PROTECTIVE EFFECT OF D-LIMONENE ON ALUMINIUM CHLORIDE INDUCED MEMORY LOSS AND LEARNING DEFICIT IN MICE

M. Sarika*, A. Lalitha Devi, Rubeena Begum and Afreen Begum

G Pulla Reddy College of Pharmacy, Mehdipatnam, Hyderabad-500028.

ABSTRACT

Introduction: Various studies suggest that free radicals play a key role in Memory and Learning impairment in various cognitive disorders. D-Limonene is a monoterpen reported to possess anti-inflammatory and anti-oxidant property. Objective: Evaluation of protective effect of D-Limonene on Aluminium chloride induced Memory loss and Learning deficit in mice. Methodology: Albino mice (20-35g) were divided into 5 groups (n=6); Group 1- received water and standard pellet feed, Group 2- received Aluminium chloride (50mg/kg, p.o.), Group 3- received Rivastigmine (5mg/kg, p.o.) + Aluminium chloride. Groups 4 & 5- received Aluminium chloride + D-Limonene (2% & 4% w/w supplement enriched with pellet feed respectively). All the groups received respective treatment for 42 days. All the behavioural studies were performed on 21st & 42nd day. On 43rd day the brain was isolated for biochemical and Histopathological evaluation. Results: Administration of Aluminium chloride significantly induced Memory loss and learning deficit by decreased latency of fall, increases transfer latency and retention latency time. Altered levels of MDA, acetyl cholinesterase, nitric oxide in brain. There were significant changes in histoarchitecture of Hippocampus and Cerebral hemispheres when compared with normal control. Treatment with D-Limonene there is a dose dependent decrease in the oxidative Stress, Cognitive disorders and Neuronal degeneration. Conclusion: The present study suggests that protective effect of D-Limonene on aluminium chloride induced Memory and learning deficit, due to its anti-oxidants property.

KEYWORDS: Various Altered levels of MDA, acetyl cholinesterase, nitric oxide in brain.
INTRODUCTION
Dementia is a brain disorder characterized by loss of intellectual ability which invariably involves impairment of memory. Memory loss is a progressive neurodegeneration of the hippocampal and cortical neurons that leads to cognitive disorders such as Alzheimer’s disease, Schizophrenia and Attention deficit hyperactivity disorder etc. Memory loss and Learning deficit is characterized by a neurochemically consistent deficit in cholinergic neurotransmission, neurofibrillary tangles and neuritic plaques. This is particularly affecting the cholinergic neurons in the basal forebrain. Acetylcholine (ACh) is a neurotransmitter that has long received much attention in memory research. It remains a fact that ACh acts on cholinergic receptors that are widely distributed throughout the brain. The decreased levels of Acetylcholine (Ach) synthesis by lowering the concentrations of acetyl coenzyme A. The evidence stems from data of several authors that demonstrated the reduction in activity of enzymes involved in the synthesis of acetylcholine, i.e. choline acetyl transferase or excess degradation of Ach by Acetyl cholinesterase (AchE). Nitric oxide synthase (NOS) containing neurons are found in cerebrum, the cerebellum, hippocampus and hypothalamus. Nitric oxide (NO) plays an important role in the control of neuronal activity. NO is extremely important and it is thought to mediate long term potentiation in hippocampus. On the other hand, abnormalities in glucose metabolism, reduced glucose utilization and levels of energy rich phosphates like ATP, ADP, etc. Disturbed energy metabolism is intricately associated with increased oxidative stress that results in oxidation of biomolecules and initiates excitotoxic neuronal cell damage. In addition Extracellular accumulation (plaques) of beta-amyloid and intracellular accumulation (tangles) of tau are thought to be the neuropathologic hallmarks of Alzheimer disease. The accumulation of these 2 proteins is thought to cause impairment of memory and loss of intellectual ability.

D-Limonene is a monoterpen is reported to possess anti-diabetic, hypolipidemic, immunomodulatory, anti-nociceptive, anti-inflammatory and anxiolytic activity by virtue of its anti-oxidant property. However, no study has been reported on protective effect of D-Limonene on Aluminum chloride induced Memory loss and Learning deficit in mice.

MATERIALS AND METHOD
Experimental animals
Male Swiss albino mice (20-35g) were procured from National Institute of Nutrition Hyderabad were acclimatized to laboratory conditions (25±3°C of temperature, 12-h
light/dark cycle), food and water was given ad libitum. After an acclimatization period of 1 week, they were randomly divided into experimental groups. All the experimental procedures were carried out in accordance with committee for the purpose of control and supervision of experiments on animal (320/CPCSCEA dated 03-01-2001) guidelines. The study was reviewed and approved by the Institutional Animal Ethics Committee (GPRCP/IAEC/07/15/2/PCL/AE-2A-MICE-M/F-30), G. Pulla Reddy College of Pharmacy, Mehdipatnam, Hyderabad, India.

**Drugs and Chemicals**

D-Limonene, Aluminium chloride, Ellman’s reagent and TBA (Sigma Aldrich), Rivastigmine (nearby pharmacy shop), TCA (HIMEDIA), Butanol, pyridine, EDTA and salts required for the preparation of buffers (SD fine chemicals).

**Experimental design**

To evaluate the protective effect of D-Limonene on Aluminium chloride induced Memory loss and Learning deficit in mice. Male Swiss albino mice weighing between (20-35g) were divided into following five groups of six rats each.

**Group 1**: Normal control (received distilled water and standard pellet feed daily for 42 days),  
**Group 2**: Disease control (received aluminium chloride p.o (50mg/kg) daily for 42 days),  
**Group 3**: Standard group (received aluminium chloride p.o + rivastigmine – 5mg/kg p.o daily for 42 days), **Group 4**: Disease treated group (received aluminium chloride p.o + 2% w/w d-limonene (supplement enriched with pellet feed respectively) daily for 42 days), **Group 5**: Disease treated group (received aluminium chloride p.o + 4% w/w d-Limonene (supplement enriched with pellet feed respectively) daily for 42 days).

All the experiments were performed in the light phase between 09:00 and 15:00. During the experimental period following behavioural parameters were evaluated.

1. Locomotor activity (Actophotometer)  
2. Retention latency (Spatial memory and learning test)  
3. Motor co-ordination (Rota Rod Test)  
4. Transfer latency (Modified Elevated Plus Maze test)  
5. Anxiety (Hole board test)
On the 43\textsuperscript{rd} day, the animals were sacrificed by cervical dislocation and the brain was dissected out for biochemical estimations of acetyl cholinesterase (Ach’ase), Nitrites, Malondialdehyde (MDA) and Histopathology test.

RESULTS

Behavioural studies

Locomotor activity on Actophotometer apparatus
As shown in table 1, fig 1 Administration of Aluminium chloride has significantly (p<0.001) decrease no. of Actophometer counts in Disease control group when compared to Normal control group. Treatment with D-Limonene (2\% and 4\% w/w) has significantly (p<0.001) increased the locomotion activity when compared to Disease control group. 4\% D-Limonene was restored to Normal levels.

Retention latency on Spatial memory and Learning test apparatus
As shown in table 2, fig 2 Administration of Aluminium chloride has significantly (p<0.001) increased retention latency time in Disease control group when compared to Normal control group. Treatment with D-Limonene (2\% and 4\% w/w) has significantly (p<0.001) reduced the retention latency when compared to Disease control group. The effect of 4\% D-Limonene has shown more pronounced activity compared to 2\% D-Limonene.

Motor coordination on Rota-rod apparatus
As shown in table 3, fig 3 Administration of Aluminium chloride has significantly decreased motor coordination in Disease control group when compared to Normal control. Treatment with D-Limonene (2\% and 4\% w/w) has significantly (p<0.001) increased the motor coordination when compared to Disease control group. Whereas 4\% d-limonene was restored to normal levels and the effect of 4\% D-Limonene has shown more pronounced activity compared to 2\% D-Limonene.

Transfer latency on Modified Elevated plus maze apparatus
As shown in table 4, fig 4 Administration of Aluminium chloride has significantly (p<0.001) increased the transfer latency in Disease control group when compared to Normal control group. Treatment with D-Limonene (2\% and 4\% w/w) has significantly (p<0.001) reduced the transfer latency when compared to Disease control group. Whereas 2\% D-Limonene was restored to normal levels and the effect of 4\% D-Limonene has shown more pronounced activity compared to 2\% D-Limonene.
Anxiety on hole board apparatus

As shown in table 5, fig 5 Administration of Aluminium chloride has significantly (p<0.001) increased the head dipping counts in Disease control group when compared to Normal control group. Treatment with D-Limonene (2% and 4% w/w) has significantly (p<0.001) reduced the head dipping counts when compared to Disease control group. Whereas 2% D-Limonene was restored to normal levels and the effect of 4% D-Limonene has shown more pronounced activity compared to 2% D-Limonene. The anti-anxiety effect has shown dose dependent action.

Biochemical parameters

Effect of D-Limonene on Malondialdehyde (MDA) level in Aluminium chloride induced Memory loss and Learning deficits in mice

As shown in table 6, fig 6 Administration of Aluminium chloride in Disease control group has significantly increased MDA levels in brain when compared with the Normal control group (p<0.001). Treatment with D-Limonene (2% and 4% w/w) has significantly (p<0.001) reduced the MDA levels compared to Disease control group. However, it was restored to normal levels in (2% w/w) D-Limonene treated mice.

Effect of D-Limonene on Nitrite levels in Aluminium chloride induced mice

As shown in table 6, fig 7 Administration of Aluminium chloride in Disease control group has significantly (p<0.001) increased Nitrite levels in brain when compared with the Normal control group. Treatment with D-Limonene (2% and 4% w/w) has shown significantly (p<0.001) decreased Nitrite levels when compared to Disease control group.

Effect of D-Limonene on Acetyl cholinesterase levels in Aluminium chloride induced mice:

As shown in table 6, fig 8 Administration of Aluminium chloride in Disease control group has significantly (p<0.001) increased Acetyl cholinesterase levels in brain when compared with the Normal control group. Treatment with D-Limonene (2% and 4% w/w) has shown significantly (p<0.001) decreased Acetyl cholinesterase levels when compared to Disease control group.
### TABLE 1: Effect of D-Limonene on Locomotor activity by Actophotometer apparatus in Alcl₃ induced Memory loss and Learning deficit in mice

<table>
<thead>
<tr>
<th>No. of actophotometer counts for 5mins</th>
<th>Days</th>
<th>Normal Control</th>
<th>Disease Control (Alcl₃)</th>
<th>Standard Control</th>
<th>Limonene (2%) + Alcl₃</th>
<th>Limonene (4%) + Alcl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21ˢᵗ</td>
<td>348.50±1.340</td>
<td>125.60±1.050ᵇ</td>
<td>251.10±0.936γᵇ</td>
<td>261.11±2.960γᵇ</td>
<td>278.87±4.504γᵇ</td>
</tr>
<tr>
<td></td>
<td>42ⁿᵈ</td>
<td>351.11±1.010</td>
<td>76.81±1.240ᵃ</td>
<td>218.61±0.880ᵇ</td>
<td>268.31±0.998ᵇ</td>
<td>295.66±3.561ᵇ</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

ᵃp<0.001,ᵇp<0.01, γp<0.05 compared to Normal control group.

ᵃp<0.001,ᵇp<0.01 compared to Disease control group.

### TABLE 2: Effect of D-Limonene on Retention latency by Spatial memory and Learning test apparatus in Alcl₃ induced Memory loss and Learning deficits in mice:

<table>
<thead>
<tr>
<th>Retention latency (in sec)</th>
<th>Days</th>
<th>Normal Control</th>
<th>Disease Control (Alcl₃)</th>
<th>Standard Control</th>
<th>Limonene (2%) + Alcl₃</th>
<th>Limonene (4%) + Alcl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21ˢᵗ</td>
<td>46.10±0.950</td>
<td>62.00±1.31ᵇ</td>
<td>37.10±0.63ᵇ</td>
<td>31.88±0.60ᵇ</td>
<td>27.11±1.06ᵃ</td>
</tr>
<tr>
<td></td>
<td>42ⁿᵈ</td>
<td>48.30±0.84</td>
<td>77.31±0.610ᵃ</td>
<td>20.55±0.67ᵃ</td>
<td>23.72±0.65ᵇ</td>
<td>16.55±0.76ᵃ</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

ᵃp<0.001,ᵇp<0.01, ćp<0.05 compared to Normal control group.

ᵃp<0.001,ᵇp<0.01 compared to Disease control group.

### TABLE 3: Effect of D-Limonene on latency of fall by Rota-rod apparatus in Alcl₃ induced Memory loss and Learning deficits in mice:

<table>
<thead>
<tr>
<th>Latency of fall (in sec)</th>
<th>Days</th>
<th>Normal Control</th>
<th>Disease Control (Alcl₃)</th>
<th>Standard Control</th>
<th>Limonene (2%) + Alcl₃</th>
<th>Limonene (4%) + Alcl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21ˢᵗ</td>
<td>67.40±1.14</td>
<td>47.11±0.58ᵇ</td>
<td>50.81±1.40ᵉ</td>
<td>62.50±0.40ᵇ</td>
<td>70.82±0.44ᵃ</td>
</tr>
<tr>
<td></td>
<td>42ⁿᵈ</td>
<td>68.10±1.41</td>
<td>39.80±0.31ᵃ</td>
<td>55.11±1.14ᵉ</td>
<td>66.33±0.54ᵇ</td>
<td>72.80±0.61ᵃ</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

ᵃp<0.001,ᵇp<0.01, ćp<0.05 compared to Normal control group.

ᵃp<0.001,ᵇp<0.01, ｃp<0.05 compared to Disease control group.
TABLE 4: Effect of D-Limonene on Transfer latency by Elevated plus maze apparatus in Alcl₃ induced Memory loss and Learning deficits in mice:

<table>
<thead>
<tr>
<th>Days</th>
<th>Normal Control</th>
<th>Disease Control (Alcl₃)</th>
<th>Standard control</th>
<th>Limonene (2%) + Alcl₃</th>
<th>Limonene (4%) + Alcl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>21&lt;sup&gt;st&lt;/sup&gt;</td>
<td>44.60±0.45</td>
<td>58.20±0.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.50±0.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.77±0.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.56±1.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>42&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>43.61±0.42</td>
<td>65.10±0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.60±0.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.90±0.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.10±1.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

<sup>a</sup>p<0.001, <sup>b</sup>p<0.01, compared to Normal control group.

<sup>a</sup>p<0.001, <sup>b</sup>p<0.01 compared to Disease control group.

TABLE 5: Effect of D-Limonene on Anxiety by Hole board apparatus in Alcl₃ induced Memory loss and Learning deficits in mice

<table>
<thead>
<tr>
<th>Days</th>
<th>Normal Control</th>
<th>Disease Control (Alcl₃)</th>
<th>Standard control</th>
<th>Limonene (2%) + Alcl₃</th>
<th>Limonene (4%) + Alcl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>21&lt;sup&gt;st&lt;/sup&gt;</td>
<td>31.50±0.88</td>
<td>38.10±0.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.00±0.96&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.11±0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.66±0.88&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>42&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>32.90±0.70</td>
<td>44.50±1.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.80±0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.55±0.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.10±0.48&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

<sup>a</sup>p<0.001, <sup>b</sup>p<0.01, compared to Normal control group.

<sup>a</sup>p<0.001, <sup>b</sup>p<0.01 compared to Disease control group.

Table 6: Effect of D-Limonene on Oxidative parameters (MDA, Acetyl cholinesterase and Nitrite) in Alcl₃ induced Memory loss and Learning deficit in mice

<table>
<thead>
<tr>
<th>OXIDATIVE PARAMETERS (BRAIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.No</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

<sup>a</sup>p<0.001, <sup>b</sup>p<0.01 compared to Normal control group.

<sup>a</sup>p<0.001, <sup>b</sup>p<0.01 compared to Disease control group.
Behavioural tests

Locomotor Activity test

![Graph showing Locomotor Activity test results for different groups.]

**Fig.1:** Effect of D-Limonene on Locomotor activity by Actophotometer apparatus in Alcl₃ induced Memory loss and Learning deficit in mice

Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

*α* p<0.001, *β* p<0.01, *γ* p<0.05 compared to Normal control group.

*α* p<0.001, *β* p<0.01 compared to Disease control group.

Spatial memory and Learning test

![Graph showing Spatial memory and Learning test results for different groups.]

**Fig.2:** Effect of D-Limonene on Retention latency by Spatial memory and Learning test apparatus in Alcl₃ induced Memory loss and Learning deficit in mice:

Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

*α* p<0.001, *β* p<0.01, compared to Normal control group.

*α* p<0.001, *β* p<0.01 compared to Disease control group.
Motor coordination test

Fig. 3: Effect of D-Limonene on latency of fall by Rota-rod apparatus in Alcl₃ induced Memory loss and Learning deficit in mice
Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

\[a p < 0.001, b p < 0.01\] compared to Normal control group.
\[a p < 0.001, b p < 0.01, c p < 0.05\] compared to Disease control group.

Transfer latency test

Fig. 4: Effect of D-Limonene on Transfer latency by Elevated plus maze apparatus in Alcl₃ induced Memory loss and Learning deficit in mice
Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

\[a p < 0.001, b p < 0.01\] compared to Normal control group.
\[a p < 0.001, b p < 0.01\] compared to Disease control group.
Anxiety test

Fig. 5: Effect of D-Limonene on Anxiety by Hole board apparatus in Alcl₃ induced Memory loss and Learning deficit in mice

Data is expressed as mean ± SEM (n=6) per group and analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s test for the comparison of means.

α p<0.001, β p<0.01, compared to Normal control group.

α p<0.001, β p<0.01 compared to Disease control group.

Biochemical parameters

MDA Levels

Fig. 6: Effect of D-Limonene on Aluminium chloride induced alteration in MDA levels.

Data is expressed as mean±SEM (n=6) and analyzed by one way (ANOVA) followed by Dunnett’s test.

α p<0.001, β p<0.01 when compared to Normal control group.

α p<0.001, β p<0.01 when compared to Disease control group.
Nitrite Levels

Fig. 7: Effect of D-Limonene on Aluminium chloride induced alteration in Nitric oxide levels. 
Data is expressed as mean±SEM (n=6) and analyzed by one way (ANOVA) followed by Dunnett’s test.  
\(^a p<0.001, ^b p<0.01\) when compared to Normal control group.  
\(^a p<0.001, ^b p<0.01\) when compared to Disease control group.

Acetyl cholinesterase Levels

Fig. 8: Effect of D-Limonene on Aluminium chloride induced alteration in AChE enzyme. 
Data is expressed as mean±SEM (n=6) and analyzed by one way (ANOVA) followed by Dunnett’s test.  
\(^a p<0.001, ^b p<0.01\) when compared to Normal control group.  
\(^a p<0.001, ^b p<0.01\) when compared to Disease control group.
Histopathology

Fig. 9(a) Normal control – hippocampus

Fig. 9(b) Disease control-hippocampus

Fig. 9(c) Standard control – hippocampus
DISCUSSION
In the present study, the effects of Aluminium exposure were investigated to describe the associated cognitive disorder and brain modifications. The pathological hallmarks of cognitive disorder are memory loss and learning deficits.\(^6\) D-Limonene is a monoterpene used as flavouring agent in addition; it is used as an important ingredient for various medicinal purposes in traditional medicine. The present study showed that protective effect on Memory loss and Learning deficit in mice, Treatment with D-Limonene (2% and 4%w/w), exhibited a significant improvement in memory and learning dysfunction in mice as
evidenced by increase in cognition. There is a significant increase in duration of sustained balance on Rota rod (improved motor coordination), increased locomotor activity on Actophotometer (improved exploratory behaviour), reduced transfer latency on Elevated plus maze test (improved cognition), reduction in anxiety on Hole board test and reduced retention latency on Morris water maze test. In addition, there is significant increase in AchE, MDA, Nitrite than Aluminium chloride induced mice. The present study was to investigate the protective and therapeutic effects of D-Limonene (2% and 4%) compared to Rivastigmine (as a standard drug). This study is performed by assessing their effects on the behavioural status of mice that represent animal model mimicking Memory loss and Learning deficit (by using AlCl₃) AchE, MDA and Nitrite levels in brain. Learning and memory can be conceived as both psychological process as well as a change in synaptic neural connectivity. Learning and memory is associated with numerous psychiatric and neurodegenerative disorders. Administration of Aluminium chloride can affect the hippocampus region that may produce reduced learning and memory processing leading to social withdrawal to avoid aversive and impairment in retention process. In other words Aluminium also functionally alters the blood brain barrier and produces changes in the cholinergic and noradrenergic neurotransmission. It causes increased free-radical generation and lipid peroxidation as well as changes in phosphoinositide metabolism and protein phosphorylation, Inflammation of the brain plays a key role in the pathogenesis of AD. In addition, excessive accumulation of reactive oxygen species and oxidative stress accompanied by depletion of endogenous antioxidants levels are implicated in the etiology of AD. There by causing severe neurotoxicity. The results of the present study indicated cholinergic neurons are positive markers for the evolution of memory and related disorders affecting acetylcholine and resulting in decreased activity of acetyl cholinesterase and choline acetyl transferase. Recent findings suggested that administration of aluminium was found to increase acetyl cholinesterase in mouse brain. We also demonstrated that chronic administration of aluminium to mice significantly increased acetyl cholinesterase, an effect that was attenuated by administration of D-Limonene for six weeks has also reversed the depletion of acetylcholine and the reduction in choline acetylase activity binding with hippocampus and cortex induced aluminium chloride. Involvement of oxidative stress is one of the causative factors for memory loss and learning deficit. Aluminium chloride may trigger the formation of reactive oxygen species (ROS). These ROS may interfere with the neuro-developmental process or cause neural damage may results in abnormal behavioural process. In the present study, altered MDA, Nitrite and altered Acetyl cholinesterase levels in the brain indicate the
involvement of oxidative stress in Aluminium chloride induced memory loss and learning deficit.\[12\] Treatment with D-Limonene has significantly reversed the altered oxidative stress markers in brain due to its antioxidant and anti-cholinesterase activity. Rivastigmine, standard drug might have acted through the cholinergic mechanism that has shown decrease in the oxidative stress and restored the antioxidant defense to protect against the Alcl\(_3\) induced oxidative stress. Rivastigmine as a protective or therapeutic agent led to protects behavioural changes by inducing Aluminium chloride. Motor clumsiness is one of the variable that is associated as a symptom which can be tested by challenging the mice using rota rod for motor coordination and balance. Performance of this skill requires neuromuscular coordination. Decreased latency in rats to fall from rotating rod and enhanced the anxiety by oxidative stress, decrease Locomotor activity assessed by Actophotometer, which indicates a possible depressant effect on the CNS and by impairment cognitive performances especially spatial learning and memory. It exerts amnesic effect equally in various behavioural models of memory including Morris water maze that can be attributed to the hippocampus and cerebral cortex damage caused due to administration of Aluminium chloride. Treatment with D-Limonene significantly ameliorates the aluminium chloride induced behavioural deficits. Histopathological studies have showed that, Aluminium chloride has damaged dentate granule cell layer, formation of infiltration of inflammatory cells and caused neuronal loss in hippocampus region. The loss of dentate gyrus neurons cell layer integrity and damaged hippocampus results in spatial memory deficits in brain. This was restored with D-Limonene treatment indicating the anti-oxidant and anti-cholinesterase activity. On the basis of these findings, we conclude that D-Limonene ameliorates aluminium chloride induced behavioural deficits, increased Acetyl cholinesterase, altered oxidative stress markers and loss of dentate granule cell, formation of inflammatory cells, due to its anti-anxiety, cognition enhancing, antioxidant and neuroprotective activity. However, the high dose of D-Limonene (4\%) exhibited a better effect than the low dose of d-Limonene (2\%). Histopathological finding in brain cells appeared more or less like the normal control group and no degeneration is observed in D-Limonene treated groups. Therefore, the improvement of spatial memory in our study might be due to the ability of D-Limonene to enhance cognition may be due to its anti-oxidant, anti-cholinesterase and anti-anxiety activity properties.

**CONCLUSION**

In the present study, treatment with D-Limonene has significantly shown protective effect on Aluminum chloride induced memory loss and learning deficit and is characterized by
increased motor coordination, locomotor activity, antioxidant profile in mice hippocampus and cerebral cortex and decreased transfer latency, retention latency, anxiety. In conclusion, present study suggests that treatment with D-Limonene has shown protective effect on Aluminium chloride induced Memory loss and learning deficit in mice, may be due to its antioxidant, anti-cholinesterase and anti-anxiety properties.

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