Anti-amnesic and Cognitive Enhancing Effects of Ethanol Leaf Extract of *Milicia Excelsa* (Moraceae) in Mice

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Abstract Objective: We investigated the anti-amnesic and cognitive enhancing effects of the ethanol leaf extract of *Milicia excelsa* in mice. Methodology: Amnesia was induced by intraperitoneal injection of scopolamine (0.4mg/kg) in Morris Water Maze; scopolamine (1mg/kg) on Y-maze test; scopolamine (0.4mg/kg) and diazepam (1mg/kg) on elevated plus maze. The effect of the extract on learning and memory of young and aged mice was also investigated on the elevated plus maze. The probable mechanism of cognitive enhancing effects of the extract was investigated using acetylcholinesterase enzyme and nitric oxide assays. Results: The extract significantly ($p<0.05$) attenuated amnesia induced by scopolamine in Morris water maze; reversed amnesia induced by scopolamine and diazepam on elevated plus maze; increased spontaneous alternation behaviour without modifying locomotor activities on Y-maze test; inhibited natural age-induced learning and memory impairment of aged mice while the learning and memory of young mice were significantly ($p<0.05$) enhanced on elevated plus maze. The extract also significantly ($p<0.05$) reduced acetylcholinesterase enzyme activity and increased nitric oxide concentration in the mice whole brain. Conclusion: This study, therefore, concluded that ethanol leaf extract of *Milicia excelsa* may possess anti-amnesic and memory enhancing effects which may involve cholinergic and nitergic pathways.

Keywords *Milicia Excelsa*, Amnesia, Acetylcholinesterase Enzyme, Nitric Oxide, Elevated Plus Maze, Morris Water Maze

1. Introduction

Amnesia is a cognitive disorder [1] characterized with a profound memory loss which may either be caused by physical injury or ingestion of toxic substances which affects the brain [2]. The cognitive deficit may also be caused by normal aging, stress and some specific neurodegenerative and psychiatric disorder such as Alzheimer's disease (AD), vascular dementia, and schizophrenia [3, 4, 5, 6].

AD is the commonest neurodegenerative disorder [7] associated with cognitive deficit [2] primarily caused by reduced cholinergic activity [8], oxidative stress, amyloid-beta peptide deposition and neuroinflammation [9] which affects the brain and subsequently memory [8]. The clinical features of AD include the amnesic type of memory impairment, deterioration of language and visuospatial deficits [10]. Individuals with AD exhibit decline in mental functions and often show non-cognitive symptoms, such as depression, apathy, and psychosis which impair their day-to-day activities [11].

It is well established that cholinergic pathways play a significant role in cognitive impairment linked to AD, aging and other neurodegenerative diseases [12]. Although there are reductions in other neurotransmitters during Alzheimer's associated neurodegeneration, the degree of brain acetylcholine (ACh) reduction directly correlates with the deterioration of cognition and of daily activity in AD patients [13]. Neuronal damage that occurs in AD has been found to result in an impairment of Nitric Oxide (NO) synthesis and a decrease in NO containing neurons in the hippocampus [14]. The functional loss of GABA_A benzodiazepine receptors and GABAergic system in AD has also been demonstrated [15]

The treatment strategies for AD have focused on the elevation of acetylcholine by cholinesterase inhibitors but the cholinergic side effects such as nausea, anorexia, vomiting, and diarrhea [16-17] associated with these medications have limited their use [18, 19]. Therefore, it is a worthwhile venture to explore the medicinal plants claimed to be used in traditional and alternative medicines for the treatment of various neurodegenerative and neuropsychiatric disorders since the World Health Organisation (WHO), has estimated the current world population of AD to be about 18 million people, the population of which is expected to nearly double by 2025 and much of the envisaged increase will be due to aging population from developing countries [20].

Milicia excelsa (welw.) C.C. Berg belongs to the family of Moraceae, otherwise known as *Chlorophora excelsa*. It is a large deciduous tree of 30 to 50 m high occurring naturally in humid forests of West Africa [21]. Its latex, leaf, stem bark, root, fruit, and ashes are used in Africa traditionally medicine to prepare local medicines for the treatment of mental illnesses [22-24], anti-aging [25], rheumatism [26], malaria [27], sexual dysfunction [28] among other ethnomedicinal uses. The antibacterial [29, 30], wound healing [31] and in-vivo anti-inflammatory properties [32] of the plant have been demonstrated. Lupeol acetate, ursolic acid, triacontyl (E)- ferulate, 2-(3,5-dihydroxyphenyl)benzofuran-5,6- diol and a benzylic diglycoside identified as 3,4- dimethoxybenzyl beta-D-xylopyranosyl (1 --> 2)- beta-D-glucopyranoside have been isolated from the leaf of *Milicia excelsa* [33].

This study aimed to investigate the anti-amnesic and memory enhancing effects of the plant extract as well as its probable mechanism of cognitive enhancing effect in mice, in order to provide scientific support for the reported traditional use of the leaf in the treatment of mental derangements in ethnomedicine [22, 23, 24].

2. Materials and Methods

2.1. Plant Materials

Milicia excelsa leaves were collected within the campus of the Obafemi Awolowo University. It was identified and authenticated by Mr. G. A. Ademoriyo of the Herbarium Unit, Department of Botany, Faculty of Sciences, OAU, Ile-Ife and herbarium number Ife 17482 was obtained.

2.1.1. Preparation of Extract

The leaves were air dried for two weeks at room temperature. The dried leaves were pulverized and 1 kg of the powder was extracted with 3 litres of seventy percent (70%) ethanol for 72 hr. The marc was re-extracted once. The extract was concentrated in vacuo at a temperature of 40°C, and subsequently, freeze-dried to yield 70 g (7.0%) crude extract coded EME (ethanol leaf extract of *Milicia*

excelsa). The test material of EME was prepared by dissolving in 2% Tween 20 in normal saline prior to oral administration to mice [34].

2.2. Animals

Male mice weighing around 20-21 g (young 3-4 months old) and aged weighing around 30-35 g (8-10 months old) were used for the behavioural assessment in this study. Male mice were used because estrogen (female sex hormone) has been shown to have affect memory; hence female animals were excluded from this study [35]. All the animals were raised and housed in a well lit and aerated room in the Animal House, Faculty of Pharmacy, Obafemi Awolowo University, Ile - Ife. The animals were fed standard commercial diet (Guinea feeds brand, Bendel Feeds Nigeria) and water *ad libitum*. The animals were acclimatized to the laboratory condition for one week before the commencement of the behavioural experiments which were done between 09:00 and 17:00 h [36]. The experimental procedures employed in this study are in accordance with the approved institutional protocols and within the ambient of internationally accepted principles for Laboratory Animal Use and Care (EEC Directive of 1986; 86/609/EEC) [37].

2.3. Drugs

Scopolamine hydrobromide, Tween 20 (Sigma Aldrich, USA), piracetam (Nootropil[®], UCB India Pvt. Ltd., Vapi, Gujarat), diazepam (Roche, Basel, Switzerland) and normal saline (Unique Pharmaceutical Limited, Lagos, Nigeria).

2.4. Methodology

2.4.1. Morris Water Maze

The spatial memory was assessed using the Morris Water-maze paradigm. The potential of EME to reverse scopolamine-induced amnesia was investigated in the Morris water-maze test [38]. Briefly, mice were pretreated orally with EME at the doses of 50, 100 and 200 mg/kg for 7 days. On the 7th day, amnesia was induced by intraperitoneal injection of scopolamine (0.4 mg/kg). Then 90 min after scopolamine injection, each animal was subjected to the acquisition test and subjected to retention test after 24 h.

The water pool apparatus consisted of a large circular bowl (diameter 65 cm and height 40 cm) filled with water to three-quarter mark (55.0 litres) and maintained at ambient temperature. Evaporated milk was added to the water to make it opaque. The pool was equally divided into four quadrants, with a removable black plastic platform (10 cm x 5 cm). The platform which served as an escape refuge from the water was submerged to a level of 1 cm below the water level in the centre of one quadrant. The platform was

invisible to the mice and remained in one location for a trial. The training phase of the Water-Maze Testing consisted of two trials per day. At the start of testing on day 1, each mouse was placed in the water with its forepaws touching the tank wall, at one of four equally spaced points around the pool. Starting release points were counter-balanced alternately near or far from the platform across subjects and groups. The mouse was allowed 120 s to locate the hidden platform. A trial terminates when the animal reaches the platform and remains on the platform for 20 s. If the animal does not locate the platform within this time frame, the animal will then be guided to the platform and made to remain there for at least 20 s. Thereafter, the animal was removed from the platform, dried and returned to its cage for the next trial [38].

The criteria/index for amnesic/cognitive activity was taken as increase/decrease in escape latency time (ELT) on retention test (second trial) in comparison with the acquisition test (first trial) [38].

2.4.2. Elevated Plus-maze (Exteroceptive Behavioural Model)

Elevated plus-maze (EPM) was used to assess learning and memory in mice. The protocol employed was as earlier described [39, 40]. The EPM used for the behavioural assay consisted of two open arms (16 cm x 5 cm) and two covered arms (16 cm x 5 cm x 12 cm) extended from a central platform (5 cm x 5 cm), which was raised to a height of 25 cm from the floor [38]. Mice were orally ingested with EME for 8 consecutive days and on the 8th day, 1 h after the last oral ingestion of EME, each mouse was placed consistently at the same end of an open arm, facing away from the central platform. The time taken in seconds for each mouse to turn and move into any of the two covered arms with its four paws was recorded as the Transfer latency (TL). The mouse that did not enter into any of the covered arms within 90 sec was gently guided into one of the two covered arms and TL was recorded as 90 sec. Thereafter, each mouse was allowed to move freely and explore the maze for another 2 min before being returned to its home cage. Retention of this learned-task (memory) was assessed 24 h after the last dose of EME (i.e. 9th day). Significant reduction in TL value for retention indicated improvement in memory.

2.4.2.1. Experimental Protocol

Male mice weighing 20 – 21 g were divided into 10 groups containing 5 mice each and were treated as follows for 8 days: Group I (Control Young): Each mouse received 10 ml/kg, p.o of 2% tween 20 in normal saline; Group II (Piracetam Young): Each mouse received 200 mg/kg, p.o of piracetam; Group III - V (Young treatment groups). Each mouse received EME (50, 100 and 200 mg/kg, p.o.) of the extract; Group VI (Control Aged): Each mouse received 10 ml/kg, p.o of 2% Tween 20 in normal saline; Group VII (Piracetam Aged): Each mouse received 200 mg/kg, p.o of piracetam; Group VIII - X (Aged treatment

groups). Each animal received EME (50, 100 and 200 mg/kg, p.o.) of the extract. TL for each mouse was recorded after 45 min of extract/drug administration on the 8th day (learning) and after 24 hr i.e on the 9th day (memory).

2.4.3. Elevated Plus-maze (Interoceptive Behavioural Models)

2.4.3.1. Scopolamine - induced Amnesia

Male mice weighing 20 – 21 g were divided into 3 groups containing 5 mice each and were treated as follows for 8 days: Group I (Scopolamine Control group): Each mouse received 10 ml/kg, p.o of 2% tween 20 in normal saline; Group II (Piracetam): Each mouse received 200 mg/kg, i.p of piracetam; Group III (Treatment group): Each mouse received the most effective dose of EME (200 mg/kg, p.o.) of the extract. On the 8th day after 45 mins of extract/drug administration, amnesia was induced in the mice by administration of scopolamine hydrobromide (0.4 mg/kg i.p) and TL noted after 45 mins and after 24 hrs [41, 42].

2.4.3.2. Diazepam - induced Amnesia

Male mice weighing 20 – 21 g were divided into 3 groups containing 5 mice each and were treated for 8 days as above in scopolamine-induced amnesia but instead of the scopolamine control group, Group I was the diazepam control group. On the 8th day after 45 mins of extract administration, amnesia was induced in the mice by administration of diazepam (1 mg/kg i.p) and TL noted after 45 mins and after 24 hrs [43].

2.4.4. Y-maze Task

The Y-maze test was conducted in a three-arm maze with angles of 120° between the arms, which were 40 cm long and 3 cm wide with walls that were 12 cm high each. The maze floor and walls were constructed from dark opaque polyvinyl plastic as previously described [44]. Mice were initially placed within one arm, and the sequence and number of arm entries were recorded manually for each mouse over an 8 min period. The sequence of arm entry was noted and recorded. The percentage of triads represented as ABC, CAB, or BCA was taken as an 'alternation' to estimate short-term memory [45]. One hour before the test, mice were administered EME (50, 100 or 200 mg/kg, p.o.) or piracetam (200 mg/kg, p.o.). Control group animals received 0.9% saline solution rather than EME or piracetam. Scopolamine (1 mg/kg, i.p.) or vehicle was introduced to induce memory impairment 30 min before the test. The alternation score (%) for each mouse was calculated by the equation: % Alternation = [(Number of alternations)/(Total arm entries - 2)] x 100. The number of arm entries was used as an indicator of locomotor activity. Arms were cleaned with 70% ethanol to remove odors and residues.

Natural aging- and scopolamine-induced amnesia are behavioural paradigms widely employed as models simulating human dementia in general and Alzheimer's disease (AD) in particular [46]. Hence, they were used in this study.

2.4.5. Biochemical Estimations

2.4.5.1. Tissue Preparation

Male mice weighing around 20-21 g were used. Five mice (n=5) per group were used. Group I served as control and was treated with 2% Tween 20 in normal saline. Group II was treated with piracetam (200 mg/kg, i.p.) and Group III-V were treated with EME (50 mg/kg, 100 mg/kg and 200 mg/kg, p.o.) respectively for 8 days. On the 9th day i.e 24 h after the last oral treatment, the animals were euthanized by cervical dislocation carefully to avoid any injuries to the tissue. For the preparation of brain homogenate for biochemical estimations, fresh whole brain was weighed and transferred into a glass homogenizer and homogenized in an ice bath after adding 10 volumes of 0.9 % sodium chloride solution. The homogenate was then centrifuged at 3000 rpm for 10 minutes and the resultant cloudy supernatant liquid was used for various biochemical estimations [47].

2.4.5.2. Estimation of Brain Acetyl Cholinesterase (AChE) Activity

AChE activity was determined spectrophotometrically using acetylthiocholine iodide (ATChI) as the substrate, by the modified method [48].

In a 96-well plate was added 240 μ l of buffer (50 mM Tris-HCl, pH 8.0,) and 20 μ l of the enzyme preparation (brain homogenate). The reaction mixture was then incubated for 30 min at 37°C, after which 20 μ l of 10 mM DTNB was added. The reaction was then initiated by the addition of 20 μ l of 25 mM ATChI. The rate of hydrolysis of ATChI was then determined spectrophotometrically by measuring the change in the absorbance per minute ($\Delta A/\text{min}$) due to the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm over a period of 4 min at 30 s interval. All assays were carried out in triplicate.

The enzyme activity was calculated as follows:

$$\text{Activity } (\mu \text{ moles/min/ml}) = \Delta A/\text{min} \times V / (\epsilon \times v \times d)$$

Where: $\Delta A/\text{min}$ = change in absorbance/minute

V = total volume of reaction mixture

v = volume of test sample in reaction mixture

ϵ = extinction coefficient of DTNB = 1.36×10^4

d = light path length (1cm).

2.4.5.3. Tissue Nitric Oxide (NO) Determination

Deproteination: 500 μ L of brain sample was mixed with 50 μ L of TCA in 1.5mL tubes. The tube was vortexed and centrifuged for 10 min at 4000 rpm. Then 100 μ L of the clear supernatant was transferred to a clean tube.

Reaction: To 100 μ L of each sample in separate, labeled Eppendorf tubes and 0.1 ml of 1% sulphanilamide in 5%

phosphoric acid was added and incubated at room temperature for 10 min., followed by addition of 0.1 ml 0.1% NED (N-1-naphthyl ethylenediamine dihydrochloride) and incubated for 10 min at 60°C. The absorbance of the chromophore formed was measured at 546nm. The standard curve was prepared using sodium nitrite (100 μ M). The amount of NO in the sample was extrapolated from the standard curve [49].

2.4.6. Statistical Analysis

All the results were expressed as mean $\pm$ SEM. The data was analyzed using ANOVA followed by Student-Newman Keuls Test, $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Effects of EME on Scopolamine-induced Memory Dysfunction in the Morris Water-maze Task

EME at 200 mg/kg, showed significant ($p < 0.05$) decrease in escape latency on day 1 when compared to the vehicle-treated control mice. Scopolamine treated control mice exhibited significant ($p < 0.05$) increase in escape latency when compared to the vehicle-treated control group. However, EME at all the doses used significantly ($p < 0.05$) reduced the escape latency in a dose-dependent manner on day 7 and day 8 when compared to the scopolamine treated control group. Piracetam, a positive control group significantly ($p < 0.05$) reduced the escape latency on day 7 and day 8. However, piracetam did not significantly affect the escape latency on day 1 when compared to the vehicle-treated control group (Figure 1).

3.2. Effect on EME on Transfer Latency (TL) on Elevated plus Maze

We evaluated the effect of EME on transfer latency (TL) on elevated plus-maze (EPM) task in mice. Aged control treated mice showed higher TL values on the 8th day (learning) and on the 9th day (memory) when compared to young treated control mice, indicating a deficit in learning and memory. Piracetam (200 mg/kg, i.p.) pretreatment significantly reduced TL on the 8th and 9th days indicating improvement in learning and memory. There was significant ($p < 0.05$) decrease in TL between the aged control group and the aged mice treated with the different doses (50, 100 and 200 mg/kg, p.o.) of the extract on the 8th and 9th day also suggesting improvement in learning and memory. Aged piracetam group pretreated with piracetam (200 mg/kg, i.p.) also showed significant ($p < 0.05$) reduction in TL when compared to control aged group.

Young mice treated with EME (100 and 200 mg/kg, p.o.) significantly ($p < 0.05$) shortened the TL compared to the young control treated mice on day 8 (learning) and EME at

all the doses used on day 9 (memory). Similarly, young piracetam group significantly ($p < 0.05$) shortened the TL on day 8 and day 9 when observed on EPM task (Figure 2).

Scopolamine at 0.4 mg/kg, i.p and diazepam at 1 mg/kg, i.p significantly ($p < 0.05$) increased TL of young mice on

day 8 (learning) and day 9 (memory) compared to young treated control group. However, the learning and memory deficit induced by scopolamine and diazepam were ameliorated by pretreatment of young mice with EME (200 mg/kg, p.o.) for 8 successive days (Figure 3).

Figure 1. Effect of ethanol leaf extract of *Milicia excelsa* (EME) on escape latencies of scopolamine-induced mice in Morris Water Maze. PRZ; piracetam (200 mg/kg, p.o.), SCOP; scopolamine (1 mg/kg, i.p.). Each bar represents mean $\pm$ SEM: ANOVA followed by Student-Newman Keuls Test, $n=5$, * $p < 0.05$ compared to scopolamine control group; $^{\#}p < 0.05$ compared to the control group.

Figure 2. Effect of ethanol leaf extract of *Milicia excelsa* (EME) on transfer latencies of young and aged mice. Each bar represents mean $\pm$ SEM: ANOVA followed by Student-Newman Keuls Test, $n=5$, *denotes $p < 0.05$ as compared to control (Young); $^{\#}$ compared to control (Aged); $^{\alpha}$ compared to the control (Young).

Figure 3. Effect of ethanol leaf extract of *Milicia excelsa* (EME) on transfer latencies of scopolamine-, and diazepam-induced amnesia in mice. SCOP; scopolamine (1 mg/kg, i.p.), DZP; diazepam (1 mg/kg, i.p.). Each bar represents mean $\pm$ SEM: ANOVA followed by Student-Newman Keuls Test, $n=5$, * $p < 0.05$ as compared to control (scopolamine); $^{\#}$ compared to control (diazepam); $^{\alpha}$ compared to control (normal mice)

3.3. Effects of EME on Scopolamine-induced Memory Deficit in the Y-maze Task

We evaluated the effect of EME on scopolamine-induced memory deficit in the Y-maze task. There was a significant reduction in spontaneous alternation (%) in scopolamine treated group when compared to the vehicle-treated control group, this reduction in spontaneous alternation was significantly ameliorated by EME (50, 100 and 200 mg/kg, p.o.) and by piracetam (200 mg/kg, p.o.) (Figure 4 Panel A). However, the numbers of the arm entries were not significantly affected in all experimental groups, which demonstrated

that locomotor activity was not affected by EME (Figure 4 Panel B).

3.4. Effects of EME on Brain Acetyl Cholinesterase (AChE) Activity in Mice

Oral administration of EME (50, 100 and 200 mg/kg) and piracetam (200 mg/kg, i.p.) for 8 consecutive days produced a significant ($p < 0.05$) reduction in brain AChE activity as compared to control treated group. The effect of EME on brain AChE activity was comparable to that of the reference drug piracetam (Figure 5).

Figure 4. The effects of the ethanol leaf extract of *Milicia excelsa* (EME) on spontaneous alternation and the total number of arm entry in scopolamine-induced amnesia in mice in the Y-maze task. PRZ; piracetam (200 mg/kg, p.o.), SCOP; scopolamine (1 mg/kg, i.p.). Spontaneous alternation behavior (A) and the total number of arm entries (B) during an 8 min session were recorded. Each bar represents mean $\pm$ S.E.M (n = 5), * $p < 0.05$ versus the control treated group; [#] $p < 0.05$ versus the scopolamine-treated group.

Figure 5. Effect of ethanol leaf extract of *Milicia excelsa* (EME) and piracetam (200 mg/kg, i.p.) on brain AChE activity of mice. Each bar represents mean $\pm$ SEM: ANOVA followed by Student-Newman Keuls Test, n=5, * $p < 0.05$ compared to the control group.

Figure 6. Effect of ethanol leaf extract of *Milicia excelsa* (EME) and piracetam (200 mg/kg, i.p.) on brain Nitric Oxide concentration of mice. Each bar represents mean $\pm$ SEM: ANOVA followed by Student-Newman Keuls Test, n=5, * $p < 0.05$ compared to control group.

3.5. Effects of EME on Brain Nitric Oxide (NO) Activity in Mice

Oral administration of EME (50, 100 and 200 mg/kg) for 8 consecutive days significantly ($p < 0.05$) increased brain NO concentration compared to the control treated group. Piracetam (200 mg/kg, i.p.) for 8 consecutive days increased brain NO concentration but not significant ($p > 0.05$) from the control treated group (Figure 6).

4. Discussion

This study investigated the anti-amnesic effects of ethanol leaf extract of *Milicia excelsa* leaf extract (EME) using exteroceptive and interoceptive models on the elevated plus-maze (EPM), Morris water-maze (MWM) paradigm and Y-maze (YMT) test in mice. This study further examined the probable anti-amnesic mechanism of EME using Acetylcholinesterase enzyme (AChE) activity and nitric oxide (NO) indices in mice.

MWM [50] and EPM [51] have been used extensively to investigate learning and memory in rodents. MWM has been used to evaluate long term memory while EPM has been employed to assess short memory [52]. Likewise, Elevated plus-maze can both serve as the exteroceptive and interoceptive behavioural model to assess acquisition and retention of memory in mice [53] by the measure of their transfer latency time (TLT).

In MWM, scopolamine increased escape latencies during the training- trial sessions, which suggested that long-term and working memory were impaired by scopolamine [38, 54] but the reverse was found in the extract treated mice indicating that EME has ameliorating effects on both short-term and long-term memory impairment in this amnesic mouse model. This is in consonance with earlier findings of medicinal plants research that ameliorated scopolamine-induced amnesia in MWM [55, 56].

Aging is a progressive process related to an accumulation of oxidative damage and behavioral retrogression of cognition [57]. Brain aging is a risk factor of neurodegenerative diseases such as AD [20]. In the findings of this study, EME attenuated age-induced learning and memory impairment of the aged mice, thus, consistent with anti-amnesic effect. EME might be acting to exacerbate the ability of the mitochondria to generate ATP which is compromised with advancing in age and/or inhibits free radical-mediated damage characteristics of old brain cells [58]. Modulation of brain aging with active phytochemicals from plant extract has been demonstrated to be useful in the aging of wild-type rodents [59]. This finding is in agreement with previous reports of medicinal plants that attenuated age-induced amnesic effects [8, 20].

Scopolamine-induced amnesic animal models are used to screen for potential treatments for cognitive dysfunction

[55]. This is simply because scopolamine- a centrally acting antimuscarinic agent impairs learning and memory in both human and experimental animals [60] and represented the main clinical symptom of AD [61].

In this study, EME inhibited scopolamine-induced amnesia in EPM suggesting that it may be acting via the enhancement of cholinergic neurotransmission to elicit its anti-amnesic effects since acetylcholine is believed to be involved in the regulation of cognitive functions [62]. This finding is further strengthened by the inhibition of AChE enzyme in the brain homogenate of EME treated mice. Since AChE plays a significant role in the regulation of the cholinergic system, inhibition of AChE, therefore, has emerged as one of the most promising strategies for the treatment of AD [63]. This finding is in consonance with earlier reports of medicinal plants that inhibited AChE activity and whose effects were ascribed to anti-amnesic and nootropic effects [64, 65, 66].

EME reversed the diazepam-induced memory impairment suggesting the probable involvement of the GABA-benzodiazepine pathway. Diazepam has earlier been reported to cause memory impairment [61]. Benzodiazepines produce amnesia in laboratory animals by activation of benzodiazepine receptors and GABAergic system [67, 68]. Hence, benzodiazepine-receptor antagonists such as flumazenil and benzodiazepine inverse agonists such as beta-carbolines have been reported to reverse diazepam-induced amnesia [69]. This finding is in accordance with the earlier finding of an agent that attenuated diazepam-induced amnesia in mice [61].

Spontaneous alternation behavior in the YMT is considered to be an indicator of short-term memory [45, 70]. In this study, EME ameliorated scopolamine-induced spontaneous alternation impairment in the YMT suggesting anti-amnesic potentials of EME. Spatial orientation and memory performance in the YMT is at least partly dependant on the hippocampus area of the brain often affected by aging [71, 72, 73]. Since EME improves spontaneous alternation behaviour in Y-maze. It is probable therefore that EME may have effect on the hippocampus. It is interesting to note that EME did not modify the locomotor activity of the mice suggesting that the observed anti-amnesic might not be due to spontaneous motor effect, thus excluding false positive result. This is in conformity with the earlier finding of a medicinal plant that elicited anti-amnesic and memory enhancing effects without modifying locomotor activity [74].

In this study, the elevation of NO concentration in the brain of EME treated mice suggests that EME may be facilitating NO release in the brain. This may be beneficial in learning and memory because previous studies have documented the synthesis of NO in the brain and its neuronal functions in learning and memory processes [75]. Furthermore, NO function as a neurotransmitter through the stimulation of soluble guanylyl cyclase to form the cyclic guanosine monophosphate (cGMP) a second

messenger molecule in the target cells [75]. Therefore, NO-cGMP pathway has been implicated in the induction of hippocampal Long-term potentiation (LTP) and long-term depression (LTD) which are known to be the predominant mechanisms of learning and memory processes [75, 76, 77, 78]. Thus, inhibitors of Nitric Oxide synthase (NOS), competing for the L-arginine substrate, can prevent the induction of LTP [79] and subsequently lead to learning and memory impairment [78, 80, 81]. In addition, there are reports that suggest a relationship between NMDA (N-methyl-D-aspartate) receptors and the NO system in learning and memory [82, 83]. LTP in the hippocampus is known to involve NMDA receptors. NOS inhibitor, L-N ω -nitroarginine, blocks LTP and that sodium nitroprusside, which releases NO, produces a long-lasting enhancement in synaptic efficacy [84]. Since the extract leads to an increase in NO in the brain, it may be inferred that the extract might be acting via activation of NMDA receptor in the hippocampus to elicit its anti-amnesic effect. NO released by the extract may also be mediating its anti-amnesic effect by enhancing cerebral blood flow since NO-mediated mechanisms have been assigned a role in cortical perfusion and cognitive function [78]. Taken together, it is plausible to suggest that NO signaling pathway might at least in part be involved in the anti-amnesic effect of EME.

Subsequently, an interaction between cholinergic and NO activities in the learning process and memory formation have been suggested. Inhibition of NO synthesis has been shown to cause a decrease in acetylcholine (ACh) release and an impairment of retention of conditioned response in rats [85]. Thus, several earlier studies have shown that cholinergic activity has an involvement in the cognitive effect of NO [85, 86, 87, 88, 89]. Besides the role of NO as a neurotransmitter [74], its antioxidant role has also been reported in the literature [90]. Therefore, the combined effects of inhibiting AChE enzyme activity coupled with the NO-releasing effect of EME and the antioxidant effects of NO may confer a therapeutic advantage in the anti-amnesic effect of EME. This finding is in line with the earlier report that NO enhances the computational ability of the brain [91].

Medicinal plants that contain ursolic acid and lupeol acetate have been found to possess anti-amnesic effects [92, 93]. Since *Milicia excelsa* leaf contains these phytochemicals in addition to other phytochemicals, it could, therefore, be suggested that ursolic acid and lupeol acetate either in additive or synergy with each other or with other phytochemicals in the leaf of *Milicia excelsa* could be responsible for the observed anti-amnesic effect of EME observed in this study.

In addition to the suggested mechanism of anti-amnesic effects of EME, it could also be suggested that EME may attenuate the accumulation of beta-amyloid, the accumulation of which is associated with the common pathological characteristics of AD [94]. Since, ursolic acid,

one of the isolated compounds from the leaf of *Milicia excelsa* leaf has been demonstrated to attenuate beta-amyloid-induced memory impairment in mice [95].

5. Conclusions

This study concluded that EME has anti-amnesic effects and cognitive enhancing effects in mice. The memory enhancing activity of EME may probably involve inhibition of brain acetylcholinesterase activity or enhancement of nitric oxide neurotransmission.

REFERENCES

- [1] H. Joshi, M. Parle. Effects of piperine on memory and behaviour mediated by monoamine neurotransmitters. *J Tradit Med* 22: 39-43, 2005.
- [2] R.V Hadaginha, V.P Tikare, K.S Patil, M.D Bhanushali, N.S Desai, A. Karigar. Evaluation of cognitive enhancing activity of *Mimusops Elengi* Linn on albino rats. *Int J. Res Ayurveda Pharm*, 1(2): 484-492, 2010.
- [3] K. Hsiao, P. Chapman, S. Nilsen, C. Eckman, Y. Harigaya, S. Younkin, F. Yang et al., Correlative memory deficits, A β elevation, and amyloid plaques in transgenic mice. *Sci* 274: 99-102, 1996.
- [4] B.S McEwen. Stress and the aging hippocampus. *Front. Neuroendocrinol.* 20: 49-70, 1999.
- [5] D.C Gooding, K.A Tallent. Nonverbal working memory deficits in schizophrenia patients: evidence of a supramodal executive processing deficit. *Schizophr Res.* 68: 189-201, 2004.
- [6] C. Haenschel, R.A Bittner, J. Waltz, F. Haertling, M. Wibrall, W. Singer, D.E Linden et al. Cortical oscillatory activity is critical for working memory as revealed by deficits in early-onset schizophrenia. *J. Neurosci.* 29, 9481-9499, 2009.
- [7] R. Scatena, G.E Martorona, P. Bottani, G. Botta, P. Pastove, B. Giardina. An update of pharmacological approaches to neurodegenerative diseases. *Expert Opin. Investig. Drugs*, 16(1): 59-72, 2007.
- [8] H. Joshi, K. Megeri. Antiamnesic evaluation of *C. phlomidis* Linn. bark extract in mice. *Braz J Pharm Sci*, 44(4): 717-725, 2008.
- [9] D.A Butterfield, S. Griffin, G. Munch, G.M Pasinetti. Amyloid β -peptide and amyloid pathology are central to the oxidative stress and inflammatory cascades under which Alzheimer's disease brain exists. *J. Alzheimer's Dis*, 4 (2002): 193-201, 2002.
- [10] D.B Madhavi, K.L.S. Kumar, V. Palanivel. Evaluation of Cognitive Enhancing Activity of *Piper Longum* Fruit Extract on Albino Rats. *Int. J. of Pharm and Chem Sci*, 1(3): 708-712, 2012.
- [11] J.L Cummings, G. Cole. Alzheimer's disease. *JAMA*,

- 287(18): 2335–48, 2002.
- [12] J.M Ellis J.M. Cholinesterase inhibitors in the treatment of dementia. *J Am Osteopath Assoc*, 3: 145–58, 2005.
- [13] D.S Auld, T.J Kornecook, S. Bastianetto, R. Quirion. Alzheimer's disease and the basal forebrain cholinergic system: relations to beta-amyloid peptides, cognition and treatment strategies. *Prog Neurobiol*, 68: 209-245, 2002.
- [14] J. Yi, L.L Horky, A.L Friedlich, Y. Shi, J.T Rogers, X. Huang. L-arginine and Alzheimer's disease. *Int J Clin Exp Pathol* 2: 211-8, 2009.
- [15] A. Limon, J.M Reyes-Ruiz, M. Ricardo Miledi. Loss of functional GABA_A receptors in the Alzheimer diseased brain. *Proc Natl Acad Sci U S A*, 109(25): 10071–10076, 2012.
- [16] S. Akhondzadeh, M. Noroozian. Alzheimer's disease: Pathophysiology and pharmacotherapy. *J Drugs* 4: 1167-1172, 2002.
- [17] R. Bullock. New drugs for Alzheimer's disease and other dementias. *Br J Psychiatry* 180: 135-139, 2002.
- [18] D.G Blazer, C.F Federspiel, W.A Ray, W. Schaffner. The risk of anti cholinergic toxicity in the elderly- A study of prescribing practices in two populations. *J Gerontol* 38: 31-35, 1983.
- [19] S.H Rogers, M.R Farlow, R.S Doody, R. Mohs, L.I Friedhoff. Donepezil study group. A 24 week, double blind, Placebo-controlled trial of donepezil in patients with Alzheimer's disease. *Neurology*, 50: 136-145, 1998.
- [20] K.B Kunte, Y. Kuna. Neuroprotective effect of *Bacopa monniera* on memory deficits and ATPase system in Alzheimer's disease (AD) induced mice. *J Sci Innov Res*, 2 (4): 719-735, 2013.
- [21] V.K Agyeman, D.A Ofori, J.R Cobbinah, MR Wagner. Influence of *Phytolyma lata* (Homoptera psyllidae) on seed growth of *Milicia excelsa*. *Ghana J. Forest*. 25:29-39, 2009.
- [22] D.A Ofori. *Milicia excelsa* (Welw.) C. C. Berg. In Louppe, D., Oteng-Amoako, A. A., and Brink M. (Eds.). PROTA (Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale), Wageningen, Netherlands, 2007.
- [23] J.A Ibrahim, I. Muazzam, I.A Jegede, O.F Kunle, J.I Okogun. Ethno-medicinal plants and methods used by Gwandara tribe of Sabo Wuse in Niger State, Nigeria, to treat mental illness. *Afr J Trad CAM* 4 (2):211–8, 2007.
- [24] M.A Sonibare, M.O Soladoye T.O Subuloye. Ethnobotanical Survey of Anti-Psychotic Plants in Lagos and Ogun States of Nigeria. *Eur. J. Sci. Res*, 19(4): 634-644, 2008.
- [25] T.O Elufioye, A.T Oladele, C.M Cyril-Olutayo, J.M Agbedahunsi, S.A Adesanya. Ethnomedicinal Study and Screening of Plants Used for Memory Enhancement and Antiaging in Sagamu, Nigeria. *Eur J Med Plants*, 2(3): 262-275, 2012.
- [26] N.R Ndah, A.E Egbe, E. Bechem, S. Asaha, T. Yengo, E.L Chia, N.M Eyenieh. Ethnobotanical study of commonly used medicinal plants of the Takamanda Rainforest South West, Cameroon. *Afr. J. Plant Sci*, 7(1): 21-34, 2013.
- [27] V.P.K Titanji, D. Zofou, M.N Ngemenya. The antimalarial potential of medicinal plants used for the treatment of malaria in cameroonian folk medicine. *Afr J Trad CAM* 5 (3): 302 – 321, 2008.
- [28] J.L Betti, O.D Yongo, D.O Mbomio, D.M Iponga, A. Ngoye. An Ethnobotanical and Floristical Study of Medicinal Plants Among the Baka Pygmies in the Periphery of the Ipassa-Biosphere Reserve, Gabon. *Eur J Med Plants*, 3(2): 174-205, 2013.
- [29] BEP Oliver. Medicinal plants in tropical West Africa. University Press. Cambridge. pp. 1-8, 1986.
- [30] T. Padayachee, B. Odhav. Antimicrobial activity of plant phenols from *Chlorophora excelsa* and *Virgilia oroboides*. *Afr J Biotechnol*, 12 (17), pp. 2254-2261, 2013.
- [31] S.O Udegbumam, T.O Nnaji, R.I Udegbumam, J.C Okafor, I. Agbo. Evaluation of herbal ointment formulation of *Milicia excelsa* (Welw) C.C berg for wound healing. *Afr J Biotechnol*, 12(21): 3351-3359, 2013.
- [32] O.A Olajide, O.T Kolawole, T.R Fagbohun, F.F Ajayi. Evaluation of the anti-inflammatory properties of *Chlorophora excelsa* stem bark extract. *Pharm Biol*, 43(9): 746–748, 2005.
- [33] Ouete JL, Sandjo LP, Kapche DW, Yeboah SO, Mapitse R, Abegaz BM, et al. Excelsoside: A new benzylic diglycoside from the leaves of *Milicia excelsa*. *Z Naturforsch C*, 69(7-8): 271-5, 2014.
- [34] L.A Akinpelu, M.A Akanmu, E.M Obuotor. Antipsychotic effects of ethanol leaf extract and fractions of *Milicia excelsa* (Moraceae) in mice. *J Pharm Res Int* 22(6): 1-10, 2018.
- [35] L.L Harburger, J.C Bennett, K.M Frick. Effects of estrogen and progesterone on spatial memory consolidation in aged females. *Neurobiol Aging*, 28(4): 602–610, 2007.
- [36] D. Dhingra, V. Kumar. Memory-Enhancing Activity of Palmatine in Mice Using Elevated Plus-maze and Morris Water Maze. *Adv Pharmacol Sci*, vol. 2012, Article ID 357368, 7 pages, 2012.
- [37] W. Waldegrave. Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes. Pg1-46.
- [38] R.G Morris. Development of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* 11, 47–60, 1984.
- [39] A.C Sharma, S.K Kulkarni. Evidence for GABA-BZ receptor modulation of short-term memory passive avoidance task paradigm in mice. *Met Find Exp Clin Pharmacol*, 12:175-80, 1990.
- [40] H. Joshi, M. Parle. Management of dementia through ayurvedic formulation Brahmi rasayana BR-2T in mice. *Alzheimer's Demen*, 1:64, 2005.
- [41] J. Itoh, T. Nabeshima, T. Kameyama. Utility of an elevated plus-maze for the evaluation of nootropics, scopolamine and electro convulsive shock. *Psychopharmacology*, 101: 27-33, 1990.

- [42] H. Joshi, M. Parle. Trikatu churna: A promising memory enhancer in mice. *Planta Indica* 1: 14-19, 2005.
- [43] J.C Plan, S.S Zhang. Antagonism of piracetam on the amnesic effect of diazepam in mice. *Yao Xue Xue Bao* 31: 91, 1996.
- [44] D.H Kim, T.M Hung, K.H Bae, J.W Jung, S. Lee, B.H Yoon, J.H Cheong et al. Gomisin A improves scopolamine-induced memory impairment in mice. *Eur. J. Pharmacol.* 7: 129–135, 2006.
- [45] M. Sarter, G. Bodewitz, D.N Stephens. Attenuation of scopolamine-induced impairment of spontaneous alternation behavior by antagonist but not inverse agonist and b-carboline. *Psychopharmacology* 94: 491–495, 1988.
- [46] L. Cahil, J. Brioni, I. Izquierdo. Mechanisms of emotional arousal and lasting declarative memory. *Trends Neurosci*, 21: 294-299, 1998.
- [47] V.S Nade, L.A Kawale, K.D Valte, N.V Shendye. Cognitive enhancing effect of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers on learning and memory. *Indian J Pharmacol.* 47(3): 263–269, 2015.
- [48] G.E Ellman, K.D Courtney, V. Andersen Jr, R.M. A new and rapid colorimetric determination of Acetylcholinesterase activity. *Biochem Pharmacol*, 7: 88–95, 1961.
- [49] M.B Grisham, G.G Johnson, J.R Lancaster Jr. Quantitation of nitrate and nitrite in extracellular fluids. *Methods Enzymol*, 268: 237–46, 1996.
- [50] R.D'Hooge, P.P De Deyn. Applications of the Morris water-maze in the study of learning and memory. *Brain Res Rev*, 36: 60–90, 2001.
- [51] J. Itoh, T. Nabeshima, T. Kameyama. Utility of an elevated plus-maze for the evaluation of memory in mice: effects of nootropics, scopolamine and electroconvulsive shock. *Psychopharmacol*, 101 (1): 27-33, 1984.
- [52] V.D Ashwlayan, R. Singh. Reversal effect of *Phyllanthus emblica* (Euphorbiaceae) Rasayana on memory deficits in mice. *Int J App Pharm*, 3(2): 10-15, 2011.
- [53] M. Parle, N. Singh. Animal models for testing memory. *Asia Pac J Pharmacol*, 16: 101-120, 2004.
- [54] C.A Barnes, W. Danysz, C.G Parsons. Effects of the uncompetitive NMDA receptor antagonist memantine on hippocampal long-term potentiation, shortterm exploratory modulation and spatial memory in awake, freely moving rats. *Eur. J. Neurosci.* 8: 565–571, 1996.
- [55] S.J Park, D.H Kim, I.K Lee, W.Y Jung, D.H Park, J.M Kim, K.R Lee et al. The ameliorating effect of the extract of the flower of *Prunella vulgaris* var. lilacina on drug-induced memory impairments in mice. *Food Chem Toxicol*, 48: 1671–1676, 2010.
- [56] P. Khare, S. Chaudhary, L. Singh, G. Yadav, S. Verma. Evaluation of Nootropic activity of *Cressa cretica* in scopolamine- induced memory impairment in mice. *Int. J. Pharmacol. Toxicol*, 2(2): 24-29, 2014.
- [57] J.A Joseph, B. Shukitt-Hale, G. Casadesus. Reversing the deleterious effects of aging on neuronal communication and behavior beneficial properties of fruit polyphenolic compounds. *Am J Clin Nutr* 81(1): 313-316, 2005.
- [58] E.C Toescu, N. Myronove, A. Verkhatsky. Age-related structural and functional changes of brain mitochondria. *Cell Calcium*, 28: 329-338, 2000.
- [59] E. Giacobini. Invited review: Cholinesterase inhibitors for Alzheimer's disease therapy: from tacrine to future applications. *Neurochem. Int*, 32(5-6): 413-419, 1998.
- [60] A. Higashida, N. Ogawa. Differences in the acquisition process and the effect of scopolamine on radial maze performance in the strains of rats. *Pharmacol Biochem Behav*, 27:483-9, 1987.
- [61] M. Parle and D. Dhingra, Ascorbic acid: a promising memory-enhancer in mice. *J Pharmacol Sci*, 93(2): 129–135, 2003.
- [62] A.B Desai, V.G Kagathara, H. Joshi, A.T Rangani, H Mungra. Evaluation of Anti-amnesic effect of Solasodine in Mice. *Int J Pharm tech Res*, 3(2): 732-740, 2011.
- [63] S. Lu, J.W Wu, H. Liu, J. Zhao, K. Liu, C. Chuang, H. Lin et al. The discovery of potential acetylcholinesterase inhibitors: A combination of pharmacophore modeling, virtual screening, and molecular docking studies. *J Biomedl Sci*, 18:8, 2011
- [64] H. Joshi, M. Parle. *Zingiber officinale*: evaluation of its nootropic effect in mice. *Afr. J. Trad. CAM*, 3 (1): 64 – 74, 2006.
- [65] H. Joshi, N. Kaur, J Chauhan. Evaluation of nootropic effect of *Argyrea speciosa* in mice. *Int J Health Sci Res*, 53(4): 382-388, 2007.
- [66] D. Saini, A.K Dhingra, B. Chopra, M. Parle. Psychopharmacological investigation of the nootropic potential of *Trigonella foenum LINN.* in mice. *Asian J Pharm Clin Res*, 4(4): 76-84, 2011.
- [67] N. Singh, A. Sharma, M. Singh. Possible mechanism of alprazolam-induced amnesia in mice. *Pharmacology*, 56(1): 46–50, 1998.
- [68] M.R Zarrindast, H. Haidari, M.R. Jafari, B. Djahanguiri. Influence of β -adrenoceptor agonists and antagonists on baclofen-induced memory impairment in mice. *Behav Pharmacol*, 15(4): 293–297, 2004.
- [69] L.H Jensen, D.N Stephens, M. Sarter, E.N. Petersen. Bidirectional effects of β -carbolines and benzodiazepines on cognitive processes. *Brain Res Bull*, 19(3): 359–364, 1987.
- [70] T. Myhrer. Neurotransmitter systems involved in learning and memory in the rat: a meta-analysis based on studies of four behavioral tasks. *Brain Res Brain Res Rev* 41: 268–287, 2003.
- [71] C.A Oomea, E. Farkas, V. Roman, E.M Vander Beek, P.G.M Luiten, P. Meerlo. Resveratrol preserves cerebrovascular density and cognitive function in aging mice. *Front Aging Neurosci*, 1: 1-9, 2009.
- [72] C.A Erickson, C.A Barnes. The neurobiology of memory changes in normal aging. *Exp. Gerontol*, 38: 61-69, 2003.
- [73] R. Havekes, M. Timmer, E.A Vander Zee. Regional differences in hippocampal PKA immunoreactivity after training and reversal training in spatial Y-maze task. *Hippocampus*, 17: 338-348, 2007.
- [74] D. Dhingra, V. Kumar. Memory-Enhancing Activity of

Palmatine in Mice Using Elevated Plus-Maze and Morris Water Maze. *Adv Pharmacol Sci*, vol. 2012, Article ID 357368, 7 pages, 2012.

- [75] J. Garthwaite. Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci*, 14: 60–7, 1991.
- [76] T.V.P Bliss, G.L Collingridge. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, 361: 31–9, 1993.
- [77] M. Zhuo, R.D Hawkins. Long-term depression: a learning related type of synaptic plasticity in the mammalian central nervous system. *Rev Neurosci*, 6: 259–77, 1995.
- [78] V. Paul, P. Ekambaram. Demonstrating the dose and time-related effects of 7-nitroindazole on picrotoxin-induced convulsions, memory formation, brain nitric oxide synthase activity and nitric oxide concentration in rat. *Pharmacol Biochem Behav*, 77:1–8, 2004.
- [79] E.M Schuman, D.V Madison. A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science*, 254(5037): 1503–1506, 1991.
- [80] J.D Robertson, J. Bonaventure, A. Kohm, M. Hiscat. Nitric oxide is necessary for visual learning in *Octopus vulgaris*. *Proc R Soc Lond Biol Sci*, 263: 1739–43, 1996.
- [81] J. Myslivecek, J. Hassmannova, J. Barcal, J. Safands, V. Zalud. Inhibitory learning and memory in new born rats influenced by nitric oxide. *Neuroscience*, 71:299–312, 1996.
- [82] J. Garthwaite. Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci*, 14:60-7, 1991.
- [83] H. Prast, A Philippu. Nitric oxide as modulator of neuronal function. *Prog Neurobiol*, 64: 51–68, 2001.
- [84] G.A Böhme, C. Bon, J. Stutzmann, D. Adam, J. Blanchard. Possible involvement of nitric oxide in long-term potentiation. *Eur J Pharmacol*, 199 (3): 379-381, 1991.
- [85] S.R Kopf, R.S Benton, R. Kalfin, M.G Giovannini, G. Pepeu. NO synthesis inhibition decreases cortical ACH release and impairs retention of conditioned response. *Brain Res*, 894: 141-4, 2001.
- [86] M. Dacher, M. Gauthier. Involvement of NO-synthase and nicotinic receptors in learning in the honey bees. *Physiol Behav*, 95: 200-7, 2008.
- [87] C.C Huang, K.S Hsu. Activation of muscarinic acetylcholine receptors induces a nitric oxide-dependent long-term depression in rat medial prefrontal cortex. *Cereb Cortex* 20: 982-96, 2010.
- [88] P.J Pistell, L.W Daffin, C.M Nelson, K.B Duffy, J.L Bowker, E.L Spangler, D.K Ingram, B.D Devan. Combined administration of subthreshold doses of the nitric oxide inhibitor, nitro-L- arginine and muscarinic receptor antagonist, scopolamine, impairs complex maze learning in rats. *Behav Pharmacol* 18: 801-5, 2007.
- [89] N. Pitsikas. The nitric oxide donor molsidomine antagonizes scopolamine and L-NAME-induced performance deficits in a spatial memory task in the rat. *Behav Brain Res*, 200: 160-4, 2009.
- [90] N. Hogg, B. Kalyanaraman. Nitric oxide and lipid peroxidation. *BBA - Bioenergetics*, 1411 (2–3): 378-384, 1999.
- [91] V. Paul, P. Ekambaram. Involvement of nitric oxide in learning & memory processes. *Indian J Med Res*, 133(5): 471–478, 2011.
- [92] Y.K Chung, H.J Heo, E.K Kim, H.K Kim, T.L Huh, Y Lim et al. Inhibitory effect of ursolic acid purified from *Origanum majorana* L. on the acetylcholinesterase. *Mol. Cells*, 11: 137-143, 2001.
- [93] A. Tanwar, P.A Bafna, A.R Bafna. Anti-amnesic effect of aqueous extract of *Crataeva nurvala* stem bark in scopolamine induced amnesia. *J Applied Pharm Sci*, 4 (09): 066-072, 2014.
- [94] Blennow K, Leon MJ, Zetterberg H. Alzheimer's disease. *Lancet*, 368(9533): 387-403, 2006.
- [95] W. Liang, X. Zhao, J. Feng, F. Song, Y. Pa. Ursolic acid attenuates beta-amyloid-induced memory impairment in mic. *Arq Neuropsiquiatr* 74(6):482-488, 2016.