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# Effect of *Ginkgo biloba* leaf extract in combination with vitamin C, E and D on Aluminum Chloride induced Alzheimer in rats.

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**Abstract**

Alzheimer's disease(AD), is a neurodegenerative ailment and the most prevalent cause of dementia. The commonly used synthetic drugs provide temporary and incomplete symptomatic treatment accompanied with severe side effects. The present study aimed at evaluating the therapeutic potential of *Ginko Biloba*, and/ or Vitamin D, E, C on aluminum chloride ($AlCl_3$)-induced neurotoxicity in rats.

AD was induced in rats by oral administration of $AlCl_3$ (17 mg/kg BW) for 1 month. Rats were treated with *Ginko Biloba* methanolic leaf extract (400 mg/kg.BW/day), vitamin $D_3$ (0.0125 mg/kg.BW/day), E(2.5mg/kg.BW/day), Vitamin C (50mg/kg.BW/day) and their combinations, respectively, for 15 days. Behavioral assessment was carried out using Y-maze test. The activity of acetylcholinesterase, Dopamine, Serotonin and Nor-Epinephrine; in addition, histopathological examination of the isolated Hippocampal brain tissues were done. It was observed that administration of *Ginko biloba* in combination with vitamin C attenuated memory impairment and improved cholinergic and dopaminergic dysfunction. Also, significant improvement in histopathological alterations and hippocampal morphology was noticed. It could be concluded that *Ginko biloba* and /or vitamin C exhibited significant improvement in memory loss and neurotoxicity and could be an effective treatment against cognitive decline in AD patients.

**Keywords:** Alzheimer's disease; Neurotoxicity; *Ginko Biloba* ; Vitamin C ;Y-maze; Neurotransmitter.

## 1. Introduction

Alzheimer's disease (AD) has recently advanced from the seventh to the sixth leading cause of death worldwide (1,2). More than 47 million people have the disease worldwide, with an expected prevalence of 131.5 million people by 2050, most of whom will be living in the developing world (3), which is greater than heart failure and cancer combined (4). AD is a devastating, progressive neurodegenerative illness that affects people, families, and society enormously personally and financially (5). Abnormalities in hippocampal structure and function are characteristics of early Alzheimer's disease (AD). Among the most common features of early (AD) is the inability to form and retain new memories, where, the integrity of hippocampus region become compromised by plaques, tangles and, eventually, the loss of synapses and neuron cell bodies (6,7). The histopathological studies of different brain regions demonstrate that decreases in hippocampal volume is a hallmark of early AD (8). The hippocampus has been shown to be particularly essential for memory tasks that require the development of configural rather than simple associations. Behavioral tests measuring hippocampal-dependent memory in rodents are often used to evaluate novel treatments for AD and other dementias (9).

Repurposing and combining of known traditional drugs can increase drug development efforts, accelerate the identification of new treatments for

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many chronic diseases including Alzheimer's disease (AD) (10). A major advantage of this approach is that the safety of the candidate compound has already been identified, eliminating the requirement for additional preclinical safety testing, chemical optimization or toxicology studies and significantly reducing the time and cost involved in advancing potential treatment into clinical studies. Another hypothesis we claimed that the usage of vitamins especially D, E and C by their own or with our drug candidates to create synergistic effect can combat oxidative stress in AD, antioxidants have been therapeutically implicated to reduce Alzheimer symptoms. Brains of people with Alzheimer's disease appear to have higher levels of natural antioxidants that clear excess free radicals, suggesting that the body is trying to combat this damage (11,12).

*Ginkgo biloba* (Ginkgo) fruits and leaves have been used since early human history as both a food and a traditional medicine(13). Ginkgo leaves and other aerial parts have been part of traditional East Asian medicine practices for at least the last 2000 years (14). Various parts of the Ginkgo tree possess bioactive compounds, including ginkgolides and bilobalides (i.e. terpene trilactones unique to the plant), as well as flavonoids (flavones, biflavones, flavonols, tannins, and associated glycosides). The dried green Ginkgo leaves have been investigated for treating several medical conditions, particularly those related to diseases of the peripheral and cerebral circulation (15). Because Ginkgo natural products encompass a variety of preparations and doses of active compounds, a comprehensive investigation of various Ginkgo natural products is warranted to determine which, if any, may be beneficial for the treatment of cognitive impairment and dementia (16). The present study aimed to evaluate the therapeutic potential of *Ginko Biloba*, vitamin D, E & C and their synergistic effect against aluminum chloride ($AlCl_3$)-induced Alzheimer's disease in rats.

## *2.Experimental*

### *2.1. Materials*

#### *2.1.1. Chemicals:*

Aluminum chloride ($AlCl_3$) was purchased from BDH Laboratory Supplies, Poole UK, vitamin D,E,C and Aricept were purchased from a commercial drug store. All other chemicals used were obtained from standard commercial suppliers and were of analytical grade.

*2.1.2.* Ginko Biloba leaf extract were a generous gift from EMA Pharm company. Cairo, Egypt. a tan powdered solid contained Ginko biloba leaf powder extract equivalent to 260mg.Vitamin C (capsules,500mg), vitamin D3 (Cholecalceferol equivalent to200000 I.U.) and vitamin E(capsules 400mg) where a gift from Al-Kahira Pharm.& Chem.IND.CO. Cairo, Egypt. The positive control drug Aricept (Donepezil HCl,5mg) was a gift from Pfizer Egypt S.A.E cairo, Egypt.

All drugs were weighed, dissolved in PBS at the required concentration then stored at -20 till work.

#### *2.1.2. Animals*

Male Wistar rats (180–200 g) procured from Central Animal House, National Research Centre (NRC). Animals were in maintained plastic cages with soft bedding. Animals were acclimatized to laboratory conditions under room temperature of 25 ± 2ºC and 12 h light/ dark cycles. The animals had free access to a standard pellet diet and normal tap water was available ad libitum to the animals throughout the experimental period. Animal handling and experimental procedures were approved by the NRC Medical Ethics Committee in accordance with the guidelines provided by the European community guidelines for the use and care of animals.

### *2.2.* ***Determination of Antioxidant Activity Using the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Method***

DPPH radical scavenging activity of *G.biloba*, vitamin D,E,C and their combinations was analyzed according to the method of Matsushige *et al* (17) with slight modification . The procedure was applied in 96 well microplates and measured in ELISA microplate reader. All samples prepared freshly and kept in the dark.100µl of methanol solution for each extract were added to 100µl of DPPH solution in each well. The prepared solutions were mixed and left for 30 min at room temperature in the dark. The hydrogen atom or electron donation abilities of the corresponding extracts were measured from the bleaching of the purple-colored methanol solution of 1, 1-diphenly-2-picrylhydrazyl (DPPH). At the end of incubation period, the absorbance was read at 520 nm. Mean of three measurements for each sample was calculated.

Percent Inhibition (I %) of DPPH free radical by different extracts was calculated by the following equation:

I % = [(A control - A sample) / A control] x 100,

where: A-control: is the absorbance of the control reaction (DPPH solution)A-sample: absorbance of the tested extract

### *2.3.* ***$AlCl_3$-induced neurotoxicity in rats***

#### *4.3.1. Animals*

Male Wistar rats (180–200 g) procured from Central Animal House, National Research Centre (NRC), were acclimatized to laboratory conditions at room temperature with food and water ad-libitum in plastic cages with soft bedding. The protocol was approved

by the NRC Ethics Committee (approval no. 19039) in accordance with the European community guidelines for the use and care of animals (18).

*2.3.2.* ***Experimental design***

Animals were randomized into 10 groups of 6 rats each distributed as follows: Group 1; normal healthy rats served as untreated negative control group, Group 2; positive control group where the rats were orally administered with $AlCl_3$ (17 mg/kg,body wt/day) (19) daily throughout the whole experiment, Group3: rats orally administered daily with standard drug (Aricept 5mg/k.gm. body wt /day) after 4 weeks intoxication with $AlCl_3$,Group 4; rats were treated with daily oral dose of *G.biloba (*400 mg/kg body wt) for 2 weeks after 4 weeks intoxication with $AlCl_3$.,Group 5; rats were treated with daily dose of by the intraperitoneal injection of 0.0125 mg/kg of vitamin D for 2 weeks after 4 weeks intoxication with $AlCl_3$ (17 mg/kg, body wt/day) ,Group 6; rats were treated with daily oral dose of vitamin E(2.5mg/kg body wt/day) for 2 weeks after 4 weeks intoxication with $AlCl_3$,Group 7; rats were treated with daily oral dose of Vitamin C (50mg/kg body wt/day)for 2 weeks after 4 weeks intoxication with $AlCl_3$ ,Group 8; rats were were administered with 400 mg/kg of Ginko Biloba orally along with 0.0125 mg/kg of vitamin D intraperitoneally for 2 weeks after 4 weeks intoxication with $AlCl_3$.,Group 9; rats were were administered with 400 mg/kg of Ginko Biloba orally along with 2.5 mg/kg of vitamin E orally for 2 weeks after 4 weeks intoxication with $AlCl_3$, Group10; rats were administered with 400 mg/kg of Ginko Biloba orally along with 50mg/kg of vitamin C orally for two weeks after 4 weeks intoxication with $AlCl_3$.

*2.3.3.* ***Behavioral study: Y-Maze Spontaneous alternation test:***

The animals' behavioral activities, including spatial learning, age related cognitive decline and memory loss, were studied using the Y maze test according to (20) with slight modifications. The maze used in the present study consisted of three arms (35 cm long, 25 cm high and 10 cm wide). Each of the three arms can be sealed off with a door, limiting the space that the rodent has to access. During the test a rat from each group is placed in an arm and one of the remaining two arms is closed off. The open arm, however, contains a food reward. The rat will roam and find the food reward. Then, in the next round, the other arm is now sealed off. During testing, when both arms are open, a mouse is to alternate between arms in consecutive trials. So, a rat is placed at the starting position, and finds reward in one arm. Then, a new trial begins and a rat is returned to the start, then it is expected to go down the other arm. All animals were tested in a randomized order at the start and end of the experimental protocol. Thirty minutes after rats treatment with either of *G.biloba*, vitamin D,E,C and their combinations (at selected doses) or reference drug (Aricept 10mg/kg b.wt./day); rats were placed at the end of one arm and allowed to move freely through the maze .The time limit in Y-maze test was 8 min, divided into 2 min of habitation and 6 min of testing where every session was stopped after 8 min or when the rat reaches the food reward (21). The maze was wiped clean with 70 % ethanol between each animal to minimize odor cues (1).

*2.3.4.* ***Blood and brain tissue sampling***

By the end of the experiment, rats were fasted overnight, blood samples were collected from the sublingual vein and blood samples were taken and serum were separated were frozen at −20 °C for biochemical analysis (22). Rats were then sacrificed by cervical decapitation. Brains were rapidly dissected, washed with isotonic saline, and dried on filter paper. Each brain was divided sagittally into two portions. The first portion was weighed and homogenized, using Omni thq - digital tissue homogenizer- USA, in ice-cold medium containing 50 mMTris/HCl and 300 mM sucrose at pH 7.4 to give a 10% (w/v) homogenate. The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was separated for biochemical analysis. The second portion of each brain was fixed in formalin buffer (10%) for histopathological investigation. The ethical conditions were applied such that the animals suffered no pain at any stage of the experiment, and the study was approved by the Ethics Committee of the NRC. Animals were disposed of in bags provided by the Committee of Safety and Environmental Health, NRC.

*2.3.5.* ***Monoamine neurotransmitters estimation***

Serum levels of dopamine, serotonin and norepinephrine were determined by enzyme linked immunoassay using BioTech- ELISA reader- USA according to (23) .

*2.3.6.* ***Acetylcholine esterase estimation***

AChE activity was estimated in the whole brain homogenates according to Ellman's method (24). Briefly, the brain homogenate was incubated for 5 min with 2.7 ml of phosphate buffer and 0.1 ml of 5, 5-dithiobis (2-nitrobenzoate) (DTNB). Further, 0.1 ml of freshly prepared acetylcholine iodide (pH 8) was added, and the change in absorbance was recorded at 412 nm.

*2.4.* ***Statistical analysis***

Analyses were performed by SPSS version 22 software for Windows (IBM-SPSS, Chicago, IL). Data are presented as mean ± SEM. Statistical analysis was carried out using two-way analysis of variance (ANOVA) followed by Tukey's test to judge the difference between the various groups followed by post-hok-LSD for the Y-Maze. Statistical significance was acceptable to a level of $P<0.05$. Data for ACHE

and Neurotransmitters were analyzed by One Way ANOVA followed by post-hok-Dunnet T3 test, significance was acceptable to a level of P<0.05.

*2.5.* ***Histopathological examination***

Autopsy samples from the rat's brains were fixed in 10% formalin saline for 24 h. Washing was done in tap water then serial dilutions of alcohol were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 °C in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin &eosin stain for examination through the light electric microscope [22].

**3.Results**

*3.1. The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity*

The antioxidant effect of *G.biloba*, vitamin D,E,C and their combinations were evaluated by DPPH in-vitro method. It is based on the reduction of methanolic DPPH solution at 520 nm in the presence of a hydrogen donor antioxidant with the subsequent formation of non-radical DPPH. The DPPH is stable organic nitrogen centered free radical with a dark purple color that becomes colorless when it reacts with antioxidant to form non-radicals [24]. The residual amount of DPPH is then measured after fixed time and is inversely correlated to the radical scavenging potential of the tested extract.From Figure 2, it was observed that *G.biloba*, vitamin D,E,C and their combinations showed variable free radical scavenging activity, whereas *G.biloba* alone showed the highest free radical scavenging activity over DPPH (at a concentration of 50µg/ml) followed by vitamin C and their combination. While vitamin E, D and their combinations showed mild moderate effect.

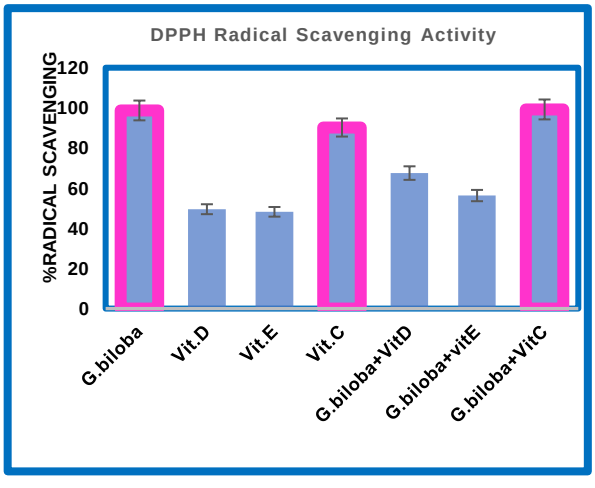


**Figure 1:** Free radical scavenging activity of *G.biloba*, vitamin D,E,C and their combinations in the DPPH radical assay. Values are expressed as mean ± SD, n = 3 at a concentration of (50µg/ml for all tested drugs).

***3.2. In-Vivo Study***

*3.2.1. Behavioral study:*

*3.2.1.1.Y-maze Spontaneous Alternation test(YM-T):*

The effect of administration of *G.biloba alone and in combination with vitamins( D,E and C)*on the acquisition of spatial recognition (working, short) memory in the AlCl3-induced AD rats, using Y-maze test is illustrated in figure (2).

The two-ways ANOVA analysis of the obtained data, indicated that treatment of rat with either G.b, Vitamin C and their combination at the selected doses were significantly able to reduce the decline in spatial recognition as expressed by the decrease in time spent by AlCl3-intoxicated rats treated by each one of the aforementioned drugs, alone or in combination, to reach food reward.

The AlCl3-induced AD rats exhibited a significantly ($P < 0.05$) more time to reach for the arm where the food reward has been located when compared to NC rats, indicating a more severe spatial recognition memory deficit. On the other hand, the combination of Gb+Vit.C administration to AlCl3-intocxicated rats significantly ($P < 0.05$) reduced time span to reach the food reward in comparison with the untreated rats, indicating an improvement in the spatial recognition memory of the rats. The timespan of rats with cognitive deficit-induced by AlCl3 after six weeks of administration compared to the control group and the donepezil (commercially available drug for treatment of cognitive dysfunction) is illustrated in Figure 2.

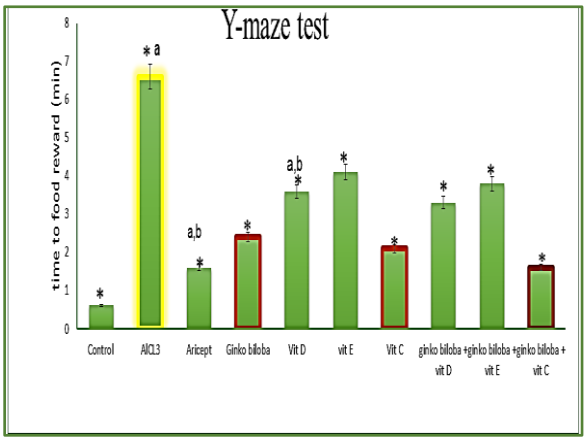


**Figure 2.** Effect of *G.biloba*, vitamin D,E,C and their combinations on short memory and learning ability (as expressed by time to food reward) in Y-maze test in AlCl3- induced AD in rats after treatments for six consecutive weeks. Data are represented as mean ± S.E.M ($n = 6$). a: Significant change at $P < 0.05$ in comparison with normal control (NC) group; b: significant change at $P < 0.05$ in comparison with AlCl3 group, as determined by Tukey's test. Statistical evaluation was done by applying two Way-ANOVA followed by Post-hok, Tukey's test followed by LSD.

*3.2.2. Acetylcholinesterase activity*

Effect of G,biloba, vitamin D,E,C and their combinations on AChE activity in serum and brain of Al-zaheimer induced rats was recorded and data is illustrated in Figure 3&4.

The administration of $AlCl_3$ for four weeks resulted in a significant increase in the AChE activity in both serum and brain with respect to normal group. Meanwhile, administration of G,biloba alone, vitamin C and their combination significantly reduced AChE activity reaching to normal value. These results show that administration of G,biloba along with vitamin C suppressed the increase in AChE activity induced by $AlCl_3$ administration (Figure3).

$AlCl_3$-induced neurotoxicity in rats was associated with a marked decrease in monoamine neurotransmitters levels (Figure4). Norepinephrine, dopamine and serotonin levels decreased significantly after four weeks of $AlCl_3$ adinmistration ($P<0.001$) as compared to normal rats. Treatment with *G.biloba*, vitamin D,E,C and their combinations resulted in a significant elevation($P<0.001$) in the serum levels of neurotransmitters; norepinephrine, dopamine and serotonin as compared to the positive control group. It was noticed that administration of *G.biloba* along with vitamin C resulted in marked elevation of all the three neurotransmitters by 94% compared to the control group (Figure 4).

*3.2.3. Monoamine neurotransmitters*

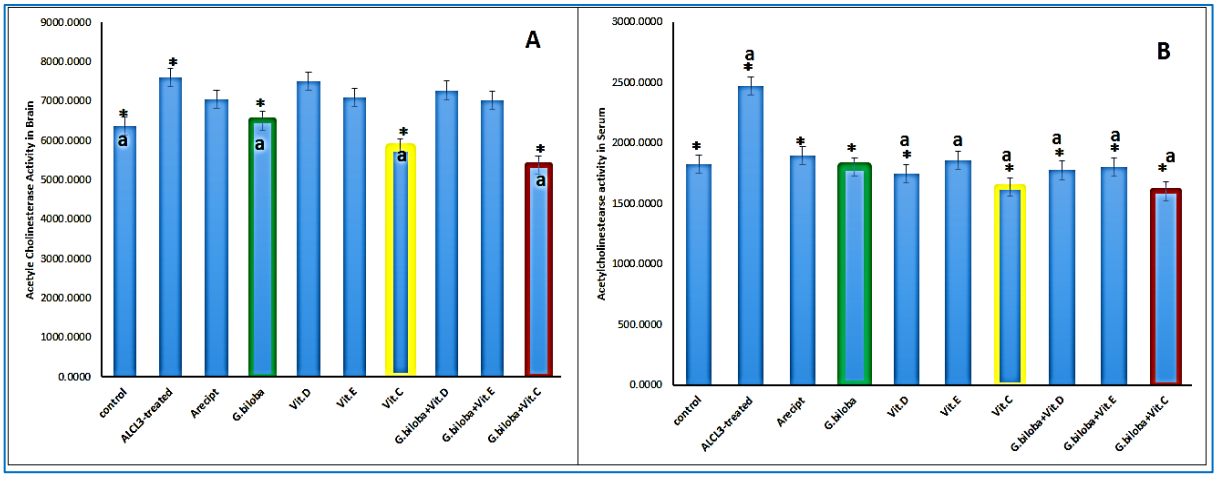


**Figure 3.** Effect of *G.biloba*, vitamin D,E,C and their combinations on *in-vivo* acetylcholinesterase activity in brain tissue homogenate(A) and Serum (B) of Al-zaheimer induced rats. Data are represented as mean ± S.E.M ($n$ = 6). a: Significant change at $P < 0.05$ in comparison with normal control (NC) group as determined by Tukey's test. Statistical evaluation was done by applying One way-ANOVA followed by Post-hok, Tukey's test followed by LSD.

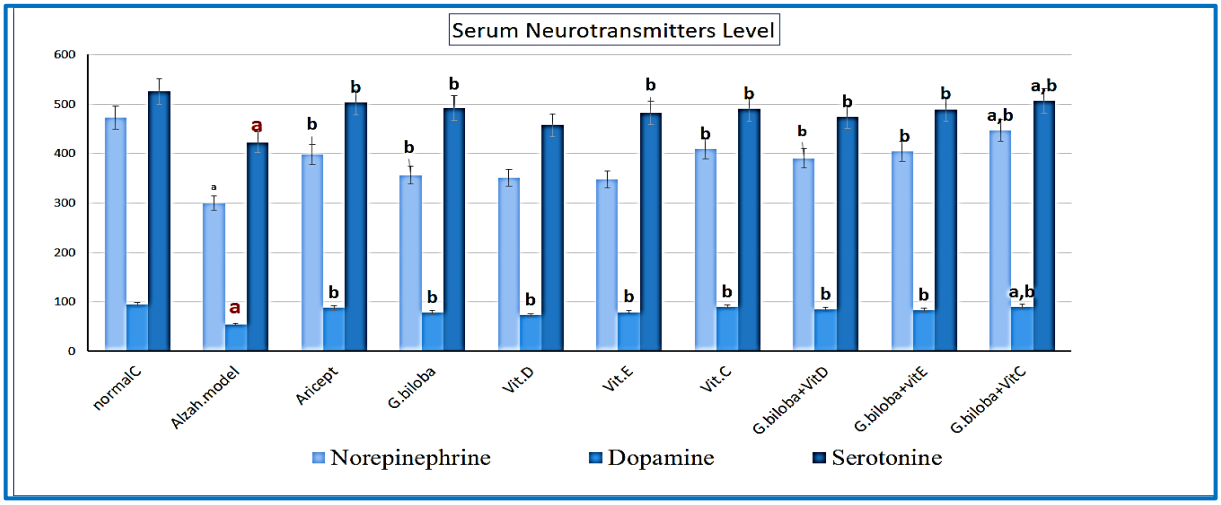


Figure 4. Effect of *G.biloba*, vitamin D,E,C and their combinations on monoamine neurotransmitters level in serum of AlCl3-induced AD rats. Data are represented as mean ± SEM. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-LSD test for multiple comparisons. (n =6). (a)Significantly different from normal control group at $P < 0.05$. (b)Significantly different from positive control group at $P < 0.05$.

### *3.3. Histopathological examinations:*

***The effect of G.biloba, vitamin D,E,C and their combinations on histopathological features induced by $AlCl_3$ in rats***

The normal control group manifested normal histological structures. Normal neurons with round nuclei were observed (Figure 5). On the other hand, the administration of $AlCl_3$ resulted in gross histopathological alterations, including the pyknosis of the nuclei, the neurodegeneration in the cerebral cortex, hippocampus, and striatum as well as the formation of Aβ plaques and neurofibrillary tangles (Figure 6). The administration of *G.biloba*, vitamin C and their combination improved such histopathological features in the subiculum in the hippocampus, and the striatum. On the other hand, such treatment did not attenuate the neurodegeneration in the cerebral cortex, and the fascia dentata in the hippocampus, in addition, pyknotic nuclei were observed in both regions (Figure8,9 and 10). The administration of vitamin D and E had no significant effect on the pyknotic nuclei and the degenerated neurons in the cerebral cortex and the subiculum in the hippocampus (Figures 11-14). The supplementation of $AlCl_3$-induced rats with the combination of *G.biloba* and vitamin C significantly improved histopathological alterations and hippocampal volume and morphology , restoring the normal architecture of the brain (Figure 10).

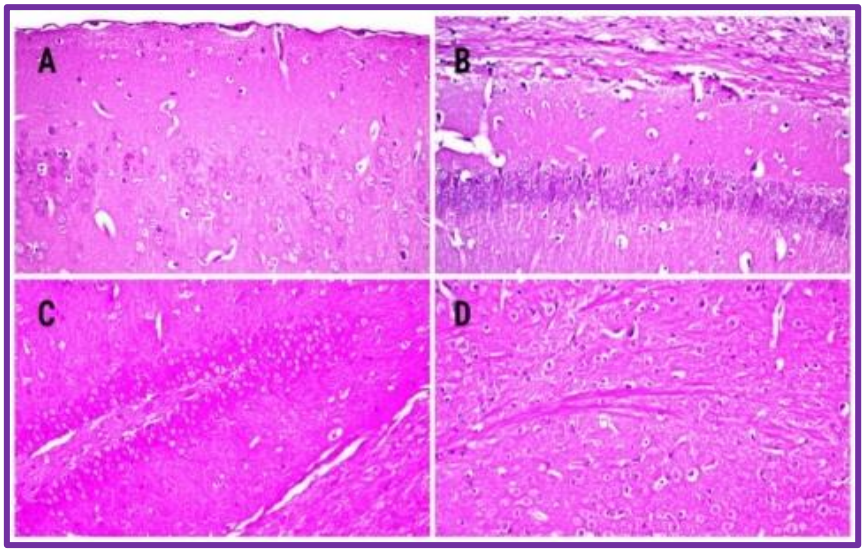


Figure 5: Brain histopathology of group 1: normal control group: (A) Cerebral Cortex ,(B)Subiculum in Hippocampus, (C)Fascia Dentata and Hilus in Hippocampus, (D) Striatum . (H&E staining, x10, scale bar=50μm)

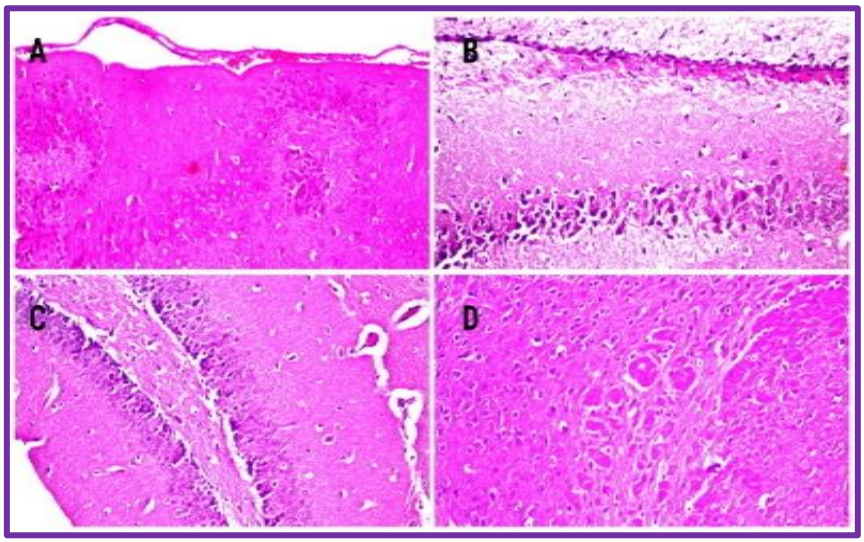


Figure 6: Brain histopathology; group 2:negative control group; rats intoxicated with AlCl3 (17 mg/kg b.wt.); Cerebral Cortex (A), Subiculum in Hippocampus (B), Fascia Dentata and Hilus in Hippocampus (C), Striatum (D). AlCl3-induced extensive neuronal vaculation and necrosis of the cerebral cortex. The hippocampus showed extensive nuclear pyknosis and degeneration . Striatum displayed multiple focal eosinophilic plagues formation with loss of the neurons. (H&E staining, x10, scale bar=50μm).

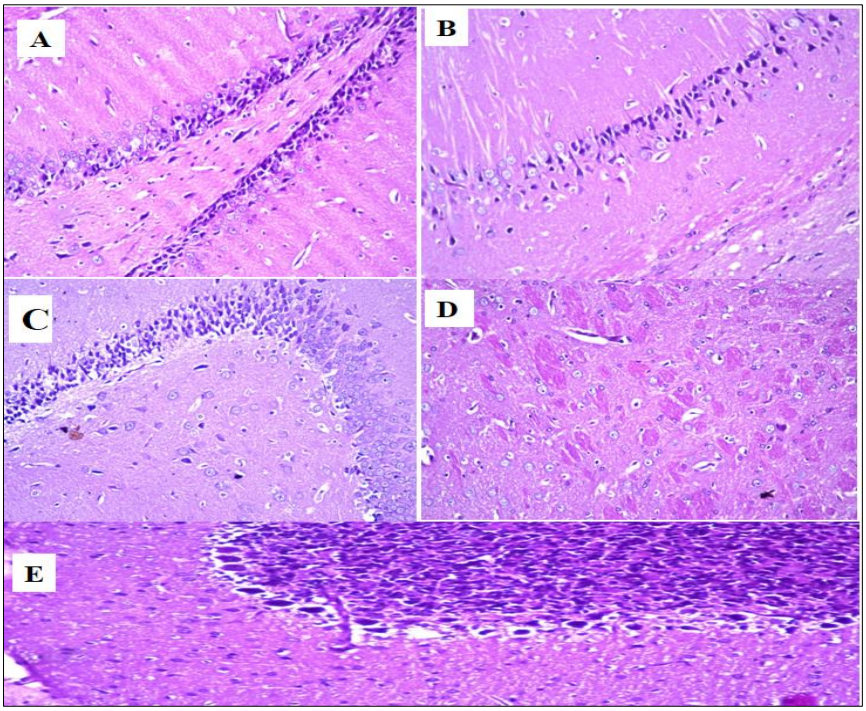


**Figure 7:** Brain histopathology of Group 4:group of rats experimentally intoxicated with AlCl3 (17 mg/kg b.wt.) and treated by Genko Biloba alone (400mg/kg). Cerebral cortex: There was no histopathological alteration (A)Hippocampus, Subiculum: There were nuclear pyknosis and degeneration in some neurons (B). Fascia Dentata and Hilus: Nuclear pyknosis and degeneration were observed in some few neurons (C). Stratum: Multiple focal eosinophilic plagues formation were detected (D). Cerebrellum: There was no histopathological alteration (E). (H&E staining, x10, scale bar=50µm)

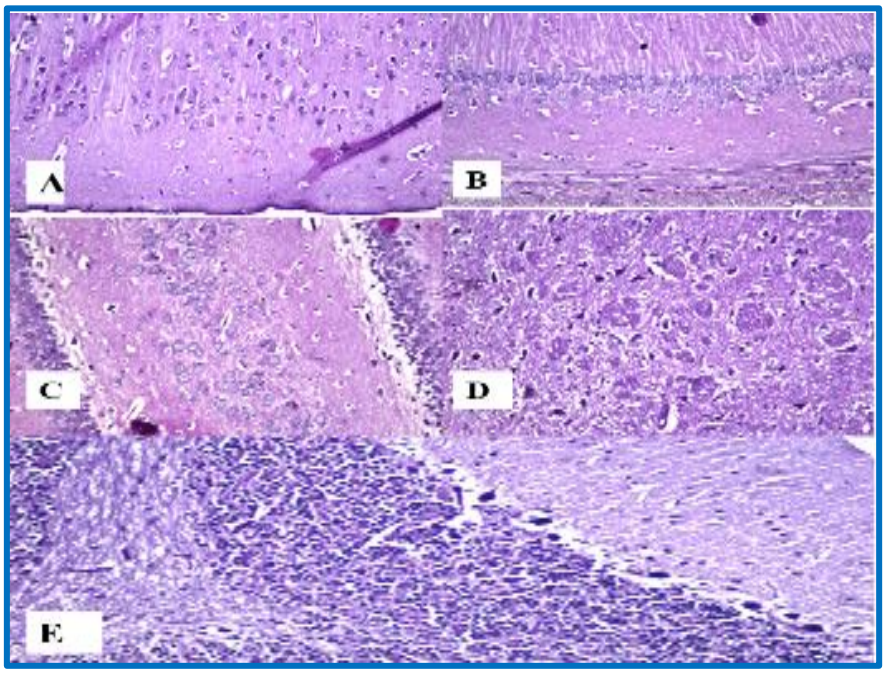


Figure 8:Brain histopathology of group 3:positive control group; rats experimentally intoxicated with AlCl3 (17 mg/kg b.wt.) and treated by reference drug(Aricept): cerebral cortex (A), subiculum in hippocampus (B), fascia dentata and hilus in hippocampus (C), striatum (D) and cerebellum (E). (H&E staining, x10, scale bar=50µm). Cerebral cortex and Subiculum in hippocampus showed no histopathological alteration. Most neurons of the Fascia dentata and hilus in hippocampus showed nuclear pyknosis and degeneration. The striatum showed multiple focal eosinophilic large plagues formation with loss of the neurons was noticed. The cerebellum recorded no histopathological alteration.

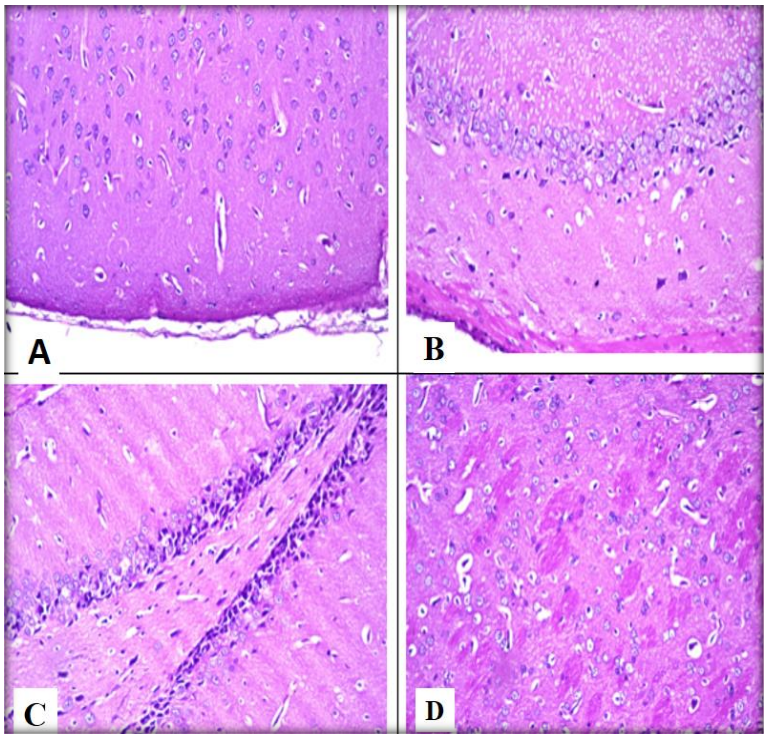


Figure 9: Brain histopathology of group (7); group of rats experimentally intoxicated with AlCl3 (17 mg/kg b.wt.) and treated by vitamin C: (A) Cerebral Cortex :there was no histopathological alteration. (B) Hippocampus:= subiculum nuclear pyknosis and degeneration were detected in most of the neurons. (C)Fascia Dentata and Hilus there were nuclear pyknosis and degeneration with atrophy in the neurons. (D)Striatum : nuclear pyknosis and degeneration were detected in the neurons). there was no histopathological alterations. (H&E staining, x10, scale bar=50μm)

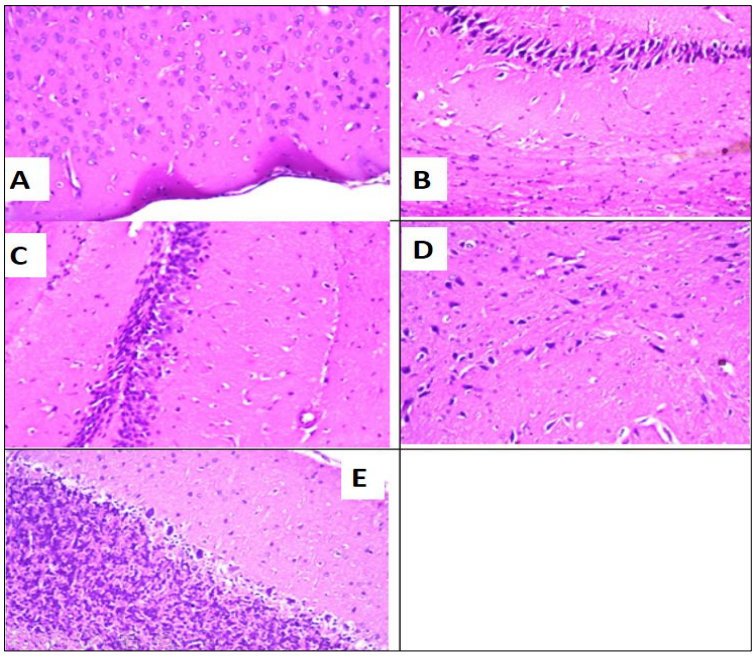


Figure 10 : Brain histopathology of group (10); Group of experimentally intoxicated with AlCl3 (17 mg/kg b.wt.) and treated by Genko biloba and vitamin C: (A) Cerebral Cortex :there was no histopathological alteration. (B) Hippocampus, Subiculum: nuclear pyknosis and degeneration were detected in most of the neurons. (C) Fascia Dentata and Hilus there were nuclear pyknosis and degeneration with atrophy in the neurons. (D) Striatum : nuclear pyknosis and degeneration were detected in the neurons). (E) Cerebrellum : there was no histopathological alterations. (H&E staining, x10, scale bar=50μm).

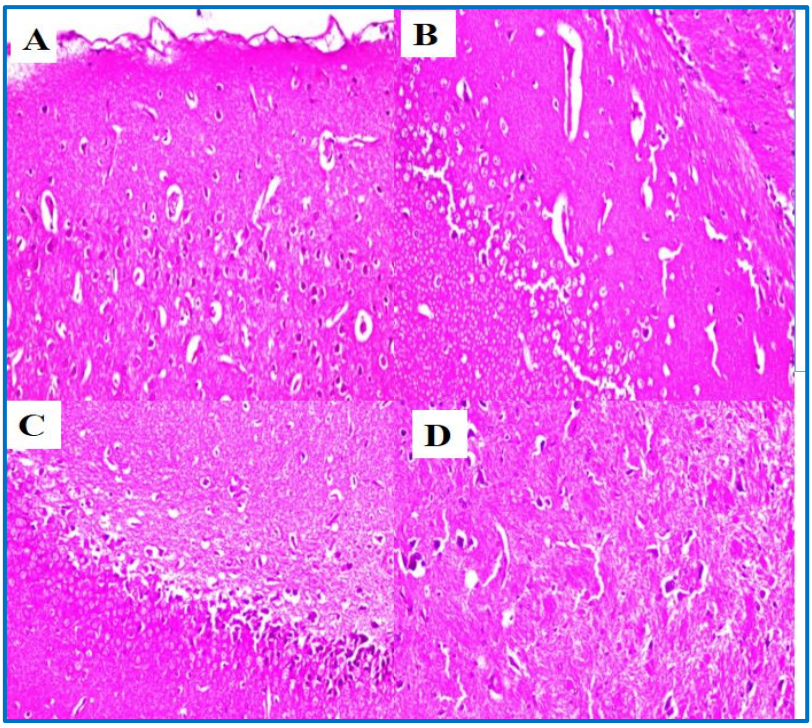


Figure 11: Brain Histopathology of Group (5): group of rats experimentally intoxicated with Alcl3 (17 Mg/Kg B.Wt.) and treated by vitamin D demonstrating (A) Pyknotic nuclei and degeneration in most of the neurons in the Cerebral Cortex, (B) Pyknotic nuclei and degeneration in most of the neurons in the Subiculum In the Hippocampus, (C) Pyknotic Nuclei and degeneration in some of the neurons in the Fascia Dentata in The Hippocampus, and (D) normal histological structure of Striatum. (H&E Staining, X10, scale bar=50µm).

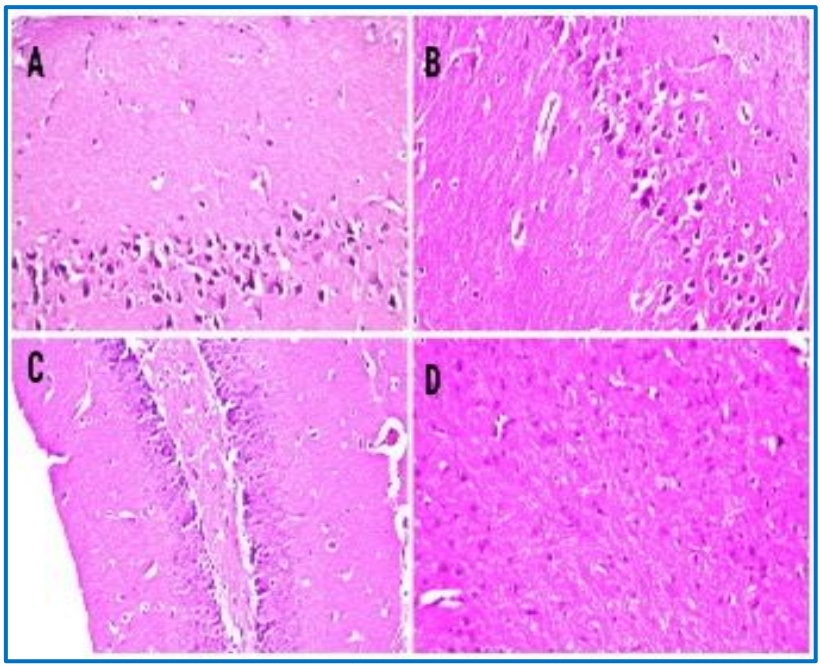


Figure 12: Brain histopathology of group (6); group of rats experimentally intoxicated with AlCl3 (17 mg/kg b.wt.) and treated by vitamin E, (A) Cerebal Cortex is showing only few neural degeneration and nuclear pyknosis after vitamin E treatment, (B) Subiculum hippocampus is showing any detected histological alterations, (C) Fascia Dentata hippocampus is showing degeneration and nuclear pyknosis in most of the neurons, and (D) Striatum has only few neural nuclear pyknosis and degeneration and eosinophilic plaques formation, (H&E staining, x10, scale bar=50µm).

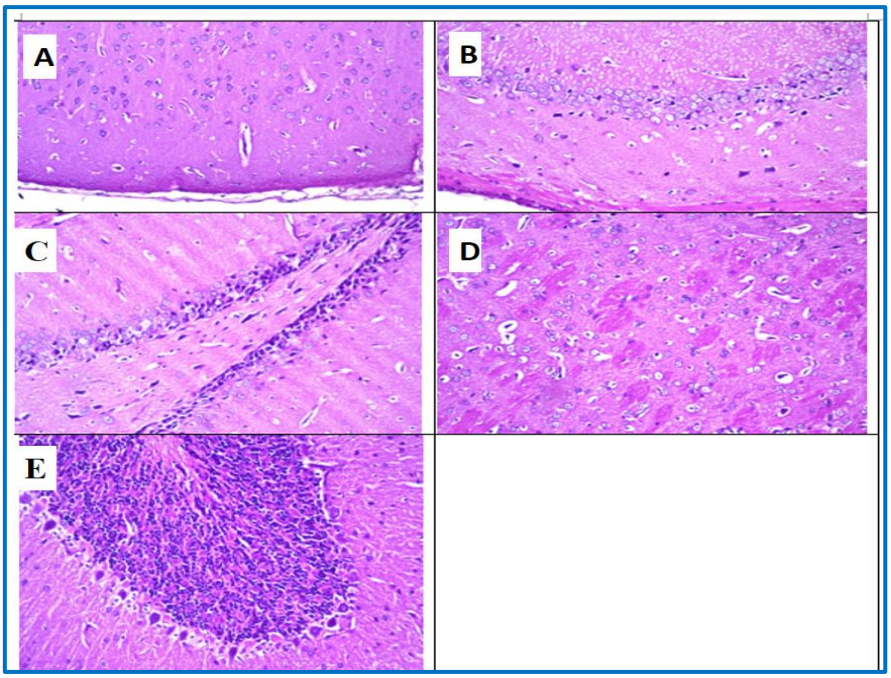


Figure 13:Brain histopathology of group (8) group of rats experimentally intoxicated with AlCl3 (17 mg/kg b.wt.) and treated by Genko biloba and vitamin D :Cerebral Cortex : (A)there was no histopathological alteration (B) Hippocampus :Subiculum there was no histopathological alteration. (C)Fascia Dentata and Hilus nuclear pyknosis and degeneration were detected in most of the neurons . (D)Striatum :there were multiple focal eosinophilic plagues formation. (E)Cerebrellum : there was no histopathological alteration. (H&E staining, x10, scale bar=50μm).

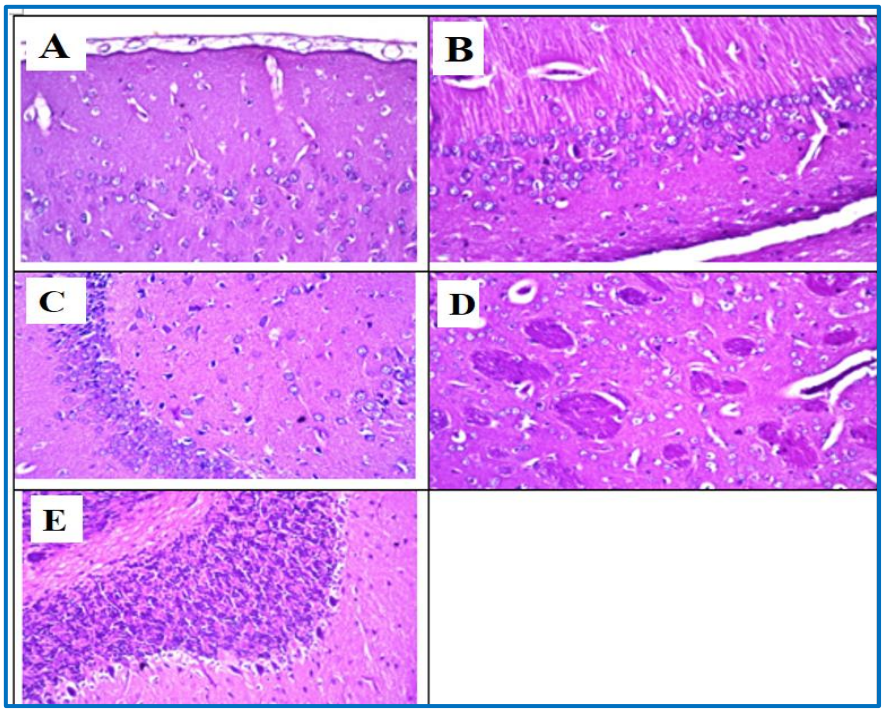


Figure 14: Brain histopathology of group (10); group of rats experimentally intoxicated with AlCl3 (17 mg/kg b.wt.) and treated by Genko biloba and vitamin E : (A) Cerebral Cortex : there was no histopathological alteration. (B) Hippocampus: = Subiculum there was no histopathological alteration.(C) Fascia Dentata and Hilus there was no histopathological alteration .(D)Striatum : multiple focal eosinophilic plagues were detected. (E) Cerebrellum : there was no histopathological alteration. (H&E staining, x10, scale bar=50μm).

## *4. Discussion*

Aging and age-related disorders can be considered as a progressive, inevitable process partially related to the accumulation of endogenous and exogenous free radicals into biomolecules (nucleic acids, lipids, proteins or carbohydrates) due to an imbalance between pro-oxidants and antioxidants in favor of the former (25,26,). A large body of evidence implicates oxidative damage in AD pathogenesis (27). It is believed that oxidative damage to critical molecules occurs early in the pathogenesis of AD and eventually leads to neuropathological alterations (28). The number of patients suffering from Alzheimer's disease (AD) all over the world is rising continually and becomes one of the biggest challenges for most societies throughout the world (29).

Aluminum has been implicated as most important risk factor in aging related changes (30) and particularly in neurodegenerative disease (31).

Al is extensively used in daily life routine as in adjuvanted vaccins, pharmaceuticals, food additives, cosmetics and cookware (32,33). Al has unknown biological function, but as a heavy metal, it is able to cross biological barriers and reach various body fluids. Once entered the cells, it can accumulate in different organs, mainly in the central nervous system causing Alzheimer's disease and dementia in humans (34, 35).

The present study aimed at evaluating the therapeutic potential of *Ginko Biloba*, and/ or Vitamin D, E, C on aluminum chloride ($AlCl_3$)-induced neurotoxicity in rats.

In the present study the aluminum chloride intoxicated rats (AD group) showed significant reduction in Ach, while significant elevation in AchE activities were reported in brain of rats, as well as the microscopic investigation for brain section revealed the presence of amyloid plaques in the hippocampus of rats. The mechanism of aluminum induced neurodegeneration is not clearly known. However, it has been reported that aluminum potentiates the activity of ferrous (Fe2+) and ferric (Fe3+**)** ions to cause oxidative damage leading to neurodegeneration (36). Moreover, aluminum promotes the formation of amyloid-β plaque and aggregation of tau protein in Alzheimer disease (37)

Numerous studies reported that daily oral administration of aluminum chloride AlCl3 (10-100mg/Kg/body weight) elicited cognitive impairments, anxiety and motor deficits in rats following a treatment of 30 days and These aluminum-induced behavioral changes are due to several mechanisms including hippocampal and cortical oxidative stress, *in vivo* neuronal death, neurotransmission disruption (38,39,40,41), cerebral Al-catecholamine complexes generation and amyloid plaques deposition (42).

Most of the AD treatment depends only on the symptomatic treatment of AD through the inhibition of acetylcholinesterase (AChE). It is known that cholinesterase inhibitors extend acetylcholine (ACh) availability after it is released from cholinergic nerve endings (43). However, their limited efficacy, adverse cholinergic side effects in the periphery, narrow therapeutic ranges, and hepatotoxicity are among the several limitations to their therapeutic success (44). Moreover, the drugs could exert several important side effects including diarrhea, nausea, insomnia, muscles cramps, vomiting, fatigue and loss of appetite (45). Thus, considering the fact that the management of AD can be a major challenge for the health care systems, all around the globe many research are now focusing in order to make use of phytopharmaceutical alternatives approaches that are widely available at low costs (46).

*Ginko biloba* leaf extract is widely used in the treatment of age-relative disorders; including failing memory, dementia due to neuronal impairment and deteriorations (47). In recent years, many articles have reported the protective effect of Ginko biloba leaf extract. It has been stated that the standardized leaf extract of Ginko biloba contain mainly flavone glycosides, terpenene lactone, and organic acids (48). The flavone glycosides consist mainly of quercetin, kaempferol, and isorhamnetin (22–27% of extract). The terpene lactones mainly contain ginkgolides and bilobalide(49).These compounds are known to possess high antioxidant activity and may explain the high antioxidant effect of Ginko biloba leaf extract. Also the diverse pharmacological effect is attributed to the synergism between the multi components present in the extract (50.51). This is in agreement with the data obtained in the current research and explains the improvement observed in $AlCl_3$ intoxicated rats treated with either Ginko biloba alone or in combination with vitamin C

Vitamin C (ascorbate) is a vital antioxidant molecule in the brain and participate as a co-factor in several enzymatic reactions including catecholamine synthesis. It was reported that the highest concentrations of ascorbate in the body are found in the brain and neuroendocrine tissues (52). Ascorbate is also considered as a neuromodulator of dopaminergic and cholinergic transmission and related behaviors. Neurodegenerative diseases typically involve high levels of oxidative stress and thus ascorbate has been postulated to play critical therapeutic roles against Alzheimer's disease (53).

It was found that Alzheimer's disease patients have lower plasma and CSF ascorbate levels despite adequate nutritional intake (54). Also, a positive relationship was shown between ascorbate supplement use and reduced disease incidence (55). Nevertheless, there is further evidence to support the use of vitamin C as a potential therapeutic avenue for Alzheimer's disease. Orally administered ascorbate protected the hippocampus in rats against oxidative stress and cytokine release in AD-induced models (56).

Furthermore, ascorbate has been shown to be an effective acetylcholinesterase inhibitor (57), however, the exact mechanism appears to be a boost to cholinergic system functioning, although this needs further investigation. These facts are in agreement with our data as it was observed that treatment with Ginko biloa along with vitamin C resulted in significant improvement in brain neurotransmitter and acetylcholine levels in AlCl3 intoxicated rats.

In animal behavior studies involving ascorbate treatments, it was observed that ascorbate at a dose of (125 mg/kg) reversed memory deficits induced by age (58,59). Another study, reported that ascorbate treatments (60-120mg/kg) either intraperitoneally for 14 days or orally for 30 days improved the food acquisition and item recognition (60).

Spontaneous alternation in a Y maze has been reported to be a reliable, noninvasive test to determine cognitive changes in Wistar rats and a measure of exploratory behavior that reflects spatial working memory that is dependent upon hippocampal function (61). This procedure involves measuring the arm choice over consecutive trials or in one continuous session, based on the assumption that deficits in alternation indicate poor spatial working memory (62). *G.biloba* administration alleviated cognitive status as deduced from behavioral profile in Y maze. Results revealed that $AlCl_3$- intoxicated rats displayed dementia and retarded learning ability whereas the administration of *G.biloba* , vitamin C and their combination resulted in the improvement of memory loss and spatial recognition as expressed by latency time. Our data is in line with a previous study that reported the protective and ameliorative effect of Ginko biloba and vitamin C against neurotoxicity in rats(63).

It is reported that cognitive processes such as concentration and learning have been related to biogenic amine neurotransmitters; norepinephrine, dopamine and serotonin. Several studies have shown that levels of brain neurotransmitters have decreased in AD. Administration of $AlCl_3$ impaired multiple neurotransmitter system via serotonergic and dopaminergic system (20,28). The present study showed that oral treatment of rats with *G.biloba* (400 mg/kg) and vitamin C (50mg/kg) and their combination for six weeks exhibited an increase in the serum levels of norepinephrine, dopamine and serotonin. On the other hand, the elevated activity of AChE leads to increased degradation of acetylcholine (Ach) neurotransmitter which in turns declines the ACh pool in the brain which is essential in learning and memory. $AlCl_3$ administration amplifies the AChE activity which is one of the major causes for the cholinergic deficit occurrence after its administration (64,65). In this study we found that treatment with *G.biloba* extract significantly reduced the AChE activity in rats' brains as compared to the $AlCl_3$ treated animals. It reveals that inhibition of AChE activity by *G.biloba*, vitamin C and the combination had a protective role in acetylcholine degradation and improved the cholinergic neurotransmission.

Thus, the *G.biloba* along with vitamin C reduced the cholinergic deficits produced by $AlCl_3$ administration resulting in an enhanced neuroprotective effect. Inhibition of acetylcholinesterase (AChE) is currently the most reliable strategy for the treatment of AD, senile dementia, ataxia, and Parkinson's disease (1).

The oral administration of $AlCl_3$ (17 mg/kg, p.o.) for four weeks resulted in extensive neuronal vacuolation and necrosis of the cerebral cortex, extensive nuclear pyknosis and degeneration of the hippocampus and the formation of multiple focal eosinophilic plagues in the striatum. The histopathological examination of the brain tissues from different sections revealed improvement in the cerebral cortex, the hippocampus and striatum of the *G.biloba* treated group with minor nuclear pyknosis and less plagues compared to the reference drug;*Aricept.*

The present study results revealed that Ginkgo biloba along with vitamin C extract improves behavioral capability, learning and memory functions and heals hippocampal neuronal toxicity induced by AlCl3 administration. The animals pretreated with ALCl3for 30 days showed memory deficits indicated by prolonged time to food reward. The vitamin C and G. biloba have antioxidants properties that counteracted the adverse effects caused by the aluminium chloride intoxication.

### *5. Conclusion*

In sight of the aforementioned findings, *G.biloba* and vitamin C combined treatment of AlCl3-induced neurotoxicity in rats represent a new therapeutic candidates for neurodegenerative disorders specifically AD. It was also observed that *G.biloba* and vitamin C combination exerts its action through the modulation of cholinergic and monoamine neurotransmission as well as through antioxidant pathways.

- **Competing interests**

The authors declare that they have no competing interests.

- **Ethics approval**

The study was approved *for the use of animals by the Medical Research Ethics Committee, National Research Centre, Dokki, Egypt*

- **Data Availability:**

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

- **Authors Contributions**

S.M.H[1]: conceived the original idea, planned the experiments, contributed to the interpretation of the results, supervised the project. A.M.[2], E.A. A[2], H.A. Kh[2], M.G. I[2], N.T. S[2], R.A. A[2] and Y.W. A[2]: Planned and carried out the experiments, contributed to sample preparation, Formal analysis and Resources. Z.A. E[3*]: contributed to planning of experiment and interpretation of the results. took the lead in writing the manuscript and Editing. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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