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Research Article

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Patho-immunohistochemical Study on the Neuro-protective Effects of Ginkobiloba against Carbamazepine-Induced Neurotoxicity in Experimental Albino Rats

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ABSTRACT

Background: *Carbamazine is a drug of choice for treatment of epilepsy, targeting its effect in particular structures of the brain tissue, mainly the cerebellum.*
Objective: *The present study was carried out to evaluate the protective effect of ginkobiloba against carbamazine induced cerebellar neurotoxicity.*
Materials and method: *To establish this goal a total number of 40 adult male Wistar rats (Rattus norvegicus) weighing between 120-170g were divided into four equal groups (Control,Tegretol treated, Ginkobiloba treated and mixed Tergetol-Ginkopiloba treated groups) .*
Results: *The histopathological lesions were recorded in different treated groups and the immunohistochemical reactivities of apoptotic P53 and Glial Fibrillary Acidic Protein (GFAP) were studied.*
Conclusion: *Using of ginkobiloba extracts could induced to a certain limits a neuroprotective effect against carbamazine induced neurotoxicity.*
Keywords: Carbamazine; Ginkobiloba; Neurotoxicity; Cerebellum; Immunohistochemistry

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INTRODUCTION

Antiepileptic drugs (AEDs) target ion channels and neurotransmitter systems in the brain; in addition, they modify bursting properties of neurons, inhibit spread of epileptic activity, and reduce synchronization [1]. Many AEDs can influence cell proliferation, neurogenesis, programmed cell death, synaptogenesis, and synaptic plasticity [2].
By Studying the Mechanisms of action of antiepileptic drugs (AEDs) at the molecular level, it is possible to discriminate at least two basic types of action of AEDs: 1. action on ion channels, i.e. on the cellular membrane, 2. interaction with receptors. Phenytoin and carbamazepine might serve as examples of AEDs directly influencing the cellular membrane, especially Na+ channels. Due to the focusing of research on the molecular mechanisms, less attention has been paid to the structural basis of the action of AEDs. The action of an AED might be exerted only in a small region (or in some parts) of the central nervous system as was shown by Gale for vigabatrin. This drug exerts its action in the midbrain, whereas injections of vigabatrin into the caudate, thalamus, superior colliculus and pontine regions are ineffective. Phenytoin primarily affects cerebellar neurones and thus exhibits its anticonvulsant action through the inhibitory output of the cerebellum. Similarly, Julien suggested the cerebellum as a target structure for carbamazepine .The estimation of the structure where the AEDs exhibit their actions is of primary importance for molecular neurobiology in order to determine which structure is to be studied. The cerebellum might be considered as a target for phenytoin and carbamazepine effects.
Ginkgobiloba is an old unique tree with no living relatives which has been flourishe during the Mesozoic era since 150 million years ago. *Ginkgobiloba L.* (*Ginkgoaceae*) been used in China for medicinal purposes for hundreds of years (Gaby 1996) and conceder one of the most popular and most studied medicinal plants used in central nervous system (CNS) disorders [3-5]. G. biloba leaf extracts (GBE) were previously used as beneficial cerebrovascular and neuroprotective effects in several preclinical and clinical studies [6-7].GBE contains about 24% flavonoid

glycosides, 6% terpene trilactones, 7% proanthocyanidins, and certain low molecular weight organic acids [8]. It has strong positive effects on brain tissue due to antioxidant properties, and can reduce the hydrogen peroxide level in cerebellar neurons [9].Additionally mechanisms which may be involved in Ginkgobiloba extract-induced neuroprotection are modulation of ion homeostasis, glucocorticoid level, Aβaggregation and synthesis of growth factors [10].

Belviranl and Okudan investigated the effects of GBE on cognitive functions as well as oxidative stress and brain-derived neurotrophic factor (BDNF) levels in aged female rats and concluded that GBE supplementation improved cognitive functions by decreasing oxidative damage and increasing the BDNF level in aged female rats.

Protective role of co-administration of Ginkgobiloba, Trifolium pretense against sodium arsenite-induced neurotoxicity was done, Sodium arsenite caused significant neurodegenerative effect , increase in tumor necrosis factor-α, thiobarbituric acid- reactive substances and lipid profile, while caused significant decrease in glutathione, total thiol content, total antioxidant capacity, acetylcholinesterase, monoamine oxidase and ATPases activities. Clearly arsenite minimized neurological damages were observed post treatment by co-administration of Ginkgobiloba, Trifolium .Neuroprotective effects of Ginkgobiloba on cerebral ischemia-reperfusion injury in ovariectomized rats was confirmed by [11]. Neuroprotective role of Ginkgobiloba on subregions of hippocampus in hypercholesterolemic rats was explored by [12]. Ginkgobiloba has potential to ameliorate .BPA-induced hippocampal neuronal damage and subsequent cognitive deficits through mechanisms involving its ability to enhance the release of biogenic amines as well as its antioxidant and adiponectin pro-secretory effects [13]. Structural changes in various brain areas followed by fluoride exposure was done and the protective effect of ascorbic acid and Ginkgo biloba against fluoride neurotoxicity was evaluated. Zaki et al., [14] reported ginkgo biloba extract can protect the rat's brain against the hazards of exposure to γ-radiation and/or lead. Chandra and Veeresham observed that noni juice and G. biloba might have altered the bioavailability of phenytoin due to induction and inhibition of CYP2C9 enzymes.

Ginkgo biloba L. attenuates spontaneous recurrent seizures and associated neurological conditions in lithium-pilocarpine rat model of temporal lobe epilepsy through inhibition of mammalian target of rapamycin pathway hyperactivation. G. biloba reduced neuronal damage in the hippocampal pyramidal layer [15]. Extract of Ginkgo biloba promotes neuronal regeneration in the hippocampus after exposure to acrylamide (Huang et al., 2017). P-glycoprotein and caspase-3 expression in the brain tissue were significantly decreased in the Epilepsy mice treated with G. biloba extract [16].

Ismail and El-Sonbaty [17] investigated neuroprotective role of fermented Ginkgo biloba (FGb) leaf extract, compared to non-fermented G. biloba (Gb) leaf extract against γ-irradiation (6 Gy) in the rats' brain. Fermentation improved the bio-activities of Gb leaf extract and thus enhanced the in-vivo antioxidant, anti-apoptotic and anti-inflammatory activities, leading to amelioration of the stress hormones and Ca level. Accordingly, the fermentation enhances the protective role of Gb against γ-irradiation induced physiological disturbance in the rat's brain.

Zhang et al., (2012) demonstrate that EGb761 had significant
therapeutic effects on ischemic stroke and it perhaps worked through activating the Akt–CREB–BDNF pathway.Ginkgo biloba (EGb-761) is a potent antioxidant that has neuroprotective effects mediated through enhancing brain-derived neurotrophic factor levels [18].

Azmy and Abd Allah [19] demonstrate the possible histological changes that may occur in the cerebellar cortex of young male albino rats as a result of prolonged exposure to mobile phones radiations and the possible role of Ginkgo biloba supplementation. Prolonged exposure to mobile phone radiations provoked degenerative changes in cerebellar cortex where Purkinje neurons revealed several structural alterations with reactive gliosis. With Ginkgobiloba supplementation, these changes were minimal.

The present work was planned to evaluate the neuro-protective effect of ginkobilopa extracts against carbamazepine-Induced neurotoxicity in experimental albino rats through histopathological and immunohistochemical studies and imaging score analysis.

MATERIAL AND METHODS

Animals and treatments:

A total number of 48 adult male Wistar rats (*Rattus norvegicus*) with average weight 150- 200 gm were obtained from the animal house of the National Organization for Drug Control and Research, Egypt. All rats were housed in a quite non-stressful environment for one week before beginning the present study. They were offered normal rat chows were composed of 20% casein, 15% corn oil, 55% corn starch, 5 % salt mixture and 5% vitaminized starch. Maintenance of animals and experimental procedures was approved by the animal ethical committee in accordance

with the guide for care and use of laboratory animals. The following treatment protocol was used for this experiment:

Table 1. Grouping and treatment of experimental animals

Groups	Treatments (orally)
I (n=12)	control group.
II (n=12)	were given Tegretol at a dose level (50mg/kg b.w/ 5 days / week) for 9 weeeks.
III (n=12)	were administrated *Ginkgo biloba* at a dose level of 6.5mg/kg b.w /5 days / week) for 9weeks
IV (n=12)	received *Ginkgo biloba* at a dose level of 6.5mg/kg b.w /5 days / week) + Tegretol at a dose level (50mg/kg b.w/ 5 days / week) for 9week

The animals were sacrificed by cervical dislocation method at three different intervals (3 ,6 and 9weeks) twenty four hours after the last administration .

Carbamazepine:

The drug was tablets come in form of Carbamazepine /Tegretol, is 200 mg tablets obtained from Novartis Pharma, S.A.E. and Cairo, Egypt. Dispersed in 10 ml distilled water, at dose level (50mg/kg b.w), the dose represent minimal and maximal therapeutic doses in humans, and were calculated for rats according to Paget and Barnes.

Ginkgo biloba Preparation:

Ginkgo biloba (GB) leaf extract made into capsules 260 mg tablets were obtained from Pharaonia pharmaceuticals Company, Alexandria, Egypt. Ginkgo biloba was dissolved in distilled water and administered to the animals orally, at dose level (6.5mg/kg b.w/ day).

Histological and Histochemical Studies:

Immediately after decapitation animals were dissected, cerebellum from different groups were quickly removed and fixed in 20 % neutral buffered formalin for histological examination and other specimens from kidney fixed in Carnoy's fluid.fluid for histochemical examination then, dehydrated among ascending series of ethanol 85% for 15 min, ethanol 95% for 1 hour and ethanol 100% in two stages 30 min for each stage , cleared in a xylene +100% alcohol for 30min, then another time in xylene for 30min , Infiltrated with xylene then placed in melted paraffin at 58-60ºC in two stages 1hour for each stage to evaporate the solvent xylene and paraffin let to enter the spaces of the tissue to allow the tissue to be filled with paraffin. Embedding: in fresh and clean paraffin by Therm Embedding Paraffin. The embedded tissue with melted paraffin was allowed to solidify and then it was ready for sectioning, Sectioning is carried out by Rotary Microtome with 5µm thick and mounted on clean microscope slide ,Staining Paraffin sections of 5 microns thickness Sections were stained with Ehrlich's haematoxylin and counterstained with eosin as a routine method after Stevens (2013) for histological study. Paraffin-embedded sections are first dewaxed to replace the wax with water; this is because most staining solutions are aqueous. For immunohistochemical study Dewaxing was usually followed by an antigen-retrieval method, which unmasks antigens that have been obscured during the fixation process.Next, depending upon the type of antibodies that will be used, a series of blocking steps take place that serve to minimize non-specific interactions of the antibody and block endogenous enzymes that can result in false positives.After blocking, the sections are incubated in antibodies; this incubation can be in directly conjugated primaries or can consist of sequential incubations in both primary and secondary antibodies (fluorescent or enzyme-linked).If an enzyme-linked secondary is used, a further incubation in substrate will be required, otherwise the sections can be mounted and imaged(http://www.kemet.com.eg-ABCAM).Slides were viewed by using Labomed ,Labo America,Inc. USA microscope and images were captured by a digital camera (Sony DSC_S5000) under 10X and 40X .

RESULT

Histopathological Findings

Control: All the layers of the cerebellum were normal. The purkinje cell layer showed large cells with prominent nuclei, the molecular and medullary cell layers showed normal vascular and cellular structure including neurons, astrocytes, microglial cells and oligodendroglia Figure 1(plate1 A,B).

Tegretol group: Examined sections in most cases revealed highly reactive astrocytes, demyelination and cerebellar congestion. Some of the neurons were degenerated and the microglia cells were seen in a variable number. satellitois and neuronophagia could also be detected in some parts. Figure 2 (plate 2 A,B,C,D). Some sections showed focal astrocytic and microglial reaction which were represented by increased number of hypertrophied astrocytes which may be a reparative Process against some demyelinated nerve fibers. Figure 3 (Plate3A,B). Moreover focal degeneration and or apoptosis of purkinje cells which completely disappeared in some parts of the cerebellum with presence of focal astrocytic reaction in the molecular layer could also be detected figure 4(plate 4,A,B).

Geinko bilopa: Examined sections revealed a comparatively more cellularity of granular and purkinje cell layer in some parts of the cerebellum.Other parts showed normal thickness and numbers of both granular and purkinje cell layers . The meningeal and cerebellar blood vessels were mildly congested .The molecular cell layer revealed normal histomorphological structures by Figure 5 (plate 5,A,B). A few number of purkinje cells were degenerated or apoptotic and represented by shrinkage with deepely eosinophilic cytoplasm. Focal astrocytic reaction with mild demyelination and increased number of gitter cell could also be detected Figure 5 (plate 5 C,D).

Tegretol and Ginkobilopa: Most of the cerebellar structures resumed a normal granular and purkinji cell layers shown in figure 6 (plate 6 B), but in a few cases the meningeal and cerebellar blood vessels were mildly congested by Figure 6 (plate6A) The molecular layers in some sections revealed focal axonal degeneration beside astrocytic and microglial reaction. The microvascular endothelial cells and the gitter cells were prominent at these areas. The endothelial cells showed deep basophilic spindle or ovoid nuclei. In some cases the purkinje cells were partially lost, degenerated or atrophied shown in figure 6 (Plate 6 C,D) and the molecular layer showed degenerated neurons beside focal vacuolation and demylination. Figure 6 (plate 6,E,F).

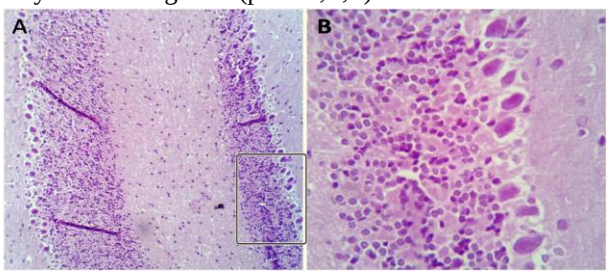

Figure 1. Plate 1 (control group): photomicrograph of H&E stained sections of brain showing: A) Normal cerebellum structures X 200. B) High power of the previous picture to show normal granular, purkinje and molecular layers.H&E X 200, 400

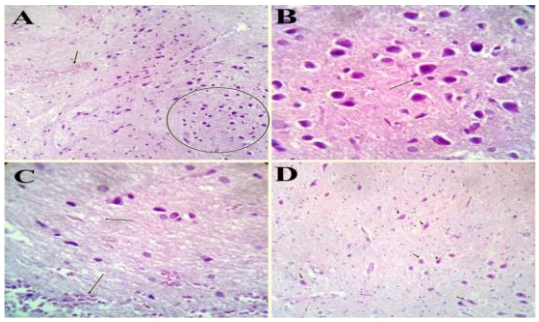

Figure 2. Plate 2 (Tegretol group): photomicrograph of H&E stained sections of cerebellum showing: A) degeneration of some neurons and congestion (arrow). B) Reactive astrocytosis, satellitois and neuronophagia. C & D) congestion, small focal hemorrhages and reactive astrocytosis(arrow head). H&E X 100,200,400

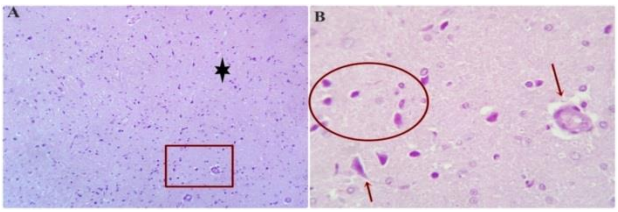

Figure 3. Plate 3 (Tegretol group): photomicrograph of H&E stained sections of brain showing: A) Normal cerebellum structures with focal astrocytic and microglial reaction X 200. B) High power of the previous picture to show the increased number of activated hypertrophic astrocytes H&EX 100, 400

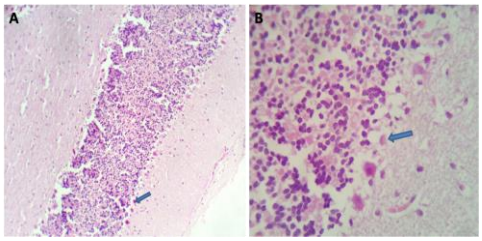

Figure 4. Plate 4 (Tegretol group): photomicrograph of H&E stained sections of brain showing: A) focal degeneration and apoptosis of purkinje cells (arrows) which completely disappeared in some parts of the cerebellum (arrow).H&E X 200, 400

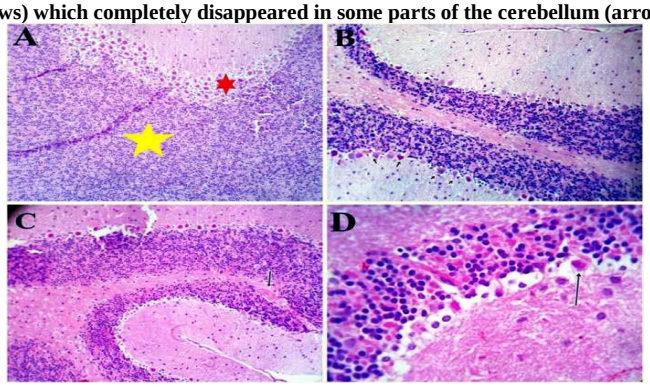

Figure 5. Plate 5 (Ginkobilopa group): photomicrograph of H&E stained sections of brain(cerebellum) showing: increase in the thickness of granular cell layer (yellow star) and increase in the number of purkinje cell layer (red star). B) Some parts showing normal thickness of granular cell layer and purkinje cells (arrow head). C) mildly congested blood vessel (arrow). D) Some of purkinje cells are degenerated , apoptotic or vacuolated and represented by shrinkage with deep eosinophilic cytoplasm (arrow) . H&E X 200,400

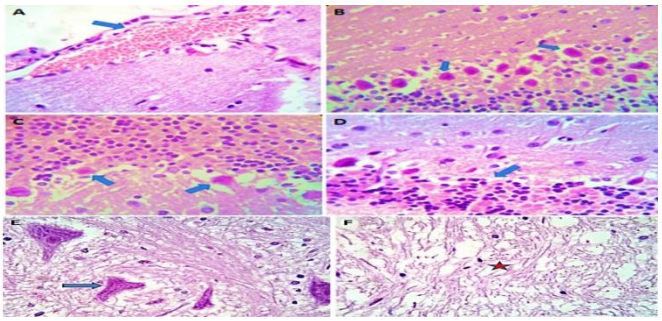

Figure 6. Plate 6 (Tegretol + Ginkobilopa group): photomicrograph of H&E stained sections of brain(cerebellum) showing: A) mild congestion of the meningeal blood vessels X 200. B) Normal and activated purkinje cells X400. C&D) Focal decrease in number of the purkinje cells (arrows) X400. E) degenerated neuron (arrow) among the demyelinated axons X400. F) Vacuolation and demyelination (star) H&E X400

Immunohistochemical Findings

Apoptotic Marker P53:

Control: All layers of the cerebellum were normal and showed negative nuclear and cytoplasmic staining reaction to the apoptotic protein p53 figure 7(plate 1A). Tegretol: Examined sections revealed a few number(2-3%) of purkinjie ,molecular and granular cells of the cerebellum , with a positive brownish nuclear and cytoplasmic staining reaction which considered to be evidence of p53 antigen expression by the affected cells. Figure 7(plate

1B,C). Ginkobilopa: A few number (1-3%) of the glial cells, including microglia and astrocytes showed positive brownish nuclear and cytoplasmic expression of p53 shown by Figure 8(plate 2A). Tegretol and Ginkobilopa: A very few number (0.5-1%) of cells of the granular cell layer showed positive brownish nuclear and cytoplasmic staining reaction to p53 shown by figure 8 (plate 2 B,C).

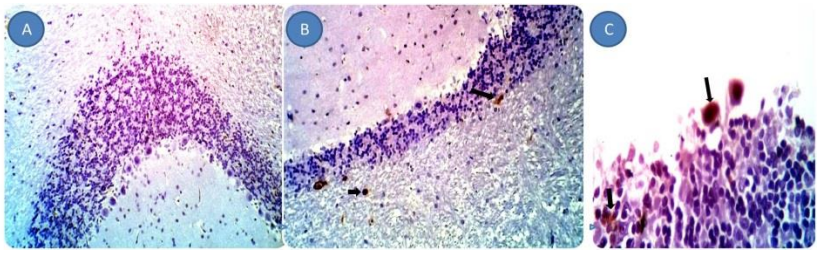

Figure 7. Plate 1: photomicrograph showing(A) negative nuclear and cytoplasmic staining reaction to the apoptotic protein (B,C) few number of purkinjie ,molecular and granular cells of the cerebellum , with a positive brownish nuclear and cytoplasmic staining reaction(arrows)X 100,200,400

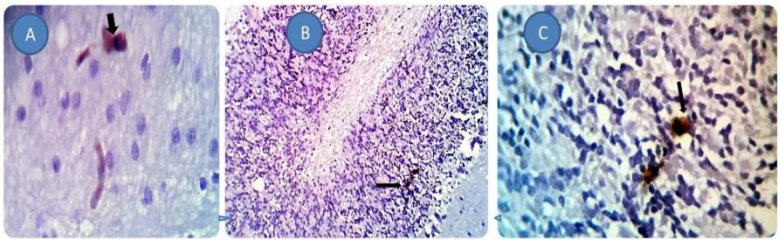

Figure 8. Plate 2: photomicrograph showing (AA few numberof the glial cells, including microglia and astrocytes showing positive brownish nuclear and cytoplasmic expression of p53.) (Arrow) (B,C) A very few number of cells of the granular cell layer showing positive brownish nuclear and cytoplasmic staining reaction to p53) (arrow)X 200,400

Glial Fibrillary Acidic Protein (GFAP):

Control: All layers of the cerebellum showed normally distributed positively stained (cytoplasmic) glial cells, mainly astrocytes Figure 9 (Plate 3A). Tegretol: Focal increase in the reactivity of positively stained astrocytes around degenerated neurons, particularly in the molecular cell layer. Figure 9(Plate3 B,C). Ginkobilopa group: Showed normally distributed positively stained glial cells, mainly astrocytes in all layers of the cerebellum.Figure 10 (Plate 4A). Tegretol and Ginkobilopa: Showed focal reactive positively stained astrocytes around some degenerated neurons and axons, especially in the molecular cell layer. Figure 10 (Plate 4B,C).

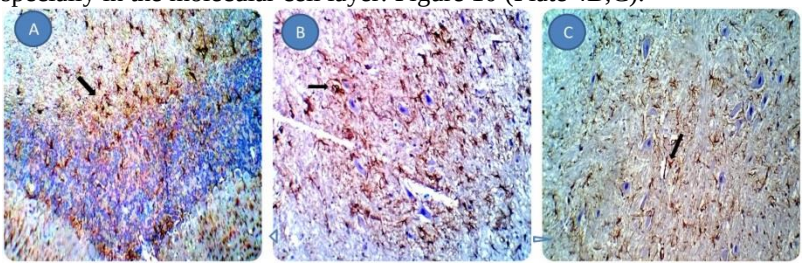

Figure 9.Plate 3: photomicrograph showing (A) normally distributed positively stained (cytoplasmic) glial cells, mainly astrocytes.)(arrow) (B,C) positively stained astrocytes around degenerated neurons, particularly in the molecular cell layer.)(arrow)X200

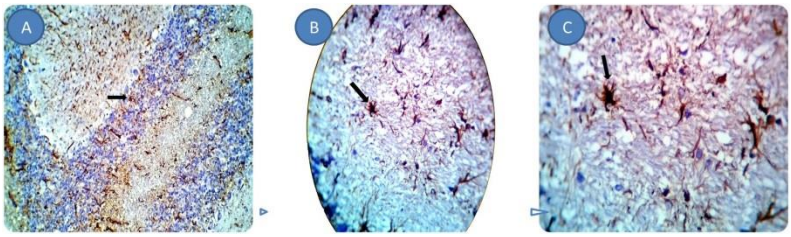

Figure 10. Plate 4: photomicrograph showing (A) normally distributed Positively stained glial cells, mainly astrocytes) (arrow) (B,C) focal reactive positively stained astrocytes around some degenerated neurons and axons , especially in the molecular cell layer)(arrow).X 200,400

DISCUSSIONS

Phenytoin and carbamazepine might serve as examples of AEDs directly influencing the cellular membrane, especially Na+ channels. The action of an AED might be exerted only in a small region (or in some parts) of the central nervous system as was shown by Gale for vigabatrin. This drug exerts its action in the midbrain, whereas injections of vigabatrin into the caudate, thalamus, superior colliculus and pontine regions are ineffective. The cerebellum might be considered as a target for phenytoin and carbamazepine effects. Many antiepileptic drugs (AEDs)can influence cell proliferation, neurogenesis, programmed cell death, synaptogenesis, and synaptic plasticity [2].

Carbamazepine could induced hematological adverse effects as erythroid hypoplasia, neutropenia and persistent thrombocytopenia.. Autoantibodies directed against the patient's red cells, granulocytes and lymphocytes were also demonstrated. In the present work carbamazine (tegretol) could induced cerebellar pathological changes as neuronal degeneration , nerve fibers demylination , satellitosis, neuronophagia , microglial reaction (increase in number of microglial cells) , increased astrocytic reactivity and congestion . Such lesions could be attributed to the effects of carbamzine on cell permeability especially sodium channel and the inhibitory effects on cerebellar neurons outpots. More over drug induced encephalopathy had been reported and decominted as pervious patho-anatomic studies showed changes in the cerebellum and temporal lobe of predominantly the pyramidal and purkinjecells in rats after chronic administration of valproate acid [20]. Those studies reported also damage to the hippocampal astrocytes and neocortex. Additionally interaction with the GABA receptor plays a role in the intrinsic toxic effects of valproic acid(antiepileptic drug)encephalopathy [21].

An indirect damaging effects could be arises due to prolonged administration

Of carbamazin as erythroid hypoplasia, neutropenia and persistent thrombocytopenia, with such changes brain anemia and neuronal degenerative disorders could be detected [22]. Immunohistochemically a few number(2-3%) of purkinjie ,molecular and granular cells of the cerebellum , showed a positive brownish nuclear and cytoplasmic staining reaction which considered an evidence of p53 antigen expression of the affected cells. The GFAP revealed focal increase in the reactivity of positively stained astrocytes around degenerated neurons, particularly in the molecular cell layer. Such observation go parallel with results of [**2**] where they reported that Many antiepileptic drugs (AEDs)can influence cell proliferation, neurogenesis, programmed cell death, synaptogenesis, and synaptic plasticity .

Treatment by ginkobiloba alone showed normal histo-morphological structures of the cerebellum with a comparatively increases cellular thickness of the granular layer in some cases or mild degenerative and apoptotic changes in the purkinge cell layer in other cases . Histochemically a few number (1-3%) of the glial cells, including microglia and astrocytes showed positive brownish nuclear and cytoplasmic expression of p^{53} .The FGAP revealed normally distributed positively stained glial cells, mainly astrocytes in all layers of the cerebellum. Our results coincide with the results recorded in many studies as Ginkgobiloba *L. (Ginkgoaceae)* been used in China for medicinal purposes for hundreds of years [23] and conceder one of the most popular and most studied medicinal plants used in central nervous system (CNS) disorders [3-5]. It has strong positive effects on brain tissue due to antioxidant properties, and can reduce the hydrogen peroxide level in cerebellar neurons [9]. Additionally mechanisms which may be involved in Ginkgobiloba extract-induced neuroprotection are modulation of ion homeostasis, glucocorticoid level, Aβaggregation and synthesis of growth factors [10]. The mild degenerative and apoptotic changes which were observed in some cases of our study could be attributed to the impurity of the extracted materials as they were used as a crude extracts. Obtaining of the most effective active ingredients (flavonoid glycosides, , terpene trilactones, proanthocyanidins, and certain low molecular weight organic acids) in a purified form may minimize any hazard effect of the crude extracts.

Co-adminestration of tegretol and Ginkobiloba showed a comparatively moderate enhancements in the provoked damaging effect of Tegretol on the cerebellar tissue as most of the cerebellar structures resumed a normal granular and purkinji cell layers. Focal axonal degeneration beside astrocytic and microglial reaction and in some cases partially lost, degenerated or atrophied Purkinjie cells were encountered. Histochemically a very few number (0.5-1%)of cells of the granular cell layer showed positive brownish nuclear and cytoplasmic staining reaction to p^{53} and FGAP revealed focal reactive positively stained astrocytes around some degenerated neurons and axons, especially in the molecular cell layer.It is clear that Ginkobilopa could be able to some extent to improve the neurotoxic effects of Tegretol . Our work go in harmony with the observation obtained by Ismail and El-Sonbat [17] , where they found that Gb leaf extract enhanced the in-vivo antioxidant, anti-apoptotic and anti-inflammatory activities, against γ-irradiation (6 Gy) neurotoxic effects in the rats' brain leading to amelioration of the stress hormones and Ca level.Moreover extract of Ginkgo biloba promotes neuronal regeneration in the hippocampus after

exposure to acrylamide [24] , P-glycoprotein and caspase-3 expression in the brain tissue were significantly decreased in the Epilepsy mice treated with G. biloba extract [16].

CONCLUSION

Finally we can conclude that using of ginkobiloba extracts could induced to a certain limits a neuroprotective effect against carbamazine induced neurotoxicity. Using of a biotechnologically purified extracts containing the most active principles should be more effective and more promising.

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