Cerebral Cortex and Hippocampal Protection Mediated by *Callistemon viminalis* in Aluminium Chloride Induced Alzheimer’s Disease

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**ABSTRACT**

**Aim:** The study was carried out to evaluate the neuroprotective effect of methanol extract of *Callistemon viminalis* in aluminium chloride induced dementia of alzheimer’s type in mice. **Methods:** Swiss albino mice of either sex were divided into 5 groups: vehicle control; AlCl$_3$ + normal saline; AlCl$_3$ + rivastigmine; AlCl$_3$ + extract (50 mg/kg; p.o); AlCl$_3$ + extract (100 mg/kg; p.o). The following parameters were analyzed in all groups: a) Behavioral parameters- morris water maze test, b) Biochemical parameters- AchE level, GSH level, SOD level, catalase level and nitrite level. Histopathological study- Hippocampus and cerebral cortex region of mice brain. **Results:** The presence of various phytoconstituents like betulinic acid, ursolic acid, β-sitosterol and lupeol reported through TLC, have a potent role as antioxidant and also known to improve blood flow as well as reduce plaque formation which proves that the extract of this plant can be used in alzheimer. Oral administration of MECV caused significant (*p*<0.01) decrease in escape latency time and significant (*p*<0.01) increase in total time in a dose dependent manner as compared to the aluminium chloride treated group. Additionally, post treatment with MECV (50mg/kg and 100 mg/kg) showed significant (*p*<0.01) decrease in AchE and nitrite level and significant (*p*<0.01) increase in the GSH, catalase and SOD level in mice brain. The histopathology of hippocampus and cerebral cortex of mice brain showed that the toxicity induced by the aluminium chloride was markedly reduced by the MECV. The normal histoarchitecture pattern of the hippocampus and cerebral cortex was also preserved. **Conclusion:** The evidence regarding the presence of phytoconstituents and analytical studies confirming their presence, along with preclinical studies on mice with conclusive results in treatment of Alzheimer shown by the extract concludes that the extract exhibits neuroprotective effect.

**Key words:** Morris water maze, AchE, GSH, Catalase, SOD, Nitrite, *Callistemon viminalis*.

**INTRODUCTION**

Dementia is not a single disease it is a group of diseases affecting memory and other cognitive abilities that alter the daily living activities.\(^1\) Alzheimer disease is characterized by progressive memory impairment, disruption in cognition, changes in personality, language difficulties, impairment of movement and other functions that lead to death from complete brain failure.\(^2\) The three major hallmarks of AD are beta amyloid plaques, neurofibrillary tangles (NFTs) and neuronal cell death which are the central factors in neurodegenerative process. Synthetic drugs that are used to treat dementia have many side effects such as nausea, vomiting, diarrhea, anorexia, abdominal pain, headache, dizziness, weight loss, fatigue, bradycardia, Urinary incontinence, insomnia, muscle cramps, hallucination, confusion.\(^3\) Traditional medicine system of India is much safer than the synthetic drugs that are unsafe to humans. Medicinal plants that enhance memory...
and reduce brain aging include ashwagandha, brahmi, caraway, coriander, turmeric, shankhpushpi, liquorice and guggul. The herbal plants have not been properly studied for their signal transduction processes and their efficacy. However, in past 10 to 20 years, the pharmacological and toxicological studies of herbs have gained much importance. Studies related to plants are receiving much attention from scientists and verified for their claimed therapeutic effects. A number of herbs traditionally employed in the Indian system of medicine have yielded positive results. The plant “Callistemon viminalis” is used for many diseases in traditional Chinese medicine. It exhibits antibacterial, antifungal, antihelminthic, antioxidant, insecticidal, haemolytic, moluscicidal, anti-platelet aggregation activities. There was no scientific data available for the fruit of Callistemon viminalis for treatment of Alzheimer disease. The present work was aimed to generate a scientific data and find the efficacy of extract of the plant.

MATERIALS AND METHODS

Chemicals: Methanol was procured from blulux laboratories private limited, Faridabad. Aluminium chloride and rivastigmine were procured from NICE Chemicals Private Limited, Cochin and Sun Pharmaceuticals, Mohali, Chandigarh. All other chemicals were of analytical grade.

Plant collection and authentication: The fruit part of Callistemon viminalis was collected from botanical garden of Rayat Group of Institutions, Ropar in the month of Jan 2019. Plant was authenticated by Mr. Ram Prasad, Herbarium Assistant in the Department of Botanical and Environmental Sciences, Guru Nanak Dev University (GNDU), Amritsar with reference no. – 1920, dated 25/02/2019.

Preparation of MECV: The ripened fruit was dried under shade for a month and seeds were removed from the fruit part. The dried fruit material was then grinded to reduce it into coarse powder with the help of a grinder. The extraction was done with methanol using soxhlet extractor. Rotary evaporator was used for evaporating the solvent.

Phytochemical evaluation: Phytochemical evaluation of Callistemon viminalis was carried out as per standard methods. Thin Layer Chromatography

Methodology for TLC: TLC Plates were made with Silica Gel-G. TLC plates were run insolvent system of Methanol: Conc. HCl (9:1 v/v) and detection was done in Iodine chamber for 10-30 min and also detected in UV chamber at 264 and 365 nm.

Animal procurement and housing

Healthy Swiss albino mice were procured from central animal facility of NIPER, Mohali. The animals were kept in propylene cage and paddy husk was used as bedding material. Husk was changed every day to clean the cages and maintain hygienic condition. 6 animals were kept in each cage and fed with standard pellet diet and water ad libitum. The room was well-aerated and a 12-hr light and dark cycle was maintained. The room temperature was maintained at 22±2°C. The study was conducted in the Department of Pharmacology, Rayat Institute of Pharmacy, Railmajra, Distt. S.B.S. Nagar, Punjab. Experiments were performed after prior approval from IAEC. (No: 874/Po/2018-19/1).

Dose selection

Dose selection of aluminium chloride (AlCl3) and rivastigmine was based on previous literature review. The dose of Callistemon viminalis was selected based on literature review in which fruit part was evaluated for other activities: 50mg/kg; p.o. and 100mg/kg; p.o.

Design of the experiment

Swiss albino mice of either sex were used having body weight 25-30 gm. They were divided into 5 groups having 6 animals each. Group I mice treated with Normal saline 0.9% w/v NaCl 10ml/kg; p.o. (5th– 42nd day), Group II mice treated with AlCl3 (70mg/kg; i.p.) from 5th to 25th day + 0.9% w/v NaCl 5ml/kg; p.o. from 26th to 42nd day). Group III received AlCl3 (70mg/kg; i.p.) from 5th to 25th day + Rivastigmine treated group (2.5 mg/kg; p.o.) from 26th day to 42nd day. Group IV was administered AlCl3 (70mg/kg; i.p.) from 5th to 25th day + test drug treated group (50 mg/kg; p.o.) from 26th day to 42nd day and Group V animals were given AlCl3 (70mg/kg; i.p.) from 5th to 25th day + test drug treated group (100 mg/kg; p.o.) from 26th day to 42nd day.

Estimation of behavioral parameters

Behavioral assessment was done with Morris water maze (MWM). In the MWM study mice were randomly selected into five groups, in each group 6 animals were present. Behavior reading was taken on 5th, 16th, 26th, 36th and 42nd day of treatment.

Spatial navigation task: Morris water maze was used to evaluate the ELT and TT of spatial navigation task. During training phase (day 1 to 4th day) mice were allowed to swim to a visible platform present in circular pool that was 180 cm in diameter and 60 cm
in height. The maximum time was 60 sec for escaping to a visible platform. If the mice did not escape from water to this platform within the given time then the mice were guided to the visible platform and allowed to remain there for 20 sec for remembering the position of the platform. At the end of the trial the mice were cleaned properly and returned back to their home cages. On 5th day (Probe day), Probe trial was conducted by removing the escape platform. The principle of MWM was that when mice escape from the water by climbing onto the platform over and over again, they learned the spatial location of the platform from any starting position in the pool and the time taken by the mice to reach the visible platform decreases. Both ELT and TT were checked for different groups on 26th day and 42nd day of the experiment. During ELT measurement the water level was 2 cm below than the level of movable circular platform. During TT measurement the water level was 2 cm above than the level of circular platform. During both the phases the platform was placed in the center of 3rd quadrant. During TT measurement platform was removed.

Estimation of biochemical parameters

After the behavioral analysis animals were sacrificed by anesthesia with diethyl ether and cervical dislocation. The brain was removed by decapitating the animal and chilled 0.1 M phosphate buffered saline (pH 7.4) was used to prepare 10% (w/v) homogenate in glass Teflon homogenizer. The solution was centrifuged at 1000 rpm for 10 min at 40°C. A supernatant was obtained after centrifuge. The solution was used to assay AchE, GSH, catalase and SOD enzyme activities.

Preparation of post-mitochondrial supernatant:
A small amount of the supernatant was again centrifuged at 12,000 rpm for 20 min. Post-mitochondrial supernatant was obtained after centrifugation the supernatant solution and was used to assay nitrite level in mice brain.

Estimation of acetylcholinesterase enzyme activity in mice brain
Acetylcholinesterase activity of brain homogenate was measured by Ellman’s photometric method with slight modification. Acetylthiocholine was used as a substrate.

Reduced glutathione estimation
Standard colorimetric method is used to determine total glutathione level.

Estimation of catalase (CAT) activity in mice brain
Catalase enzyme converts the hydrogen peroxide into water and oxygen, using iron or manganese cofactor as a catalyst. 10% w/v 0.05 ml of post mitochondrial supernatant was added into cuvette containing 1.95 ml of 0.05 M phosphate buffer (pH-7). Then 1 ml of 0.019 M H₂O₂ was added into the cuvette and absorbance was measured at 240nm for 30 sec. Control cuvette contains all the components except Substrate.

Estimation of superoxide dismutase (SOD) activity in mice brain
SOD enzyme inhibits the oxidation of oxymine by inhibiting xanthine-xanthine oxidase system. Hydroxylamine nitrite was produced by the oxidation of oxymine showed absorbance peak at 550 nm. 0.5 ml supernatant was added into the cuvette and 1 ml of 50 mM Sodium carbonate, 0.4 ml of 25 µM NBT and 0.2ml of 0.1 mM EDTA was added into it. Then 0.4 ml of 1 mM hydroxylamine hydrochloride was added into it and absorbance was measured at 560 nm. Control cuvette is simultaneously run without homogenate. The SOD activity is expressed as units per mg of brain protein.

Histopathology study of mice brain
Histopathology was done by fixing the cerebral cortex and hippocampus region of mice brain in 4% paraformaldehyde and then embedded in paraffin for slicing the brain parts. Section cutter (Leica, Germany) was used to slice the brain into 5 mm sections and the sections were stained with haematoxylin and cosin reagent. After that sections were examined by a light microscope. Haematoxylin produces blue color and eosin produces violet and red color.

Statistical analysis
Values are represented as mean ± SEM, n=6. Data were analyzed by one-way ANOVA followed by Dunnett’s post hoc comparisons test in Graph Pad Prism version 8.0.2. *p< 0.05, **p<0.01, ***p<0.001, ****p>0.05, a compared with control group, b compared with aluminium treated group.
RESULTS

Preliminary phytochemical screening: MECV was subjected to preliminary phytochemical screening and the results obtained have been presented in Table 1. The extract showed the presence of glycosides (saponin, steroid, pentacyclic triterpenoid) and polyphenols

Thin layer chromatography (TLC)

Isolation of plant extract: TLC chromatogram of MECV using solvent system [Hexane: Ethyl acetate (1:1)] with long and short UV light, iodine as detection reagent showed six spots in TLC plates. The Rf value of the extract with inference are showed in Table 2.

Effect of Callistemon viminalis on behavioral studies

Effect of MECV in aluminum chloride induced behavioral alteration (Morris water maze) in mice.

Results are described as per the performance on Day 5th, 16th, 26th, 36th and 42nd in morris water maze. During the retention trial conducted on day 5th (Probe day) all mice spent more time in the target quadrant (Q3) as compared to other quadrants. The vehicle control group showed normal retrieval of memory during ELT and TT to find the platform. Significant (p<0.01) difference in ELT and TT between control and the aluminium chloride (AICl3 70mg/kg/i.p.) treated group, indicate that chronic oral administration of AICl3 cause deterioration of learning and memory skills in swiss albino mice. MECV (50mg/kg/p.o; 100 mg/kg/p.o) and rivastigmine (2.5mg/kg/p.o) have produced significant decreases in ELT and increase in TT in morris water maze when compared with aluminium chloride treated group. Result is summarized in Table 3; Table 4; Figure 1; Figure 2.

Effect of MECV on biochemical parameters

a) Effect of MECV on AchE level in the mice brain

The level of AchE in the control group (Group I) animals was found to be 1.65± 0.10 nM/L/min/gm of tissue. Aluminium chloride treatment resulted in a significant increase in AchE levels (p<0.01) as compared to control group. Post treatment with standard drug rivastigmine and MECV (50mg/kg and 100 mg/kg) showed significant (p<0.01) decrease in AchE levels when compared with aluminium chloride treated animals. The results are summarized in Table 5; Figure 3.

b) Effect of MECV on GSH level in the mice brain

The level of GSH in Control group (Group I) was found to be 2.68±0.11nM/mg of protein. Aluminium chloride treatment resulted in a significant decrease in GSH level in the mice brain (p<0.01) as compared to control group. Post treatment with standard drug rivastigmine and MECV (50mg/kg and 100 mg/kg; p.o), showed significant (p<0.01) increase GSH level in the mice brain when compared to aluminium chloride treated animals. The results are summarized in Table 5; Figure 4.

c) Effect of MECV on catalase level in the mice brain

The level of catalase in control group (Group I) animal was found to be 1.75±0.11 µM of H2O2 decomposed/min/mg of protein. Aluminium chloride treatment resulted in a significant decrease (p<0.01) in catalase level in mice brain as compared with the control group. Post treatment with standard drug rivastigmine and MECV (50mg/kg and 100 mg/kg; p.o), showed significant (p<0.01) increase in catalase level in the brain of mice when compared to aluminium chloride treated animals. The results are summarized in Table 5; Figure 5.

d) Effect of MECV on SOD level in mice

The level of SOD in Control group (Group I) animal was found to be 4.68±0.10 units/mg of protein. Aluminium chloride treatment resulted in a significant decrease in SOD level in the mice brain (p<0.01) at the end of the experiment (42th day) as compared to control group. Post treatment with standard drug rivastigmine and MECV (50mg/kg and 100 mg/kg; p.o), showed significant (p<0.01) increase in SOD level in the brain of mice.
when compared to aluminium chloride treated animals. The results are summarized in Table 5; Figure 6

e) Effect of MECV on nitrite levels in the mice brain

The level of nitrite in control group (Group I) animal was found to be 1.80±0.21 nM/mg of protein. Aluminium chloride treatment resulted in a significant decrease in nitrite level in the mice brain (p<0.01) as compared to control group. Post treatment with standard drug rivastigmine, showed significant (p<0.01) decrease in nitrite level in the brain of mice when compared to aluminium chloride treated animals. Post treatment with MECV (50mg/kg and 100 mg/kg; p.o) showed significant (p<0.01; p<0.01) decrease in nitrite level when compared to aluminium chloride treated animals. The results are summarized in Table 5; Figure 7

Histopathological reports of mice brain which showing neuronal degeneration in hippocampus

Observations: From the histopathological study of the hippocampus part of mice brain it has been observed that, Figure 8A vehicle control group (0.9% NaCl): In the group I, animals which were treated with normal Saline, the hippocampus of the mice brain showed minimal normal histological structure. Figure 8B positive control group (AlCl₃): In group II animals which were treated with aluminium chloride showed neuronal loss, gliosis and typical vacuolar degeneration in hippocampus. Figure 8C comparative control group (rivastigmine): Group III animals which were treated with standard drug (rivastigmine) along with aluminium chloride revealed slight neuronal damage. Figure 8D MECV (50 mg/kg): Group IV animals which were treated with MECV (50 mg/kg/p.o) and aluminium Chloride showed minimal neuronal damage. Figure 8E MECV (100 mg/kg): Group IV animals which were treated with higher dose of MECV (100 mg/kg/p.o) and aluminium chloride showed minimal neuronal damage and severity of lesions.

Histopathological reports of mice brain which showing neuronal degeneration in cerebral cortex

Observations: From the histopathological study of the cerebral cortex part of mice brain it has been observed that; Figure 9A vehicle control group (0.9% NaCl): Normal histological structures and well-formed neurons. Figure 9B positive control group (AlCl₃): Neuronal degeneration with abnormal cellular morphology, recruitment of macrophages, with damaged cerebral cortex. Figure 9C comparative control group (rivastig-
mine): No histopathological alterations were observed. Figure 9D MECV (50 mg/kg): No histopathological changes and sections show reduced morphologic abnormalities in all regions with well-formed nuclei without irregular features. Figure 9E MECV (100 mg/kg): Sections showed normal architecture of brain regions like control group with no histopathological alterations.

**DISCUSSION**

In the present study, an attempt has been made to evaluate the anti-alzheimer’s activity of MECV in aluminium chloride induced neurotoxicity in experimental animal model. Alzheimer disease is the most common form of dementia and 90% of the patients suffer from AD type of dementia. Alzheimer’s disease (AD) is a critical neurodegenerative disease characterized by memory loss and diminished performance, language and visuospatial skills. The neuropathological features of AD involve the injury and death of neurons. It starts from the hippocampus region of the brain which is mainly involved in

| Table 1: Phytochemical screening of MECV. |
|---|---|---|
| S No. | Class of compound | Present (+) / Absent (-) |
| 1. | Glycoside | + |
| 2. | Polyphenol | + |

Figure 7: Effect of MECV on nitrite level in mice brain.

Figure 8: Histopathology of hippocampus in mice brain; A: Normal histological structures were observed; B: neuronal loss, gliosis and typical vacuolar degeneration in hippocampus; C: slight neuronal damage; D: showed minimal neuronal damage and severity of lesions.

Figure 9: Histopathology of cerebral cortex in mice brain; A: Normal histological structures and well-formed neurons; B: Neuronal degeneration with abnormal cellular morphology, recruitment of macrophages, with damaged cerebral cortex; C: No histopathological alterations were observed; D: No histopathological changes and sections showed reduced morphologic abnormalities in all regions with well-formed nuclei without irregular features; E: Sections showed normal architecture of brain regions like control group with No histopathological Alterations.
memory and learning, then affects the entire brain. The presence of senile plaques and neurofibrillary tangles is mostly seen in the hippocampus, cerebral cortex of the brain.25

Aluminium chloride model is used because it is most reliable model as compared to others. Aluminium is a toxic metal present in the drinking water and food, which passes into the brain and causes oxidative damage and neurodegeneration in the brain resulting in learning and memory deficits. The chronic aluminium treatment causes oxidative stress and deposition of the amyloid beta in the hippocampus and cerebral cortex region of the mice brain that causes memory impairment and neurobehavioral deficits. Chronic administration of MECV reversed the cognitive deficit produced by aluminium chloride and showed its neuroprotective action.

The Morris water maze is the most widely used behavioral tests for studying spatial learning and memory of the experimental animals. Initially rodents are allowed to swim to a visible platform to escape from a pool of water. After that platform is hidden under the surface of water, so that the animal remembers its location in order to escape from the water. It is used to check the cognitive function, study animal models of neurodegenerative disease (AD and PD) and test potential drug therapies. In the present study, chronic administration of aluminium chloride for 6 weeks showed cognitive impairment in mice on performance day. Mice treated with aluminium chloride showed increased ELT and TT

<table>
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<th>S. No.</th>
<th>Plant Extract</th>
<th>Solvent system</th>
<th>R, value</th>
<th>Inference</th>
<th>Reference</th>
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<td>1.</td>
<td>Methanolic extract of <em>Callistemon viminalis</em></td>
<td>Hexane: Ethyl acetate</td>
<td>0.5</td>
<td>Lupeol</td>
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<td>Ursolic acid</td>
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<td>0.3</td>
<td>Betulinic acid</td>
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<td>β-sitosterol</td>
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<td>Group I 0.9% w/v NaCl 10ml/kg; p.o.</td>
<td>4.90±0.37</td>
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<tr>
<td>Group II AlCl₃ 70mg/kg; i.p + 0.9% w/v NaCl 10ml/kg; p.o</td>
<td>4.79±0.35</td>
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<tr>
<td>Group III AlCl₃ 70mg/kg; i.p + rivastigmine 2.5 mg/kg; p.o</td>
<td>4.75±0.29</td>
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<td>Group IV AlCl₃ 70mg/kg; i.p + MECV 50 mg/kg; p.o</td>
<td>4.92±0.30</td>
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<tr>
<td>Group V AlCl₃ 70mg/kg; i.p + MECV 100 mg/kg; p.o</td>
<td>4.75±0.34</td>
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<tr>
<th>Treatment</th>
<th>5th day</th>
<th>16th day</th>
<th>26th day</th>
<th>36th day</th>
<th>42nd day</th>
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<tr>
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<td>26.09±0.11</td>
<td>28.13±0.14</td>
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<td>30.20±0.10</td>
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<td>Group II AlCl₃ 70mg/kg; i.p + 0.9% w/v NaCl 10ml/kg; p.o</td>
<td>24.23±0.10</td>
<td>17.18±0.13**</td>
<td>15.25±0.14**</td>
<td>12.01±0.23**</td>
<td>10.63±0.10**</td>
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<td>Group III AlCl₃ 70mg/kg; i.p + rivastigmine 2.5 mg/kg; p.o</td>
<td>22.53±0.14</td>
<td>19.37±0.13**</td>
<td>21.28±0.16**</td>
<td>23.33±0.19**</td>
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<td>Group V AlCl₃ 70mg/kg; i.p + MECV 100 mg/kg; p.o</td>
<td>24.89±0.24</td>
<td>19.16±0.23**</td>
<td>21.72±0.04**</td>
<td>21.85±0.17**</td>
<td>22.19±0.19**</td>
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Singh and Prashar.: Callistemon viminalis Role in Aluminium Chloride Induced Alzheimer’s Disease

2.68±0.11
0.73±0.05
1.89±0.21
4.68±0.19
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AchE (nM/L/
2.23±0.09
2.01±0.08
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4.33±0.16
2.27±0.13
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agent to prevent the development and progression of dementia. However, further investigations are necessary to establish its clinical efficacy and potential toxicity before its use as a medication for the treatment of AD.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

ABBREVIATIONS

MECV: Methanolic Extract of *Callistemon viminalis*; AchE: Acetylcholinesterase; GSH: Reduced Glutathione; SOD: Superoxide Dismutase; DTNB: 5, 5’-dithiobis-(2-nitrobenzoic acid).

REFERENCES

Aluminium chloride treated animal showed remarkable increase in the escape latency time (ELT) and decrease in the total time (TT). Aluminium chloride significantly increases in the level of AchE enzyme and nitrite in the hippocampus and cerebral cortex and decrease the level of antioxidant enzymes (GSH, catalase and SOD). Animals treated with MECV showed improvement in cognitive function which was assessed by morris water maze apparatus. The plant extract improved the deteriorative effect of aluminium chloride on AchE enzyme and increase the levels of antioxidant enzymes (GSH, catalase and SOD). Our results suggested that MECV might be beneficial to prevent the development and progression of dementia.