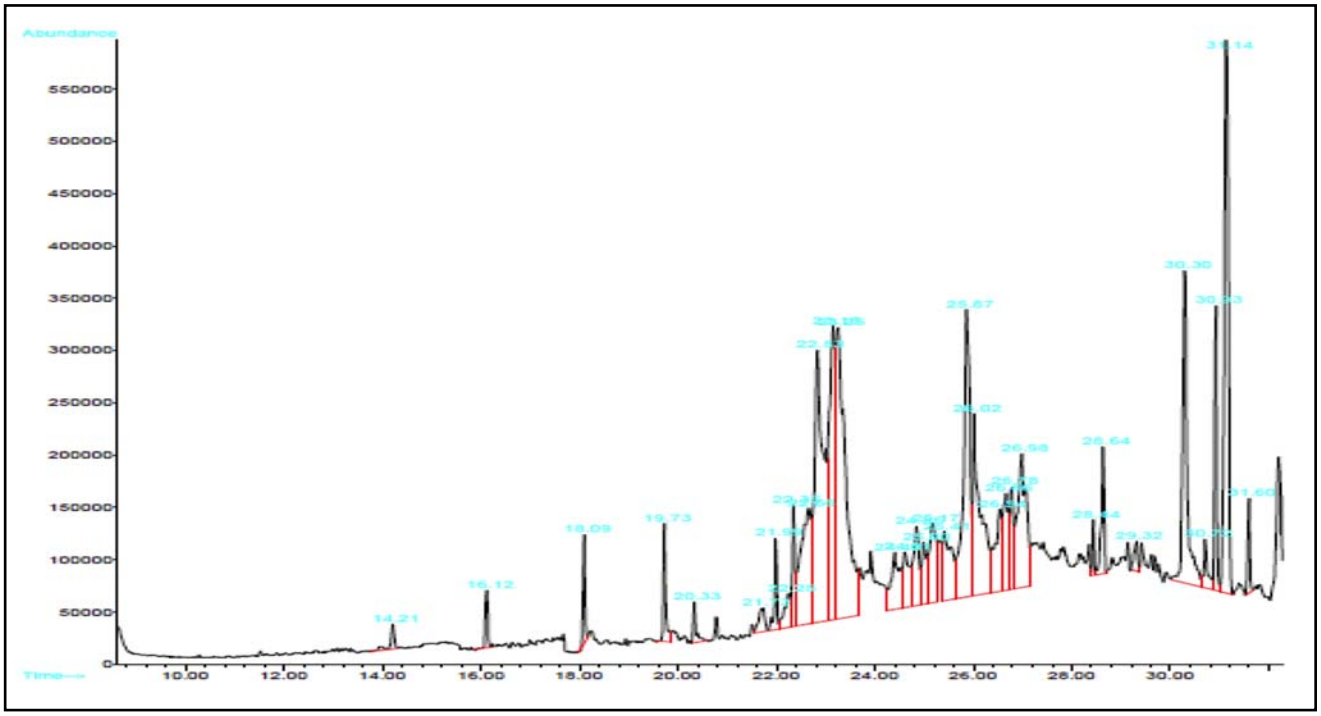

Fig. 2. GC-MS chromatogram of 98% ethanolic extract of *Moringa oleifera*

in the target chamber after they reached it, and the escape box was placed on that chamber for 90 seconds. The probing trial (day 5) assessed the extent to which spatial learning had been retrieved (short term memory). The process was similar to the learning phases. The maze was washed down between each trials (with a 5% alcohol solution) to remove any lingering scents that might serve as signals (Hatef *et al.* 2023).

Elevated plus maze (EPM) test

The elevated plus maze test evaluated the anxiolytic activity of AE and EE extracts. This test assessed mice anxiety-like behavior due to their innate distaste for open and elevated places. The tool comprised two closed and two open arms, constructed from acrylic material and positioned 50 cm above the floor. The elevated plus maze was made of a central platform (10 cm × 10 cm), two opposite open arms (50 cm × 10 cm × 1 cm height), and two closed arms (50 cm × 10 cm × 40 cm height) (Rafati *et al.* 2015; Sestakova *et al.* 2014; Tarsaei *et al.* 2022). At the start of each test, the animal was put in the center, facing open arms, and given five minutes to wander freely. The motion path of the animals was recorded using a video-tracking system (Borj Sanat, Tehran, Iran). Total arms entries (open and closed), open-arms, and closed-arms entry percentages were examined. In addition, the percentage of open arm entries [open entries/(open + closed entries) 100] and the proportion of time spent in open arms [(open time/300) 100] were calculated. The maze was wiped using an alcohol solution before each experiment (Rafati *et al.* 2015). The anxiety index was determined by Mazor *et al.* and Rao *et al.* methods (Mazor *et al.* 2009; Rao & Sadananda 2016), which considered the frequency and length of time spent in the open arms in proportion to the overall quantity of time spent exploring the apparatus (the higher the index, the lower anxiety). The anxiety index was computed using the formula below:

$$\text{Anxiety index} = 1 - \left(\frac{\text{Open} - \text{arm time}}{\text{Total time}}\right) + \left(\frac{\text{Open} - \text{arm entries}}{\text{Total entries}}\right)/2$$

Statistical Analysis

All results are presented as the mean ± standard deviation (SD). All statistical analysis was analyzed using GraphPad Prism® 8.1 software for Windows (GraphPad Software, San Diego, CA, USA). The significance level was accepted at $p < 0.05$ after Dunnett's test using one-way ANOVA.

Results

GC-MS analysis

The results of the GC-MS analysis of *Moringa oleifera* ethanolic extract (EE) lead to the identification of several compounds. These chemicals are classified by the mass spectrometry coupled to the GC. As shown in Figure 2 and Table 1, the GC-MS spectra and the potential bioactive compounds of 98% ethanolic extract (crude extract) to determine the chemical groups of the biomass indicated the presence of several chemicals with varying retention times.

The fragmentation of the large compounds into more minor compounds produces peaks with various m/z ratios. These mass spectra constitute the compound's traceable fingerprint data library. However, we found 33 peaks from GC-MS analysis, and then we selected 12 peaks that showed a higher 50% in the data in the Wiley library. Some important constituents detected include n-eicosane, hexadecenoic (palmitic) acid, and tetradecane.

Body weight, food and water intake

Several patterns of body weight, water, and food intake are shown in Fig. 3. According to Fig. 3A, the rates of body weight gain in all groups increased comparatively considerably from day 1 to day 30. However, the body weight in the treated groups did not show a significant difference compared with the control group. Both ethanolic extracts exhibited the highest body weight and weight gain; however, no significant difference was observed in the treated groups compared to the control group ($p > 0.05$, Fig. 3A and B). The AE 200 mg/kg group demonstrated a decrease in body weight that was not statistically significant compared to the control group.

Based on Fig. 3C, the graph showed the fluctuation pattern from day 1 to day 25 in groups EE 5 mg/kg and EE 200 mg/kg. However, groups including control, AE 5 mg/kg, and AE 200 mg/kg exhibited steady lines without major fluctuations. During the study, both AE extracts (5 and 200 mg/kg) showed high food intake from day 5 to 25. On day 25, no significant difference between groups was detected. Finally, in all groups at day 30, food consumption decreased compared to the control group (AE 5 mg/kg, AE 200 mg/kg, and EE 200 mg/kg at $p < 0.001$, while EE 5 mg/kg at $p < 0.05$). For the analyses depicted in Fig. 3D, animals in different groups consumed significantly more and less water, especially between days 15 and 25. However, at day 30, all treated groups, showed high consumption water compared to the control group (AE 5 and 200 mg/kg at $p < 0.001$; EE 5 and 200 mg/kg at $p < 0.05$).

Barnes maze test

The learning and memory assessment of mice were evaluated between the twenty-fifth and thirtieth days after i.p. injection. The effect of dose-related extracts on spatial memory was analyzed using the Barnes maze. In all doses of the administration of aqueous and ethanolic extracts, there was a significant difference in the time to reach the target hole (Fig. 4B) and the error rate (Fig. 4B) compared to the control group ($p < 0.05$), as shown in Fig. 4.

Direct strategies were increased in the EE 200 mg/kg group during the learning phase; however, other groups showed a less direct strategy during these training days. On the probe trial, we observed a random strategy in AE 5 mg/kg, EE 5 mg/kg, and EE 200 mg/kg. Only the AE 200 mg/kg showed 25% serial strategy on the probe trial, respectively. The EE 200 mg/kg group's results were completely different from the other groups. Thus, considering the search strategies in the probe trial (day 5), we categorized our groups into the aqueous extracts (AE 5 and 200 mg/kg groups) and ethanolic extracts (EE 5 and 200 mg/kg groups). On the other hand, we also categorized low and high doses of extracts. We selected the direct strategy as a spatial strategy, while random and serial strategies are included in the non-spatial strategy for analysis

Tab. 1. Compound investigated in the 98% ethanol extract of *Moringa oleifera* in GC-MS

RT	Name of compound	Molecular formula	Molecular weight g/mol	%	Compound nature
14.22	3-Isopropoxy-1,1,1,7,7,7-hexamethyl	$C_{18}H_{52}O_7Si_7$	577.2	56	organosilicon compound
16.11	3,3′:5,3″-bis(trimethylene)-2,6-di(1′,8′-naphthyrid-2′-yl)pyridine	$C_{27}H_{21}N_5$	415.5	50	Naphthyridines
18.09	Cyclohexasiloxane, 2,2,4,4,6,6,8,8,10,10,12,12-dodecamethyl-	$C_{12}H_{36}O_6Si_6$	444.92	90	Cyclomethicone
19.72	Tetradecane,629-59-4, n-tetradecane	$C_{14}H_{30}$	198.39	96	Alkanes
22.36	n-Eicosane	$CH_3(CH_2)_{18}CH_3$	282.56	53	Alkanes
22.66	Hexatriacontane	$C_{36}H_{74}$	506.97	64	Oligomer of polyethylene
23.15	Eicosane	$C_{20}H_{42}$	282.54	97	Alkanes
23.25	Eicosane	$C_{20}H_{42}$	282.54	95	Alkanes
30.30	n-Hexadecanoic (palmitic) acid	$C_{16}H_{32}O_2$	256.42	98	Fatty acid
30.93	Hexadecanoic acid, ethyl ester (Ethyl palmitate)	$C_{18}H_{36}O_2$	284.47	97	Fatty acid
31.15	Eicosane	$C_{20}H_{42}$	282.54	97	Alkanes
31.59	Bis(trimethylsilyl)acetylene	$C_8H_{18}Si_2$	170.39	64	Organosilicon

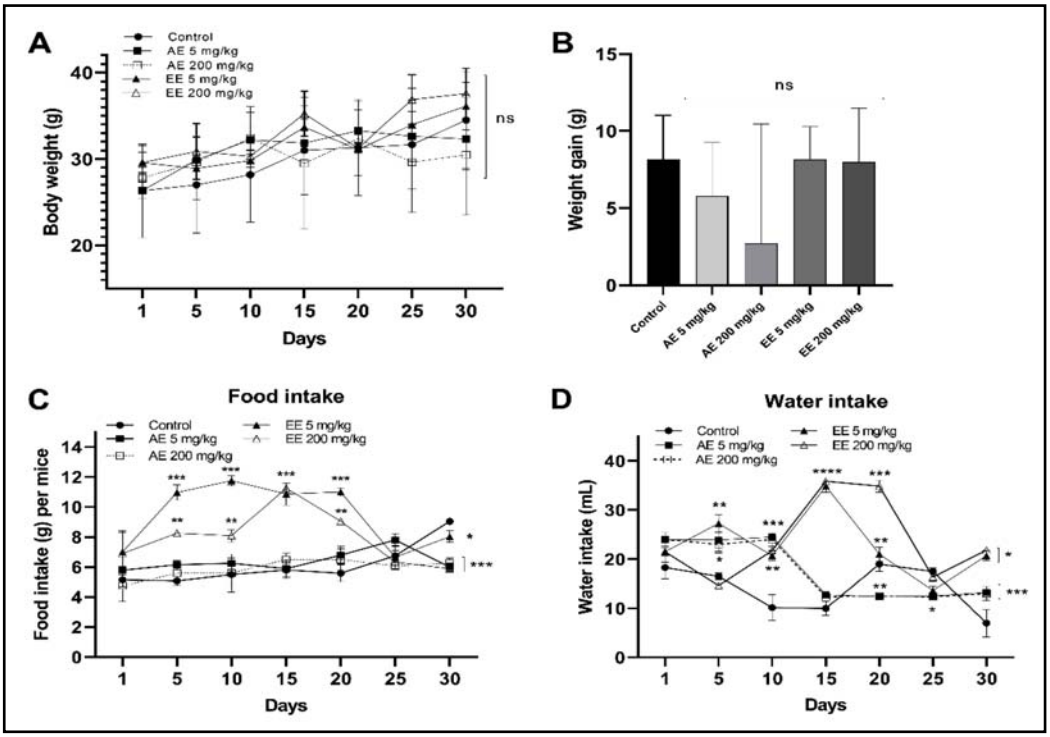

Fig. 3. Effects of various crude extracts of *M. oleifera* on body weight, food and water intake. **A)** Body weight of mice subjected to administration of water and ethanolic extract of *M. oleifera*, **B)** weight gain after end of the experiment, **C)** food intake (g), and D) water intake (mL). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ against the sham group; one-way ANOVA followed by Dunnett's multiple comparison test. ns = the experiment groups and control group showed no statistically significant differences.

using Fisher's exact test. Based on Fisher's exact test, no significant difference was observed between AE and EE extracts during the learning phase ($p = 0.0573$), the relative risk was 0.5385, 95% CI: 0.2962 to 0.9699, and the odds ratio was 0.4818 (95% CI: 0.2350 to 0.9425). We did not find a significant difference ($p > 0.05$) between AE vs. EE extracts groups for the probe trial. As we mentioned, we tried to analyze low and high doses of those extracts. According to Fisher's exact test during the training session based on 5 vs. 200 mg/kg of those extracts, we found a significant difference at $p = 0.0244$, with relative risk = 0.4815 (95% CI: 0.2614 to 0.8772) and odds ratio = 0.4229 (95% CI: 0.2125 to 0.8370). The percentages for a total of 5 mg/kg extracts (AE and EE) were 5.08% and 44.92% (spatial and non-spatial, respectively), and for 200 mg/kg, they were 10.55% and 39.45% (spatial and non-spatial, respectively). In addition, for the probe trial, we did not observe a significant difference between low and high doses of extracts ($p > 0.05$).

During the learning phase of all groups, the latency to escape has not been changed except between groups ($F_{(4,140)} = 8.85$, $p < 0.0001$, Bonferroni post hoc, Fig. 4B); however, for days ($F_{(3,140)} = 1.73$, $p = 0.1627$) and for interaction ($F_{(12,140)} = 1.50$, $p = 0.1301$), there were no significant differences. Based on time spent on the probe trial using one-way ANOVA using Dunnett's test, all groups showed a significant difference compared to the control group ($p < 0.0001$). It was concluded that all treated groups did not affect the time and memory required to find a target hole. Fig. 4C depicts the overall number of errors. As with the latency, the total number of errors followed by the same pattern over learning phases (groups) for all groups significantly ($F_{(4,140)} = 25.2$, $p < 0.0001$), except for interaction ($F_{(12,140)} = 0.302$, $p = 0.9881$, and for days ($F_{(3,140)} = 1.35$, $p = 0.2612$). In the probe trial, there was a significant difference between the groups compared to the control group ($p < 0.0001$).

Elevated plus maze test

To further assess the effect of various extracts on stress in mice, an EPM test was conducted. The amount of time (sec) spent in the open and closed arms of the elevated plus maze test was measured for male mice in the control and treatment groups. The EPM test is depicted in Fig. 5, representing the percentage of entries in open and closed arms and the percentage of time spent in open and closed arms. However, when compared to the control group, the EE 200 mg/kg extract spent more time (21.8%) in the open arms ($p = 0.9369$). However, the AE 5 and 200 mg/kg groups showed less time spent than others ($p = 0.0005$, 10.49%, and 10.53%, respectively), followed by group EE 5 mg/kg ($p = 0.0053$) at 12.80%. Also, a similar pattern of significant differences was observed in the time spent on open arms and the entries to the open arms (Fig. 5 A and D). Regarding the percentage of open-arms entries, the one-way ANOVA exhibited a significant difference between the AE 200 mg/kg and EE 5 mg/kg compared to the control group ($p < 0.0001$ and $p = 0.0481$, respectively), which showed no positive effect on stress. Meanwhile, the EE 200 mg/kg and AE 5 mg/kg groups showed a similar result, control group but no significant difference as compared with the control group (Fig. 5D). However, we observe a higher percentage of time spent in closed

arms in AE 5 mg/kg at 56.93% compared to the control group ($p = 0.0275$).

Open-arm duration and open-arm entries may be used to compute an anxiety index score ranging from 0 to 1 based on the total number of entrances, including those with open and closed arms, and cumulative time spent on this test. In this study, the anxiety index ranged from 0.6 to nearly 0.9, with a significant difference between groups. However, only the EE 200 mg/kg group showed a lower anxiety index with no significant difference compared to the control group ($p = 0.5306$).

Discussion

Moringa oleifera leaves are widely used in the treatment of a variety of disorders across the world, including neurological diseases, diabetes, hypertension, inflammation and hypercholesterolemia(Ademosun *et al.* 2022). Scientific proof needs to be improved for the medical applications of natural products, which in many cases are based exclusively on traditional knowledge. This result satisfies a crucial need for *in vivo* examination of the alleged tradition of using laboratory animals as a model (Mahdi *et al.* 2018). The present study examined the influence of *M. oleifera* leaves extracts on spatial memory, learning, and stress. The results indicated conclusively that both extracts of *M. oleifera* leaves considerably enhanced spatial memory according to the number of errors.

Hence, we performed the EPM test to evaluate anxiety-like behavior. After 30 days of i.p. administration at doses 5 and 200 for ethanol and aqueous extracts, a rise in the percentage of entries was observed in the EE 5 mg/kg group in the open arms ($p = 0.0481$), but the percentage of time spent was lower than in the control group ($p = 0.0053$). However, treatment with EE (200 mg/kg) did not significantly change the number of entries in the open arms as well as time spent in open arms, indicating high dose of ethanol extract of *M. oleifera* has no anxiotic effect. However, the AE extract showed a significantly reduced number of entries and time spent with open arms. Based on Sutalangka *et al.* (2013), hydroalcoholic (50% water/50% ethanol) extract of *M. oleifera* treated groups exhibited lower escape latency but enhanced retention time in MWM test (Sutalangka *et al.* 2013). Another study by Afrin *et al.* (Afrin *et al.* 2022) showed considerably fewer working memory errors were made by the experimental group using ketamine along with 200 mg/kg of *M. oleifera* than by memory-impaired group (only induced by intraperitoneal ketamine). Additionally, based on their study, the working memory variability is lowest in the experimental group. The spatial memory encoding process is related to the hippocampal subregions CA1, CA3, and dentate gyrus (DG), particularly the dorsal hippocampus. Whereas the recall procedure is linked to CA3, the function of CA2 remains poorly understood (Sutalangka *et al.* 2013). Researchers observed that the NR2B subunit in the brain's prefrontal cortex and hippocampus was improved by the omega-3 polyunsaturated fatty acid present in *M. oleifera* leaves (Hauber & Bareiss 2001). *M. oleifera* polyunsaturated

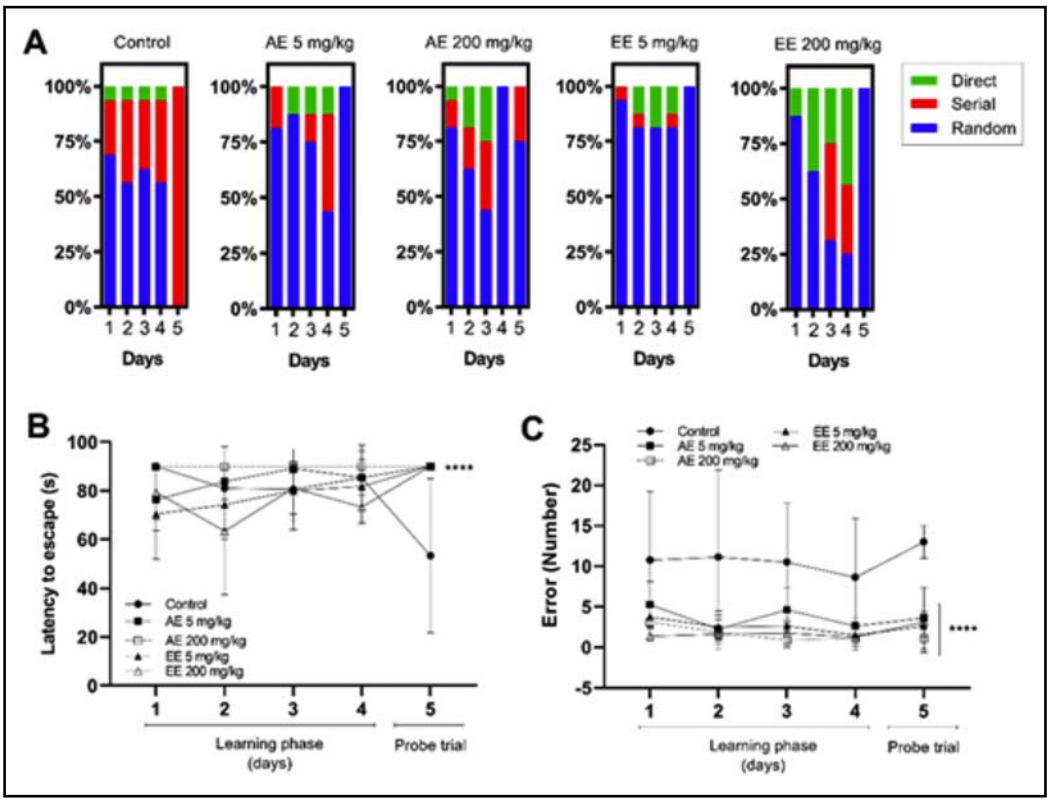

Fig. 4. The effect of aqueous and ethanol extract of *M. oleifera* at 5 and 200 mg/kg to evaluate memory and to learn in the Barnes Maze task. **A)** Percentage of search strategy, **B)** latency to escape in the learning phase and probe trial, **C)** number of errors in the learning phase and probe trial.

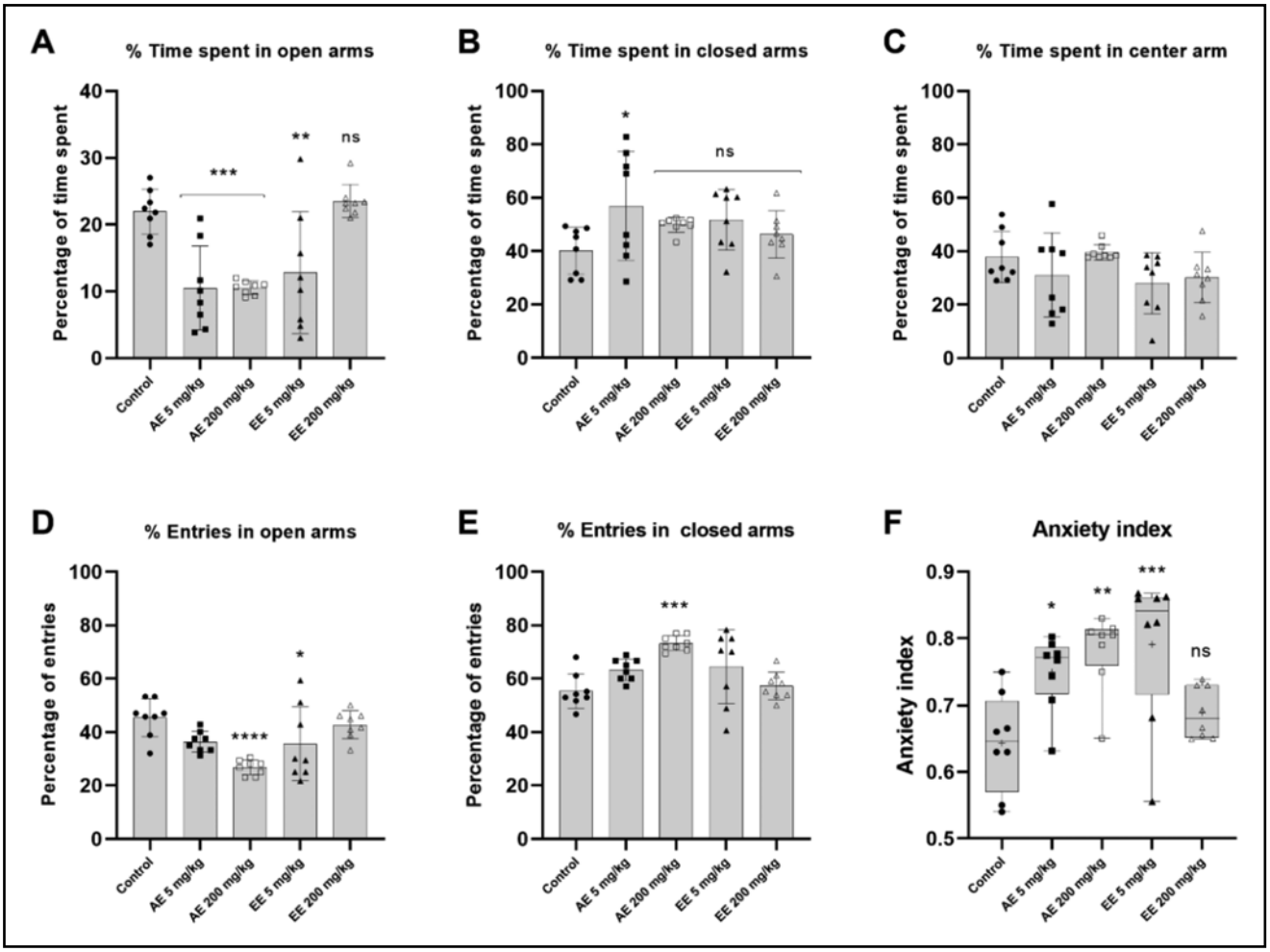

Fig. 5. Effects of two different extracts in various doses in the EPM test in open and closed arms. **A)** percentage of time spent in the open arms; **B)** the percentage of time spent in the closed arms; **C)** the percentage of time spent in the center zone; **D)** the percentage of the entries into the open arms; **E)** the percentage of entries into the closed arms, and **F)** anxiety index, a correlation exists between the anxiety index and open arms. The whiskers of the box plot indicate minimum to maximum. Data represent the mean ± SD (n = 8 per group). * $p < 0.05$, *** $p < 0.001$, ns = no significant. One-way ANOVA followed by Dunnett's multiple comparison test.

fatty acids increased NR2A and NR2B subunit expression in the hippocampus (Shen *et al.* 2018). Based on GC-MS, the ethanolic extract showed hexadecenoic acid (palmitic acid), categorized as a fatty acid. However, dietary palmitic acid influences on learning because palmitic acid alters multiple essential neuronal proteins via palmitoylation, including GAP-43, NMDA receptor, and AMPA receptor, all of which play a crucial role in learning (Hosseinzadeh *et al.* 2007). However, structural fatty acids have a detrimental effect on cognitive outcomes in stroke patients (Kotlega *et al.* 2021). Because we discovered two fatty acids in our ethanol extract (n-Hexadecanoic (palmitic) acid and Hexadecanoic acid, ethylester (Ethyl Palmitate), this could explain why increasing NMDAR subunits prevented working memory deficits in *M. oleifera*-treated mice in our study. In a study by Afrin *et al.* using a ethanol *M. oleifera* extract mixture with ketamine, the extract could prevent ketamine-induced memory impairment, and NMDAR may be implicated in this preventive efect (Afrin *et al.* 2022).

Abijo *et al.* (2019) found that large doses of aqueous *M. oleifera* caused damage to specific areas of the frontal cortex of growing Wistar rats (Abijo *et al.* 2019). However, Bakre *et al.* (2013) found that the ethanol extract of *M. oleifera* doses ranging from 250 to 2000 mg/kg were shown to significantly reduce rearing, grooming, head dips, and locomotion ($p < 0.001$). It also increased anxiogenic effect and enhanced learning and memory (Barke *et al.* 2013). Based on Sutalangka's study, the memory-enhancing effect of *M. oleifera* leaf extract may be mediated in part by a reduction in oxidative stress and an improvement in cholinergic performance. Also *M. oleifera* leaves extract may have a crucial role in improving cognitive performance through processes including vasodilation, which increased regional blood flow, and the reduction of monoamine oxidase (MAO), which led to improved dopaminergic function (Dangi *et al.* 2002; Sutalangka *et al.* 2013).

According to Onasanwo *et al.* (2021), a supplement diet from *M. oleifera* dramatically reduced oxidative and

inflammatory stress, restored cholinergic transmission by inhibiting acetylcholinesterase (Ache), and maintained neuronal integrity in the brains of mice. Their findings show that a diet supplemented with *M. oleifera* may serve as a viable therapeutic and pharmacological macromolecule for reducing neuronal cell death and managing Alzheimer's disease (Onasanwo *et al.* 2021). In another study, Zeng *et al.* (2019) found that the seeds of *M. oleifera* exhibit neuroprotective properties in both the acute and chronic phases of ischemic stroke (Zeng *et al.* 2019). In another study by Mahaman (2018), an additional 14 days of *M. oleifera* gavage were administered as curative therapy. In addition to preventing hyperhomocysteinemia-induced oxidative stress and cognitive deficits, the extract from *M. oleifera* was also found to be able to reserve them (Mahaman *et al.* 2018). However, more research is still necessary into the extract's active component(s) and the mechanism's finer points.critical protective mechanism may promote hippocampus neurogenesis and synaptic plasticity and enhance

Conclusion

M. oleifera leaves are neuroprotective effects and improve the cognitive function of brain. However, the cognitive positive effect of *M. oleifera* ethanol extract is more than its aqueous extract.

Conflict of interest

The authors declare no conflicts of interest.

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