Original Research Article

Ginkgo biloba Ameliorates Aluminum Induced Neurotoxicity in Rats

Abstract

Ethnopharmacological relevance

Ginkgo is a large tree with fan-shaped leaves. The leaves are often orally taken by individuals with memory deficits such as Alzheimer's disease and to improve blood flow to the brain in older people.

Aim of the study

We evaluated the protective effects of Ginkgo biloba against aluminum chloride ($AlCl_3$)-induced neurotoxicity

Study design

Eighty male albino rats were divided into four main groups (n = 20 per group) and provided with varying doses and combinations of AlCl3 and/or Ginkgo biloba (GB) in drinking water, DW. The treatments were administrated daily for 12 weeks.

Results

Ginkgo biloba extract caused a significant increase in brain neurotransmitters contents [Norepinephrine (NE), Serotonin (5-HT) and Dopamine (DA)] of intoxicated adult male albino rats. The plant extract also improved aluminum induced disruption of tissue architecture and significantly reduced DNA damage as indicated by reduction in different comet assay parameters in the brain of intoxicated rats during the entire experimental period.

Conclusions

Gingko biloba has protective effects against aluminum-induced neurotoxicity. Its mechanisms of action appears to be mediated by increasing monoamine neurotransmitter synthesis, and improving the integrity of DNA and tissue architecture in the brain.

Keywords

Aluminum chloride**;** Ginkgo biloba**;** Neurotoxicity; Neurotransmitter

1. Introduction

Aluminum is considered most toxic in its soluble ionic form [1].. It enters the human body at all developmental stages of life [2]. Although, the highest concentrations are found in young rats than old rats [2], aluminum has been associated with neurobehavioral changes in mammals. Chronic exposure to aluminum ions leads to mood changes, dysmnesia, convulsions, and muscular weakness. The preferred accumulation sites are bones, spleen, liver and lungs [3] and exposure causes tissue oxidative stress. The latter involves alterations in antioxidant enzymes activity and generation of reactive oxygen species [4, 5] and reduced mRNA expression of endogenous antioxidants [6].

Other pathological effects of aluminum include induction of DNA fragmentation [7], and lesions in the brain such as neuronal degeneration and hemorrhage [8] and pericellular edema [9]. Aluminum also increases lipid peroxidation and interferes with normal metabolism and distribution of minerals. It displace biologically important cations such as calcium, iron, zinc, copper and magnesium from their binding sites [10]. The neurotoxic effects of aluminum are well documented in human and experimental animals [11].

The leaves and seeds of Ginkgo biloba contain bioactive compounds such as flavonoid and terpenoid that have neuroprotective effects and therapeutic roles

against many neurodegenerative disorders [12]. The organic acid extracts of the plant such as kynurenic, hydroxykynurenic, and vanillic have antioxidant, anti-allergic, anti-inflammatory, anti-tumorigenic, anti-anxiety, anti-carcinogenic effects [13]. Ginkgo biloba extract (EGb 761) is viewed as a polyvalent agent with a doable therapeutic use within the treatment of neurodegenerative diseases of complex origin, e.g. Alzheimer's disease (AD) EGb 761 has potential effectiveness against toxicity induced by β-amyloid (Aβ) derived peptides (Aβ25−35, Aβ1−40 and Aβ1−42) on hippocampal primary cultured cells, this space being severely affected in AD. These results recommend that the neuroprotective effects of EGb 761 [14]. The effects of EGb 761 on the CNS underlie one among its major therapeutic indications i.e., people plagued by deteriorating cerebral mechanisms associated with age-associated impairments of memory, attention and different psychological feature functions. EGb 761 is presently used as symptomatic treatment for cerebral insufficiency that happens throughout traditional ageing or which can result to chronic degenerative dementia, vascular dementia, and for neurosensory disturbances. Depressive symptoms of patients with illness {Alzheimer's} disease (AD) associated aged non-Alzheimer patients may reply to treatment with EGb 761 since this extract has an anti-stress result. Basic and clinical studies, conducted each in vitro and in vivo, support these useful neuroprotective effects of EGb. EGb 761 has many major actions it improves blood natural philosophy and tissue metabolism, and opposes the prejudicial effects of anemia. In animals, EGb 761 possesses inhibitor and free radical-scavenging activities, it reverses age-related losses in brain alpha1-adrenergic, 5-HT1A and muscarinic receptors, protects against anemia somatic cell death, preserves the operate of the hippocampal mossy fiber system, will increase hippocampal high-affinity B-complex vitamin uptake, inhibits the down-regulation of hippocampal corticoid receptors and enhances somatic cell malleability known chemical constituents of EGb 761 are related to bound actions. Each flavonoid and ginkgolide constituent's area unit

concerned within the free radical-scavenging and inhibitor effects of EGb 761 that decrease tissue levels of reactive oxygen species (ROS) [15]. Neuroprotective effects of Ginkgo biloba in central nervous system include protection of neurons against ischemia, free-radical-induced apoptosis, and preservation of hippocampal mossy fibers and neural plasticity, and prevention of cognitive deficits subsequent to traumatic brain injury and stress [16, 17]. Administration of Ginkgo biloba extract is also associated with improved spatial memory and changes in the neurotransmitter levels in several regions of the brain [18]. The plant is also neuroprotective against several neuronal insults [19], promotes regeneration and survival of neural tissue [20, 21].

The antioxidant activity of ginkgo biloba is associated with caspase-3 activation [22]. Its neuroprotective effects are expressed through inhibition of monoamine oxidase (MAO) A and B in the presence of kaempferol and subsequent increased in the levels of serotonin, noradrenaline (NA), and dopamine (DA) increased in the brain [23, 24, 25]. Additional protective effects of the plant extract against age-related memory impairment may be associated with the inhibition of β-amyloid peptide production, , lowering free cholesterol levels, acceleration of acetylcholine release, and modulating neurotransmitter receptors of the central nervous system [12,26, 27, 28]. Cells are permeable to Ginkgo biloba extracts; hence, the extracts have cytoprotective effects at both nuclear and cytoplasmic levels [29].

The objective of the present study was to determine aspects of the mechanisms of aluminum-induced neurotoxic effects and if such effects could be ameliorated by Ginkgo biloba.

2. MATERIALS AND METHODS

A. Chemicals and Diagnostic kits:

Aluminum in the form of anhydrous aluminum chloride (AlCl3) was purchased from Al Gomhuria Company, Egypt. Ginkgo biloba extract in a powder form was

obtained from Xiamen Forever Green Source Biochem Tech. Co., Ltd. (FGS). China. All chemicals used for estimation of amine levels were analytical grade.

Full botanical plant names

Family: *Ginkgoaceae* Engl.

Genus: *Ginkgo* L.

The plant list → *Gymnosperms* → *Ginkgoaceae* → *Ginkgo* → *Ginkgo biloba L.*

Product name	Ginkgo biloba Extract
Latin Name	*Ginkgo biloba Linn.*
Active ingredients	Ginkgolic Acid, Lactone, Flavone
Appearance	Brown fine powder
Part used	Leaf
Specification	24% Ginkgoflavoglycosides; 6% Terpene lactones; Ginkgolic acid < 5ppm <24/6, Ginkgolic Acid 1ppm max, USP>, <10:1 TLC (Water-soluble)>, <24/6, Ginkgolic Acid 1ppm max, CP2010>, <24/6, Ginkgolic Acid 5ppm max, DAB10>, <Flavone 24%Min,Lactone 6%Min HPLC，USP/EP>, <Flavone 24%Min,Lactone 6%Min HPLC，Ginkgolic Acid 5ppm max，CP05>,< Flavone 24%Min,Lactone 6%Min HPLC，CP05>
Test Method	HPLC, TLC

B. Animals

We used 80 male Wister albino rats (weighting 100-120 gm). Animals were purchased from Al-Zyade experimental animal production center, Giza, Egypt. During the experiment, they were housed in polyethylene cages, with stainless steel wire lids (bedded with wood shavings), and kept at room temperature (20-25 °C) and under 12 h light/dark cycle. Balanced ration diet and water were supplied ad libitum. The study was approved by the Research Ethical Committee of the Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Egypt. The initial 10 days were used to quarantine the animals and as period of acclimatization.

C. Experimental design

Rats were randomly divided into four experimental groups consisting of twenty animals each (n = 20). The specific treatments are:

Group I (Control): Rats were given tap water and feed ad libitum throughout the experiment and kept as a control.

Group II (Aluminum group): Rats received aluminum chloride ($AlCl_3$) in drinking water at a concentration of 1.43 g/L (290 mg/L Al) for 12 weeks. This corresponds to a dose of 40 mg /kg B.W [31].

Group III (Ginkgo group): Rats were supplemented with Ginkgo biloba extract at dose of 100 mg/kg body weight [32] dissolved in D. W. daily for 12 weeks.

Group IV (Aluminum-Ginkgo group): Animals were given Ginkgo biloba extract at dose of 100 mg/kg (dissolved in D. W.) orally daily, together with aluminum chloride at concentration of 1.43 g/L (290 mg/l Al) in drinking water for 12 weeks.

D. Tissue sampling:

Ten rats were sacrificed from each group after six and twelve weeks. Fresh brain tissues were immediately washed in saline and divided into 3 parts: one part was kept in PBS (phosphate buffered saline) and then stored at -80 C for Comet assay, the second part was stored at -80 C for estimation of monoamine contents (Serotonin, Norepinephrine and Dopamine), and the third part was kept in 10% neutral formalin for the histopathological examination

I-Estimation of brain neurotransmitters

- Brain tissue sample weighing ≤300 mg was homogenized in 3 ml of cold acidified N-*butanol* ; [33]. Dopamine, norepinephrine and serotonin (5-HT) levels in the forebrain were estimated using the fluorometric method [34].

II- Comet assay (Single cell gel electrophoresis)

Slides were prepared by cleaning in methanol and burning over a blue flame. They were then immersed in hot 1.0% normal melting agarose (NMA) and air-dried before storing at room temperature. To isolate cells, a small piece of brain tissue was placed in 1 ml cold HBSS containing 20 mM EDTA and 10% DMSO. The piece was minced into fine pieces, and the Pellet? resuspended in 1% low melting point agarose (LMPA). A 10 µl suspension containing about 10,000 cells was placed on a slide and subjected to cell lysis and electrophoresis. The slides were subsequently stained with Ethidium bromide [35]. The fluorescent stain was visualized (magnification 400 x) using an automated fluorescence microscope and the images were captured on a computer, equipped with Comet Score software (Komet IV). Three parameters were adopted as indicators of DNA damage: Tail

length (TL; length of DNA migration), the percent of DNA in the comet tail (% Tail DNA) and Tail moment (TM) [36].

III- Methods used for histopathological study:

Brain tissue samples intended for histopathological investigation were fixed in 10 % neutral formalin, and then embedded in paraffin. After deparafinnization, tissue sections that were 5-µm in thickness were prepared and stained by Haematoxyline and Eosin staining [37] for subsequent evaluation.

IV- Statistical analysis:

Data were analyzed by using a one-way analysis of variance (ANOVA). Duncan's post hoc test was used to determine the significant differences between treatment means. The differences between means were considered statistically significant at $P \leq 0.05$.

3- Results:

3.1. Brain neurotransmitters:

The effects of AlCl3 or/and Ginkgo biloba on norepinephrine, serotonin and dopamine levels are shown in Table 1. Levels of norepinephrine, serotonin and dopamine in the forebrain of the rats were significantly decreased ($p<0.05$) in AlCl3 administrated rats (2nd group) as compared with control group (1st group) after 6th and 12th week. Ooral administration of Ginkgo alone (3rd group) or with AlCl3 (4th group) elevated norepinephrine, serotonin and dopamine levels in the brain of adult male albino rats significantly as compared to aluminum treated rats (2nd group) after 6th and 12th week but still lower than control group (1st group).

Table (1): Effect of $AlCl_3$ and Ginkgo biloba on norepinephrine (NE), dopamine (DA) and serotonin (5-HT) level in the brain of four groups of adult male albino rats (n= 10rat/group)

Treatment groups	NE		DA		5-HT	
	6W	12W	6W	12W	6W	12W
I	0.70±0.01[a]	0.67±0.01a	0.86 ± 0.01a	0.87 ± 0.01[a]	0.38±0.01[a]	0.35±0.01[a]
II	0.56±0.01[c]	0.51±0.01[c]	0.71 ± 0.01c	0.70 ± 0.02[c]	0.28±0.01[c]	0.26±0.01[c]
III	0.66±0.01[ab]	0.63±0.01[ab]	0.87 ± 0.01a	0.85 ± 0.02[a]	0.36±0.003[ab]	0.33±0.001[ab]
IV	0.63±0.02[b]	0.61±0.02b	0.82 ± 0.02b	0.80 ± 0.02b	0.34±0.01[b]	0.31±0.02[b]

-Mean value ± SE

-The mean difference is significant at p <0.05-The values in the same raw carrying different letters were significantly different.

3.2. Effect of $AlCl_3$ or/and Ginkgo biloba on DNA damage observed by comet assay in the brain of adult male albino rats

- The effects of AlCl3 or/and Ginkgo biloba on DNA damage observed by comet assay assessed as (Tail length (TL), %DNA in tail and Tail moment (TM)) in the brain cells of adult male albino rats are presented in **Table (2)**. Administration of $AlCl_3$ to rats of the 2nd group significantly increased DNA damage index observed by different comet assay parameters as compared with control group (1st group) after 6th and 12th week. Oral administration of Ginkgo biloba alone (3rd group) or with $AlCl_3$ (4th group) significantly reduced $AlCl_3$ induced DNA damage as indicated by reduction in some comet assay parameters after 6th and 12th weeks.

Table (2): Effect of $AlCl_3$ and Ginkgo biloba on DNA damage observed by comet assay in the brain of four groups of adult male rats (n=10rats/group)

Treatment groups	Tail length		%DNA in tail		Tail moment	
	6w	12w	6w	12w	6w	12w
I	0.57±0.07[b]	0.51±0.07[b]	1.40±0.33[c]	1.12±0.29[b]	0.01±0.002[b]	0.01±0.001[b]
II	4.37±0.99[a]	5.14±0.98[a]	14.99±1.28[a]	16.81±1.99[a]	0.64±0.15[a]	0.89±0.25[a]
III	0.72±0.08[b]	0.67±0.09[b]	1.50±0.31[b]	1.64±0.37[c]	0.01±0.001[b]	0.01±0.003[b]
IV	1.65±0.41[b]	1.39±0.43[b]	5.15±0.86[b]	3.69±0.87[b]	0.11±0.004[b]	0.06±0.002[b]

-Mean value ± SE

-The mean difference is significant at p <0.05

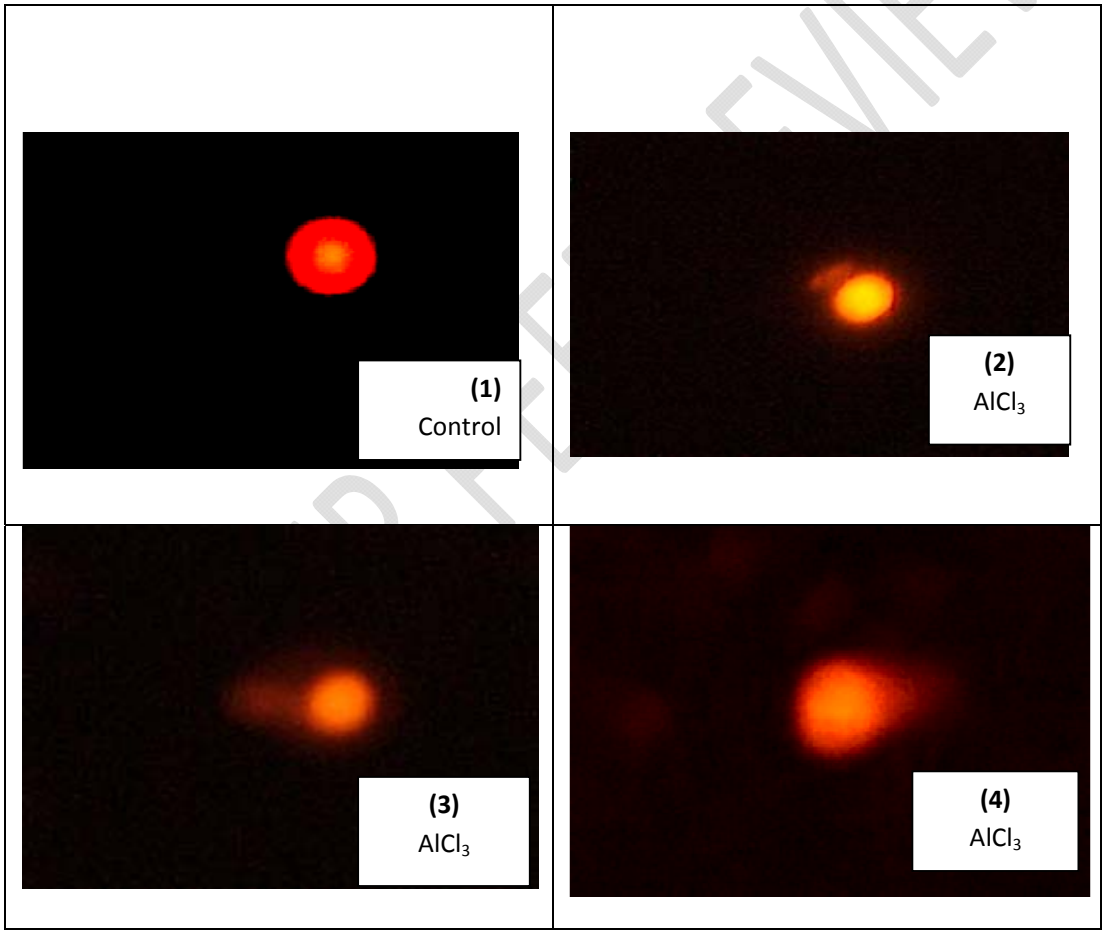
-The values in the same raw carrying different letters were significantly different.

3.3. Effect of $AlCl_3$ or/and Ginkgo biloba on DNA damage observed by photomicrographs of comets in the brain cells of adult male albino rats:

The Comet assay results of $AlCl_3$ and or Ginkgo biloba observed by photomicrographs in different experimental groups are shown in Figures 1 to 16. Undamaged DNA is recognized as a fluorescent core while the presence of strand breaks in the chain (damaged DNA) causes DNA to migrate and form a tail comet during the electrophoresis. There was no DNA damage in brain of control (**Fig. 1 & 9**). Rats in 2nd group intoxicated with $AlCl_3$ showed severe DNA damage in the

brain cells after 6th and 12th week **(Figures 2, 3, 4, 10, 11 and 12).** No DNA damage was resulted in **Gingo-treated rats** after 6th and 12th week by microscopic examination **(Figure 5 and 13).** Oral administration of Ginkgo biloba along with exposure to $AlCl_3$ (4th group) showed slight DNA damage in the brain after 6th and 12th week **(Figure 6, 7, 8, 14, 15 and 16).**

Fig. 1-8. Photomicrographs of comets in the brain cells stained with ethidium bromide in different experimental groups after 6th week (x400)



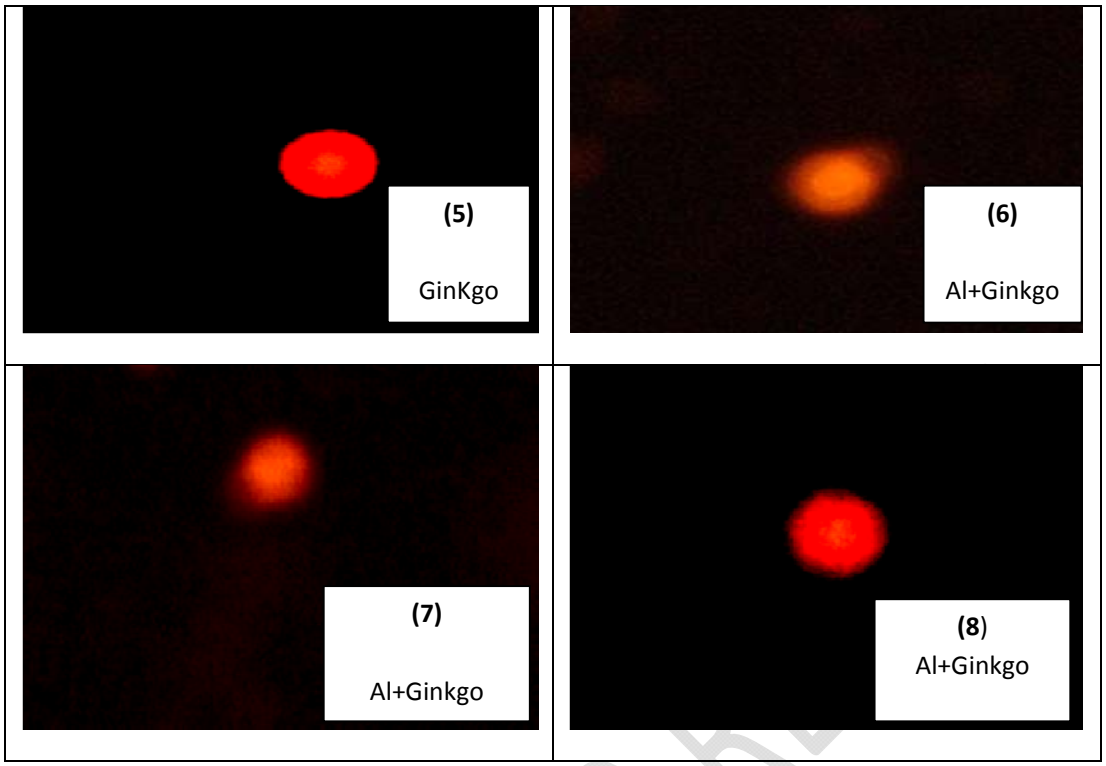


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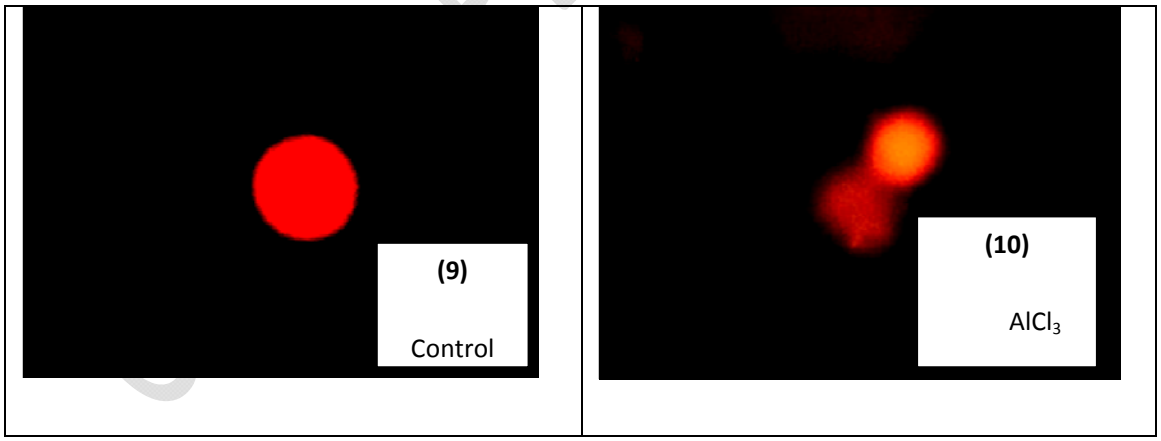
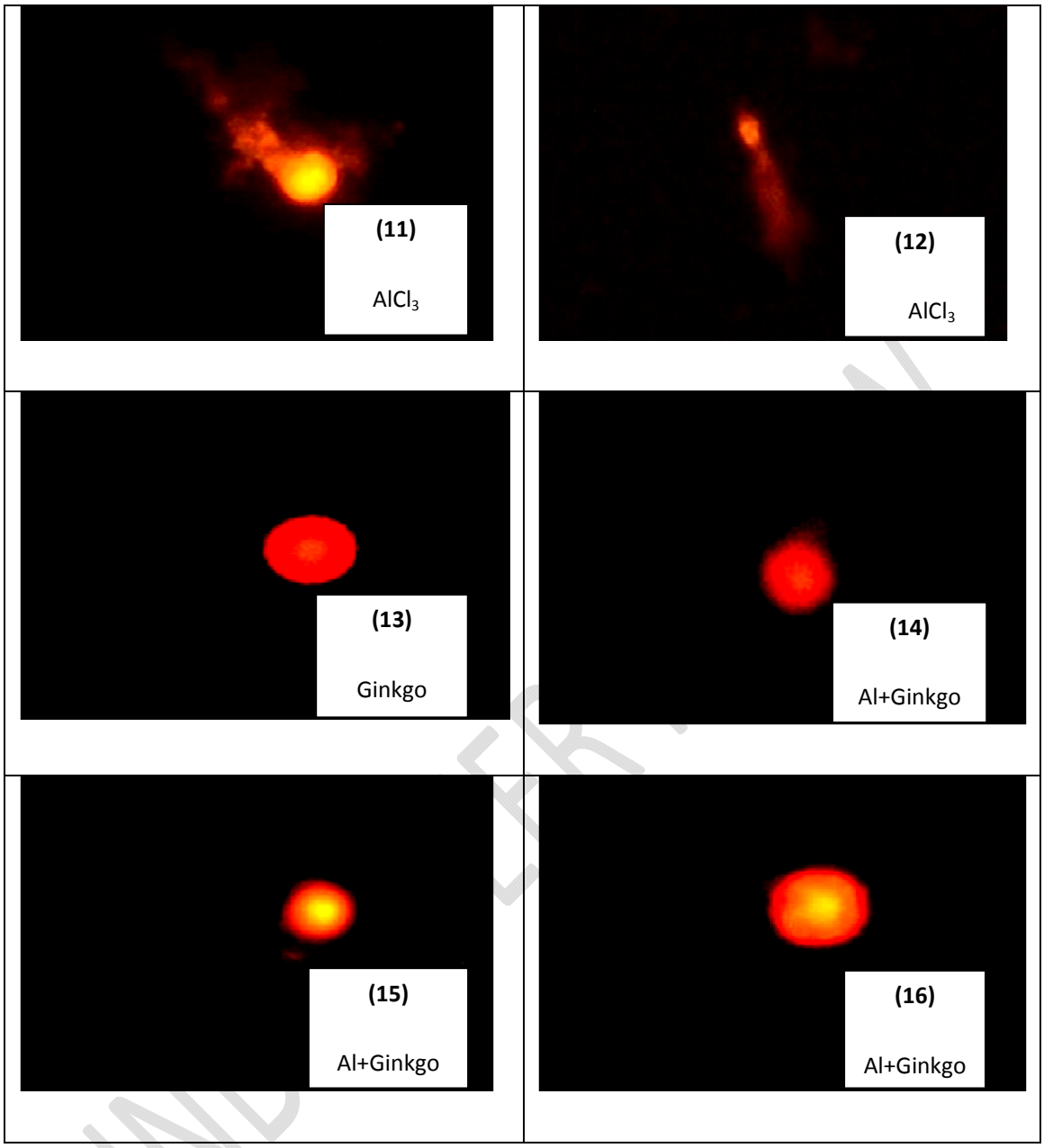
Fig. 9-16. Photomicrographs of comets in the brain cells stained with ethidium bromide in different experimental groups after 12th week (x400)





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3.4.2. Effect of $AlCl_3$ and Ginkgo biloba on the brain histoarchitecture

248 Within 6 weeks, Aluminum induced alteration in brain histoarchitecture.
249 Neurons with cork screw shaped neurofibrillary tangles were
250 characteristically demonstrated in cerebral cortex **(Fig. 17).** It caused
251 neurodegenerative lesions consisting of deposition of abundant amyloid
252 plaques particularly in the cerebrocortical **(Fig. 18)** and hippocampal regions

(Fig. 19) associated with neuronal degeneration and proliferation of glia cells **(Fig. 20).** Brain of aluminum chloride treated rat for 6 weeks showing cork strew shaped neurofibrillary tangles **(Fig. 21).** Other frequently demonstrated lesions were degeneration of pyramidal nerve cells **(Fig. 22)** and intense inflammatory reactions associated with focal gliosis **(Fig. 23)** as well as cerebral hemorrhage **(Fig. 24)**.

Brain showed normal neuronal cells with large round nuclei after 6 weeks **(Fig. 25).** After 12 weeks of aluminum treatment, the brain had more deposition of amyloid plaques associated with congestion of cerebral blood vessels, perivascular cuffing, glia cells and neuronal degeneration **(Fig. 26).** After 6 and 12 weeks with less frequent cerebral hemorrhage and decreased frequency of amyloid plaque deposition after 12 weeks **(Fig. 27).** Cerebral blood vessels in most examined sections revealed intravascular aggregation of leukocytes with perivascular edema and cuffing with glia cells **(Fig. 28).** Focal cerebral tissue necrosis associated with reactive gliosis was also demonstrated **(Fig. 29)**.

Histopathological examination of brain aluminum and Ginkgo treated rats showed improvement of the brain histoarchitecture.

The brain of Ginkgo treated rats alone showed normal cerebral cortex and hippocampus similar to those demonstrated in the control ones. Brain showed normal neuronal cells with large round nuclei after 12 weeks **(Fig. 30**). Brain of aluminum chloride and Ginkgo biloba treated rat for 12 weeks showing less deposition of amyloid plaques **(Fig. 31**). Brain of Ginkgo treated rats together with Al intoxication revealed marked reduction of the histopathological lesions compared to aluminum treated one. Brain showed lowered number of degenerated neurons **(fig. 32)**.

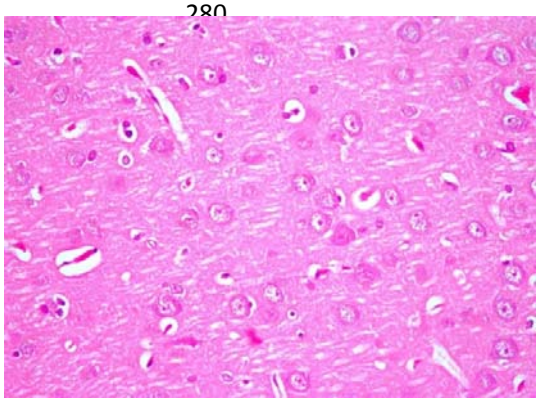


Figure 17: Brain of control rat showing normal cerebral cortex. The neuronal cells appeared normal with large round nuclei and prominent nucleoli (H&E X400).

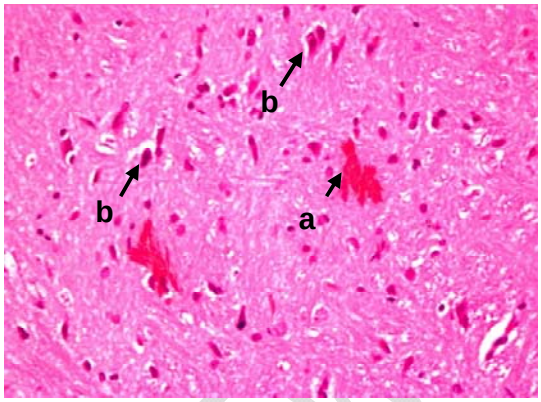


Figure 18: Brain of aluminum chloride treated rat for 6 weeks showing deposition of abundant amyloid plaques **(a)** in cerebral cortex associated with neuronal degeneration **(b)** (H&E X400).

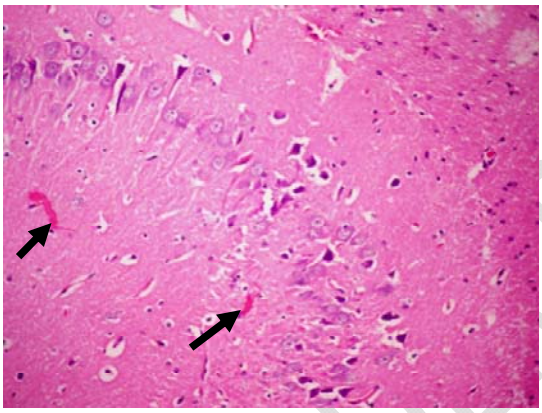


Figure 19: Brain of aluminum chloride treated rat for 6 weeks showing deposition of abundant amyloid plaques (arrow) in hippocampal region (H&E X200).

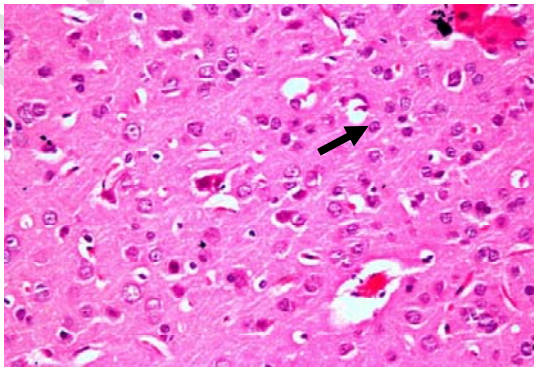
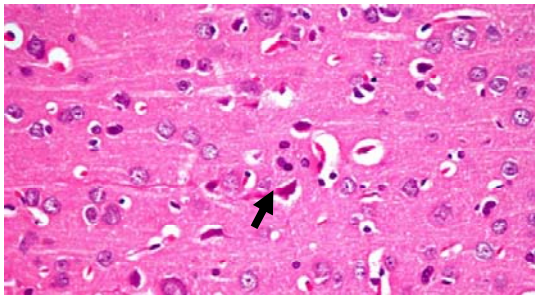


Figure 20: Brain of aluminum chloride treated rat for 6 weeks showing neuronal degeneration associated with proliferation of glia cells (arrow) (H&E X400).



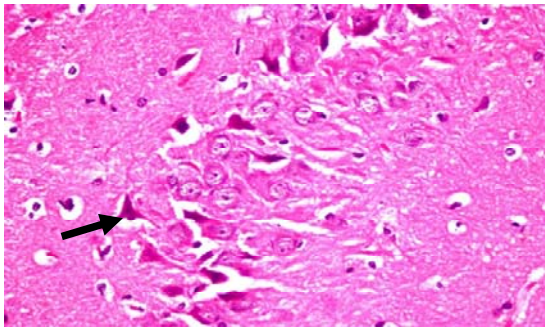


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Figure 21: Brain of aluminum chloride treated rat for 6 weeks showing cork strew shaped neurofibrillary tangles (arrow) (H&E X400).

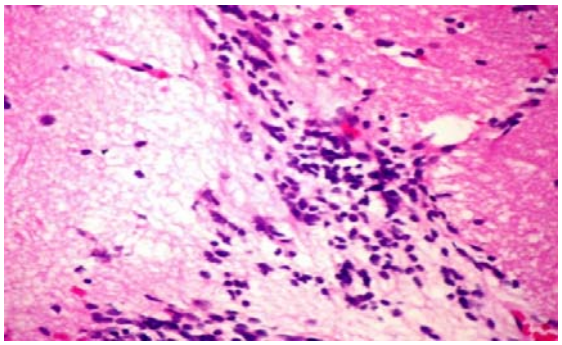

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Figure 22: Brain of aluminum chloride treated rat for 6 weeks showing degeneration of pyramidal nerve cells (arrow) (H&E X400).

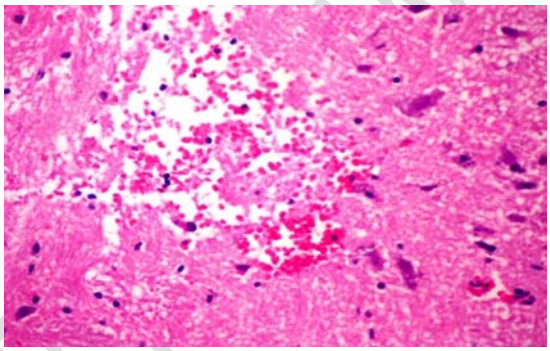

Figure 23: Brain of aluminum chloride treated rat for 6 weeks showing intense inflammatory reactions associated with focal gliosis particularly microglia cells (H&E X400).

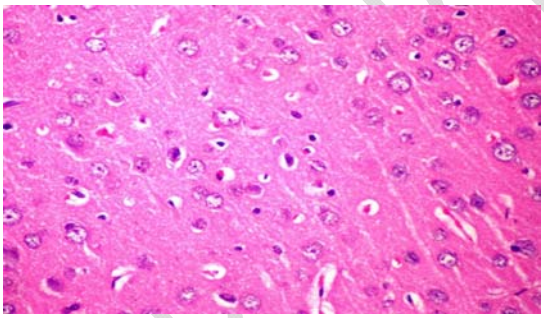

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Figure 24: Brain of aluminum chloride treated rat for 6 weeks showing cerebral hemorrhage (H&E X400).

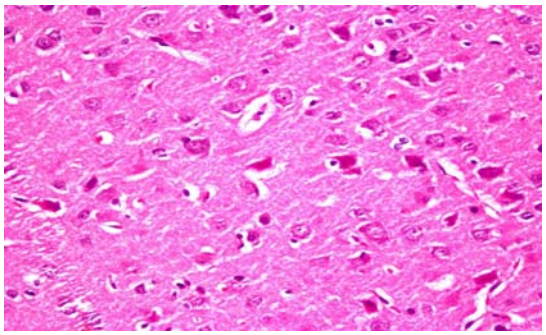

Figure 25: Brain of Ginkgo biloba treated rat for 6 weeks showing normal cerebral cortex (H&E X400).

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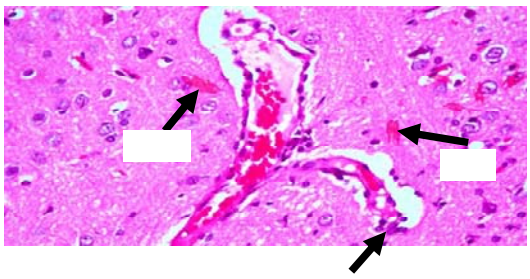

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Figure 26: Brain of aluminum chloride and Ginkgo biloba treated rat 6 weeks showing less neuronal degeneration (H&E X400).

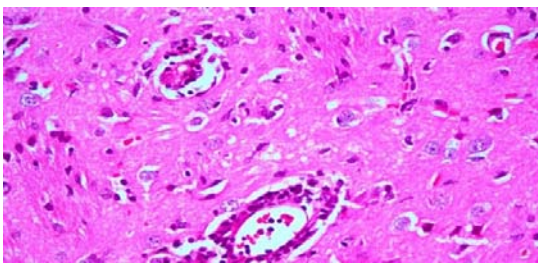

Figure 27: Brain of aluminum chloride treated rat for 12 weeks showing deposition of amyloid plaques **(a)** associated with congestion of cerebral blood vessels with perivascular cuffing with glia cells **(b)** and neuronal degeneration **(c)** (H&E X400).

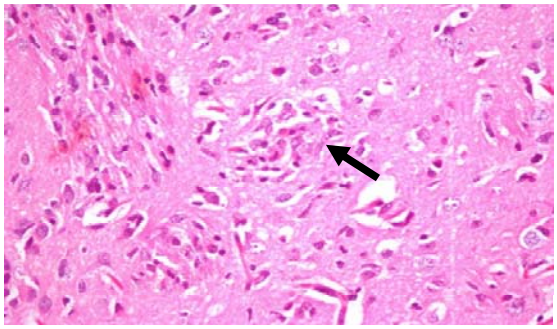

Figure 28: Brain of aluminum chloride treated rat for 12 weeks showing intravascular aggregation of leukocytes with perivascular edema and cuffing with glia cells (H&E X400).

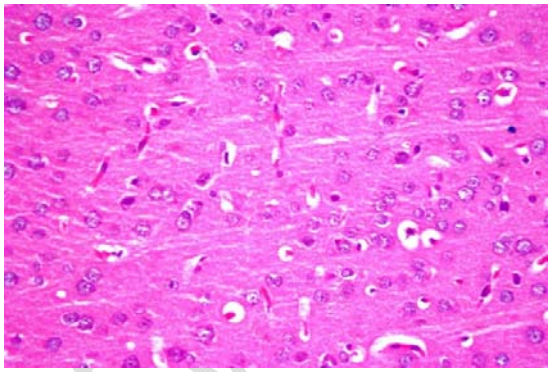

Figure 29: Brain of aluminum chloride treated rat for 12 weeks showing Focal cerebral tissue necrosis associated with reactive gliosis (arrow) (H&E X400)

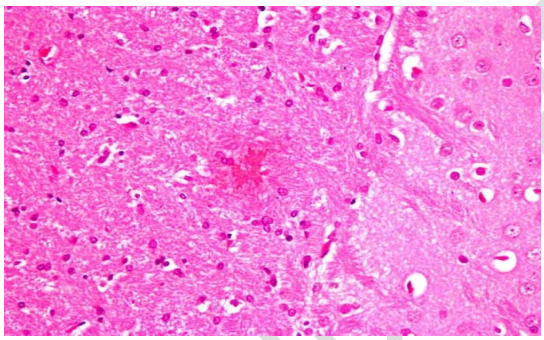

Figure 30: Brain of Ginkgo biloba treated rat for 12 weeks showing normal neuronal cells with large round nuclei (H&E X400).

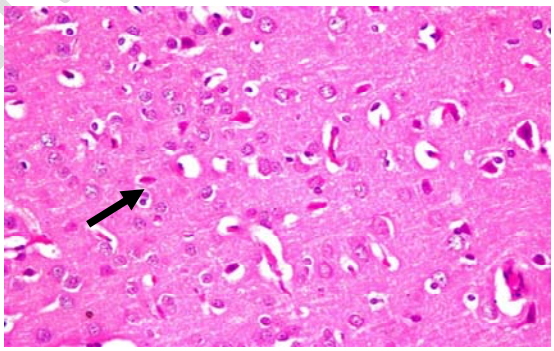

Figure 31: Brain of aluminum chloride and Ginkgo biloba treated rat for 12 weeks showing less deposition of amyloid plaques (H&E X400).

Figure 32: Brain of aluminum chloride and Ginkgo biloba treated rat for 12 weeks showing sparse degenerated neuronal cells (arrow) (H&E X400)

Disscussion

In the forebrain, such as the thalamus, hypothalamus and hippocampus; neurotransmitters play key roles in the regulation functions such as emotion and

behavior. The level of these chemical also changes as a result of neurotoxicity [38].

The present study demonstrated that $AlCl_3$ induced a significant decrease in the brain level of neurotransmitters (Norepinephrine (NE), Serotonin (5-HT) and Dopamine (DA) than control group during 6 or 12 weeks of treatment. The changes in brain neurotransmitters contents were also associated with degenerative changes in brain of Al-treated rats (Figures 17-24, 27-29).These results are consistent with the findings of Xiu et al. [39] who showed that aluminum administration reduced norepinephrine content in the hypothalamus from rats. Eraza et al. [40] attributed that the reduction of NE content might be due to inhibition effect of aluminum on the enzymes activity related to NE synthesis, including dopamine-beta-hydroxylase and tyrosine hydroxylase (the rate-limiting enzyme of NE synthesis)

The protective effect of Ginkgo biloba extract is demonstrated by the significant increase in brain neurotransmitters contents of NE, 5-HT and DA of intoxicated rats (Fig. 26, 31 and 32). This may be attributed to the ability of Ginkgo extracts to stabilize mitochondrial function [41]. Our results are also similar to those reported by Blecharz-Klin et al. [19] who showed that administration of high doses of Ginkgo biloba extract caused significant elevation of noradrenaline, dopamine and serotonin in rat brain.

We demonstrated that $AlCl_3$ induced a significant increase in different comet assay parameters. These results are consistent with the findings of Rui & Yongjian [42] who reported that $AlCl_3$ induced DNA damage in mice hippocampus or cortex cells. Similarly, Sumathi et al. [43] showed that DNA of Al treated cells showed a comet tail indicating the DNA damage arising from the genotoxicity in the Al-treated brain cells as compared to DNA of control cells. Deleterious effects of aluminum may be attributed to increased levels of reactive oxygen species [44] as well as nitrogen species [45].

On the other hand, prophylactic treatment with Ginkgo biloba extract significantly reduced $AlCl_3$-induced DNA damage as indicated by reduction in different comet assay parameters in the brain of intoxicated rats during the entire experimental period. These results are consistent with the findings of El Mesallamy et al. [46] who found that Ginkgo biloba extract supplementation significantly diminished DNA damage caused by N-nitrosodiethylamine (NDEA) as indicated by a significant decrease in the comet assay parameters compared to control group. Similarly, Alam et al. [47] showed that Ginkgo biloba extract significantly diminished the level of DNA damage caused by the Technetium (^{99m}Tc). The protective effect of Ginkgo biloba extract is attributed to its cytoprotective effects such as its high free radical scavenging ability, which can be exerted in the nuclear, cytoplasmic and extracellular compartments [30, 48].

In conclusion, the neurotoxic effects of aluminum are mediated by inhibition of the synthesis of monoamine neurotransmitters, induction of DNA damage and disruption of brain tissue and neural histoarchitecture. Gingko biloba exerts protective effects against the described consequences of aluminum toxicity.

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