

## Oleuropein Attenuates Deltamethrin-induced Apoptosis in Rat Cerebellar Purkinje Neurons

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### Abstract

**Background:** Deltamethrin (DM) is a synthetic pyrethroid insecticide that can elicit neurotoxicity, and lead to apoptosis. There is accumulating evidence that oleuropein (OE) has anti-apoptotic effect. This study aimed at determining the DM toxicity and anti-apoptotic effect of OE pretreatment in cerebellar Purkinje neurons.

**Materials and Methods:** Rats were randomly divided into four groups as follow: DM treated group (12.5 mg/kg; single dose), OE treated group (20 mg/kg per day), DM plus OE treated group, and vehicle group. Sections of cerebellum were taken 24 hours after deltamethrin injection and studied for histopathological and immunohistochemistry assessments.

**Results:** Further characteristics of degeneration in Purkinje neurons were observed in DM group compared with DM plus OE group. Compared with DM group ( $9.56 \pm 1.69$ ), the positive staining for Bax in Purkinje neurones decreased in DM plus OE group ( $2.99 \pm 0.50$ ) but upper than OE ( $0.72 \pm 0.15$ ) and vehicle ( $0.57 \pm 0.03$ ) groups. Compared with DM group ( $0.50 \pm 0.05$ ), the positive staining for Bcl-2 in Purkinje neurons increased in DM plus OE group ( $3.29 \pm 0.18$ ) but lower than OE ( $4.38 \pm 0.80$ ) and vehicle ( $5.87 \pm 1.93$ ) groups.

**Conclusions:** Our results suggest that DM induces apoptosis in Purkinje cells which is subsided by oleuropein.

**Keywords:** Deltamethrin; Oleuropein; Purkinje; Apoptosis

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### Introduction

Pyrethroids are comparatively safe insecticides that have been classified as type I or type II based upon their chemical structure and clinical manifestations of acute exposure (1). Deltamethrin is a type II synthetic pyrethroid insecticide used worldwide as a major class of insecticides in agriculture (2). Acute exposure to deltamethrin can elicit neurotoxicity, characterized by ataxia, loss of coordination, hyperexcitation, convulsions, and paralysis (3). Neurotoxicity of deltamethrin mediated by a series of cellular, molecular, and biochemical cascades, including modification of sodium channels kinetics (4), increasing neurotransmitter release (5), S100 $\beta$  upregulation (6), induction

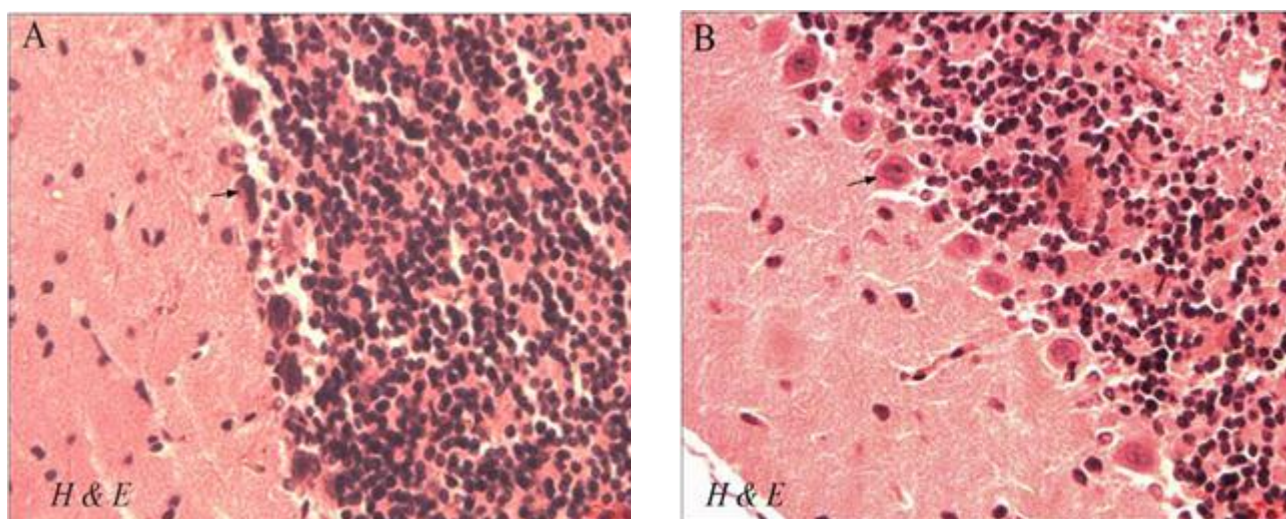
of oxidative damage (7), and induction of cytochromeP450s (8). Moreover, in vitro and in vivo studies suggest an important role played by apoptosis in neurotoxicity of deltamethrin (9, 10). Apoptosis or programmed cell death is a key mechanism of neurodegenerative diseases which is triggered by toxins, radiation, hypoxia, oxidative stress, and ischemia-reperfusion, loss of survival /trophic factors, and DNA damage (11). A number of studies revealed that exposure to deltamethrin significantly affected the survival of neurons in rat brain and induced mitochondria-mediated apoptosis (12, 13). The progressive loss of neurons in central nervous system, leads to various neurodegenerative diseases.

In previous decades, a rapidly growing number of natural polyphenol compounds with anti-apoptotic effects have been described. One of the main sources of these molecules is olive oil. Olive oil is a rich source of polyphenolic components such as its main component oleuropein (3, 4 dihydroxyphenylelenolic acid), which are able to cross the blood-brain barrier and which have many beneficial health effects in human (14-16). There is accumulating evidence that attributed the beneficial effects of oleuropein and its derivatives to a variety of biological activities, including free radical scavenging/antioxidant actions, anti-inflammatory effects, anti-carcinogenic properties, and anti-apoptotic properties (16, 17). In this regard, some experimental studies documented that oleuropein

and its derivatives have anti-apoptotic effects against intestinal ischemia/reperfusion injury (18), 6-hydroxydopamine-induced PC12 cell apoptosis (19), and doxorubicin-induced cardiomyopathy (20).

Purkinje cells are among the largest neurons, and are responsible for most of the electrochemical signaling in the cerebellum. These cells can be harmed by a variety of causes such as toxic exposure, autoimmune diseases, genetic mutations, and neurodegenerative diseases.

In the present study, we evaluated the effect of deltamethrin on histopathology and immunohistochemistry of Purkinje neurons, and investigated the role of oleuropein in alleviating the harmful effects of deltamethrin on these cells.



**Figure 1.** Hematoxylin eosin staining of paraffin sections from the cerebellum of DM (A) and DM+OE (B) treated-rats. Many Purkinje cells showed characteristics of degeneration with pyknosis of nuclei and shrinkage of cytoplasm in DM group (arrow), 400 $\times$ . Little or no signs of degeneration were seen in DM + OE group (arrow), 400 $\times$ .

## Materials and methods

### Animals

Female adult Sprague–Dawley rats weighing 180–200 g (Pasteur Institute, Tehran, Iran) were used. They were kept under standard conditions and were fed a standard rat chow and drinking water ad libitum throughout the study period.

### Experimental groups

The rats were randomly allocated in four groups of five rats each: (I) deltamethrin (DM) treated group (a single intraperitoneal dose of 12.5 mg/kg, 24 hours before sampling; Sigma) (13); (II) oleuropein (OE) treated group (intraperitoneally for 7 days at 20 mg/kg per day; Sigma) (21); (III) DM plus OE treated group was given pretreatment of oleuropein for 7 days at 20 mg/kg per day with a single intraperitoneal dose of 12.5 mg/kg deltamethrin on the seventh day; (IV) vehicle group (the same volume of DMSO).

### Histopathological assessment

Cerebellum samples were taken 24 hours after deltamethrin injection (10), fixed in 10% (wt. /vol.) PBS-buffered formaldehyde and embedded in paraffin. Five-micrometer coronal sections were prepared from the paraffin-embedded blocks using microtome. For histopathological assessment, some tissue sections were deparaffinized with xylene, stained with Hematoxylin eosin and cresyl violet, and studied by light microscopy (DME; Leica Microsystems Inc., Buffalo, NY, USA). All the histological studies were performed in a blinded fashion.

### Immunohistochemistry

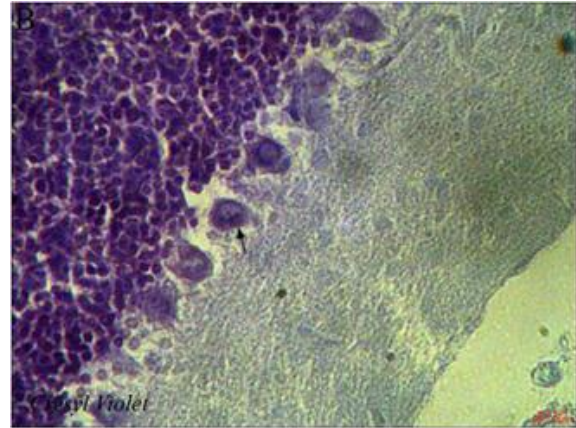
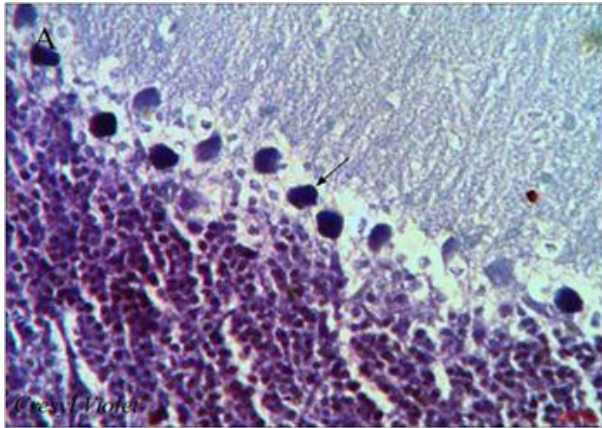
For immunohistochemistry, sections were incubated in goat serum (in order to block nonspecific site), and anti-Bax rabbit polyclonal antibody (1:50 in PBS, vol. /vol., Abcam), or anti-Bcl-2 rabbit polyclonal antibody (1:100 in PBS, vol. /vol., Abcam) overnight at 4 °C. Sections were washed with PBS and then



incubated with secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit IgG peroxidase, Abcam) for 2 hours and demonstrated with diaminobenzidine tetrahydrochloride for 5 minutes. Afterwards, they were dehydrated and mounted. For negative controls, primary antibodies were omitted. For quantitative analysis, immunohistochemical photographs were assessed by densitometry using MacBiophotonics Image J 1.41a software.

### Statistical analysis

Statistical analysis was carried out in SPSS (Version 15, Chicago, IL, USA). Results were presented as mean values ( $\pm$ SD). The K-S test was used in order to evaluate the normality of the data. Also, the Tukey's multiple comparison tests and the analysis of the variance were used to compare each two groups and data among the groups, respectively. A value of  $p < 0.05$  was considered significant.



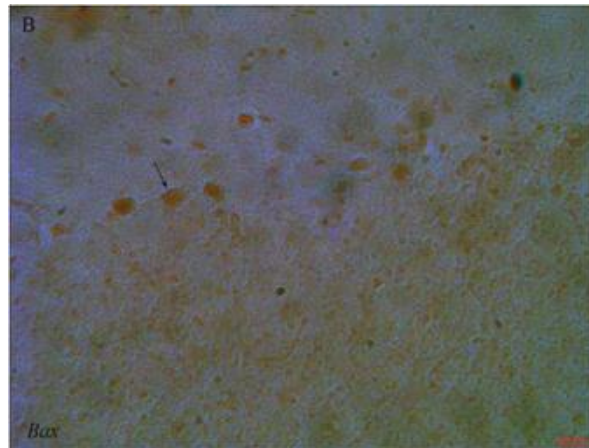
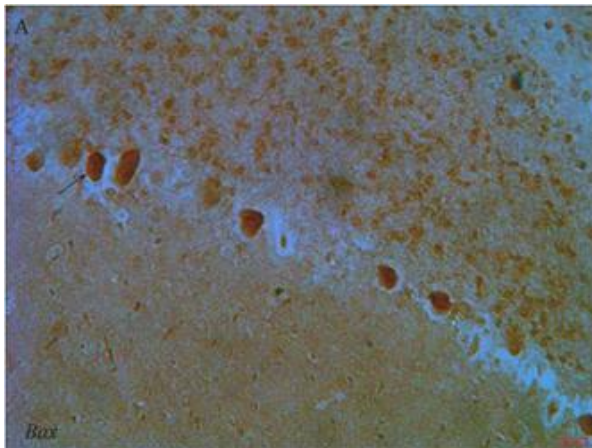
**Figure 2.** Cresyl violet staining of paraffin sections from the cerebellum of DM (A) and DM+OE (B) treated rats. Many Purkinje cells showed characteristics of degeneration with shrinkage and strong staining of Nissl bodies in DM group (arrow), 400 $\times$ . Little or no signs of degeneration were seen in DM + OE group (arrow), 400 $\times$ .

## Results

### Histopathological assessments

To observe the morphological characteristics of purkinje cells in rat cerebellum of all experimental groups, the hematoxylin-eosin and cresyl violet staining were used in the present study. Histopathological study showed some degenerative changes with

hematoxylin-eosin staining in purkinje cells (pyknosis of nuclei and shrinkage of cytoplasm) (Figure. 1A) and with cresyl violet staining (shrinkage and strong staining of Nissl bodies) (Figure. 2A) in the cerebellum of DM- treated rats. Whrease, little or no signs of degeneration were seen in other groups (Figure. 1B & 2B).



**Figure 3.** Light photomicrographs show immunohistochemical expression of Bax in DM (A) and DM+OE (B) groups (arrow), 400 $\times$ . The positive staining of Bax is presented by a brown color of cytoplasm.

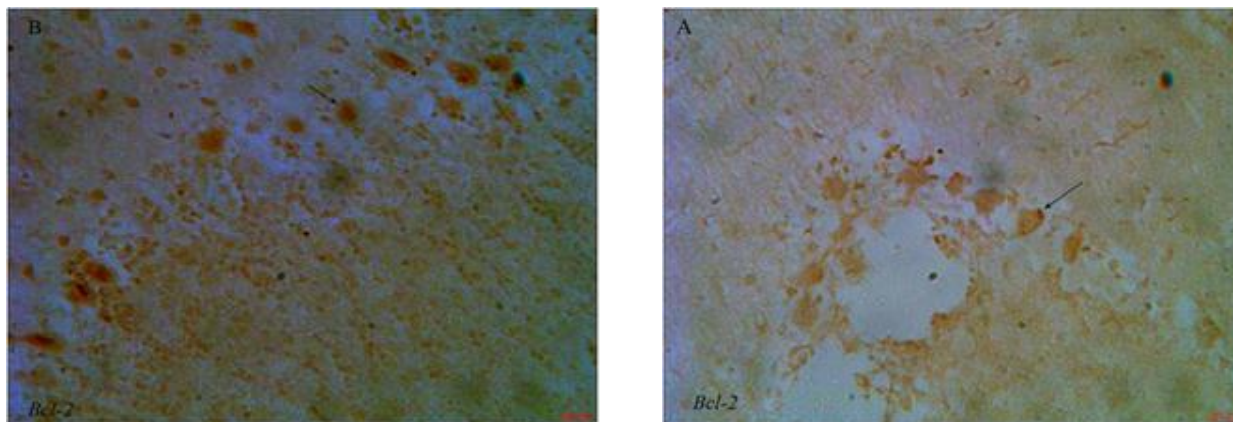
### Immunohistochemistry for Bax and Bcl-2

Figure 3 shows the immunohistochemical staining of Bax. Purkinje cells of the cerebellum from DM

treated-rats exhibited a strong positive staining for Bax ( $9.56 \pm 1.69$ ) (Figure 3A). Oleuropein treatment in DM plus OE treated group reduced the degree of

positive staining for Bax ( $2.99\pm 0.50$ ) (Figure 3B). Purkinje cells of the cerebellum from OE ( $0.72\pm 0.15$ ) and vehicle ( $0.57\pm 0.03$ ) treated-rats showed weak

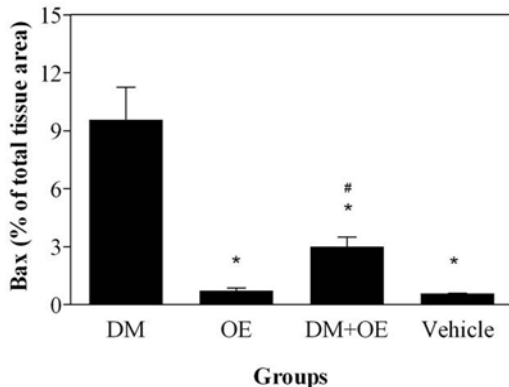
positive immunoreactions for Bax. Figure 4 shows the immunohistochemical staining of Bcl-2.



**Figure 4.** Light photomicrographs show immunohistochemical expression of Bcl-2 in DM (A) and DM+OE (B) groups (arrow), 400 $\times$ . The positive staining of Bcl-2 is presented by a brown color of cytoplasm.

The expression of Bcl-2 was weak in the DM treated-rats ( $0.50\pm 0.05$ ) (Figure 4A) compared to the up-regulation of Bcl-2 in the DM plus OE treated-rats ( $3.29\pm 0.18$ ) (Figure 4B).

The expression of Bcl-2 in purkinje cells of the cerebellum in the OE and vehicle treated-rats were  $4.38\pm 0.80$  and  $5.87\pm 1.93$ , respectively.



**Figure 5.** Densitometry analysis of immunohistochemical photomicrographs for Bax. Data are expressed as a percentage of total tissue area. \* $P < 0.001$  versus DM group; # $P > 0.05$  versus OE and Vehicle groups. Bars indicate the standard deviations of the mean (SDM).

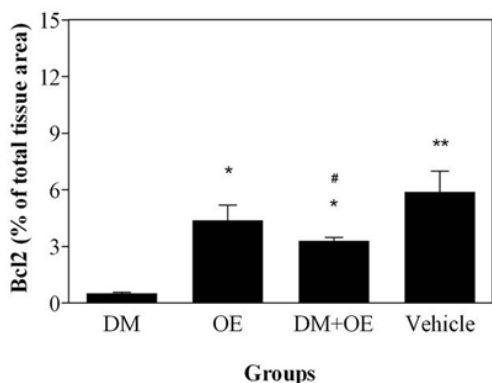
Quantitative analysis showed that Bax levels significantly reduced in DM plus OE treated-rats compared to DM group (Figure 5). Meanwhile, Bcl-2 levels significantly increased in DM plus OE treated-rats compared to DM group (Figure 6).

## Discussion

Neurotoxins are well known risk factors for chronic neurodegenerative diseases. Although molecular

mechanisms involved in the pathogenesis of neurodegenerative diseases remain unclear, oxidative stress, excitotoxicity, inflammation, and apoptosis have been implicated as possible causes on neurodegeneration (22). Apoptosis is a key molecular mechanism of neurodegenerative diseases that is regulated by the Bcl-2 family proteins (22). Among these proteins, Bcl-2 and Bax play anti-apoptotic and pro-apoptotic roles, respectively (23). The ratio of Bax to Bcl-2 determines the cell fate; excess Bcl-2 leads to survival of cells, while Bax induces apoptosis (24, 25). In vitro and in vivo studies have shown that apoptosis is a key mechanism of deltamethrin neurotoxicity which is mediated by altered expression of P53, Bax and Bcl-2, and caspases (9, 13, 26). Caspases are a group of cysteine proteases that play critical roles in apoptosis (27). P53 is a tumor suppressor gene which can induce apoptosis (28). Also, Chen et al. (12) showed that deltamethrin may have an effect on mitochondria-mediated apoptosis of nerve cells in rat brain by altered expression of cytochrome c. The cytochrome c is a small heme protein which is found associated with the inner membrane of the mitochondrion, involved in initiation of apoptosis (29). Deltamethrin causes apoptosis by interaction with  $\text{Na}^+$  channels which is leading to calcium overload and activation of the ER stress pathway (9). Results of our immunohistochemical assessment showed that the treatment with deltamethrin increased positive staining for Bax, whereas exhibited a decreased positive staining for Bcl-2 in Purkinje neurons of DM group. These represent a potentially avoidable event by pharmacological interventions. To date, the majority of epidemiological studies involving olive

oil is linked to a decreased incidence of certain types of neurodegenerative diseases such as Alzheimer's (30), multiple sclerosis (31), and aging (32).



**Figure 6.** Densitometry analysis of immunohistochemical photomicrographs for Bcl-2. Data are expressed as a percentage of total tissue area. \* $P < 0.05$  versus DM group; \*\* $P < 0.01$  versus DM group; # $P > 0.05$  versus OE and Vehicle groups. Bars indicate the standard deviations of the mean (SDM).

Animal and human studies demonstrated that olive oil phenolic compounds are highly bioavailable, the first requirement for a dietary compound to be a potential neuroprotective effect is that it enters the blood circulation. In this regard, a recent study showed that after administration of olive oil phenols, these were absorbed, metabolized and distributed through the blood stream and across the blood-brain barrier (33). On the other hand, in vitro studies have suggested that anti-apoptotic properties of oleuropein and its derivatives, is a pivotal potential neuroprotective mechanism against neurodegenerative diseases (34). Results of our immunohistochemical assessment showed that treatment with oleuropein reduced positive staining for Bax; while on the contrary, it increased positive staining for Bcl-2 in the DM plus OE treated group. Conversely, oleuropein inhibited the expression of proapoptotic protein Bax and induced that of the antiapoptotic protein Bcl-2, thereby provided the molecular evidence for the neuroprotective activity of oleuropein. In this regard, González-Correa et al. (35) documented that lactate dehydrogenase efflux, as a marker of brain cell death, inhibited in a concentration-dependent manner after 7 days of oral treatment with hydroxytyrosol in rat brain slices subjected to hypoxia-reoxygenation. In vitro study has shown that the olive oil phenolic extract and one of its constituents, gallic acid, exert anti-apoptotic effect against H<sub>2</sub>O<sub>2</sub>-induced apoptotic cell death in Hela cells with reduction of time-dependent caspase 9 activity (36). Also, another study documented that incubation of PC12 cells with oleuropein could decrease cell damage and reduce biochemical

markers of apoptotic cell death including activated caspase 3, Bax/Bcl-2 ratio, and DNA fragmentation in 6-hydroxydopamine-induced PC12 cell apoptosis (19). Histological and molecular examinations demonstrated that oleuropein aglycone modulated apoptosis pathway, as shown by tunel staining and Bax/Bcl-2 expressions, in a murine model of intestinal ischemia/reperfusion injury (18). A recent study has shown that oleuropein prevents doxorubicin-induced cardiomyopathy through modulation of kinases such as Akt (20), a serine/threonine-specific protein kinase that plays a key role in apoptosis and cell proliferation (37).

In the present study, it is clear that deltamethrin exposure resulted in alternations of Bax/Bcl-2 expressions and apoptosis in cerebellar Purkinje neurons. Oleuropein pre-exposure provided protection against deltamethrin-induced apoptosis in terms of histopathological and immunohistochemical expression of the pro- and anti-apoptotic protein. In conclusion, this study suggested that oleuropein has modulatory effects against deltamethrin-induced apoptosis in rat cerebellar Purkinje neurons.

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#### Authors' Contributions

RA supervised the study, participated in designing and conducting the study. MB and GhE carried out the study and collected the data.

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#### Conflict of Interest

The authors declare that they have no conflict of interest in this article.

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