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Original Article

Role of Azadirachta indica leaf extract on Working and Reference Memory of ketamine Induced Memory Impaired Male Wistar Rats in Morris Water Maze

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ABSTRACT

Background: *Ketamine, a known NMDA receptor antagonist, induces memory impairment in experimental models. Azadirachta indica leaf extract (AILE), rich in bioactive compounds, has shown potential neuroprotective effects. This study evaluates the effects of AILE on ketamine induced memory impairment using the Morris Water Maze (MWM) test in male Wistar rats.* ***Methods & Materials:*** *This experimental study aimed to investigate the effects of AILE on working and reference memory in ketamine-induced memory-impaired male Wistar rats. 24 rats were selected for the animal selection. Male wistar rats were divided into normal memory (oral normal saline treated,5ml/kg/day for 26 days), memory impaired (intraperitoneal ketamine treated, 15mg/kg/day during 5 days of acquisition phases) and experimental (oral AILE treated, 300mg/kg/day for 26 days and intraperitoneal ketamine, 15mg/kg/day during 5 days of acquisition phases). The study was conducted at the Memory Laboratory, Department of Physiology, Bangabandhu Sheikh Mujib Medical University (BSMMU), duration from March 2020 to February 2021. Ethical approval was obtained following a thorough review by Institutional Review Board (IRB) of BSMMU.* ***Results:*** *Ketamine administration significantly impaired spatial working and reference memory, evidenced by increased escape latency and reduced target crossings. AILE co-administration significantly improved these parameters, reducing escape latency and increasing target crossings. Remarkably, AILE enhanced reference memory retrieval beyond normal levels, indicating its therapeutic potential.* ***Conclusion:*** *AILE mitigates ketamine-induced memory deficits and enhances spatial memory retrieval, likely through NMDA receptor modulation, oxidative stress reduction, and apoptotic pathway attenuation. These findings highlight the potential of AILE as a therapeutic agent for memory impairment disorders.*

Keywords*: Azadirachta indica, Morris Water Maze, ketamine-induced memory impairment, working memory, reference memory*

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INTRODUCTION

Memory is a complex cognitive process involving the acquisition, storage, and retrieval of information. It is broadly categorized into working memory, which temporarily holds and manipulates information for immediate use, and reference memory, which stores long-term contextual information. Working memory plays a pivotal role in tasks such as comprehension, learning, and reasoning by maintaining a limited amount of spatial or non-spatial information for short-term use [1]. Reference memory, on the other hand, is a long-term declarative memory system associated with consistent recall of environmental or procedural cues, such as an animal's navigation of its territory [2]. These two forms of memory represent key aspects of episodic memory: the "what" (content) and "where" (spatial context) dimensions.

Memory formation involves distinct neural mechanisms, with working memory relying on persistent neural activity and reference memory involving synaptic plasticity, such as long-term potentiation (LTP). N-methyl-D-aspartate (NMDA) receptors, critical for synaptic plasticity, mediate excitatory postsynaptic potentials (EPSPs) that underlie memory functions. Blockade of NMDA receptors disrupts these processes, leading to memory impairment [3]. Ketamine, a noncompetitive NMDA receptor antagonist, inhibits persistent neural activity and impairs both working and reference

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memory at sub-anesthetic doses [4,5]. Animal models treated with ketamine demonstrate impairments in memory acquisition, encoding, and retrieval, as evidenced by performance in spatial tasks such as the Radial Arm Maze (RAM) and Morris Water Maze (MWM) [6]. The MWM is particularly effective for evaluating spatial working and reference memory, as it relies on an animal's ability to navigate using spatial cues to locate a hidden platform. This task has been strongly correlated with hippocampal synaptic plasticity and NMDA receptor function, making it a robust tool for studying memory impairment [7].

Given the limitations and adverse effects of pharmacological treatments for memory impairment, there is growing interest in alternative therapeutic strategies, including the use of medicinal herbs. *Azadirachta indica*, commonly known as neem, has demonstrated a range of pharmacological properties, including anti-inflammatory [8], antioxidant [9,10], antiviral [11], and neuroprotective effects [12]. Notably, neem leaf extract (AILE) has shown potential in preventing memory deficits in experimental models, with doses of 300 mg/kg/day [13] and 500 mg/kg/day [12] proving effective without adverse effects.

Additionally, sex hormones play a critical role in learning and memory processes, further emphasizing the need for comprehensive studies addressing both hormonal and neurochemical factors in memory modulation [14].

OBJECTIVE

The present study aims to evaluate the effects of AILE on spatial working and reference memory in male Wistar rats subjected to ketamine-induced memory impairment. Using the MWM test, we assess whether AILE can ameliorate the deficits in spatial memory caused by ketamine administration, providing insights into its potential therapeutic applications.

METHODS & MATERIALS

Study Design and Settings

This study was conducted at the Memory Laboratory, Department of Physiology (BSMMU), and spanned from March 2020 to February 2021. The study population consisted of male Wistar rats weighing 200 ± 50 g, selected for their suitability for memory-related experiments and homogeneity in weight and age. Male wistar rats were divided into normal memory G1 (oral normal saline treated, 5ml/kg/day for 26 days, memory impaired G2 (intraperitoneal ketamine treated, 15mg/kg/day during 5 days of acquisition phases and experimental G3 (oral AILE treated, 300mg/kg/day for 26 days and intraperitoneal ketamine, 15mg/kg/day during 5 days of acquisition phases).

Animal selection

Twenty-four (24) rats were obtained from the central animal house of BSMMU, Dhaka. All rats were kept in the rat laboratory of the department of Physiology, BSMMU, and were housed in specially built plastic cages with 4 rats per cage under a 12/12-hour light/dark cycle. The ambient room temperature was maintained at around 27° to 28°, corresponding to the thermoneutral zone for rodents [15]. All the rats had free access to the standard laboratory food, cooled boiled water *ad libitum*, during acclimatization. To avoid circadian influences, all the experiments were performed during the day between 08.00 and 16.00.

Study Procedure

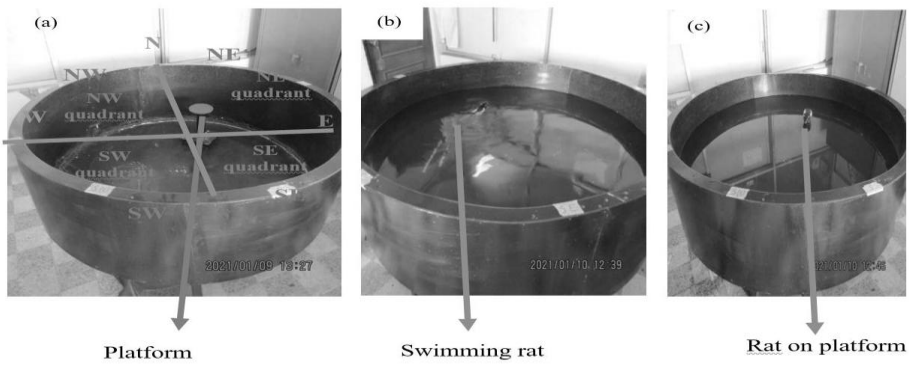
Apparatus

The MWM was a circular pool with a diameter of 150 cm and a height of 50 cm, filled with water at a depth of 30 cm, maintained at a temperature of (24–26) °C (Figure 1). The pool was divided into four quadrants: northwest (NW), northeast (NE), southeast (SE), and southwest (SW) [16,17]. A black platform (28 cm in height) was placed at the center of one quadrant, submerged 2 cm below the water surface to serve as the escape platform. Both the pool and the platform were painted black to eliminate visual cues. The pool featured eight labeled start locations: NW, NE, SE, SW, south (S), north (N), east (E), and west (W).

Procedure

The test was conducted in a well-lit room containing various external visual cues such as racks, windows, doors, shelves, computers, cameras, and the experimenter. Test was performed according to the methods of previous research [16,17]. Eight (8) rats from each group (total 24) were room acclimatized for 7 days. This test was divided into reference memory rest and a working memory test.

Every day each rat was brought into the memory lab for reference and a working memory test. The initial trial was started 30 minutes after administering the prefixed treatment based on their group assignment.



W: west; NW: northwest; SW: southwest; S: south; SE: southeast; E: east; NE: northeast; N: north.

Figure – I: Morris water maze pool (a) without water (b) with swimming rat (c) with rat on platform.

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Reference Memory Test

The reference memory test consisted of three phases: habituation, acquisition (training), and probe trial (Figure – II).

1. Habituation Phase (Days 19–21)

Each rat was introduced to the water pool for 3 minutes daily over three consecutive days without the escape platform. This allowed the rats to familiarize themselves with the pool environment.

2. Acquisition Phase (Days 22–27)

During the acquisition phase, each rat underwent four swimming trials daily for six consecutive days. The platform was submerged 2 cm below the water surface and placed in a specific quadrant, consistent across all trials for each day. The starting locations for each trial were randomized. For the first trial on Day 22, the platform was placed in the NE quadrant, and the rat was released into the pool from the SW quadrant, facing the pool wall. The rat was allowed 60 seconds to locate the platform. If it failed, it was gently guided to the platform and allowed to remain there for 20 seconds before being returned to its cage. A 30-second interval was maintained between trials. Subsequent trials on the same day involved start locations in the SE, S, and W quadrants, respectively. Escape latency, defined as the time taken for a rat to locate the platform, was recorded for each trial. The sequence of start locations changed daily as detailed in Table I and Platform position and sequence of start locations of the working memory test in MWM as detailed in Table II.

3. Probe Trial (Day 28)

Twenty-four hours after the last acquisition trial, the platform was removed from the pool. Each rat was released from a distal starting point and allowed to swim for 60 seconds. The number of crossings over the former platform location was recorded as an indicator of spatial memory retention.

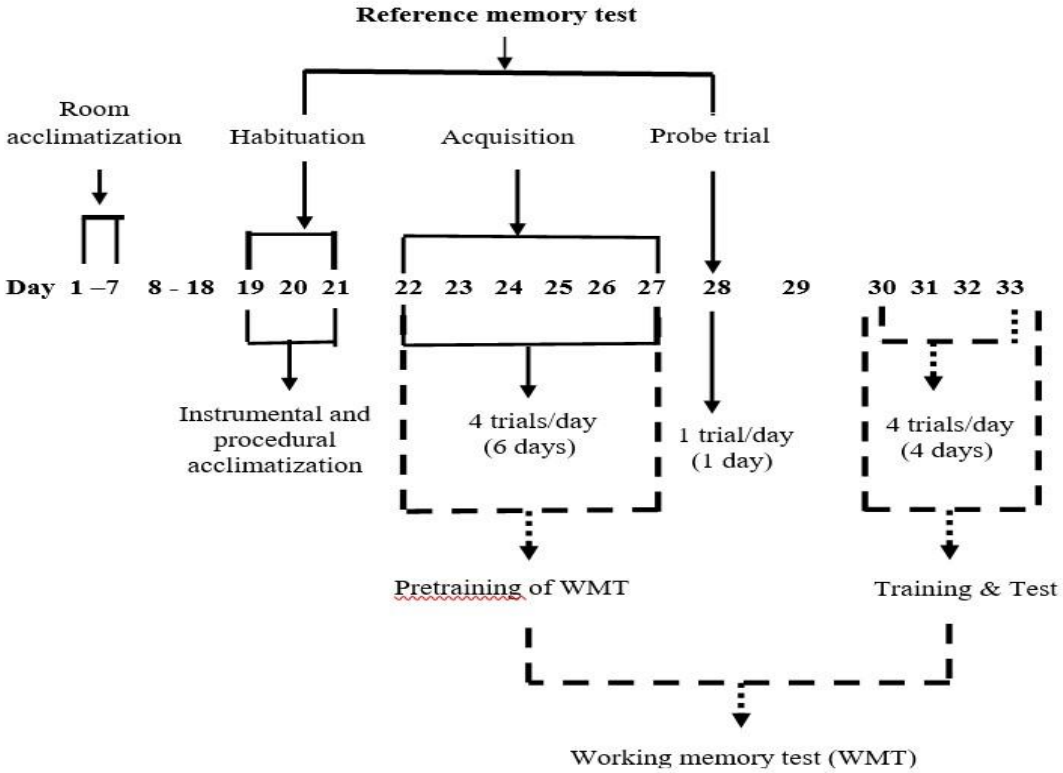

Figure - II: Work plan in different days of Morris water maze (MWM)

Table – I: Sequence of start locations in the acquisition phase of the reference memory test in MWM

Days	Sequence of start locations				Platform
	1st trial	2nd trial	3rd trial	4th trial	
Day 22	SW	SE	S	W	
Day 23	SE	S	W	SW	
Day 24	S	W	SW	SE	NE
Day 25	W	SW	SE	S	
Day 26	SE	SW	W	S	
Day 27	W	SE	S	SW	

SW: South-west; SE: North-east; NE: North-east; W: West; S: South

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Table – II: Platform position and sequence of start locations of the working memory test in MWM

Days	Sequence of start locations				Platform
	1st trial	2nd trial	3rd trial	4th trial	
Day 30	SE	SW	S	W	NE
Day 31	NE	NW	N	E	SW
Day 32	SE	E	NE	S	NW
Day 33	NW	W	N	NE	SE

SW: South-west; SE: North-east; NE: North-east; W: West; S: South

Working Memory Test

The working memory test comprised two phases: pre-training and training.

1. Pre-training Phase (Days 22–27)

The pre-training phase overlapped with the acquisition phase of the reference memory test, providing rats with initial exposure to the task and pool environment.

2. Training and Test Phase (Days 30–33)

After a rest period of four days, each rat underwent four trials daily for four consecutive days. On the first day, the platform was placed in the NE quadrant, and the starting locations were sequentially chosen from SE, SW, S, and W quadrants. The platform position changed daily, and the sequence of start locations was randomized for each trial. The rats were subjected to the same procedures as the acquisition phase, and escape latency was recorded.

Preparation of AILE

The fresh leaves of *Azadirachta indica* extract were collected from Bangladesh Agricultural University (BAU), Mymensingh, and identified by an expert taxonomist. Fresh green leaves of *A. Indica* was washed, and diseased/dried leaves were discarded. The clean leaves were shade-dried for 3 days. The dried leaves were crushed and soaked in double-distilled water in a 1:4 ratio for 3 days. The mixture was then filtered using Whatman No.1 filter paper. The filtrate was heat-evaporated to remove water and concentrate the extract. The concentrated extract was stored in a refrigerator until use. It was filtered, and the filtrate was concentrated over a water bath to obtain a solidified extract.

Treatment Plan

The treatment plan involved distinct phases of room acclimatization, habituation, acquisition, and testing. Each group received either *Azadirachta indica* leaf extract (AILE), ketamine, or normal saline (NS) as per group assignments. (Table – III).

Table – III: Treatment plan for Morris water maze test

Phase	Duration	Day	Treatment	Platform
Room acclimatization	7 days	Days 1–7	No treatment	Without platform
Instrumental acclimatization	11 days	Days 8–18	AILE or NS	Without platform
Habituation	3 days	Days 19–21	AILE or NS	Without platform
Acquisition (reference memory)	6 days	Days 22–27	AILE or NS + Ketamine	With platform (4 trials/day)
Probe trial	1 day	Day 28	AILE or NS	Without platform
Working memory training & test	4 days	Days 30–33	AILE or NS + Ketamine	With platform (4 trials/day)

Sacrifice

Each rat was placed in a large glass desiccators and 5 to 6 ml of di-ethyl ether (99%) was poured into it. The following 5 to 10 minutes the rat was observed closely. Painless death of the deeply anaesthetized rats was ensured by decapitation. Later, sacrificed animals were incinerated by prism, Bangladesh.

Data analysis

Results were expressed as mean ± SEM (standard error of mean). Statistical analysis was done by SPSS (version 16.0). Statistical tests were carried out by ANOVA followed by Bonferroni post Hoc test and paired Student's t test. $p \leq 0.05$ was considered as statistically significant.

Study Variables

Study Variables for Morris Water Maze (MWM) Test shown in Table IV.

Table – IV: Study Variables for Morris Water Maze (MWM) Test

Memory Type	Aspect of Memory	Variable	Unit
Working Memory	Acquisition & Retrieval	Escape latency in training & test	Seconds/trial
Reference Memory	Acquisition & Retrieval	Escape latency in the acquisition phase	Seconds/day
	Retrieval	Target crossings	Frequency/minute

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Ethical Considerations

All procedures involving animal subjects adhered to the guidelines set by the Animal Experimentation Ethics Committee of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Ethical approval was obtained following a thorough review by the Institutional Review Board (IRB) of BSMMU.

RESULTS

Escape latency of working memory test in MWM

In the present study, mean±SEM EL at 1st trial were 22.53±1.87, 40.62±1.59 and 21.65±2.16 seconds in Group 1 (G1) normal memory, Group 2 (G2) memory impaired, and Group 3 (G3) experimental, respectively.

Similarly, at 2nd trial the variable were 11.53±0.74, 27.34±i.29, 11.00±2.16 seconds; at 3rd trial, 6.84±0.51, 18.96±1.98, 7.87±1.61 seconds; at 4th trial 6.84±0.37, 14.00±0.83 and 6.65±1.37 seconds in the G1, G2 and G3 respectively.

Here, mean values of this variable were significantly different among all groups in all the experimental trials. Moreover, the mean±SEM EL of the G2 was significantly (p≤0.001) higher in comparison to those in group G1 in all trials of all the experimental days.

In addition, mean values of this variable in G3 were significantly (p≤0.001) lower in comparison to those of G2 in all trials of all the experimental days. However, the difference of mean values was statistically nonsignificant between G1 and G3 in all trials of all experimental days.

Escape latency in acquisition phase of MWM: reference memory acquisition

In the present study, mean±SEM EL at day 22 were 24±0.90, 46.84±1.59 and 27.65±2.66 seconds in G1, G2 and G3 respectively.

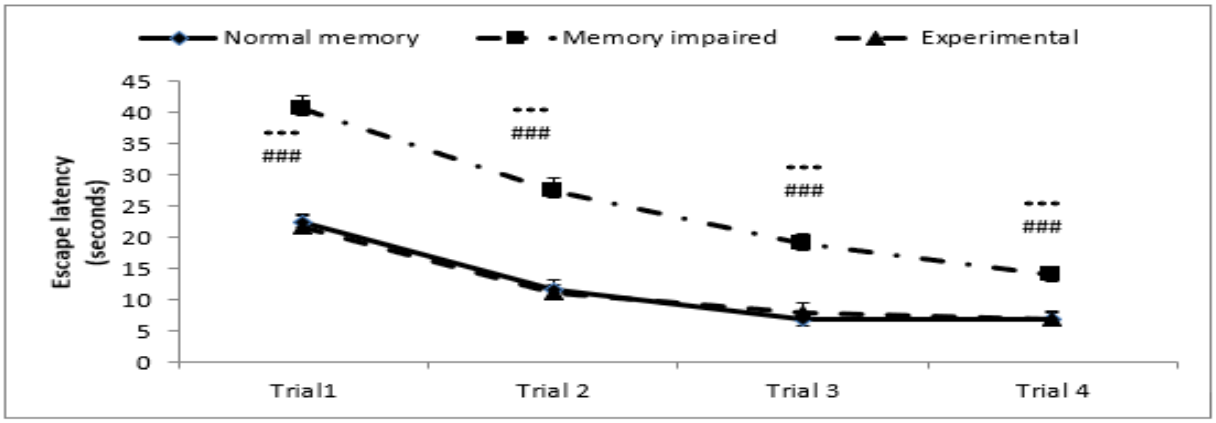
Similarly, at day 23 the variables were 20.18±1.57, 47.03±3.61 and 18.34±2.85 seconds; at day 24, 11.62±1.07, 47.06±1.24 and 11.68±2.05 seconds; at day 25, 11.50±1.49, 35.34±1.92 and 7.34±0.84; at day 26, 7.50±0.62, 29±40±1.99 and 7.25±0.69; at day 27, 5.96±0.38, 27.56±2, 37 and 5.43±0.45 seconds in G1, G2 and G3 respectively.

Here, mean values of this variable were significantly (p≤0.001) different among all groups in trials of all the experimental days (from day 22 to day 27).

In addition, mean±SEM EL of the G2 was significantly (p≤0.001) higher in comparison to those of G1 in all trials of all the experimental days.

Moreover. mean values of this variable in the G3 was significantly (p≤0.001) lower in comparison to those of G2 in all trials of all the experimental days. However, these differences of mean values were statistically non-significant between G3 and G1 in all the experimental days.

A. Escape latency (EL) in training and test



B. Esacpe latency (EL) in acquisition phase

Trials

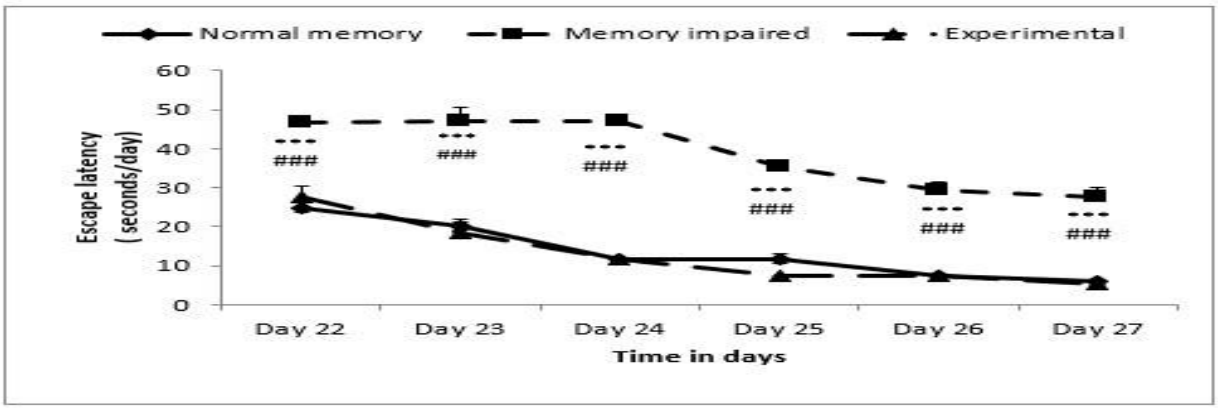


Figure – III: A. Escape latency (EL) in training and test B. Escape latency in acquisition phase in different trials and different days of Morris water maze test in different groups of rats.

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Trial 1: mean± SEM of 4 T1s (trial 1) of 8 rats in 4 days of training and test. Trial 2: mean± SEM of 4 T2s (trial 2) of 8 rats in 4 days of training and test training and test. Trial 3: mean± SEM of 4 T3s (trial 3) of 8 rats in 4 days of training and test. Trial 4: mean± SEM of 4 T4s (trial 4) of 8 rats in 4 days of training and test. Each day symbolizes mean ±SEM escape latency of 4 trials in that day of acquisition phase for 8 rats. Statistical analysis was done by ANOVA (among group) followed by Bonferroni's post hoq test (between trial). *: Normal memory vs Memory impaired; #: Memory impaired vs Experimental; $: Normal memory vs Experimental. In the interpretation of results, p≤0.05 was considered as significant. */#/$: p≤0.05;**/##/$$: p≤0.0;, ***/###/$$$: p≤0.001.

Target crossings in MWM

In the present study, mean±SEM target crossings at day 28 were 8.20±0.35, 4.5±0.26 and 9.87±0.66 frequency/min in the G1, G2, and G3, respectively.

Here, mean values of this variable in the G2 were significantly lower (p≤0.001) in comparison to the G1 in the probe trial day. Moreover, mean±SEM target crossings in G3 were significantly higher than those of G2 in the probe trial. Furthermore, there was a significant (p≤0.042) difference of mean values between G1 and G2 and G3 on day 28. (Table – V)

Table – V: Number of target crossings in probe trial in MWM in different groups of rats

Experimental day	Groups	Target crossings (frequency/minute)
Day 28 (Probe trial)	Group 1 (G1) normal memory	8.20±0.35 (7 to 9)
	Group 2 (G2) memory impaired	4.50±0 .26(3 to 5)
	Group 3 (G3) experimental	9.87±0 .66 (7 to 12)

Each column symbolizes mean±SEM for 8 rats. Values in parenthesis indicate ranges. G1 = rats with oral normal saline (5 ml/kg) for consecutive 26 days (day 8 to day 33). G2 = rats with intraperitoneal (ip) ketamine (15 mg/kg) for consecutive 6 days of acquisition phase (day 22 to day 27). G3= rats with oral *Azadirachta indica* leaf extract (300 mg/kg) for consecutive 26 days (day 8 to day 33) and ip ketamine (15 mg/kg) for consecutive 6 days of acquisition phase (day 22 to day 27). MWM: Morris water maze.

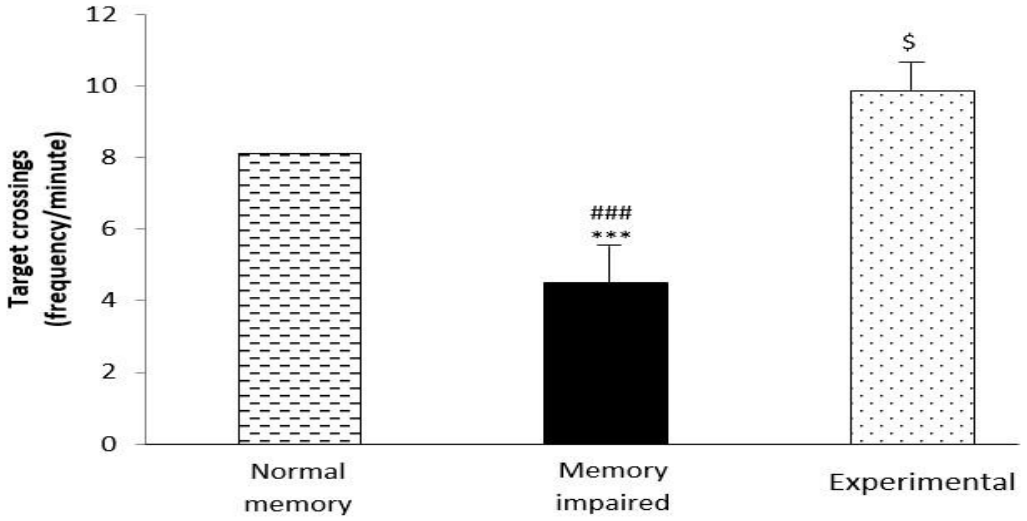

Figure – IV. Number of target crossings on probe trial at day 28 of MWM in different groups of rats.

Each bar symbolizes a number of mean±SEM target crossings for 8 rats. Statistical analysis was done by ANOVA (among groups) followed by Bonferroni's post hoq test (between groups), *: Normal memory vs Memory impaired; #: Memory impaired vs Experimental; $: Normal memory vs Experimental. In the interpretation of results, p≤0.05 was considered as significant. */#/$:p≤0.05,**/##/$$: p≤0.01, ***/###/$$$: p≤0.001.

DISCUSSION

The present study was undertaken to assess the effects of AILE on memory and its potential involvement in modulating NMDA receptor activity. Using ketamine-induced memory-impaired male Wistar rats, the effects of AILE on the acquisition and retrieval of both working and reference memory were evaluated.

Our findings revealed that sub-anesthetic doses of ketamine (15 mg/kg) significantly impaired working and reference memory in rats alongside prolonged escape latency and reduced target crossings in the Morris water maze, compared to normal rats. These results align with prior studies, suggesting that ketamine exerts its effects by blocking NMDA receptors on the postsynaptic membrane of pyramidal neurons in the prefrontal cortex [18]. This blockade likely

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disrupts persistent neural activity essential for working memory maintenance [19].

Additionally, ketamine's antagonistic effect on NMDA receptors of GABAergic interneurons in the cerebral cortex and hippocampus may lead to disinhibition of glutamatergic presynaptic pyramidal neurons. This cascade could cause a transient surge in glutamate release, initiating pathways involving AMPA receptors and voltage-gated calcium channels (VGCC) [20,21]. The subsequent intracellular calcium accumulation might disrupt calcium homeostasis, promote mitochondrial dysfunction, and activate apoptotic pathways [22,23]. These processes, coupled with increased oxidative stress, likely underlie the memory impairments observed in ketamine treated rats [24].

Interestingly, our study found that AILE co-administration prevented ketamine-induced memory deficits. Rats treated with AILE showed increased target crossings in the Morris water maze compared to ketamine-only rats. These observations are consistent with previous studies reporting AILE's neuroprotective properties [12]. Although limited data were available to compare other variables, potential mechanisms underlying AILE's effects include its ability to reduce proapoptotic proteins such as cytochrome c and caspase in the hippocampus and to mitigate oxidative stress [25].

AILE's effects may also involve quercetin, a flavonoid constituent, which enhances NR2A and NR2B subunit expression of NMDA receptors in mice [26]. This suggests that AILE might counteract ketamine-induced NMDA receptor antagonism by enhancing NMDA receptor function. As ketamine's primary mechanism involves NMDA receptor antagonism, the prevention of memory impairment by AILE in this study highlights its potential role in modulating NMDA receptor activity.

Notably, AILE not only prevented memory deficits but also enhanced the retrieval of reference memory in experimental rats compared to normal memory rats after a 24-hour interval. To our knowledge, this is the first study to report this unique beneficial effect of AILE on memory enhancement. However, the lack of relevant literature highlights the need for further investigation to elucidate the underlying mechanisms of this observation. The findings of this study provide evidence for the protective and memory-enhancing effects of AILE in ketamine-induced memory impairment, potentially mediated through modulation of NMDA receptor function, reduction of oxidative stress, and suppression of apoptotic pathways. These results highlight the therapeutic potential of AILE in managing memory-related disorders.

CONCLUSION

The present study demonstrates that AILE significantly mitigates ketamine-induced memory impairment in male Wistar rats, as assessed through the MWM. Ketamine administration caused deficits in both working and reference memory, evidenced by increased escape latency and reduced target crossings. However, AILE effectively reversed these impairments, leading to a significant improvement in escape latency and an increase in target crossings, indicating enhanced spatial learning and memory retrieval.

Additionally, AILE not only prevented memory deficits but also enhanced reference memory retrieval beyond normal levels, a novel and promising finding of this study. Further investigations are needed to explore the molecular pathways involved and to evaluate the potential therapeutic applications of AILE in memory-related disorders.

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