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Neuroprotective Effect of Hesperidin Methyl Chalcone against Aluminium Chloride Induced Alzheimer's Disease: In-Silico, In-Vitro, and In-Vivo Studies

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Keywords: Alzheimer's disease, Hesperidin methyl chalcone, *Acetylcholinesterase*, Tau protein, Flavonoid.

ABSTRACT

Objective: To evaluate Neuroprotective activity of Hesperidin methyl Chalcone on Aluminium chloride-induced Alzheimer's disease model in rats. **Methods:** The research work was divided into two models that are Nootropic model and the Aluminium chloride model. Male Wistar Albino rats (200–225 g; 10–12 weeks old) were used for the screening of anti-Alzheimer's activity. Control group animals received normal saline by i.p. route, Negative control groups receives Aluminium chloride (100 mg/kg b.w. i.p.) as inducing agent, Positive control groups receive Donepezil (1 mg/kg b.w. i.p.) and Test groups receive Hesperidin methyl chalcone(HMC 30 mg/kg b.w. i.p.) along with the inducing agent. The duration of the experiment was 18 days. The behavioral analysis tests for the Nootropic model were carried out on day 6^{th}, $7^{th,}$ and 8^{th} day, for the Aluminium chloride model it was carried out on the day of 15, 16 & 17. At the end of the study, the animals were sacrificed by excess anesthesia and the brain was isolated to carry out various biochemical estimations. **Results:** Compared to the Negative control group, the Novel object recognition, Elevated plus maze, Morris water maze as well as levels of Acetylcholine, Dopamine, tissue antioxidants **(**SOD, CAT, GSH, Gpx, and GST) were increased significantly in the treatment group and MDA as well as MPO were decreased significantly. **Conclusion:** The HMC has exhibited a significant neuroprotective effect in Aluminium chloride-induced Alzheimer's disease in rats. The antioxidant properties of HMC were revealed from the estimation of tissue antioxidants. The neuroprotection can be attributed to its ability to increase the Acetylcholine as well as dopamine thereby it enhances the memory.

1. INTRODUCTION:

Alzheimer's disease (AD) is a gradual neurodegenerative condition and the primary cause of dementia.[1] presently, more than 50 million people are affected by dementia worldwide, including AD, and it affects people aged 65 or older. It is already predicted that it will expand to 74.7 million by 2030.[2]an approximate 2.1 million patients with Alzheimer's age of 85 years or older were documented in 2017.[3] AD is a gradual and recurring neurodegenerative condition that causes global cognitive impairment that affects memory, focus, judgment, and reasoning, according to Tanzi and Bertram (2005).[4]

The important features of pathogenesis of AD are indeed the progressive aggregation of the Beta-amyloid protein fragment (plaques) and twisted tau protein tangles, external and internal brain neurons, respectively.[5]

Conversely, the memory dysfunction is associated with impairment of the cholinergic system involving neurotransmitters and their neurons. Dysfunction of the cholinergic system results from the loss of cholinergic neurons in the hippocampus and the forebrain.[6]

Available pharmacological intervention for AD have only limited impact and impaired nerve function, causing disease associated with the symptoms and debilitating complications of Alzheimer's. Many of the market-based medicines focus primarily on cognitive enhancement by inhibiting the enzyme AChE. Also, AD is not the result of individual causes such as AChE but is a multifactorial disorder that must be considered even before a medication is produced.

Other factors include oxidative damage and synaptic damage play a crucial role in deficits of cognition in AD. Herbal remedies can be another source of neuroprotective medicines since they can maintain adequate brain cell connectivity and the loss of neuronal functions under dysfunctional conditions.[7]

Metal ions equilibrium in the brain is essential with proper cognitive function, and its deregulation has always been evident as among the critical factors in neurodegeneration development.[8] Neuroinflammation may involve different metals, including aluminum, lithium, copper, zinc, lead, silica, fluoride, mercury, and iron.

Aluminum (Al) has been identified as a potential cause of Alzheimer's disease development (AD).[9]Aluminum enters the brain by food, antacids, cosmetics, toothpaste, fumes/particles

inhaled, and drinking water and can be accumulated in the cortex, hippocampus,[10,] and cerebellum essential for learning and memory.[11]

In such regions, aluminum causes oligomerization and accumulation of AD-associated pathologies such as amyloid-beta (Aβ), over phosphorylated tau aggregation, lipid peroxidation, impaired calcium ion exchange, and apoptosis.

Hesperidin(3, 5, 7-trihydroxy flavanone 7 glycosides), a phytoflavanone present in abundant citrus, is effectively documented because of its lipophilic nature the blood-brain barrier is penetrated and Offers Neuroprotection against Parkinson's disease, Huntington's disease, immobilization pressure, cerebral ischemia/reperfusion and strokes related to their antioxidant properties are anti-inflammatory and anti-apoptotic.

Although Hesperidin is not very water-soluble in its original form, Hesperidin Methyl Chalcone (HMC) is extremely water-soluble for higher absorption in GIT.[12] as all HMC Flavonoids are antioxidants; it also supports stabilizing effects of blood vessels. HMC treatment may reduce pro-and anti-apoptotic marker expression. Moreover, far there has been no investigation into the anti-apoptotic function of Hesperidin methyl chalcone in $AlCl_3$-induced AD rats.

The purpose of this research was to evaluate the therapeutic effect of HMC on learning disabilities and memory impairment, oxidative stress, and apoptosis in rats with AlCl3 induced AD hippocampus, cortex, and cerebellum.

The molecular docking research for Hesperidin methyl chalcone and Donepezil was carried out using the Drug Discovery Studio 3.5. To gain insight into the molecular interactions.

For Molecular docking studies, Human Butyrylcholinesterase, Human Acetylcholinesterase, Tau protein kinase, and also inhibitor BACE 1 are selected as Targets. Hesperidin methyl chalcone and Donepezil acts as a Ligand.[13]

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals:

Hesperidin methyl chalcone (Sigma Aldrich, India), Donepezil (Gift sample from Apotex Research Pvt. Ltd), All the reagents which are used for the study were of analytical grade and freshly prepared on the day of experiments.

2.2 Experimental Animals:

The research was conducted in compliance with the guidance of the Committee for the Control and Supervision of Animal Experiments (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethical Committee (IAEC) of Acharya & BM Reddy College of Pharmacy Bengaluru with Reference no. IAEC Ref No; IAEC/ABMRCP/2018-2019/13.

Male Wistar Albino rats (200–225 g; 10–12 weeks old) were used for the screening of anti--Alzheimer's activity. The animals were housed in standard environmental conditions and with feed pellets, water ad libitum, less than 12 h of light / dark period, temperature 25±2ºC, and controlled humidity. Before the study started, the rats were permitted to habituate to reduce the stress for one week.

2.3 Experimental Design:

With the evolutions of pharmacological activity, the research was split into two models, the Nootropic model and the Aluminum chloride model, respectively.

2.3.1 Nootropic Model

Male albino Wister rats were divided into three groups with each comprising of six animals.

(i) Group1: Control (Saline i.p.)

(ii) Group2: Positive control [Donepezil (DPZ) 1 mg/kg; i.p.]

(iii) Group3: Hesperidin methyl chalcone [(HMC) 30 mg/kg; i.p.]

All groups undergo pretreatment through an eight-day intraperitoneal route for Nootropic activity. All these rats conducted behavioral training from day six on until day eight.

2.3.2 Aluminium chloride Model

Male albino Wister rats were divided into four groups with each comprising of six animals.

(i) Group1: Control (Saline; i.p.)

(ii) Group 2: Negative control [AlCl3 (100 mg/kg; i.p.) dissolved in saline]

(iii) Group 3: Positive control {[donepezil (DPZ) 1 mg/kg; i.p.] + (AlCl3 100 mg/kg; i.p.)}

(iv) Group 4: Hesperidin methyl chalcone {[(HMC) 30 mg/kg; i.p.] + (AlCl3 100 mg/kg; i.p.)}

For AlCl3, amnesia was caused by frequent AlCl3 100 mg/kg intraperitoneal injections for nine days after pretreatment with HMC (day 9 to day 17) in all groups, except for the control group. Half an hour after administration of $AlCl_3$, Day 15 was NOR and Day 16 was EPM, and MWM was on the 17th day of research. The rats were sacrificed at the end of the experiment; the Separated brains were used for further biochemical and immunohistochemical research.

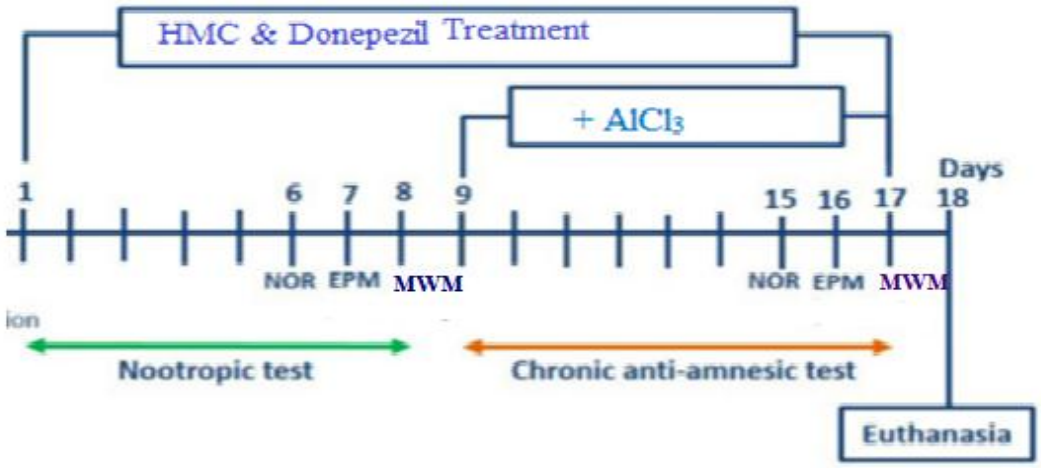


Figure No. 1: Schematic representation of experimental procedure (NOR- Novel object Recognition, EPM- Elevated plus maze, MWM- Morrie's water maze).

2.4 *In silico* studies:

In silico studies were performed for Hesperidin methyl chalcone on comparing with standard drug Donepezil using drug discovery studio 3.5.

Human tau proteins (1J1C), Human Acetylcholinesterase (4PQE), Human butyrylcholinesterase (1P0I), and Human BACE 1 bound inhibitor (2NTR) are selected as targets. For selected targets, related proteins were downloaded from the Protein data bank.

The protein targets are docked with HMC and Donepezil individually and C-Docker interaction and C-Docker interaction energy are recorded along with amino acid interactions.

2.5 Behavioral analysis

2.5.1 Novel Object Recognition (NOR)

An open field box covered in black acrylic material (40x40x20 cm) was used for object recognition testing as the experimental apparatus. Two similar height Lego toys were the items scrutinized. The texture, color, and scale of both types of items shown during the test session varied. There are three steps to this assessment: (i) habitual cases; (ii) training and (iii) screening. Each rat was allowed the first day to get acquainted with the open field box for about 10 min without an object being around every rat was placed for 5 min in the open field on the second day and was allowed free exploration of the two identical objects. After a post-training session of 90 min interval, some of the old objects used was replaced by a new one and rats were exposed to a 2-minute test run. It documented and evaluated the time spent with each object. Clean the open field box to reduce scent traces with 70 percent ethanol between runs. The recognition index has been determined Using the [TB / (TA+TB)] formula for TA and TB to examine common object A and new object B. Development of an object was observed when a rat with its nose or forepaws sniffed or approached the object.[14] The Novel object recognition task for the Nootropic model was evaluated on day 6 and for the aluminum chloride model on day 15.

2.5.2 Elevated plus maze (EPM) test

The device of EPM consists of four arms sharing the same length, i.e. two open arms (50 x 10 cm) cross two closed arms with walls 40 cm high. All these arms were joined using a central square (10 x 10 cm), which gave the device plus signs an appearance.

Also, the EPM had been raised 50 cm above ground level. Memory evaluation was conducted during two phases via EPM. Each rat was placed at the end of an open arm throughout the training period and the transfer of latency time (s) was noted by using a stopwatch, Which is the time every rat had to reach either closed arm (with all four paws). With 70 percent ethanol between races, the maze was cleaned to eliminate scent traces. Further to a training session, a test was performed 24 h (retention) to assess memory retention. In both phases (training and testing) for each rat, the time cut off to explore the maze was 90 s. During test sessions, a reduction in the transfer latency time was taken as an indicator of memory improvement.[15] The Elevated plus-maze task was assessed on day 7 for the Nootropic model and day 16 for the aluminum chloride model.

2.5.3 Morries Water maze test:

The maze was designed as a method to assess spatially or place learning and was named the Morris water maze. Water filled up the tank with a 3/4th amount. The platform can be square or circular. Water temperature is one common concern. Initially, it was proposed that rats require warmer water than standard ambient air temperatures found in most laboratories (19–22 ° C), but this was not commonly carried out. Evaluated rats perform well in water adjusted to 19–22 ° C ambient temperatures and show no evidence of serious fatigue or hypothermia at normal test times. In the maze place the rat in the ideal starting position, facing the wall of the tank. At water level, the animal is released into the water (not dropped). The moment the animal is released a timer or device monitoring software starts. If the animal hits (touches) the board, stop the timer. Maximum duration of 1 or 2 min per trial is standard; for rats usually 2 min. Animals are usually given multiple trials per day. The most frequently numbered is 4.[16] finally percentage time spent in the pool is calculated.

The Morris water maze test was assessed on day 8 for the Nootropic model and day 17 for the aluminum chloride model.

2.6 Biochemical Estimations:

Brains were isolated from rats (n=5), Brain tissue was homogenized to obtain 10 % w / v homogeneous with a Tris ice-cold buffer. The homogenate was centrifuged at 5000 rpm for 30 min at 4ºC. BCA kits were measured for the concentration of total protein. Aliquot of supernatant was used for the estimations of superoxide dismutase[17], Catalase[18], Glutathione peroxidase[19], Reduced glutathione[20] activities and to estimate the levels of Thio Barbituric Acid Reductive Substances (MDA) [21] & Myeloperoxidase (MPO) [22].

2.7 Neurotransmitter estimations:

The main neurotransmitter involved in Alzheimer's is Acetylcholine. It is estimated by using the colorimetric method. Along with Acetylcholine, the role of Dopamine was also estimated.

The main principle involved in Acetylcholine estimation includes It is a photometric method for the determination of *Acetylcholinesterase a*ctivity of tissue homogenate.

The activity of the enzyme is calculated by increasing the yellow color derived from thiocholine when it reacts with the ion dithiobisnitrobenzoate. It is based on those reactions being coupled;

Acetylthiocholine ⟶ Thiocholine + Acetate

Thiocholine + dithiobisnitrobenzoate ⟶ Yellow color

Based on Acetylcholinesterase enzyme activity the level of Acetylcholine was estimated.[23]

The principle behind the Estimation of dopamine was based on the inhibitory effect of DA on thionine oxidation by bromate in acidic media. Spectrophotometrically, the improvement in absorbance was accompanied at 601nm.[24]

2.8 Inflammatory Markers estimations:

Cytokines are important regulators of physiological inflammation. The aberrant cytokine expressions contribute to pathogenesis in inflammatory associated diseases. The cytokine IL-1 α and IL-6 are important pro-inflammatory cytokines; it plays a key role in the immune system to transition from innate to acquired immunity. Quantification of IL-1α & IL-6 levels in brain tissue was done by Rat IL-1α & IL-6 ELISA kit as per the procedure.

2.9 Immunohistochemical Stain Analyses:

Immunohistochemical stain analysis was performed in the hippocampus with 4-hydroxy-2-nonenal (4HNE) staining using Doublecortin (DCX) and lipid peroxidation to assess neurogenesis. Every group's brain sample was overnight soaked in 4 percent paraformaldehyde. Methodically, the samples were cryoprotected for 24 h in 10, 20, and 30 percent sucrose.

The brains were then placed into 15 percent polyvinylpyrrolidone (PVP), frozen with dry ice, and cut with a Leica CM3050 cryostat into 40 µM of frozen coronal sections. They subsequently stored all parts in an anti-freeze buffer. Endogenous quenching was conducted on the free-floating parts using 1 percent H_2O_2 in methanol for 30 minutes. The samples were treated using a blocking buffer (1.0 % bovine serum albumin in PBS and 0.3 % Triton X-100) for 1 hour after phosphate buffer saline (PBS) wash, samples were incubated with a primary DCX (1:500, Abcam) and 4HNE (1:250, Abcam) antibodies overnight at 4 ° C. Upon washing with PBS the tissue was once again incubated for 2 h with a secondary anti-rabbit biotinylated goat antibody (Abcam). The tissues were then exposed to an avidin-biotin-peroxidase complex for 2 h. Peroxidase activity is viewed using a stable diaminobenzidine solution. All immunoreactions were microscopically controlled, and tests were measured.

3.0 Statistical Analysis:

All data were expressed as Mean ± SEM (n = 6) rats in each group. Statistical analysis was performed using one-way ANOVA followed by the Dunnett test. ***P < 0.05, **P< 0.01 is considered as significant on comparing treatment groups with the control group for Nootropic model and treatment groups are compared with $AlCl_3$ group for $AlCl_3$ model.

3 RESULTS:

3.1 *In silico* studies using Drug Discovery studio 3.5

Ligands: Hesperidin methyl chalcone & Donepezil

Targets: 4PQE, 1P0I, 1J1C & 2NTR

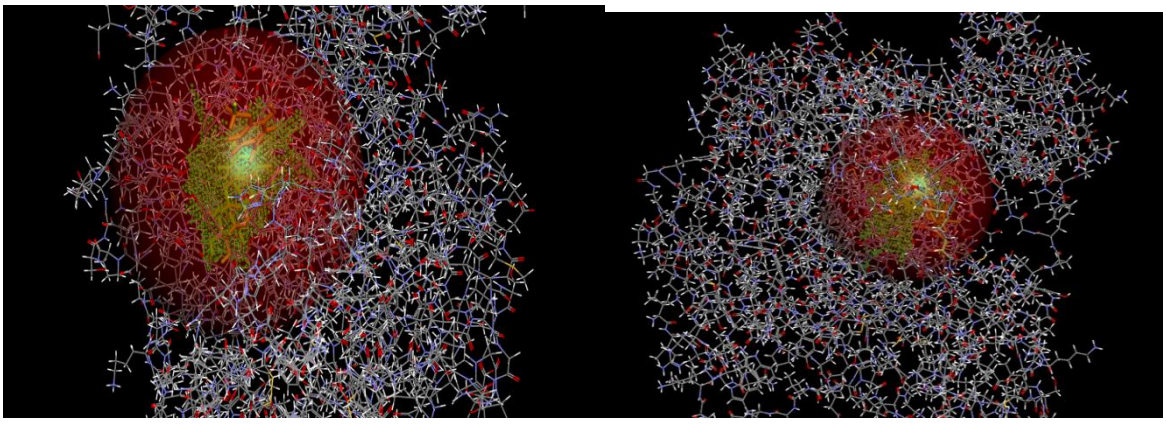
Table No. 1: Docking study Results

Targets	Compounds	-C Docker Energy	-C Docker Interaction Energy	Interactive Amine acids
4PQE	HMC	61.82	55.20	TYR-A341, TRP-A86, TRA-A286, TYR-A37 & TYR-133
	DPZ	10.21	46.34	TRP-A286, TYR-A341
1P0I	HMC	36.92	67.47	HIS-A438, SER-A287 & LEU-A286
	DPZ	07.15	48.94	
1J1C	HMC	69.62	52.54	CYS-A199, GLY-A202, LYS-A85, ARG-A141 & ILE-A62
	DPZ	06.87	44.82	VAL-A70
2NTR	HMC	66.62	83.59	GLN-A73, GLY-A34, ILE-A126, ARG-A128, GLY-A230 & ARG-A235
	DPZ	08.80	52.80	

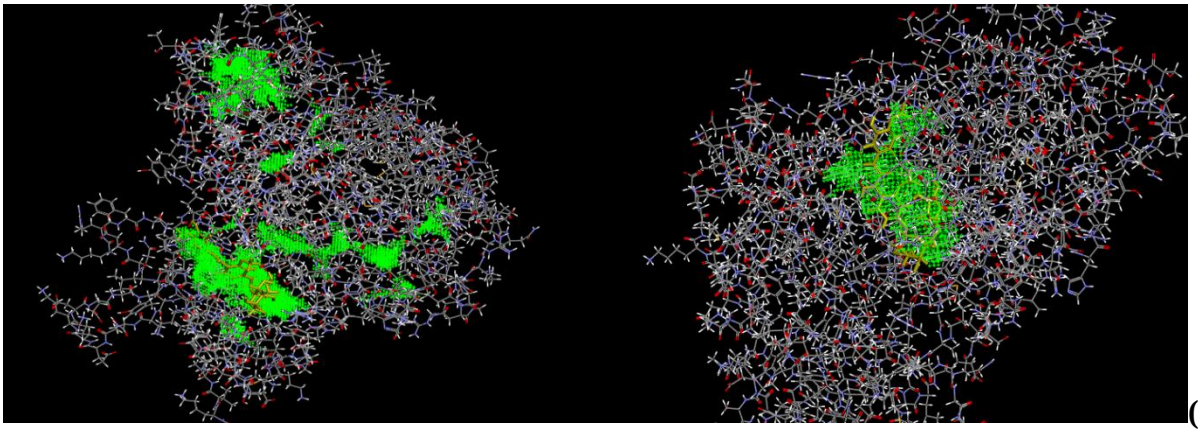
Hesperidin methyl chalcone has been docked into several known enzyme conversion targets AChE, BuChE, Tau protein kinase 1, and β-amyloid i.e. Inhibitor of BACE-1. HMC demonstrated high –C Docker energy and –C Docker energy interaction with all targets

comparable with a well-known standard drug such as donepezil and shows strong binding. HMC interactions in the corresponding binding pockets.

The commonest amino acids that interact with standard compounds are TRP-A286, TYR-A341 in the case of molecular docking into 4PQE. HMC interacted with them all in different docking positions. HMC interacted with most of amino acids including TYR-A341, TRP-A86, TRA-A286, and TYR-A37 & TYR-133. The No amino acids which interact with standard compounds were found while molecular docking into 1P0I. The amino acid interactions of HMC were found in HIS-A438, SER-A287 & LEU-A286. The main interacting amino acid for the standard was identified through molecular docking into 1J1C is VAL-A70. Whereas for HMC docking the interactions of amino acids were found to be CYS-A199, GLY-A202, LYS-A85, ARG-A141 & ILE-A62. When the standard was docked with 2NTR there no such interacting amino acids were observed. However, HMC interacts with the highest number of amino acids which form hydrogen bonds with GLN-A73, GLY-A34, ILE-A126, ARG-A128, and GLY-A230 & ARG-A235. The oxygen atom of HMC has in many cases formed several hydrogen bonds to the same or different amino acids. These interactions may be responsible for the powerful binding leading to better interaction of energy, due to the presence of several oxygen atoms. The –C Docker energy, -C Docker interaction energy, and the interactive amino acids from the different docking studies were summarized in Table 1 where the docking interactions are illustrated in the following Figure.



(A) 4PQE **(B) 1P0I**



C) 1J1C **(D) 2NTR**

Figure No. 2: Docking the HMC images into specific AD (A) AChE targets (PDB ID: 4PQE), (B) BuChE (PDB ID: 1P0I), (C) Tau protein kinase 1(PDB ID: 1J1C), and (D) BACE-1 (PDB ID: 2NTR) Describing the binding reactions in the binding compartment of various amino acids.

3.2 Nootropic effect of Hesperidin methyl chalcone

Results obtained from the NOR HMC Nootropic activity test are shown in Figure. After 7 days of pretreatment, the influence of HMC on memory function was evaluated. The outcomes for the novel object were expressed as an index (%) of recognition. Focused on the results, the pretreated HMC groups reported an increase in the novel object recognition index compared to the control group and the donepezil groups. In EPM, when compared with the control (Figure), the inflection ratio in HMC treated groups was significantly increased. The results were expressed as Inflexion ratio for Elevated plus-maze. In Morris's water maze the time taken to reach the Hidden platform was measured. The HMC and DPZ pretreated group showed less time to reach the platform on comparing with the control group. The results were expressed as a percentage of time spent in the pool for Morris water maze test.

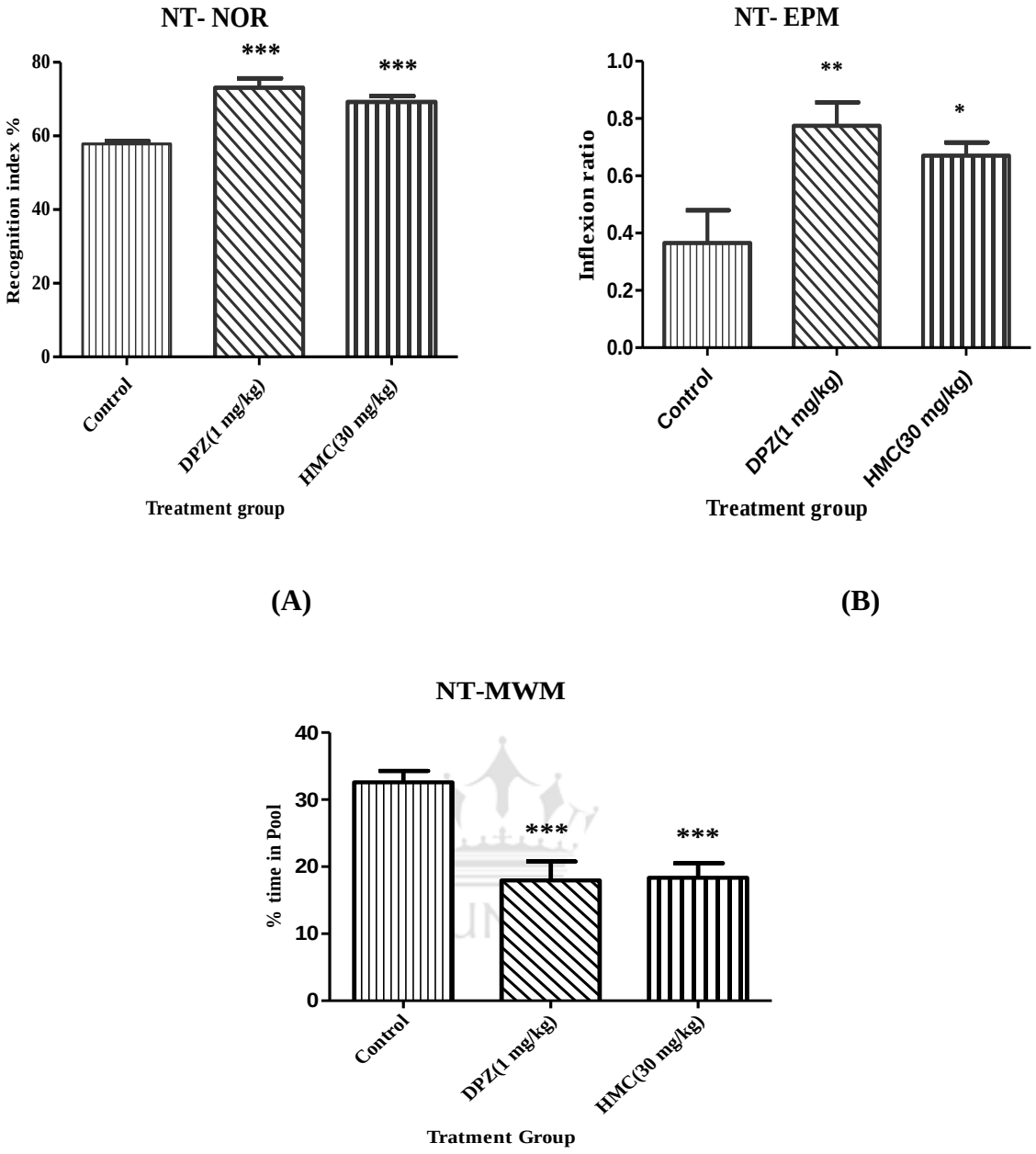
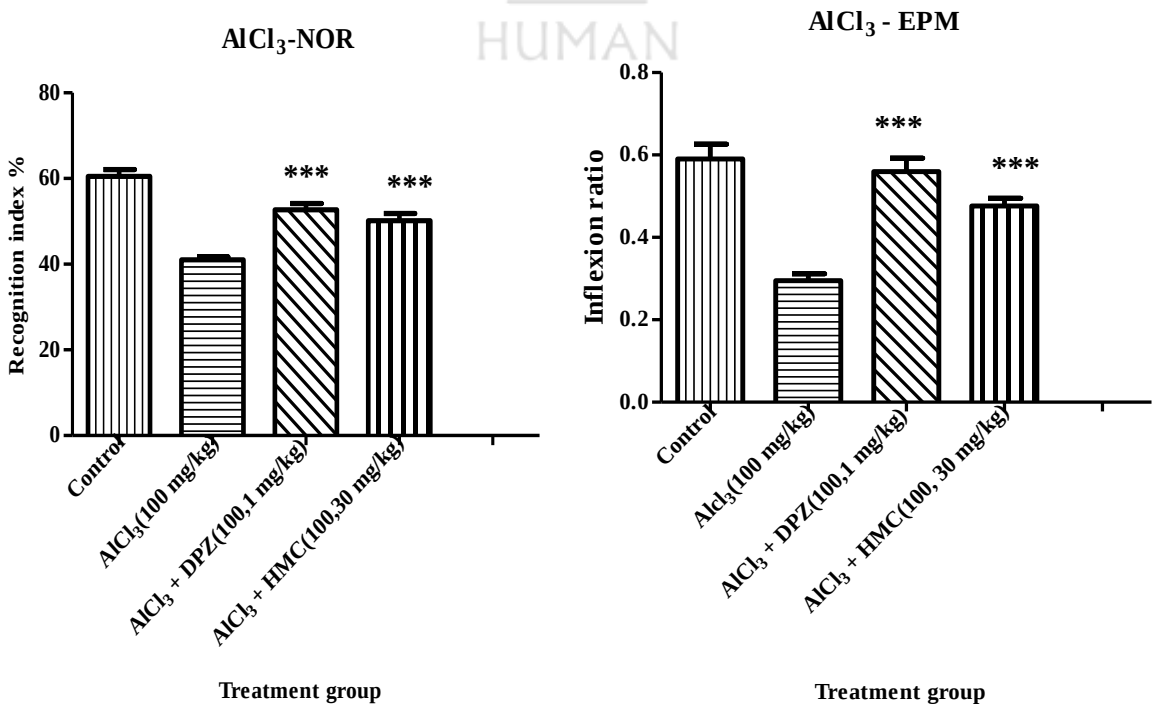


Figure No. 3: Behavioral analysis for the identification of novel objects (NOR), Elevated plus maze (EPM) and Morries water maze (MWM). (A) Display a graph chart for the Nootropic model recognition index in NOR (B) Symbolizes a graph plot for the Nootropic model inflection ratios in EPM and (C) represents percentage time in pool for MWM for Nootropic model.

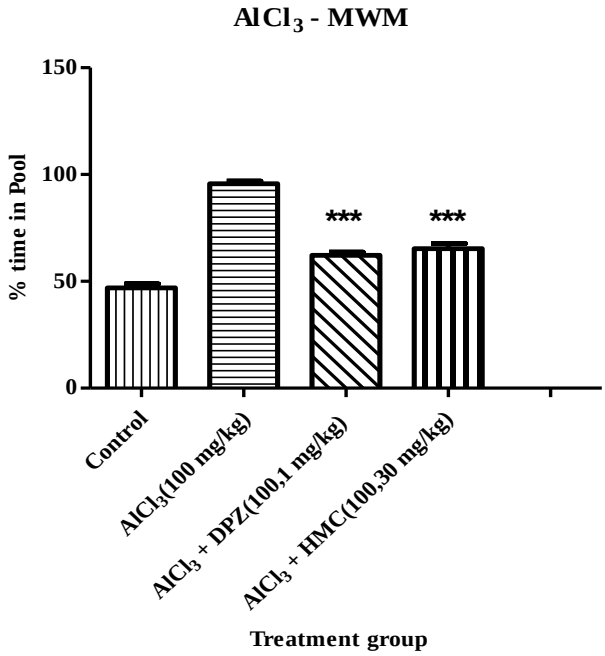
Values were given as Mean ± SEM (n = 6). One way ANOVA was to analyze data, followed by Dunnett test. ***P < 0.05, **P< 0.01 is considered as significant on comparing treatment groups to control groups.

3.3 Anti-amnesic Effect of Hesperidin methyl chalcone in Rats with Aluminium chloride-Induced Amnesia

The NOR test for the chronic aluminum chloride model showed a decrease in the percentage recognition index for the group treated with aluminum chloride (AlCl3 100 mg/kg) (Figure 4A). By comparison, for all groups treated with HMC, the percentage of the recognition index (30 mg/kg) was high and comparable with that of donepezil (1 mg/kg). A statistical difference in the percentage of the recognition index among treated HMC and the AlCl3 model was observed. In EPM, the inflection ratio analysis showed that the retention memory in groups treated with HMC improved compared to the $AlCl_3$ group; however, this was statistically significant (Figure 4B). In MWM test showed that the AlCl3 group has spent more percentage time in the pool comparing with results of the HMC and Donepezil treated group and the results were statistically significant on comparing the entire treatment group with the AlCl3 group (Figure 4C).



(A) **(B)**



(C)

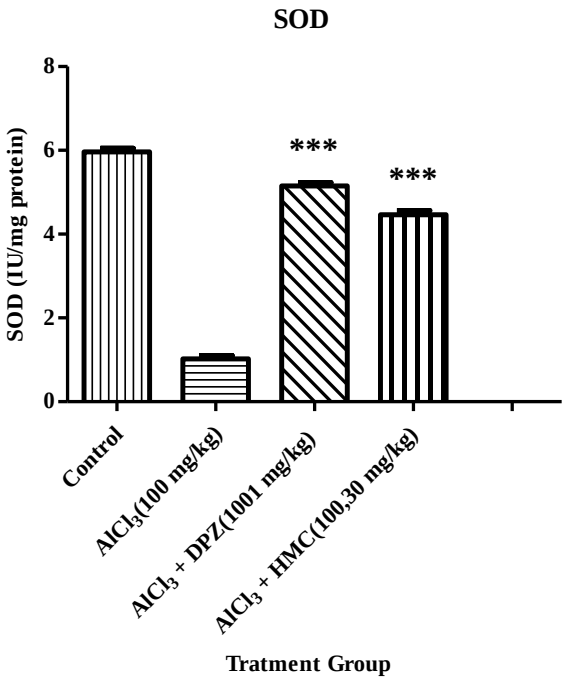
Figure No. 4: Behavioral analysis for NOR, EPM, and MWM (A) Describes the recognition index graph plot in NOR for the $AlCl_3$ model (B) Symbolizes a graph plot for $AlCl_3$ model's inflection ratios in EPM. The behavior study for (A, B, and C) was compared with the group of aluminum chloride ($AlCl_3$ 100 mg/kg).

Values were given as Mean ± SEM (n = 6). One way ANOVA was to analyze data, followed by Dunnett test ***P < 0.05, **P< 0.01 is considered as significant on comparing treatment groups to $AlCl_3$ groups.

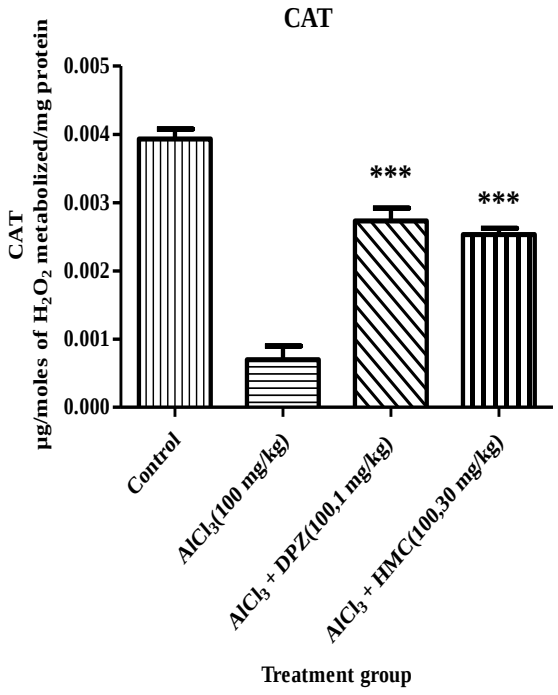
3.4 HMC Suppresses Oxidative Stress and Inflammation in $AlCl_3$ model

In Alzheimer's physiopathology, oxidative stress and inflammation play a central role. Therefore, to validate the neuroprotective effects of HMC against AD, we further investigated the effects of HMC on oxidative stress and inflammation.

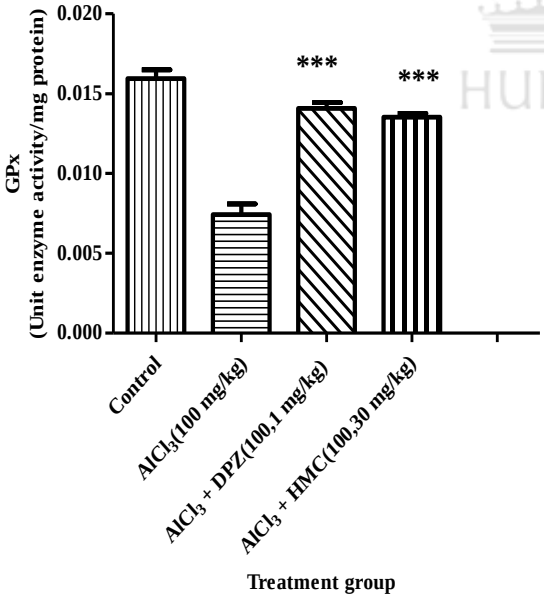
The Antioxidant enzymes such as SOD, CAT, and GPx and GSH levels were remarkably decreased in AlCl3 group, these levels were significantly increased in HMC treated group and results are comparable with Donepezil group. Moreover, the level of Lipid peroxidation and Reactive oxygen species levels are high in AlCl3 group it is indicated by increased level of MDA and MPO level in AlCl3 group and these levels were significantly decreased by treatment with HMC and Donepezil. All these results were shown in (Figure 5).


SOD
SOD (IU/mg protein)
8
6
4
2
0
***
***
Control
AlCl3(100 mg/kg)
AlCl3 + DPZ(1001 mg/kg)
AlCl3 + HMC(100,30 mg/kg)
Tratment Group

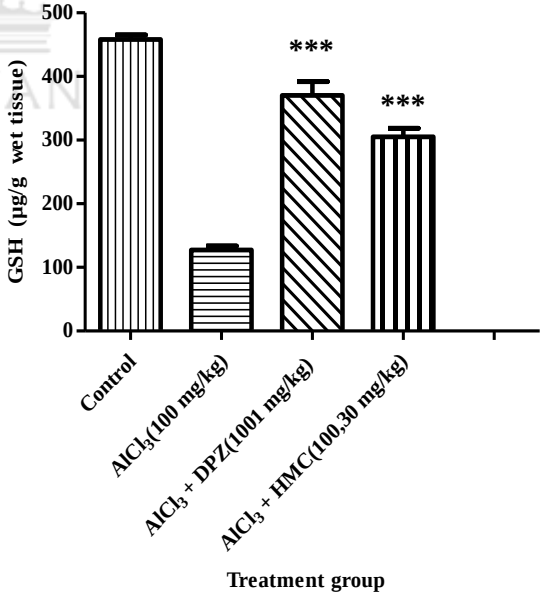
(A)


CAT
CAT
µg/moles of H2O2 metabolized/mg protein
0.005
0.004
0.003
0.002
0.001
0.000
***
***
Control
AlCl3(100 mg/kg)
AlCl3 + DPZ(100,1 mg/kg)
AlCl3 + HMC(100,30 mg/kg)
Treatment group

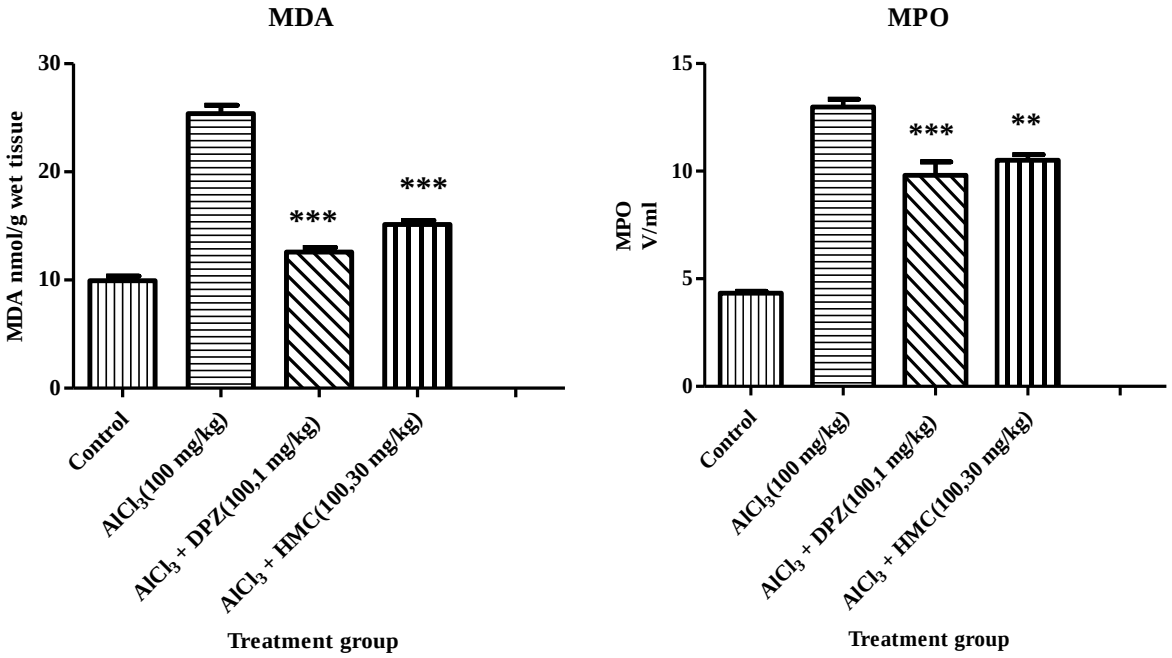
(B)


GPx
GPx
(Unit enzyme activity/mg protein)
0.020
0.015
0.010
0.005
0.000
***
***
Control
AlCl3(100 mg/kg)
AlCl3 + DPZ(100,1 mg/kg)
AlCl3 + HMC(100,30 mg/kg)
Treatment group

(C)


GSH
GSH (µg/g wet tissue)
500
400
300
200
100
0
***
***
Control
AlCl3(100 mg/kg)
AlCl3 + DPZ(1001 mg/kg)
AlCl3 + HMC(100,30 mg/kg)
Treatment group

(D)



(E) **(F)**

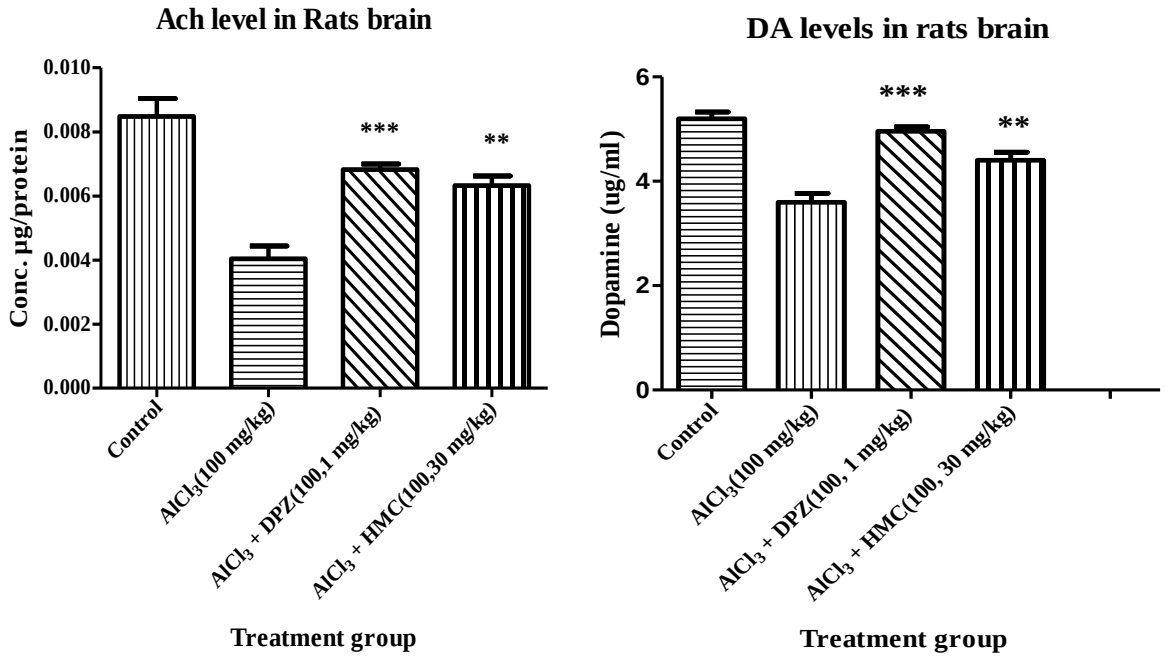
Figure No. 5: Antioxidant enzymes and Lipid peroxidation enzyme concentration in rat brains.

(A) Represents the concentration of enzyme Superoxide dismutase in isolated rat brain, (B) represents the concentration of Catalase, (C) represents Glutathione peroxidase, (D) represents Glutathione concentration, (E & F) represents oxidative stress markers such as Malondialdehyde and Myeloperoxidase in AlCl3 model.

Values were given as Mean ± SEM (n = 6). One way ANOVA was to analyze data, followed by Dunnett test. ***P < 0.05, **P< 0.01 is considered as significant on comparing treatment groups to AlCl3 groups.

3.5 Estimation of Neurotransmitters:

The AlCl3 administration significantly reduces the expression of Acetylcholine because it increases the Acetylcholinesterase expression. The treatment with HMC significantly reduces the expression of Acetylcholinesterase and increases the level of Acetyl choline. The AlCl3 administration not significantly affects the dopamine but the level of Dopamine was slightly reduced in AlCl3 administration and it was significantly increased in treatment with HMC and Donepezil (Fig 7).



(A) **(B)**

Figure No. 6: Neurotransmitter levels in isolated rat brains

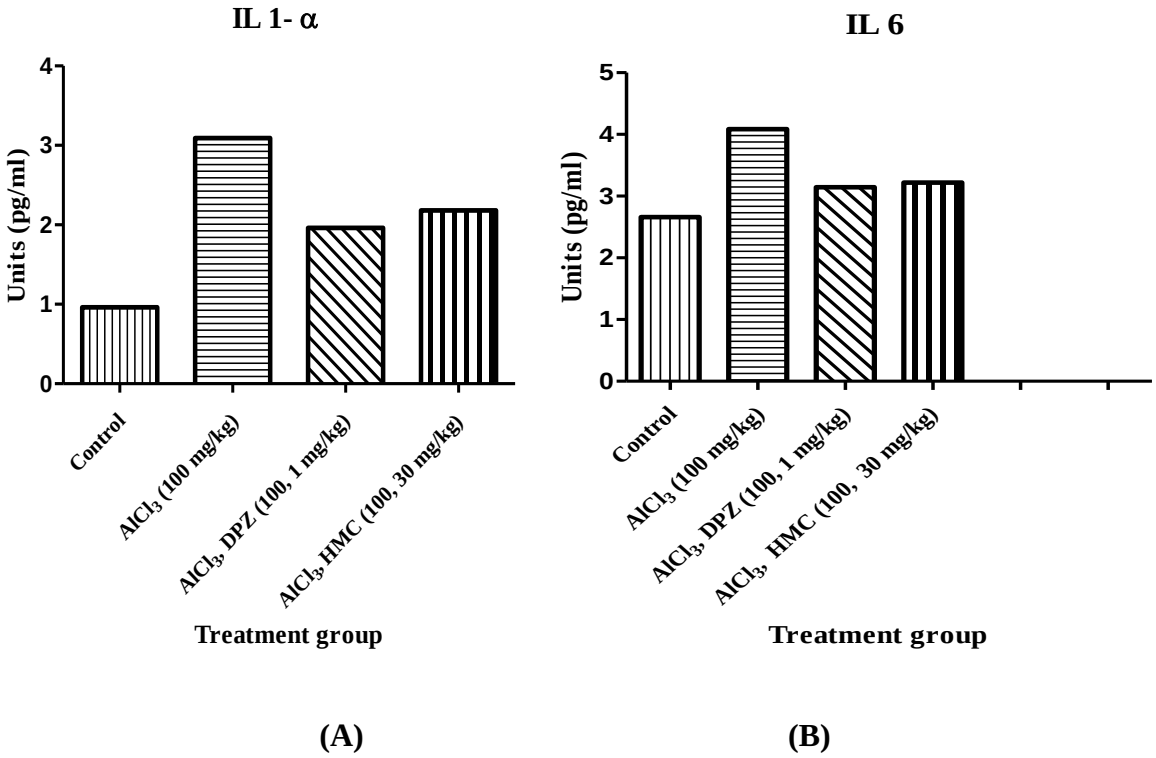
(A) Represents the level of Acetylcholine in the rat brain, (B) Represents the Dopamine level in isolated rat brain in the AlCl3 model.

Values were given as Mean ± SEM (n = 6). One way ANOVA was to analyze data, followed by the Dunnett test. ***P < 0.05, **P< 0.01 is considered as significant on comparing treatment groups to $AlCl_3$ groups.

3.6 Estimations of Inflammatory markers:

The IL-1 α level was significantly increased in AlCl3 treated group when compared with the Control group. It is an indication that more inflammation has occurred with AlCl3 treatment. This level was significantly decreased by treatment with Donepezil & HMC.

The IL-6 level was more in Alzheimer's disease, so in AlCl3 treated group the level of IL-6 is high when comparing with the control group. The Standard drug treatment and HMC treated group shown significantly decreased IL-6 level when comparing with $AlCl_3$ alone (Fig 8).



(A) **(B)**

Figure No. 7: inflammatory markers estimations in isolated rat brains

(A) Represents the IL-1 α level in isolated rat brain, (B) Represents the IL-6 level in isolated rat brain in $AlCl_3$ model.

Values were given as Mean ± SEM (n = 6). One way ANOVA was to analyze data, followed by the Dunnett test. ***P < 0.05, **P< 0.01 is considered as significant on comparing treatment groups to $AlCl_3$ groups.

3.7. Immunohistochemical Stain Analysis:

Doublecortin immunohistochemical analysis for assessment of neurogenesis in the hippocampus of AD brains. Neurogenesis was decreased in the negative control group [Arrow] but neurogenesis was increased in both the standard and test groups. [Star]

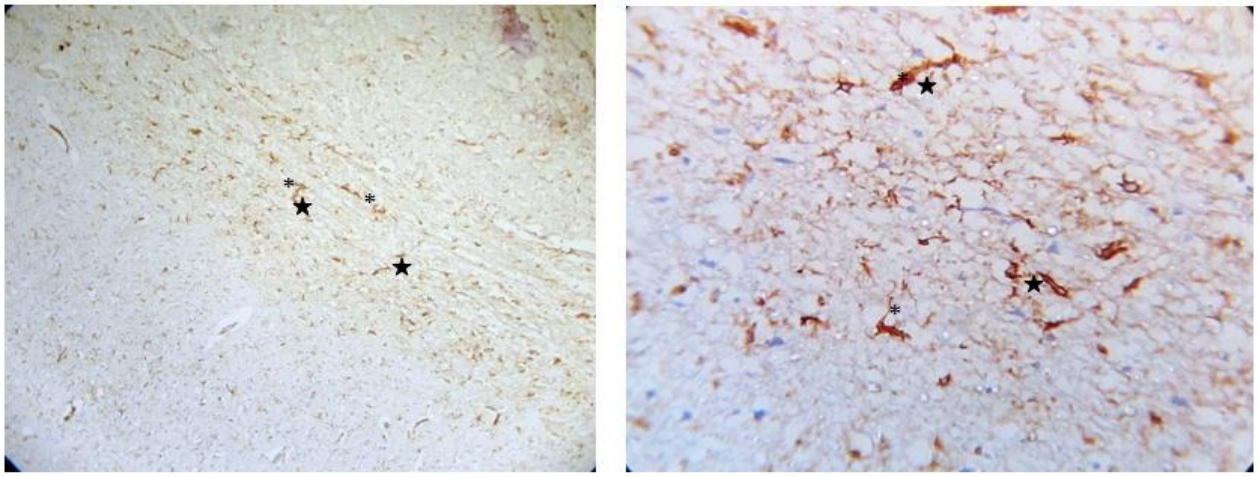


Figure No. 8: Control group: (Normal saline)

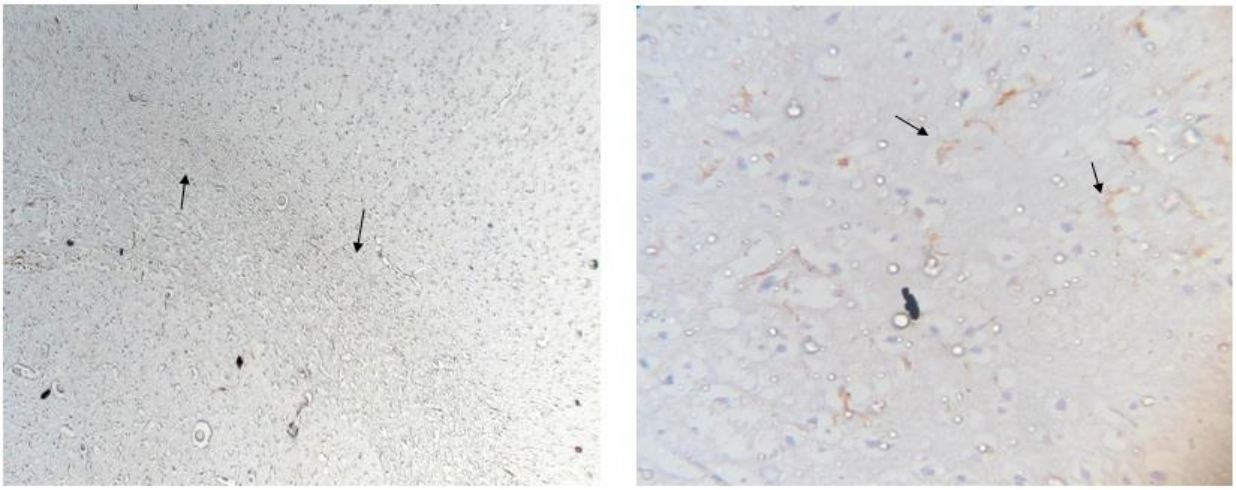


Figure No. 9: Negative control: ($AlCl_3$ 100 mg/kg b.w.)

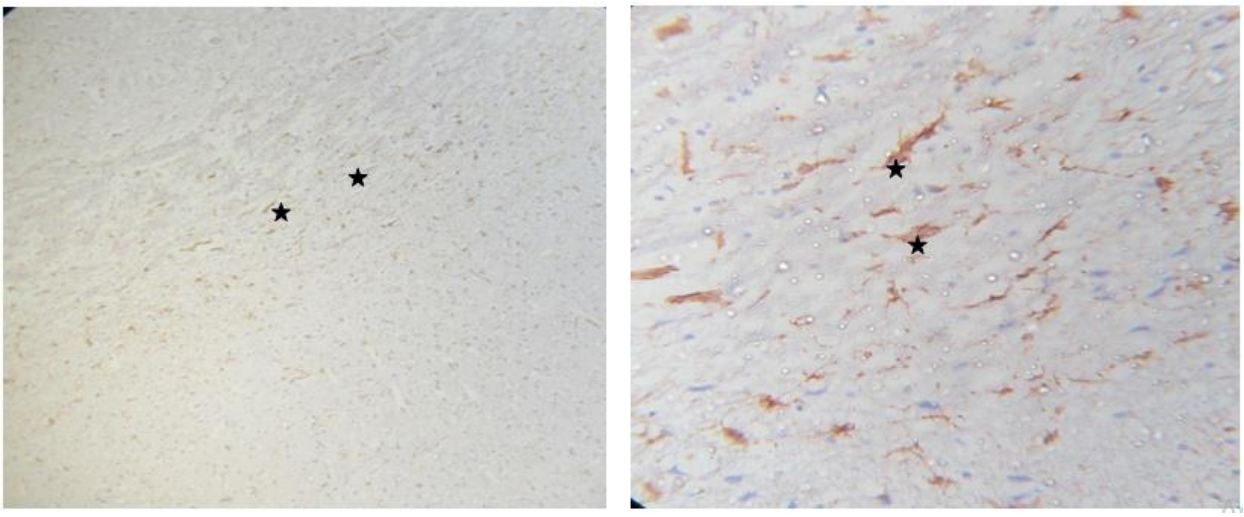


Figure No. 10: Positive control: (Donepezil 1 mg/kg b.w.)

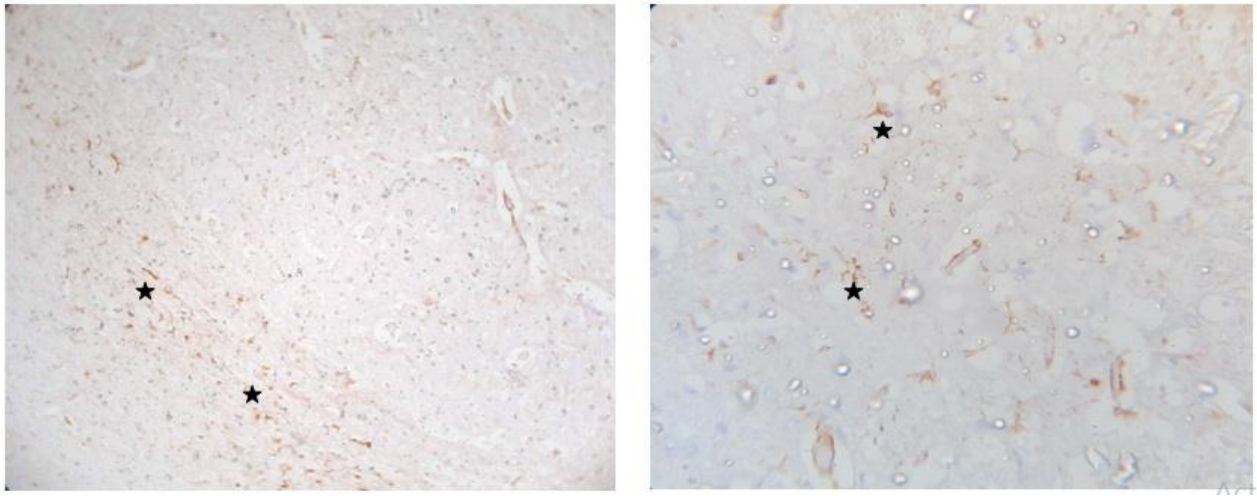


Figure No. 11: Test: (HMC 30 mg/kg b.w.)

DISCUSSION:

The current study investigated the neuroprotective action of HMC on learning and memory impairment in Alzheimer's model caused by aluminum chloride. Because of its lipophilic existence, Phytoflavanone hesperidin, which is abundant in citrus fruits, is known to easily pass the blood-brain barrier and provide neuroprotection. Although Hesperidin is not very water-soluble in its original form, methylation with Hesperidin and chalcone enhances Solubility and may show Synergistic neuroprotection activity because chalcone is also reported as a drug to treat oxidative stress.[25]

The formation of neurofibrillary tangles, cholinergic neuronal terminal loss in the hippocampus and cortex, β amyloid protein aggregation (AB), production of oxidative stress and neuronal apoptosis in the hippocampus, a site for memory formation, and synaptic plasticity occurs during learning that is close to AD pathogenesis, have been shown by aluminum exposed animals.[26] By considering this Aluminium chloride is selected as an inducing agent to induce Alzheimer's disease.

Flavonoids are powerful metal ion chelators and form stable products with ions such as beryllium, aluminum, iron and zinc.[27] we examined the neuroprotective effects of HMC in our understanding that oxidative stress and inflammation play a crucial role in the development of AD in AlCl3-induced rats based on its in vivo antioxidant property model.

Centered on outcomes of in silico studies on different targets it is confirmed that HMC is having multi-target capacity to treat Alzheimer's disease (Fig 3). The outcomes of Behavioral

studies such as Novel object recognition Elevated plus maze and Morrie's water maze reveals that HMC (30 mg/kg) is having Neuroprotection and Memory enhancing properties (Fig 4 & 5).

Moreover, the Biochemical parameter results showed that the HMC is having good Antioxidants properties by enhancing the Antioxidant enzymes like SOD, CAT, GPx, and GSH and also having Anti-inflammatory properties by reducing the concentration of MDA and MPO levels in the brain, so it directly indicating that HMC is having Neuroprotective activity to treat Alzheimer's disease(Fig 6).

AChE inhibition is an effective beneficial technique in Alzheimer's disease treatment.[28] By rolling up its destruction AChE inhibitors improve the accessibility of acetylcholine, thereby helping to manage the symptoms of AD by improving cholinergic transmission in the brain.[29] In this study, HMC treatment provides neuroprotection by decreasing AChE activity and raises the amount of acetylcholine in the hippocampus of rats.

The inflammatory estimations show that AlCl3 treatment increases the level of cytokines i.e. IL-1 α & IL-6. The treatment with Donepezil & HMC significantly reduces the level.

The Immunohistochemical analysis has revealed that the HMC has offered protection against neurodegeneration induced by $AlCl_3$. Degenerative changes in the Hippocampus of the rat brain seen with the negative control group. The treatment with Standard drug Donepezil & Test drug HMC has shown a positive result i.e. Neurogenesis seen.

CONCLUSION:

In conclusion, the results of this study have shown that HMC provides rats with Nootropic and neuroprotective abilities in AlCl3-induced Alzheimers. Nootropic results in NOR, EPM, and MWM might be due to greater visual recognition and spatial memory.

HMC possessing Anti-amnesic activity by increasing the expression of antioxidant enzymes like SOD, CAT, GPx, and GSH and decrease the expression of MDA and MPO. All obtained results are comparable with a standard drug such as Donepezil. The inflammatory markers such as IL-1α & IL-6 levels were decreased on the treatment with HMC 30 mg/kg b.w dose. The Immunohistochemical stain analysis revealed that HMC prevented neurodegeneration in the hippocampus of the rat brain & promotes neurogenesis.

The present study highlights that HMC possesses neuroprotective action against AlCl3 toxicity. However, more research is required to study the molecular mechanism of action of HMC against AlCl3 induced neurotoxicity.

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