

## Neuroprotective Role of Esculetin, A Coumarin Derivative Against Aluminium Chloride Induced Cognitive Deficits in Rats

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### Abstract

Esculetin, a hydroxycoumarin, has been reported to have neuroprotective action against several neurotoxins. Aluminum, a nonessential element and potent neurotoxin, has been suggested to have a link with the etiology and progression of Alzheimer's disease (AD). In this experiment, the possible neuroprotective role of esculetin on aluminium chloride mediated cognitive impairments in rats was investigated. Rats were orally treated with aluminum chloride (100mg/kg/day) and esculetin (25 and 50mg/kg/day) for 42 days. Cognitive functions were analysed by performing several behavioural tests: Passive avoidance test, Morris water maze test, modified elevated plus maze test, radial arm maze, and open field test. The results of the present study indicated that esculetin attenuated aluminum-induced memory deficits in rats. Therapeutic approaches for AD mainly target the inhibition of acetylcholine esterase, thereby preventing the damage of acetyl choline and improving the cholinergic transmission in the CNS. Previous studies demonstrated the potent AChE inhibitory activity of esculetin may be due to the presence of hydroxyl groups (6, 7, or 8 positions) and o-dihydroxyl (catechol) group. Hence, the AChE inhibition property of esculetin may be responsible for its memory enhancing activity.

Key words - Alzheimer's disease, aluminium, esculetin, memory, locomotion.

## Introduction

Approximately 50 million people are affected with dementia globally, owing to the enhanced aging population and it is predicted to raise three times by 2050, which enhances the threat of incapacity, burden of illness and health care costs [1]. Alzheimer's disease (AD), the prevalent form of dementia, is characterised by a stable loss of cognitive abilities because of neurodegeneration. The presence of senile plaques and neurofibrillary tangles are two pathological characteristics of AD. The cortex and hippocampus, which are primarily involved in cognitive tasks, are notably impacted in the early stages of AD [2]. Nearly 80% of the neurons in the hippocampus die during the course of the disease and cognitive impairment particularly mild to severe memory loss; behavioural abnormalities along with decreased capacity to meet out the daily activities were manifested.

Aluminium (Al), a universally abundant nonessential element, has been related to both acute and chronic disorders, and it accumulates in many mammalian tissues, including the brain, bone, liver, and kidney [3,4]. It is also reported to have a link with several neurodegenerative diseases including AD, dialysis encephalopathy and amyotrophic lateral sclerosis [5]. Additionally, it facilitates the development of the two primary histological hallmarks of AD [6]. It can also disrupt the activity of the enzymes involved in the metabolism of acetylcholine, which impairs memory [7,8].

Rodents are currently subjected to a variety of behavioural tests to assess sensory-motor function, learning and memory impairment, anxiety and depressive-like behaviour, chemical dependence, and many forms of cognitive function [9]. Several aspects of memory loss including deficits in learning memory (modified elevated plus maze), working and reference memories (radial arm maze, Y-maze), long-term memory (radial arm maze) [10], spatial memory (Morris water maze) and motor functions (open field and rotarod test) [11] in Al intoxicated rats were analysed.

Unfortunately, to date no pharmacological interventions that effectively prevent or stall AD have been developed. Many experiments and clinical trials have shown that traditional herbal medicine, which has multiple targets, can provide effective treatment of neurological diseases including AD. Various medicinal plants such as *Artemesia capillaris*, *Citrus limonia*, *Cichorium intybus* and *Ceratostigma*

*willmottianum* are reported to contain phenolic compounds particularly coumarins [12, 13]. Among the several coumarins, esculetin (6,7-dihydroxycoumarin) is reported to have protective actions against renal dysfunctions, cardiovascular diseases, dysmetabolic syndromes, cancer and neurological disorders [14]. Previous experiments indicated the capability of esculetin to penetrate the blood–brain barrier thereby attenuating mitochondrial dysfunction, oxidative stress, inflammation and neuronal loss, in various *in vivo* models of psychiatric diseases, Parkinson's disease and cerebral ischemia [15, 16, 17, 18, 19]. However, the neuroprotective role of esculetin against behavioural indices linked with AD still remains unanswered.

## MATERIALS AND METHODS

### Chemicals

Aluminium chloride and esculetin used in this study were purchased from Sigma- Aldrich, Bangalore, India.

### Animals

Male Albino rats weighing 180–220 g was obtained from Biogen Laboratory Animal Facility, Bangalore and maintained in Central Animal House, Rajah Muthiah Medical College & Hospital (RMMC&H), Annamalai University (AU). The animals were maintained under standardized conditions with a room temperature of 28 C, air moisture of 65%, free access to water and food with a 12 h dark/ light cycle adaptation. The institutional ethical committee approved this study. The Proposal number was AU – IAEC/1260/11/19). The experimental procedures were executed adherence to the recommendation of Institutional Animal Ethical Committee of RMMC&H, AU.

### Experimental Design

Thirty rats were randomly separated into five groups of 6 animals each.

Group 1: Control animals were treated with carboxyl methyl cellulose

Group 2: Rats were orally treated with  $\text{AlCl}_3$  solution in water (100 mg/kg of b.w) daily for 42 days [20].

Group 3: Animals were orally treated with  $\text{AlCl}_3$  as group II and esculetin at a dose of 25 mg/ kg of b.w daily for 42 days [17].

Group 4: Animals were orally treated with  $\text{AlCl}_3$  as group II and esculetin at a dose of 50 mg/ kg of b.w) daily for 42 days.

Group 5: Rats were administered with esculetin alone (50mg/ kg of b.w) daily for 42 days.

At the end of experimental period, various behavioural tests such as Passive avoidance test, Morris water maze test, modified elevated plus maze test, radial arm maze, and open field test were performed.

## **Behavioural Assessment Methods**

### **Passive avoidance task**

The apparatus contains a metal grid floor and light and dark chambers that were separated by a door containing wall. This experiment was conducted on two continuous days. In the acquisition test, individual rats were kept separately in the light chamber. An electric shock (0.5 mA and 40 V for 1 s) was provided to the feet of the rats through the grid, soon after its entry into the dark chamber. Animals were taken out immediately from the dark chamber and placed into the cage. Each rat was kept again in the light chamber of the apparatus after 24 h and the time consumed to its entry into the dark chamber was noted as step-through latency (STL) time. The test was stopped and the STL score was noted as 300 s, if any animal not entered into the dark room of the apparatus within a 5 min test period [21].

### **Morris water maze test**

The water was filled into a big circular (150 x 45 cm; radius x height) swimming pool, up to the depth of 30 cm at  $28 \pm 1^\circ\text{C}$ . The tank was divided into four equal quadrants (North East, North West,

South East and South West). During the acquisition trail (0<sup>th</sup> day), a small platform was kept 1 cm above the water surface. The entire individual rat was participated in four consecutive trials with a 5 min break. Each rat was smoothly kept in the all four divisions in each trial and provided 120 s to reach the platform by swimming. It was permitted to persist for 20 s in the visible platform. Animals were allowed to reach the platform by gentle pushing, if failed to reach the platform within 120 s and allowed to stay there for next 20 s. On 42<sup>nd</sup> day (Probe trials), the mean time to reach the hidden platform for each animals was recorded [22].

### **Modified elevated plus maze (EPM) test**

The modified EPM test is mainly utilized for the evaluation of spatial learning and memory [23]. The maze contains two opposite closed arms (50 cm × 10 cm) with 40 cm high walls on sides and two open arms of similar size without side walls. These four arms are linked to central (10 cm × 10 cm) square platform. The entire apparatus is kept 50 cm above the ground. Each and every animal were kept individually at the extreme end of an open arm on 0<sup>th</sup> day. The time required by the rats to move from the open arm to closed arm was calculated as the initial transfer latency (ITL). Rats were allowed to stay for 20 s after calculating the ITL and then replaced to the animal cages. After the completion of the experiment (42<sup>nd</sup> day), the test was repeated by placing the animals in an open arm and the required time for the animal to move to the closed arm is noted as the retention transfer latency (RTL).

### **Radial arm maze test**

The apparatus contains a central compartment, which is having octagonal connections with 8 similar spaced arms (15 × 15 × 70 cm). Four arms were provided with sugared cereals in furrow which were located 2 cm away from the end. The presence of sweets were only limited to furrows. In the trial period, timings began after the animals were kept in the central octagonal platform and allowed to move freely. If the rats entered at least half the distance of the arm, then it is recorded as arm preferences. If the rat visited all the baited arms or stayed in the maze for 10 min, then it was concluded as the test was completed. The reference and working memories were calculated by observing the number of frequent entries to tempted arms and the non-tempted arms respectively [24].

## Open field test

The resin cover drawn with 25 ( $5 \times 5$ ) squares was spreaded over the floor of wooden square box (L  $100 \times 100 \times H$  40 cm). Animals were kept individually into a corner of open field apparatus and its behavior was noted for 5 min. Central and peripheral movements are counted when the rats visited into 9 central squares and 16 peripheral squares adjacent to the walls with both its forelimb respectively. Total number of grooming (licking the fur and face, scratching behavior *etc*) and rearing (standing with hind limbs, leaning on the walls, sobbing and looking around *etc*) behaviours [25] were also counted.

## Results

### Esculetin administration nullified $AlCl_3$ induced behavioral paradigm

#### Passive avoidance task

In the passive avoidance task, animals should learn to escape from an electric shock exposure in darkness. Although the nocturnal animals choose the dark environment naturally, it has to suppress this tendency by remembering the negative stimulus. Rats treated with  $AlCl_3$  exhibited reduced step-through latency than the control animals. In contrast, co-administration of esculetin significantly reversed the memory and learning deficits as compared to  $AlCl_3$  alone treated rats. Esculetin alone treated animals displayed no significant variances in memory in comparison to control group rats (Figure 1).

#### Morris water maze test

MWM test was performed before the initiation of the experiment (acquisition trial - 0<sup>th</sup> day) and at the end of the experiment (probe trial - 42<sup>nd</sup> day).  $AlCl_3$  consumption significantly decreased the time taken to reach the platform (probe trial- 42<sup>th</sup> day) than control group in MWM test. Esculetin co-treatment significantly augmented the time taken to reach the platform as compared to  $AlCl_3$  alone treated rats (Figure 2).

#### Modified elevated plus maze test

The mean ITL exhibited no significant variation among the control and experimental animals.  $AlCl_3$  treated animals showed significant alterations in RTL when compared to ITL and exhibited

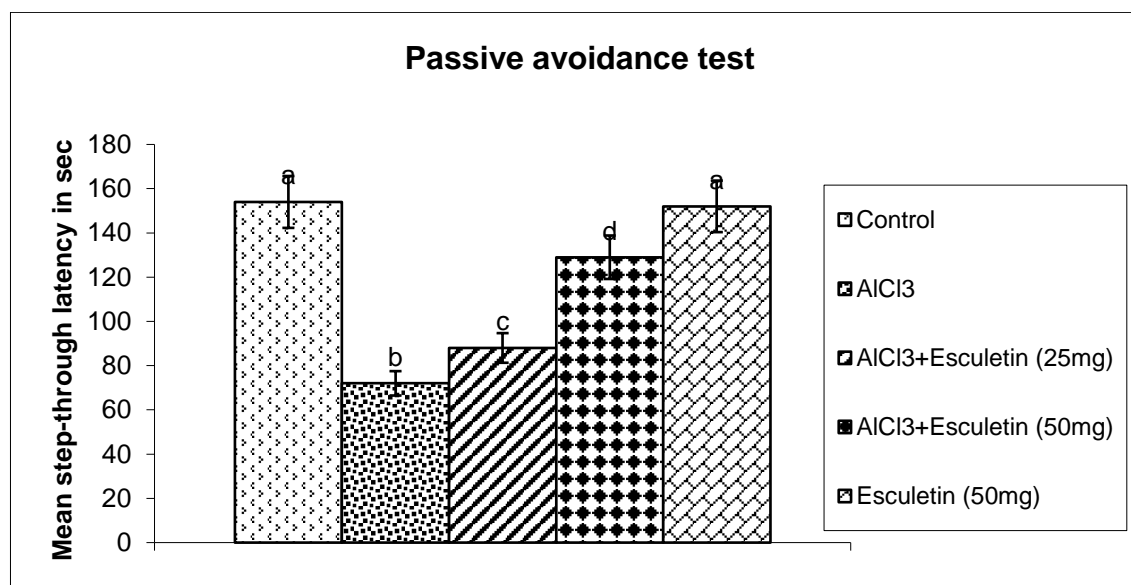
memory dysfunction. Supplementation of esculetin attenuated the  $\text{AlCl}_3$  induced mean RTL on 42<sup>nd</sup> day. No significant differences were found in the mean RTL between control and esculetin alone treated group (Figure 3).

### Radial arm maze test

Animals administrated with  $\text{AlCl}_3$  required much time to end the radial arm maze test with more errors in the working and reference memory task than the control group. Esculetin supplementation nullified the  $\text{AlCl}_3$  induced cognitive impairment ( $p < 0.05$ ), whereas esculetin alone treatment exhibited no significant changes than control animals (Figure 4).

### Open field test

Al-exposure suppressed the movement and activities (numbers of peripheral and central squares crossed, rearing and grooming) than control animals. C-treatment of esculetin significantly improved  $\text{AlCl}_3$ -altered hypo-movement, grooming and rearing activities (Figure 5).



**Figure 1.** Effect of esculetin on STL measurement in passive avoidance test in control and experimental rats. Data are expressed as mean  $\pm$  SEM (one-way ANOVA followed by DMRT) for six rats in each group. Values not sharing the same alphabets differ significantly ( $p < 0.05$ )

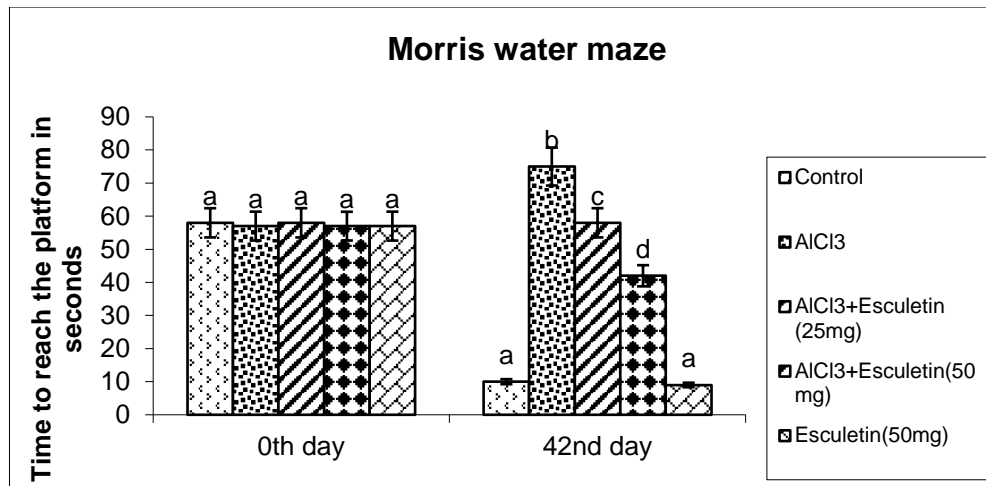


Figure 2. Effect of esculetin on acquisition (0<sup>th</sup> day) and probe (42<sup>th</sup> day) trials in Morris water maze test. Data are expressed as mean  $\pm$  SEM (one-way ANOVA followed by DMRT) for six rats in each group. Values not sharing the same alphabets differ significantly ( $p < 0.05$ ).

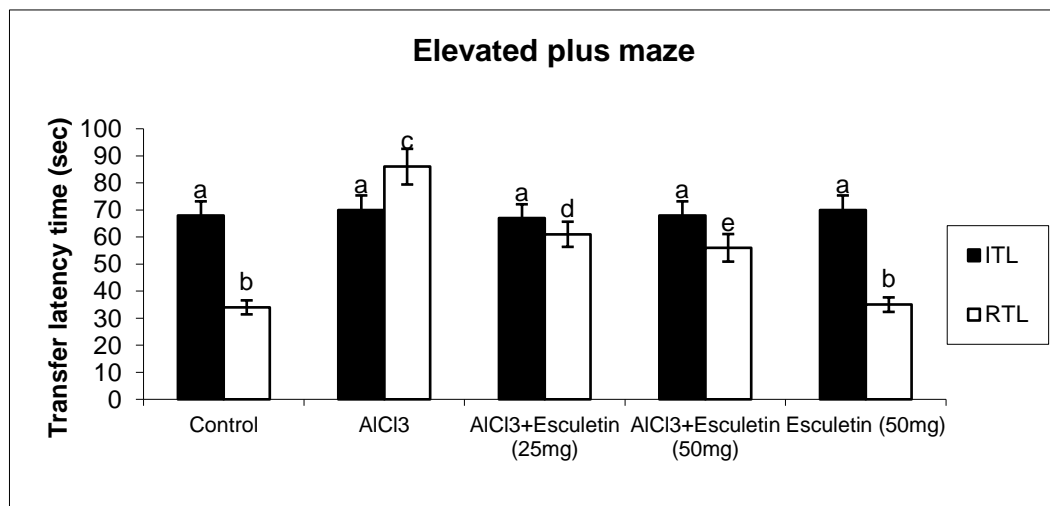
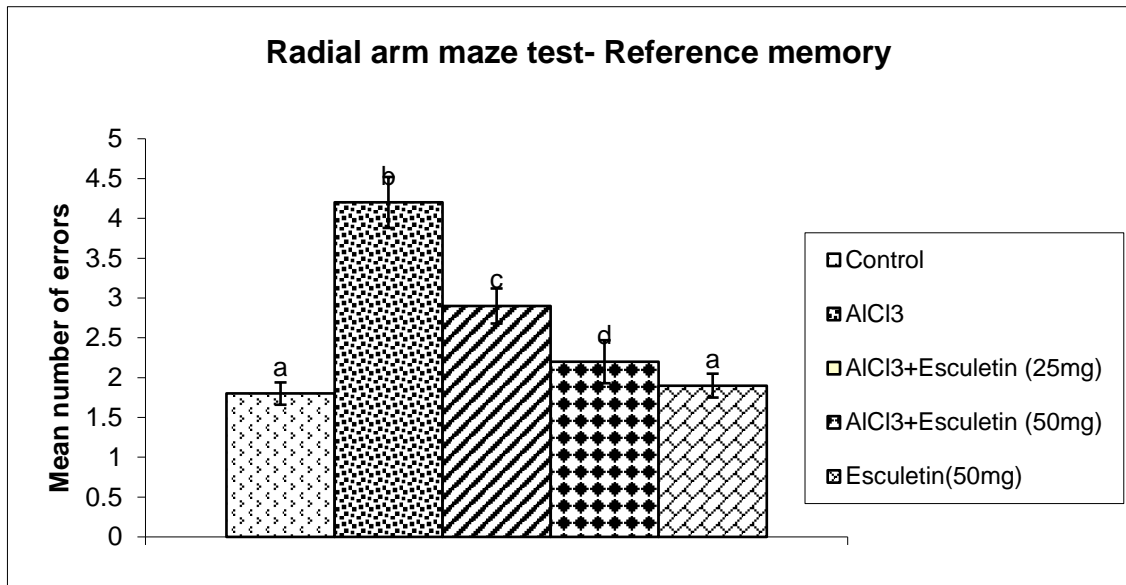


Figure 3. Effect of esculetin on mean ITL and RTL in modified elevated plus maze test. Data were presented as mean  $\pm$  SEM (one-way ANOVA followed by DMRT) for six rats in each group. Values not sharing the same alphabets differ significantly ( $p < 0.05$ ).



A



B

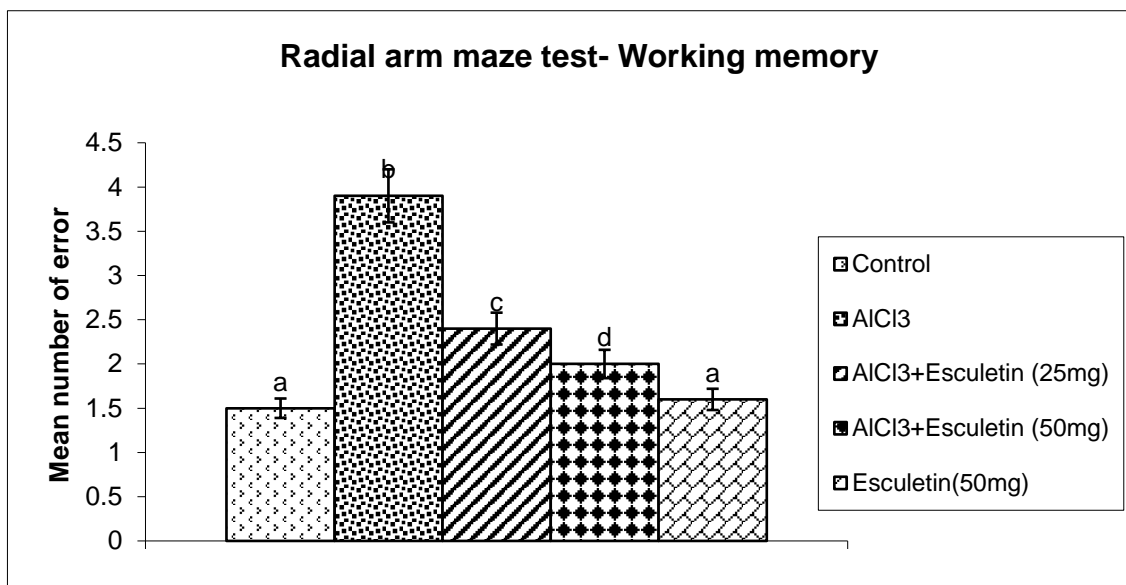
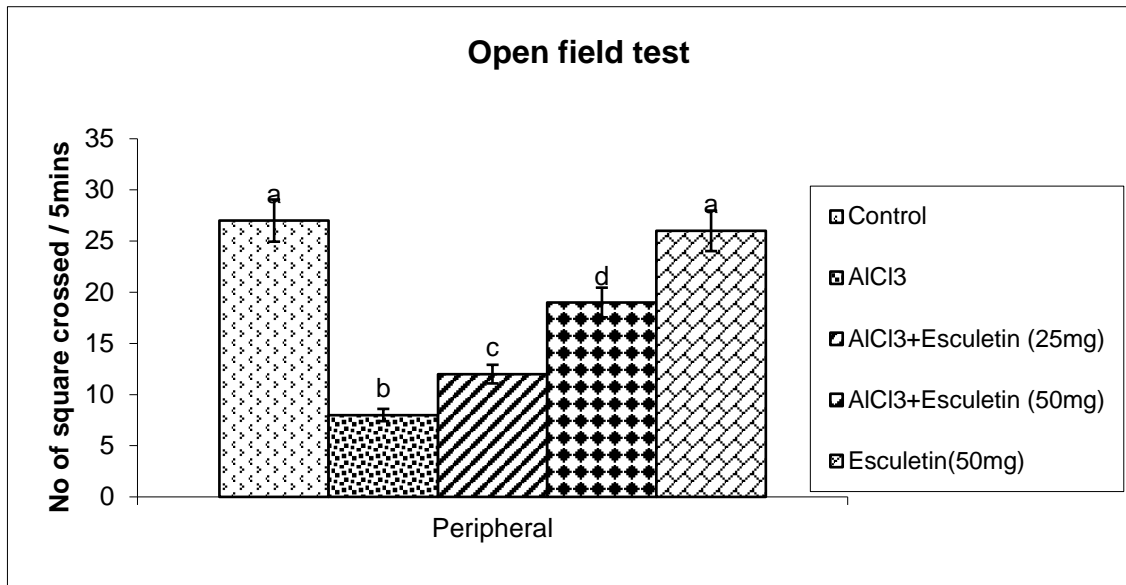
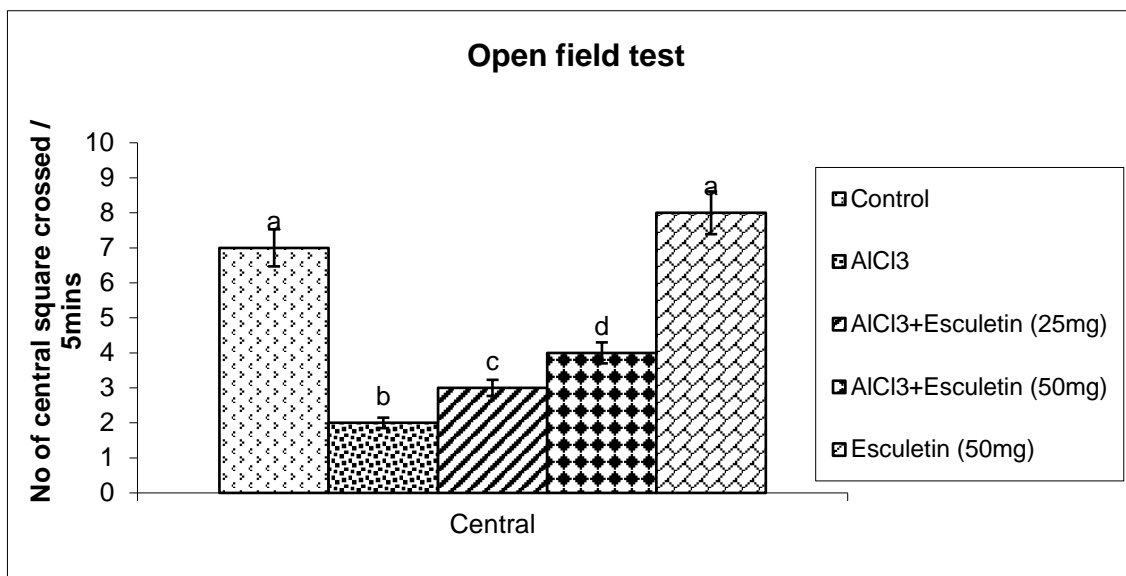


Figure 4 (A&B). Effect of esculetin on memory impairment in radial arm maze test. Data were presented as mean  $\pm$  SEM (one-way ANOVA followed by DMRT) for six rats in each group. Values not sharing the same alphabets differ significantly ( $p < 0.05$ ).

A



B



C

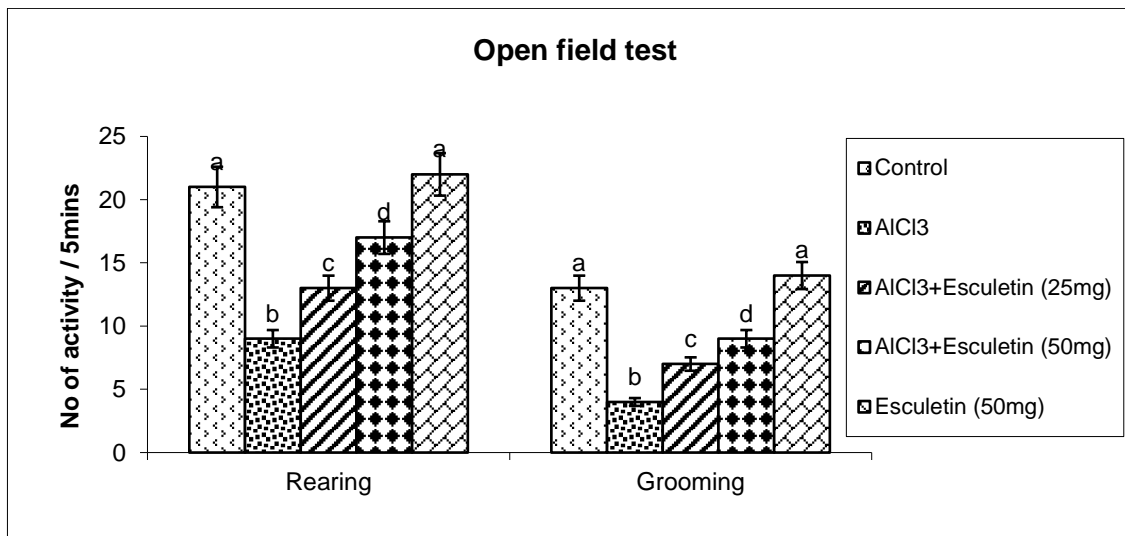


Figure 5 (A-C). Effect of esculetin on locomotion and activities in open field test. Data were presented as mean  $\pm$  SEM (one-way ANOVA followed by DMRT) for six rats in each group. Values not sharing the same alphabets differ significantly ( $p < 0.05$ ).

## Discussion

As there is no direct procedure used to measure the memory and learning processes, it can be evaluated by determining behavioural changes. Behavioural measurement is a sensitive marker of Al induced neurotoxicity [26]. The impaired hippocampal function is measured by Morris water maze test, which is the mostly vulnerable region during AD [27]. The results of our study indicated that the administration of AlCl<sub>3</sub> significantly diminished the spatial memory in Morris water maze test. Previous experiments from our lab indicated that the intraperitoneal and oral administration of AlCl<sub>3</sub> significantly reduced the spatial memory in Morris water maze test [8, 20]. Singh et al., [28] demonstrated that the oral administration of esculetin enhanced latency to reach the platform in Morris water maze test in reserpine-induced mice model of fibromyalgia, which is in consistent with our results.

EPM test is performed for analysis of spatial learning and memory by quantifying both the initial (ITL) and retention transfer latency (RTL). The result of the present study indicated that the oral administration of AlCl<sub>3</sub> enhanced the RTL as compared to control indicating a reduction in spatial learning

and memory. In contrast, reduction in RTL after the determination of ITL showed enhancement in recognition memory of rodents. Oral administration of esculetin to  $\text{AlCl}_3$  treated rats enhanced the memory processes by significantly reducing RTL of rats as compared to neurotoxin alone exposed animals.

Passive avoidance test is a fear-induced task used to assess both the long and short term memory by measuring pre and post-shock latencies. The  $\text{AlCl}_3$  treated rats indicated the decrease in retention latency (RL) because of anxiogenic-like behavior in rodents, whereas esculetin administration indicated enhancement in RL. In the passive avoidance test, the enhancement in the post-shock latency after the pre-shock latency showed progress in recognition memory of rodents. This may be owing to neuroprotective potential of esculetin.

In open field test, the movement (peripheral and central) and activities (grooming and rearing) of the rats are assessed to quantify the neuroprotective functions of pharmacological agents in central nervous system. In corroborate with our and other previous studies [11,29],  $\text{AlCl}_3$  administered animals showed a reduction in locomotion and activities, due to Al induced depression, whereas co-treatment with esculetin attenuated the Al mediated motor dysfunction. Previous experiments indicated the protective role of esculetin by attenuating locomotor and activity dysfunction in open field test against reserpine-induced mice model of fibromyalgia [28], lipopolysaccharide mediated anxiety- and depressive-like behaviour in mice [16], 3-nitropropionic acid induced neurotoxicity [30] and aluminium induced neurodegeneration [31].

Olton et al., [32] indicated that the reference and working memories were measured by radial arm maze test. If the rat enters into each and every new arm from the central compartment, it is referred as working memory, while if it enters into the arm containing food is considered as reference memory. The dysfunction in the working and reference memory are measured if the rats, repeatedly enters into same arm or unable to enter into the food containing arm respectively. The  $\text{AlCl}_3$  treated rats showed an increase in the RTL and along with a diminished working and reference memories.

Administration of aluminium disrupts the memory and learning processes in animals, due to their adverse effect on cholinergic system [10, 11]. Acetylcholine (ACh) is the main neurotransmitter that is reported to play a vital role in the maintenance of learning and memory functions. Therapeutic approaches for AD mainly converging on cholinergic hypothesis, primarily by target the inhibition of AChE, thereby preventing the damage of ACh and improving the cholinergic transmission in the CNS [33]. Ali et al., [34] indicated that the potent AChE inhibitory activity of esculetin may be due to the presence of hydroxyl groups (6, 7, or 8 positions) and o-dihydroxyl (catechol) group. As to conclude, the AChE inhibition property of the esculetin is responsible for its memory enhancing action.

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