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RESEARCH ARTICLE

Ameliorative potential of *Punica granatum* peel extract against Fipronil induced neuronal toxicity in Male Wistar Albino rats

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ABSTRACT

Fipronil is a second-generation broad spectrum phenyl pyrazole insecticide used in veterinary, households, topical pet care products and agricultural practices. Indiscriminate and improper use of this insecticides leads to a range of harmful effects in both humans and animals. So the present study was carried out to explore the effect of fipronil toxicity in brain of wistar albino rats and to check the potential ameliorative effect of pomegranate peel extract *(Punica granatum)* in it. For this study 24 male Wistar albino rats that were randomly assigned to 4 groups with 6 rats in each group. Fipronil was gavaged @ 10 mg/kg b.wt. orally using distilled water as a vehicle to group II animals. Peel extract of *Punica granatum* @ 200 mg/kg b.wt. was fed to group IV along with fipronil for exploring the ameliorative effects for a period of 6 weeks. Group I and III served as vehicle control and *Punica granatum* control respectively. Oxidative stress estimation in brain tissue revealed significant increase ($P<0.05$) in the levels of lipid peroxidation (LPO) and decreased levels of superoxide dismutase, catalase, reduced glutathione and glutathione peroxidase in fipronil treated rats. Histopathological examination of same group showed sub-meningeal hemorrhages, capillary proliferation, neuronal degenerative changes like shrinkage, central chromatolysis, satellitosis and neuronophagia, gliosis, spongiosis in the cerebral cortex and rounding, shrinkage and loss of Purkinje cells of the cerebellum were also observed. Co-administration of *Punica granatum* (pomegranate peel extract) along with fipronil resulted in restoration of oxidative stress parameters and histological architecture of brain near to normal.

Keywords: Amelioration, fipronil, male Wistar albino rats, oxidative stress, *Punica granatum*

INTRODUCTION

The use of pesticides in modern agriculture is a double-edged sword, boosting crop yields and ensuring food security, yet also presenting serious threats to both environmental and human health[1]. Pesticides play a vital role in reducing substantial agricultural losses, which are estimated to exceed 45 percent annually due to pest infestations[2], while also playing an important role in controlling vector borne diseases. Beyond agricultural applications, they are also found in various consumer products[3]. These chemicals are categorized according to their intended use and chemical structure, allowing for targeted pest control that promotes productivity and supports food security in a cost-effective manner. In the realm of modern agriculture and pest management, pesticides are commonly divided into four primary groups based on their chemical structure and mechanism of action: organochlorines, organophosphates, carbamates and pyrethroids[4].

Fipronil, a commonly used insecticide in agriculture, is effective in managing pests and maintaining public hygiene[5]. Classified under the phenyl pyrazole group, it demonstrates efficacy against over 250 insect species. However, extensive use of fipronil has been linked to negative impacts on animal health, human well-being and plant systems[6]. Fipronil has been shown to exert various toxic effects on both humans and animals, including cytotoxicity, reproductive toxicity, liver damage, neurotoxicity and neurodegenerative outcomes across both vertebrate and invertebrate species[7]. These effects are primarily linked to its action on γ-aminobutyric acid (GABA) receptors which are key components in the central nervous system and the main inhibitory neurotransmitter in mature vertebrates. There are two major subtypes of GABA receptors: GABAA and GABAB[8]. GABAA receptors act as ligand-gated ion channels that allow chloride ions to enter the neuron upon activation by GABA, thereby contributing to inhibitory synaptic transmission[9]. Fipronil disrupts this process by blocking GABA-gated chloride channels, particularly those associated with GABAA receptors, leading to nervous system excitation,

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neuronal hyperactivity, paralysis and ultimately death in insects[10]. Although both vertebrates and invertebrates possess GABA-regulated chloride channels, fipronil exhibits a significantly higher binding affinity for insect GABA receptors than for those in vertebrates, especially the GABAC subtype, which it binds with much lower affinity[11]. This selective binding profile contributes to its heightened insecticidal activity and offers a broader safety margin for mammals.

Recent findings suggest that glycine receptors in vertebrates may represent an additional target through which fipronil exerts its toxic effects. These receptors, which are ionotropic and responsive to the neurotransmitter glycine, mediate inhibitory neurotransmission by allowing chloride ions to flow into neurons. They are predominantly located in the brainstem and spinal cord and play a crucial role in maintaining neural inhibition within the central nervous system[12]. Fipronil binds strongly to several human glycine receptor subtypes such as α1, α1β, α2 and α3 which effectively inhibiting their function[13]. These subtypes share structural and functional similarities with vertebrate GABAA receptors, suggesting that all isoforms of human glycine receptors may contribute to fipronil's toxic potential in humans[13].

Fipronil may enhance oxidative stress, which leads to the generation of reactive oxygen species and is related to its toxic effects in different organs. Oxidative stress occurs when the generation of reactive oxygen species (ROS) exceeds the usual antioxidant capability of target cells[14]. Thus, measuring antioxidant indicators such as catalases, superoxide dismutase, glutathione peroxidase and lipid peroxidase can assist in determining the extent of oxidative damage to various tissues[15].

Recently, herbal medicine got more attention in seeking their complete potential in curing various toxicity cases in humans and animals[16]. Plant-derived antioxidants are composed of both essential nutrients with proven free radical-scavenging properties and a range of non-vitamin, non-mineral compounds. Alongside well-known antioxidants like alpha-tocopherol (vitamin E), ascorbic acid (vitamin C), carotenoids and zinc, many herbal remedies also contain bioactive compounds such as flavonoids, polyphenols and flavoproteins. Additionally, certain individual plants or specific herbal combinations used in traditional formulations may function as antioxidants by neutralizing superoxide radicals or by enhancing the activity of superoxide dismutase (SOD) in various tissues[17].

Pomegranate (*Punica granatum*) is known for its abundance of bioactive compounds that exhibit strong antioxidant properties[18]. In recent years, growing evidence has highlighted its wide range of health benefits, leading to increased scientific interest in exploring its therapeutic potential. The health-promoting effects of pomegranate pulp are largely credited to its rich content of phenolic compounds, including gallic acid and the distinctive polyphenols punicalagin α and β, the latter being unique to this fruit[19]. Punicalagin, a type of hydrolysable ellagitannin, is particularly noted for its antioxidant, anti-inflammatory and antiproliferative effects[20]. Most of the research explored the protective effect of fruit, leaves and seed part of pomegranate, hence current study aimed to explore the ameliorative effect of peel extract of pomegranate in neurotoxic effects induced by fipronil in wistar rats.

MATERIALS AND METHODS

Purchase of lab animals, fipronil and pomegranate peel extract

A total of 24 male Wistar albino rats were procured from Sri Venkateswara Enterprises, Bangalore, for this study. Following a two-week acclimatization period, the animals were randomly assigned to groups and housed in standard polypropylene cages. They were maintained under controlled environmental conditions at 25 ± 1^{O}C with a 12:12 hour light/dark cycle throughout the 6-week experimental period. Standard laboratory hygiene practices were followed, and the rats were provided with laboratory animal feed and water *ad libitum*. Institutional Animal Ethics Committee (IAEC) approval was obtained prior to the commencement of the experiment (Approval No. 281/go/ReBi/S/2000/CPCSEA/CVSc/TPTY/010/ Veterinary Pathology/2023, dated 08.05.2023). Technical-grade fipronil (99% pure; Batch No. FIP92B5266) was obtained from Gharda Chemicals Ltd., Mumbai. *Punica granatum* (pomegranate) peel extract (Product No. Dadim LC23030077) was procured from Chemiloids Life Science Pvt. Ltd., Vijayawada, Andhra Pradesh.

Experimental design

Groups	No. of Rats per group	Doses
Group I: Negative control	6	Adlibitum feed & distilled water
Group II: Fipronil control	6	Fipronil @ 10 mg/kg body weight (daily)
Group III: *Punica granatum* control	6	*Punica granatum* orally in distilled water @ 200 mg/kg body weight (daily)
Group IV: Fipronil + *Punica granatum*	6	Fipronil @ 10 mg/kg body weight orally in distilled water + *Punica granatum* orally in distilled water @ 200 mg/kg body weight (daily)

Table 1. Mean and SD values of LPO (nM MDA/g of tissue), SOD (U/mg of protein), Catalase (nM of H_2O_2 decomposed/min/mg of protein), Reduced glutathione (μmoles GSH/g of tissue) and Glutathione peroxidases (U/mg of protein) in brain of rats of different experimental groups.

	At the end of experimental Period (6th week)			
Oxidative stress parameters in brain	**GROUP I**	**GROUP II**	**GROUP III**	**GROUP IV**
LPO (nM MDA/g of tissue)	166.15 ± 0.94^{c}	348.84 ± 3.16^{a}	171.53 ± 1.88^{bc}	174.61 ± 1.60^{b}
SOD (U/mg of protein)	12.3 ± 0.21^{a}	8.8 ± 0.30^{c}	12.2 ± 0.33^{a}	11.2 ± 0.10^{b}
Catalase (nM of H_2O_2 decomposed/min/mg of protein)	0.23 ± 0.04^{a}	0.09 ± 0.02^{b}	0.21 ± 0.02^{a}	0.19 ± 0.01^{a}
Reduced glutathione (μmoles GSH/g of tissue)	18.64 ± 2.62^{a}	10.11 ± 0.98^{b}	17.89 ± 0.93^{a}	16.22 ± 0.84^{a}
Glutathione peroxidase (U/mg of protein)	24.89 ± 1.41^{a}	16.35 ± 0.75^{c}	22.36 ± 0.71^{ab}	20.09 ± 0.47^{b}

A total of 24 male Wistar albino rats were randomly assigned into four groups with six animals in each group.

Parameter studied

Oxidative stress

Tissue pieces of brain were minced and homogenized in 0.05 M ice cold phosphate buffer (pH 7.4) by using a Virtis homogenizer to make 10% homogenate. 0.2 ml of the homogenate was used for lipid peroxidation assay[21]. The remaining part of homogenate was mixed with 10% trichloroacetic acid in the ratio of 1:1, centrifuged at 5000 rpm for 10 min at 4^{O}C and supernatant was used for estimation of reduced glutathione[22]. The remaining part of the homogenate was further centrifuged at 15000 rpm for 60 min at 4^{O}C and the supernatant obtained was used for estimation of superoxide dismutase[23], catalase[24] and glutathione peroxidase[25] in brain of all rats in all groups.

Gross and histopathology

A detailed post-mortem examination was conducted on all the sacrificed rats in all the experimental groups. The gross lesions were recorded and representative tissue pieces from brain was collected and preserved in 10% neutral buffered formalin for histopathological studies. Fixed tissues were processed by routine paraffin embedding technique. Sections of 5-6 (μ) thickness was cut and stained with routine Hematoxylin and Eosin method (H&E)[26].

Statistical analysis

The results were statistically analysed by performing one-way ANOVA[27].

RESULT

Oxidative stress

Lipid peroxidation (LPO)

The mean brain LPO values in Group I to IV were 166.15±0.94^{c}, 348.84±3.16^{a}, 171.53±1.88^{bc} and 174.61±1.60^{b} (nM of MDA/g of tissue) respectively, and are given in Table 1 and Fig. 1a. A significant increase (P<0.05) in the brain LPO values was observed in fipronil treated rats

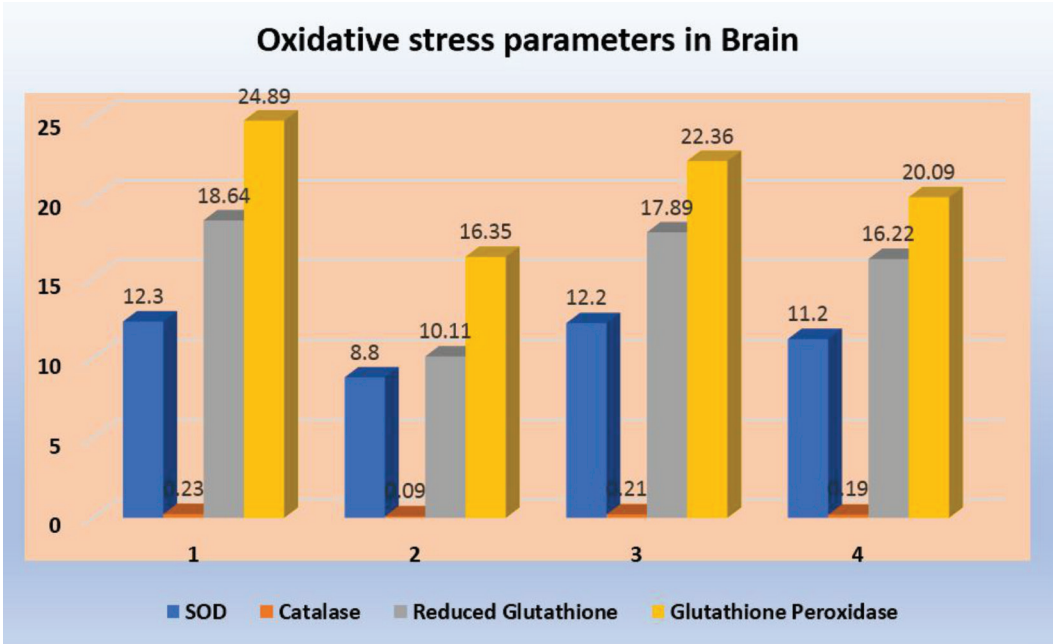

Fig. 1a. Mean and SD values of SOD (U/mg of protein), Catalase (nM of H_2O_2 decomposed/min/mg of protein), Reduced glutathione (μmoles GSH/g of tissue) and Glutathione peroxidases (U/mg of protein) in brain of rats of different experimental groups.

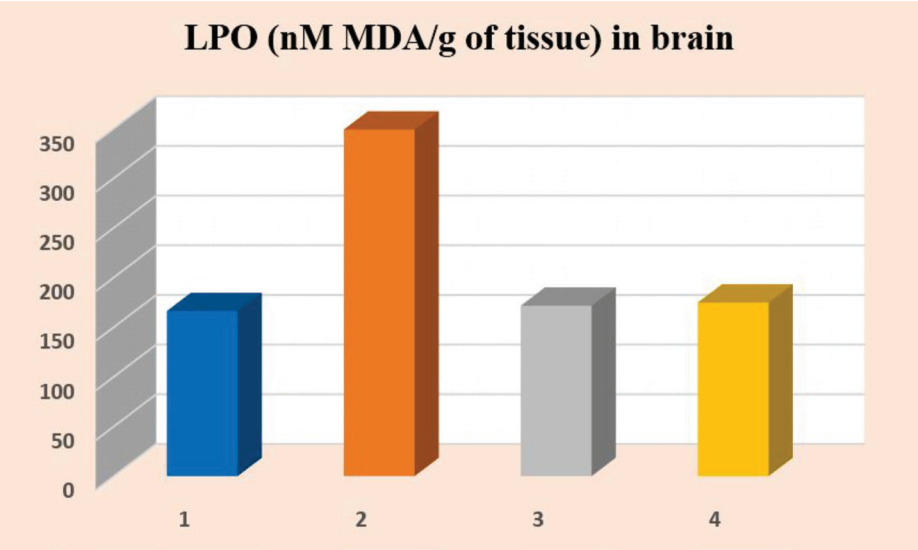

Fig. 1b. Mean and SD values of LPO (nM MDA/g of tissue) in brain of rats of different experimental groups. (Created separate graph for LPO as the LPO value ranges from 170-345 much higher compared to other parameters hence found difficulty to keep all in single graph, as Y axis value ranges differ in different parameters).

compared to the control group. However, no significant difference was noticed in LPO values among *Punica granatum* treated rats (Group III) compared to control rats (Group I). At the same time, a significant decrease in LPO values was noticed in ameliorated group rats compared to fipronil treated rats.

Superoxide dismutase

The mean SOD values in Group I to IV rats were 12.3 ± 0.21^{a}, 8.8 ± 0.30^{c}, 12.2 ± 0.33^{a} and 11.2 ± 0.10^{b} (U/mg of protein) respectively and are given in Table 1 and Fig. 1b. A significant ($P<0.05$) decrease in Group II (fipronil treated) rats was recorded in the SOD values of the brain when compared to control rats (Group I) and *Punica granatum* ameliorated (Group IV) rats. There was no significant difference in SOD values of *Punica granatum* treated rats (Group III) compared to control rats (Group I).

Catalase

The mean brain catalase values were 0.23 ± 0.04^{a}, 0.09 ± 0.02^{b}, 0.21 ± 0.02^{a} and 0.19 ± 0.01^{a} (nM of H_2O_2 decomposed/min/mg of protein) in Group I to IV respectively, and shown in Table 1 and Fig. 1b. Statistically, a significant ($P<0.05$) decrease was observed in catalase values in fipronil treated rats (Group II) when compared to control rats (Group I). A Significant ($P<0.05$) increase was observed in *Punica granatum* ameliorated rats (Group IV) when compared to fipronil treated rats. There was no significant difference in the catalase level among *Punica granatum* treated (Group III) rats compared to control rats.

Reduced glutathione

The overall mean of reduced glutathione values of the brain in Group I to IV rats were 18.64 ± 2.62^{a}, 10.11 ± 0.98^{b}, 17.89 ± 0.93^{a} and 16.22 ± 0.84^{a} (µmoles GSH/g of tissue) respectively, and are given in Table 1 and Fig. 1b. There was a significant ($P<0.05$) decrease in GSH values of fipronil treated rats (Group II) when compared to the control group. No significant difference was noticed in

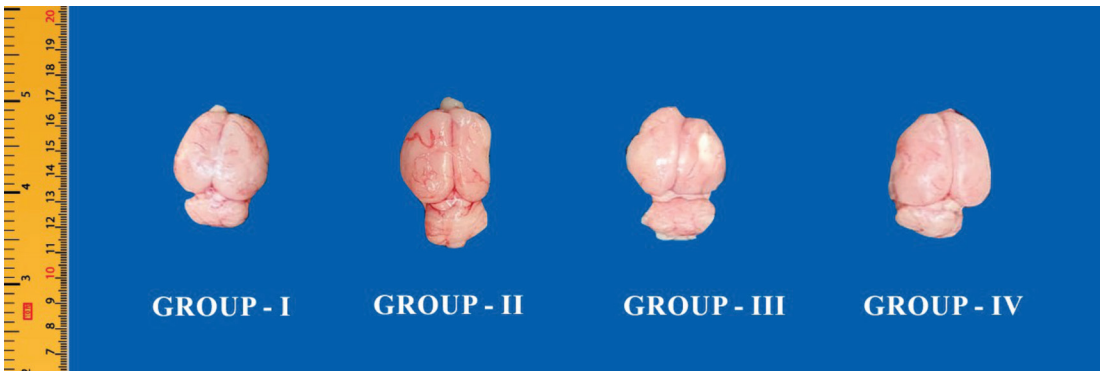

Fig. 2. Brain: Group II 6th week; note congestion of cerebral blood vessels.

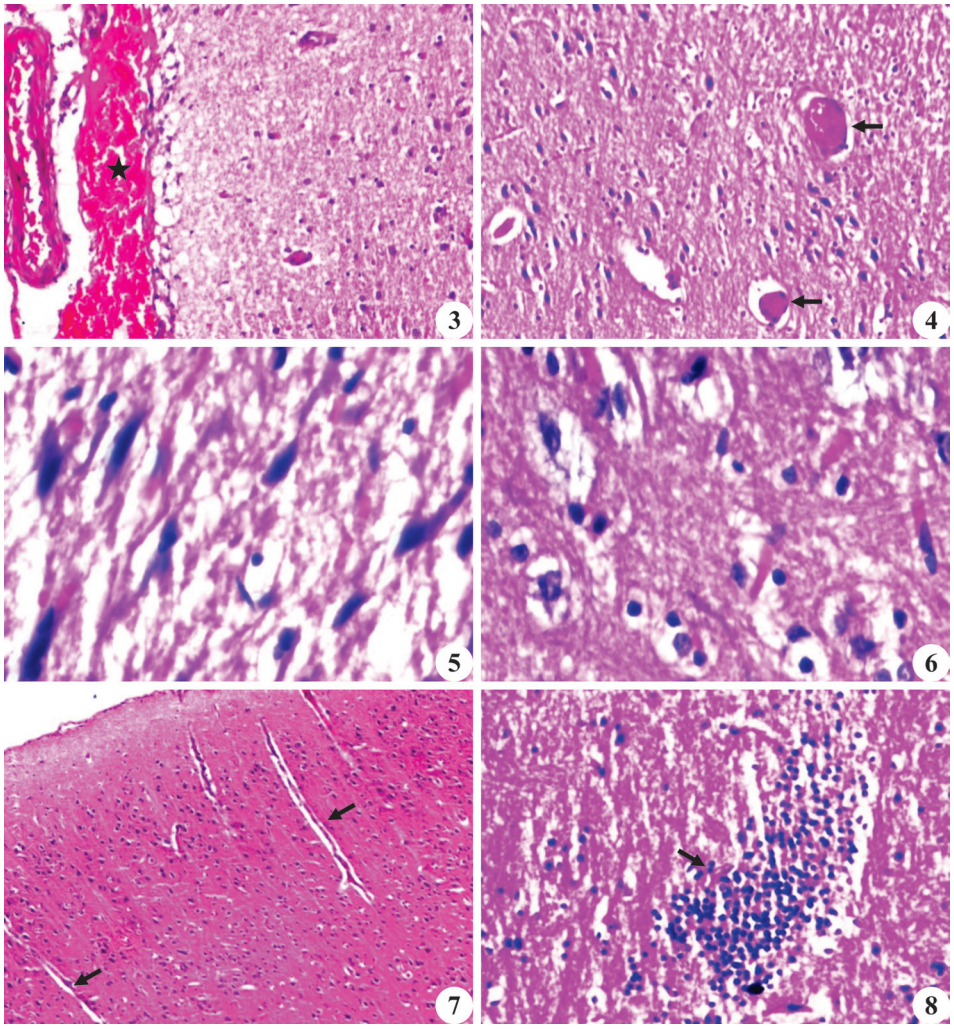

Fig. 3. Brain: Group II: Section showing sub meningeal haemorrhages (asterisk) and congested blood vessels (H&E x100); **Fig. 4.** Brain: Group II: Section showing cerebral blood vessel congestion (arrowed) (H&E x100); **Fig. 5.** Brain: Cerebral cortex: Group II: Section showing shrinkage of neurons with hyperchromatic nuclei (H&E x400); **Fig. 6.** Brain: Cerebral cortex: Group II: Section showing satellitosis and neuronophagia (H&E x400); **Fig. 7.** Brain: Cerebral cortex: Group II: Note moderate capillary proliferation in cerebrum (arrowed) (H&E x40); **Fig. 8.** Brain: Cerebral cortex: Group II: Section showing gliosis (arrowed) (H&E x100).

the reduced glutathione among *Punica granatum* treated (Group III) and control rats.

Glutathione peroxidase (GPx)

The mean GPx values were 24.89±1.41^{a}, 16.35±0.75^{c}, 22.36±0.71^{ab} and 20.09±0.47^{b} (U/mg of protein) in Group I to IV rats respectively, and are given in Table 1 and Fig. 1b. There was a significant ($P<0.05$) decrease in the mean brain GPx values of fipronil treated (Group II) rats compared to ameliorated rats (Group IV) and control rats (Group I). At the same time, the brain GPx values in Group I and Group III rats were statistically insignificant.

Pathology

After the end of the experimental period (6th week), both control (group I) and *Punica granatum* (group III) treated rats showed no gross and microscopic lesions of pathological significance. The brain of fipronil-treated rats (group II) showed cerebral blood vessel congestion (Fig. 2), whereas *Punica granatum* treated rats (group IV) did not show any prominent gross changes.

Histopathological examination of brain of fipronil treated rats revealed submeningeal haemorrhages (Fig. 3), congested cerebral blood vessels (Fig. 4) and choroid plexus. Most of the rats showed various neuronal degenerative changes like shrinkage and hyperchromatic neuronal cells (Fig. 5), central chromatolysis, satellitosis and neuronophagia (Fig. 6) in the cerebral cortex. Moderate to severe proliferation of capillaries (Fig. 7), gliosis (Fig. 8), spongiosis and demyelinating changes were conspicuous findings in the cerebral cortex of all treated rats. Majority of fipronil treated rats showed cerebellar degenerative changes like rounding, shrinkage (Fig. 9) and even complete loss of Purkinje cells in the Purkinje cell layer (Fig. 10). Hyperplasia of Purkinje cells was observed in the Purkinje cell layer of the cerebellum,

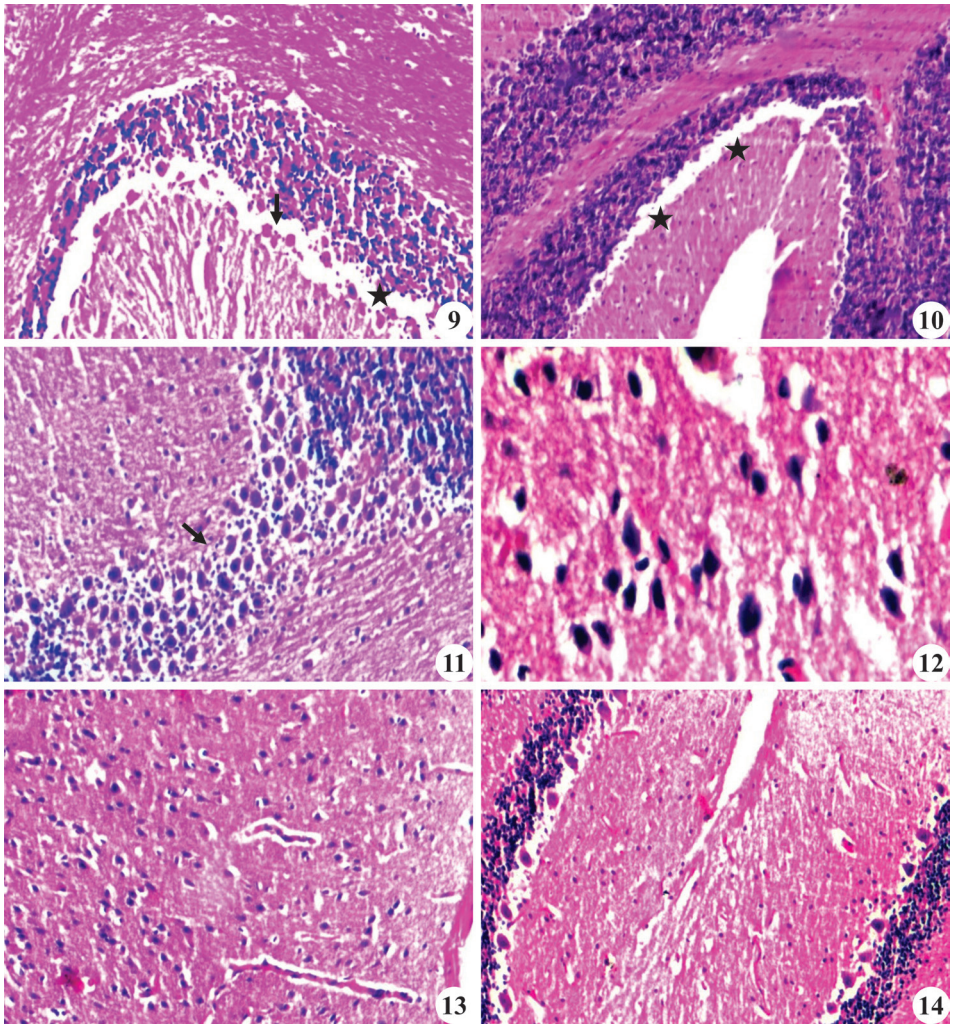

Fig. 9. Section showing rounding (arrowed), shrinkage (asterisk) and loss of Purkinje cells in Purkinje cell layer (H&E x100); **Fig. 10.** Brain: Cerebellum: Group II: Section showing complete loss of Purkinje cell layer (asterisk) (H&E x100); **Fig. 11.** Brain: Cerebellum: Group II: Note hyperplasia of Purkinje cells of Purkinje cell layer (arrowed) (H&E x100); **Fig. 12.** Brain: Cerebral cortex: Group IV: Section normal appearance of neurons (H&E x400); **Fig. 13.** Brain: Cerebral cortex: Group IV: Section showing mild proliferations of capillary blood vessels (H&E x100); **Fig. 14.** Brain: Cerebellum: Group IV: Note normal appearance of Purkinje cell layer (H&E x100).

with these hyperplastic cells extending into the granular layer and forming multiple layers (Fig. 11) in some rats. Neuronal degeneration, spongiosis and demyelinating changes in the molecular layer were more evident in the cerebellum by the end of 6th week of experimental period.

In Group IV rats, similar lesions like group II were observed in ameliorated rats by the end of 6th week of the experimental period with reduced intensity like mild neuronal degenerative changes, reduced spongiosis (Fig. 12) in both cerebrum and cerebellum, reduced capillary blood vessel proliferation (Fig. 13), mild congestion and normal appearance of Purkinje cell layer (Fig. 14).

The brain of *Punica granatum* treated rats (Group III) appeared normal and closely resembled those of the control group.

DISCUSSION

In the present study, a significant ($P<0.05$) increase in LPO (lipid peroxidation) value was noticed in the brain of fipronil treated rats (group II). When compared to corresponding control rats (group I). The present findings were in accordance with the results reported by earlier authors[28]. The increased LPO value might be due to alterations of lipid bilayer configuration in the cell membrane by ROS generated by fipronil, which in turn produces an excess of lipid peroxides (malondialdehyde) from the damaged membrane[29]. Hence, malondialdehyde which is a secondary product of lipid peroxidation, is used as a marker of cell membrane damage.

In the present research, a significant ($P<0.05$) decrease in superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione was observed in the brain of fipronil treated animals when compared to corresponding

controls. These findings were in agreement with[30]; who observed decreased levels antioxidant enzymes in liver of mice teated with fipronil. The decreased antioxidant enzymes activities in different organs in the present study might be due to fipronil induced excessive generation of ROS and reduced cell defence mechanism.

Significant reduction in LPO values along with increased levels of SOD, CAT, GPx, GST and GSH was observed in the *Punica granatum* ameliorated group (Group IV). The restoration of these values near to normal range in *Punica granatum* ameliorated rats might be due to anti-lipid peroxidation property of *Punica granatum*[31] and its antioxidant activity[32].

In the present study, brain of fipronil treated rats showed cerebral blood vessel congestion grossly. Microscopical examination of cerebral cortex revealed submeningeal hemorrhages, neuronal degenerative changes, moderate to severe proliferation of capillaries, gliosis, spongiosis and demyelinating changes. In cerebellum, rounding, shrinkage, loss of Purkinje cells in Purkinje cell layer, neuronal degeneration, spongiosis of molecular layer and hyperplasia of Purkinje cell layer were observed. Similar findings like shrinkage and loss of Purkinje cells, depletion of granular cell layers were reported by[33] in his work. These lesions might be caused by fipronil-induced oxidative damage in the brain. Because fipronil and fipronil sulfone are lipophilic, they cross the blood-brain barrier and cause brain damage and they also inhibit GABA-gated chloride ion channels and mitochondrial function, activating apoptotic proteins (caspases-3), ROS and the release of anti-inflammatory cytokines leads to neuronal necrosis and other neurobehavioral and histological changes in the brain[34].

In *Punica granatum* ameliorated rats (group IV), similar changes were noticed but with reduced intensity when compared to fipronil treated rats (group II). These improved brain changes might be due to the neuroprotective action[35] and antioxidant properties of *Punica granatum*[31].

CONCLUSION

The observations made in this study indicates that fipronil @ 10 mg/kg b.wt. / day orally for a period of 6 weeks induces toxic effects on brain in rats due to accumulation of fipronil and associated oxidative damage in brain tissue. Treatment with *Punica granatum* peel extract @ 200 mg/kg b.wt. / day concurrently with the fipronil was shown to have ameliorating effect on different pathological alterations induced in brain which suggest the neuroprotective effect.

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