Comparative Study between the Neuroprotective Effect of Curcumin and Vitamin C against Neurotoxicity Induced By Acetamiprid on the Cerebellum of Male Adult Albino Rats: A Histological Study

Amira Shahat Mohamed[a]*, Maha Abdelbaki Ahmed[a], Dorreia AbdAllah Mohamed[b]
[a]Human Anatomy and Embryology Department, Faculty of Medicine, South Valley University, Qena, Egypt.
[b]Human Anatomy and Embryology Department, Faculty of Medicine, Assiut University, Assiut, Egypt.

Abstract
Background: Acetamiprid (ACE) is a neonicotinoid insecticide, known in the trade market as: Mospilan, Assilan and Chipco. It is used to get rid of sucking insects. Its intake might cause toxic effects on the cerebellum.
Objectives: The study aimed to determine the pathological effects of acetamiprid on the cerebellar cortex of male adult albino rats and the role of both Curcumin and Vitamin C in altering those pathological effects.
Materials and methods: Forty male adult albino rats were used in this study. The rats were divided into four groups (n=10 each): group I (control) and received corn oil, group II (treated with acetamiprid orally), group III (treated with acetamiprid + Curcumin orally), group IV (treated with acetamiprid + Vitamin C orally). Cerebellar tissue samples were used for histopathological examination using light and transmission electron microscopy. Morphometric and statistical studies were done to measure Purkinje cell (PC) number and the thickness of the granule layer.
Results: There was a significant structural damage of cerebellar cortical tissue in group II. Group III showed restoring of the pathological structure of cerebellar cortex compared to group II. Group IV showed less improvement compared to group III.
Conclusion: Curcumin and vitamin C could have a protective effect against neurotoxicity induced by acetamiprid via their antioxidant property.
Keywords: Acetamiprid; Neurotoxicity; Curcumin; Vitamin C; Cerebellum.
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***Correspondence**: Amiraalsharif5@gmail.com
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Introduction

Acetamiprid is a neonicotinoid that acts as an insecticide to get rid of pests, but it may have toxic effects on mammals **(El-Hak et al., 2022).**

It is used in manufacture fungicides and insecticides for controlling pests and fleas on cats and dogs **(Brunet, 2005)**.

Insecticides including acetamiprid are known to contain free radicals named reactive oxygen species (ROS) which has the ability to infiltrate DNA to cause multiple changes. ROS is mainly consisted of super oxide anion, H2O2 and singlet oxygen (**Butterfield, 2020**).

All neurons in the spinal cord and brain, and muscle connections have post-synaptic dendrites that contain nicotinic acetylcholine receptors, which acetamiprid binds. The result of this reaction is production of a state of muscular spasm and even subsequent death. Acetamiprid is highly toxic to insects due to its preferential affinity for binding to their receptors **(Tomizawa et al., 2000)** and of less toxicity to mammals because of the change in the form of receptor in vertebrates **(Sanchez-Bayo, 2012)**.

Acetamiprid exposure impairs Purkinje and granule cells in the cerebellum and causes the degeneration of cognitive and behavioural abilities. As the primary components of the cerebellar information processing system, PCs can negatively impact motor coordination, the hallmark of Parkinson's disease **(Baltazar et al., 2014)**.

Due of its high oxygen consumption rate, elevated quantities of polyunsaturated fatty acids, and relatively low quantity of antioxidative enzymes, the cerebellum is vulnerable to oxidative stress **(Fahn and Cohen, 1992).** Numerous studies have been conducted to find the neuroprotective compounds that can lessen the harm that pesticides do to the central nervous system (CNS). A non-toxic polyphenol having antioxidative, antimitogenic, and anticancer effects is called curcumin. **(Lonare et al., 2014; Sahu et al., 2016 and Marzouki et al., 2017).** Curcumin can penetrate the blood-brain barrier and treat a variety of neurological conditions, including Parkinson's and Alzheimer diseases **(Chattopadhyay et al., 2004)**.

Curcumin is known to protect mitochondria against both in vivo and in vitro oxidative stress **(Bayomi et al., 2015)**.

Vitamin C is an antioxidant that dissolves in water and has been indicated to react with superoxide, hydroxyl radicals and singlet oxygen decreasing cell damage **(Bowman, 2012)**. Moreover, vitamin C simply passes through blood brain barrier (BBB) **(Agus et al., 1997)** and deactivates extracellular and intracellular free radicals, as it is a rich store of electrons which stick to free radicals to restrain their activity **(Bendich, 1990)**.

Material and methods

Forty adult male albino rats weighting 150 ±20 gm (10 weeks old) were obtained from the animal house at South Valley University. Two rats were kept in each spotless plastic cage so they could get acquainted to the lab setting. In a mass air displacement room with a 12-hour light/12-hour dark cycle, animals were kept at 24 2 °C and 50 10% relative humidity. The animals received ad libitum access to a balanced diet and clean water. We bought acetamiprid technical 90% pure (MOSPILAN) from the Egyptian Ministry of Agriculture. The rules in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health were strictly followed when conducting this study. The Principles for the Ethical Control and Supervision of Animal Care and Use were strictly followed during the conduct of animal research. Every effort was made to decrease suffering.

Experimental design: Four groups of ten rats each were created randomly. Group I (control) received an equal number of vehicles (corn oil). A dose of 40 mg/kg of acetamiprid was administered

orally to Group II for 21 days in a row. For 21 straight days, Group III was given an oral dose of 100 mg/kg of curcumin and 40 mg/kg of acetamiprid. For 21 days straight, Group IV was given an oral dose of 50 mg/kg of vitamin C and 40 mg/kg of acetamiprid.

Handling of cerebellar tissue: At the conclusion of the experimental period, the animals were deprived for a night (3 weeks). Diethyl ether inhalation was used to anaesthetize the rats before they were sacrificed. The cerebellum was taken out of the brain and divided into two hemispheres.

For light microscopic study: One hemisphere from each rat was fixed in bouin solution for 24 hours and then was washed well and kept in alcohol 70% then placed in paraffin to be examined by light microscope. Hematoxylin and eosin (H&E) was used to stain slices of 5 mm thickness **(Bancroft and Gamble, 2008).** Semithin sections (1μm thick) were prepared and stained with toluidine blue, examined and photographed under the light microscope.

For electron microscopic study: One hemisphere from each rat was kept in glutraldehyde 5% for electron microscopic examination. The cerebellum was dissected, sliced into thin slices about 2mm thick, and preserved in osmium tetroxide solution that was 1% concentrated. The samples were immersed in Epon resin and ethanol in increasing concentrations **(Bancroft and Gamble, 2008)**.

Morphometric study: At the Histology Department, Faculty of Medicine, South Valley University, the morphometric. Using a computer-aided picture analyser, measurements were made using the touch count approach (soft imaging system-An Olympus Company). In ten randomly selected parts from six separate animals for each group, five non-overlapping fields, and a 10 objective lens were used for the measurements. The mean number of PCs and the mean thickness of the granular layer were the characteristics that were examined.

Statistical analysis

SPSS version 19 was used for the analysis. Each animal group's morphometric data were statistically examined, and the investigated animal groups were compared using the ANOVA test. The mean ± SD was used to present the information. The importance of the data was assessed using p values, where p 0.05 was considered significant.

Results

Light microscopic results using H&E stain

Group I (control group):

The H&E-stained slices of the cerebellar cortex from the control group displayed normal architecture under light microscopy. There are three layers that make up the grey matter of the cerebellar cortex: the middle Purkinje cell layer (PCL), the inner granular cell layer (GCL), and the outer molecular cell layer (MCL). There are few neurons in the outer MCL. At the intersection of the MCL and the GCL, the PCL reveals a single row of large PCs with the shape of flasks. In between the PCs, which come in a variety of shapes and have pale nuclei and cytoplasm, are these cells having a distinctive centrally located rounded open-face nucleus with a prominent nucleolus and were surrounded by Bergmann astrocytes that were dispersed in the superficial part of the granular cell layer. The granular nerves in the GCL were organised in a dense pattern. With their dark stained nuclei and very little cytoplasm around it **(Fig.1)**.

Group II (acetamiprid-treated rats):

Cerebellar cortex revealed marked histological alterations markedly on the Purkinje cell layer (PCL). The PCs appeared distorted, disarranged with irregular size and shape. Few astrocytes were noticed. The GCL showed many vacuoles with areas of patchy cell loss **(Fig.2A).** A few diminished PCs with fractured nuclei and eosinophilic cytoplasm were seen **(Fig.2B)**.

Disturbed shrunken displaced PCs in the granular layer with dark cytoplasm and surrounded by halos of empty space were also noticed **(Fig.2C)**. The MCL appeared with shrunken basket and stellate cells being surrounded by prominent perineural spaces **(Fig.2D)**.

Group III (acetamiprid + Curcumin):

The molecular layer shows normal stellate and basket cells. The PCL is normally distributed in single row. No degenerative changes appear. It restored its flask shaped appearance with deeply stained nuclei and cytoplasm. The GCL shows small granular cells which appear normal **(Fig. 3)**.

Group IV (acetamiprid + Vit C): The cerebellar cortex of the rats who got acetamiprid and vitamin C at the same time showed that all layers of the cerebellar cortex had returned to normal in comparison to the control group. The PCL seemed mostly normal and linear, and the majority of the PCs reclaimed their flask shape with darkly stained nuclei and cytoplasm. However, a small number of cells appeared degenerated and others disappeared leaving vacuoles **(Fig. 4-A)**. Both molecular and granular layer show normal stellate and basket cells **(Fig.4-B)**.

Light microscopic results using toluidine blue stain

Group I (control group):

Examination of semithin section of a cerebellar rat of group I showed cerebellar cortex with its purkinje, granular and molecular layers. PCs had oval shaped cells of vesicular nuclei and were arranged in a single row. They were surrounded by glial cells **(Fig.5)**.

Group II (acetamiprid-treated rats):

As regard group II, examination of the semithin section showed perineuronal spaces around the cells in the molecular layer. There were no longer any functional PCs. Other PCs with asymmetrical, heavily pigmented nuclei were visible. Additionally, granular layer vacuolation was observed. **(Fig. 6)**.

Group III (acetamiprid +Curcumin):

Group III displayed the three strata in their typical state. Vesicular nuclei and the form and organisation of PCs were preserved. Glial cells were everywhere around the PCs. There were no vacuolation was found in GCL **(Fig. 7)**.

Group IV (acetamiprid + Vit C):

The semithin section of cortical cerebellum in group IV showed that molecular layer contained some spaces. PCs retained its shape and arrangement. They were oval in shape and have vesicular nuclei. PCs were surrounded by glial cell. GCL showed vacuolation **(Fig.8)**.

Electron Microscopic results

Group I (control group):

Numerous small, dark granular cells with somewhat dark oval nuclei and the distinctive condensed chromatin were present in the layer of granular cells, which also contained rough endoplasmic reticulum (RER), lysosomes and mitochondria. The granular cells were interspersed with oligodendrocytes **(Fig. 9A)**.

The PCs in the cerebellar cortex were found to have irregular shapes and big eccentric euchromatic nuclei surrounded by cytoplasm with well-defined mitochondria and lysosomes. On the inner side of the nuclear envelope, which had mild invagination, the Purkinje cells' and their nuclei's enormous blocks of condensed chromatin were scattered (**Fig.10 A)**.

Group II (acetamiprid-treated rats):

Variable-sized granular cells were visible. While other cells displayed pyknotic nuclei, some displayed condensed nuclear chromatin and erratic nuclear envelopes. Their cytoplasm was torn up, the mitochondria enlarged. The granular layer's oligodendrocytes had condensing, degenerating alterations, either with electron dense clumped chromatin or pyknotic nuclei with apoptotic modifications **(Fig.9 B)**.

Rats in group II's cerebellar cortex showed that most of the PCs were distorted and

shrunk. Pyknotic nuclei, vacuolated cytoplasm, dilated RER cisternae and degenerated or abnormal mitochondria were present in some PC nuclei (**Fig.10B**).

Group III (acetamiprid +Curcumin): The granular layer was composed of a large number of small, black granular cells with distinctive compacted chromatin, somewhat dark oval nuclei, and very little cytoplasm that contained rough endoplasmic reticulum, mitochondria, and lysosomes **(Fig. 9C**.)

The cerebellum's PCL and GCL had reclaimed their usual form and appearance. The PCs had an atypical form, big eccentric euchromatic nuclei, and nucleoli surrounded by cytoplasm that contained distinct mitochondria, lysosomes, and RER. On the inner side of the nuclear envelope, which had mild invagination, the Purkinje cells' and their nuclei's enormous blocks of condensed chromatin were scattered (**Fig.10C**).

Group IV (acetamiprid +Vit C):

A very small number of cells did, however, still have active cytoplasm, an abundance of mitochondria, and an active RER (**Fig. 9D).**

A histological image of the cerebellar cortex that was similar to the normal one was visible. The majority of Purkinje nerve cells had a typical appearance, and their nuclei had blocks of condensed chromatin that were dispersed on the inside of the nuclear envelope with a minimal amount of invagination. Activated mitochondria and lysosomes were visible in its cytoplasm **(Fig.10D**). The granular cells resembled those of the control group almost exactly.

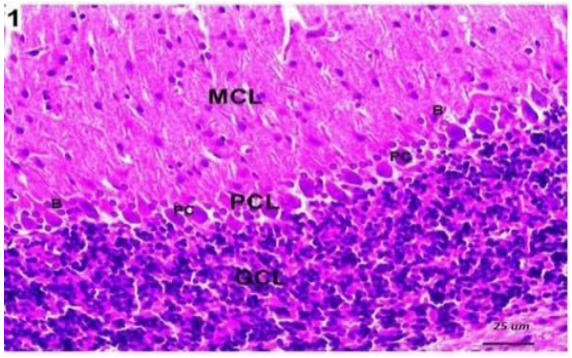


Fig.1. MCL, the middle PCL, and the Bergmann astrocytes are all visible in photomicrographs of a section of the group I cerebellar cortex. PCL has large flask-shaped PCs with large vesicular nuclei in the centre (B). The inner GCL is made up of a large number of densely packed, highly pigmented cells (H&E, x400).

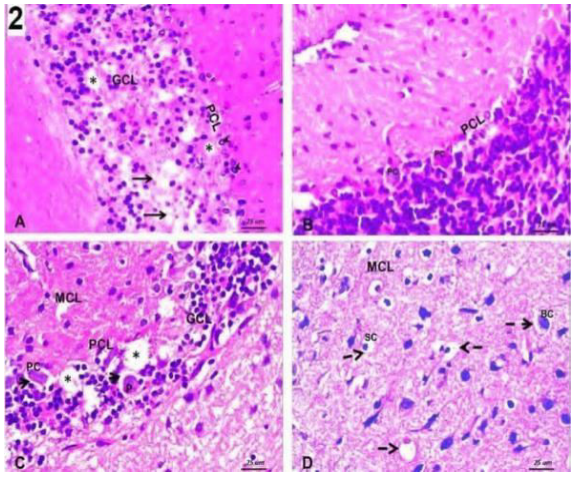


Fig.2. Sections of group II's cerebellar cortex. PCL exhibit severe deformation and have few astrocytes (head arrows). With areas of patchy cell loss and many vacuoles (asterisks) in the GCL (black arrows). (B) Eosinophilic, fragmented PCs with shrunken cytoplasm. Disturbed PC in the GCL (C) seemed to be shifted downward (P), some of which had black cytoplasm and flat surfaces. Vacuoles are many (asterisk). (D) the MCL, is bordered by substantial perineural gaps (detached arrows) and contains smaller basket cells (BC) and stellate cells (SC) (H&E, x400).

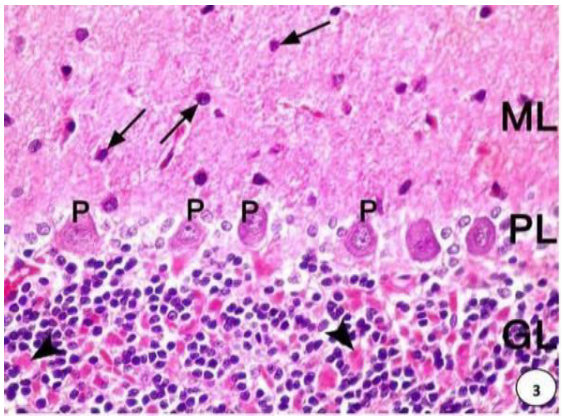


Fig. 3. The middle PCL displays large pyriform cells (P) with vesicular nuclei and apparent nucleoli, while the outer MCL contains few nerve cells (arrows). This image is a photomicrograph of a section of the adult rat cortical cerebellum from group III. Small, spherical cells that are densely packed make up the inner GCL. Between the granular cells, there are acidophilic regions (arrowheads) (H&E, x400).

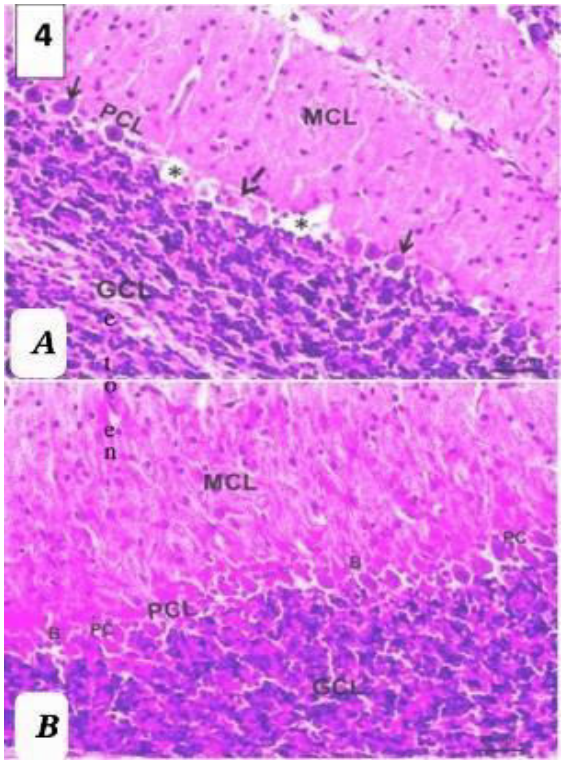


Fig.4. Cerebellar cortex of group IV showed (**A**) Relatively normal linear appearance of PCL. PC restore their normal flask shape (arrows) with darkly stained nuclei and cytoplasm. Few vacuoles (asterisks) and degenerated PC (dashed arrow) are still seen. Both MCL and GCL appeared normal. (**B**) Normal linear appearance of PCL with greater numbers of normal flask shaped PC with aggregations of excess astrocytes around them. MCL and GCL layers appear as control group (H&E, x400).

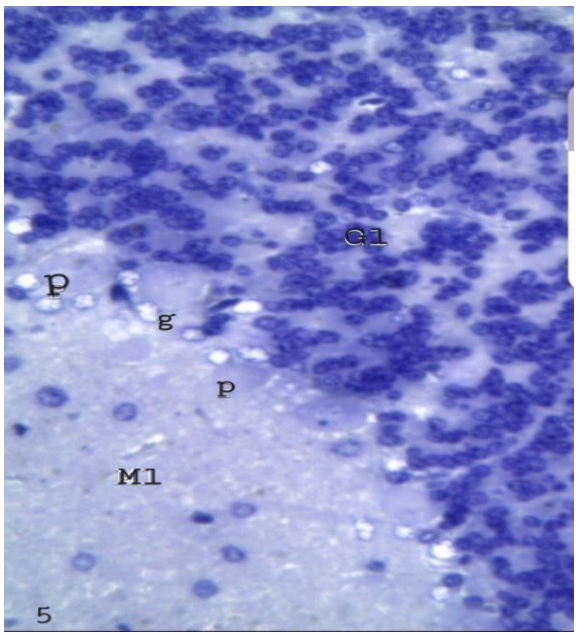


Fig.5. The MCL and the GCL are seen in a photomicrograph of a semithin section taken from a group I cerebellar rat (GL). The glial cells (g), GCL, and oval-shaped PC (P) with vesicular nuclei that are organised in a single row (Toluidine blue, x1000).

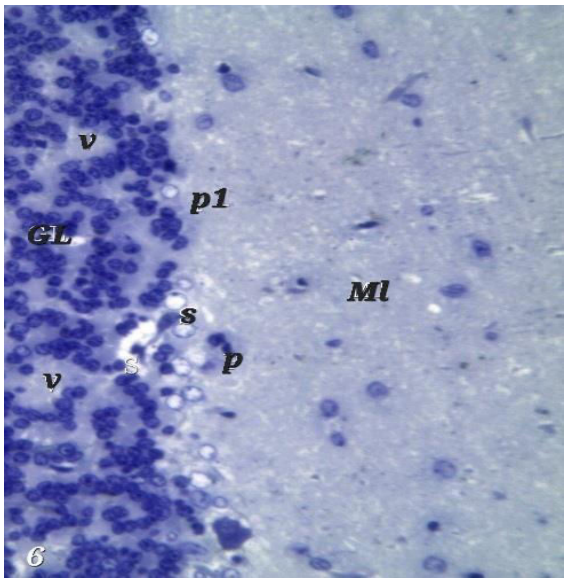


Fig. 6. A picture of semithin section of cortical cerebellum in rat of group II showing perineuronal space around the cells in the MCL (S). PC are absent and degenerated (P1). One PC appears with irregular densely stained nucleus (P). There are many vacuolations (V) in the GCL (Toluidine blue, x1000).

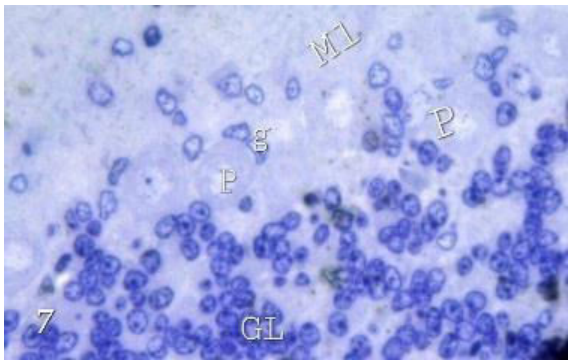


Fig.7. A photomicrograph of semithin section of cerebellar cortex in group III showing the three layers appear normal. PCs (P) retain their shape and arrangement with vesicular nuclei. PCs are surrounded by glial cells (g) (Toluidine blue, x1000).

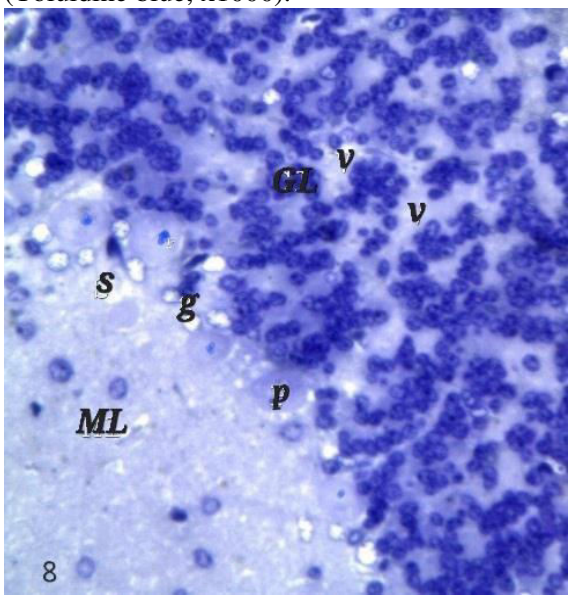


Fig.8. Imaging of semithin section of cerebellar cortex in group IV which showing MCL contains some spaces (S). PCs (P) retain its shape and arrangement. They are oval in shape with vesicular nuclei. PCs are surrounded by glial cell (g). GCL contains vacuolations (V) (Toluidine blue, x1000).

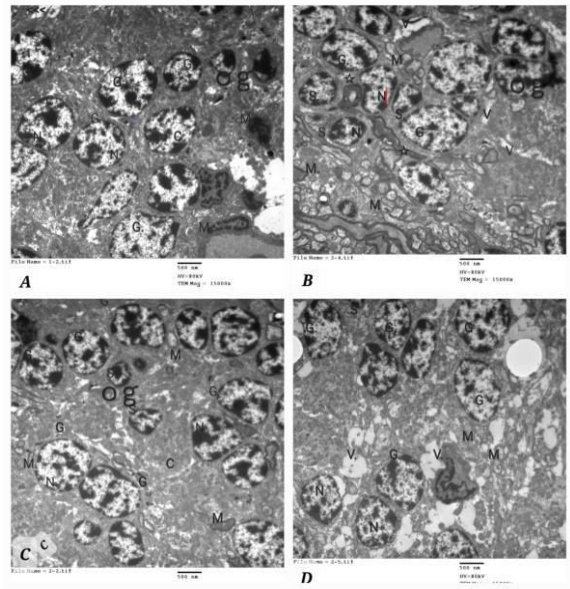


Fig.9. Electron imaging of cerebellar cortex of rats: (A) Group I showing that granule cells (G) appear normal oval in shape with large deeply stained nuclei (N) and clumped chromatin (C). Mitochondria (M) are also found in cytoplasm. Oligodendrocytes are detected with deeply stained nuclei. (B) Group II showing that some of the granule cells appear normal (G) with the others appear shrunken and necrotic (S). The nucleus (N) appears irregular in shape with clumped chromatin. Cytoplasm contains vacuolations (V) with multiple mitochondria (M). Blood capillaries also surround granule cells. (C) Group III showing that multiple granule cells (G) appear normal with oval or rounded well defined nucleus (N) with chromatin clumps (C). Few granule cells appear degenerated and necrotic (S). The cytoplasm shows mitochondria. (D) Group IV showing that granule cells (G) appear normal. The nucleus is oval in shape with clumped chromatin. Cytoplasm contains vacuolations (v). Mitochondria (M) are detected in the cytoplasm (TEM×15000).

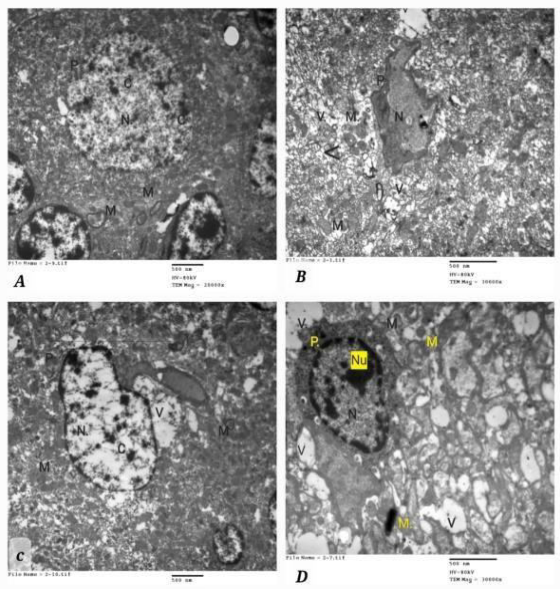


Fig.10. Electron imaging of cortex of cerebellum of (A) Group I: showing PC (P) with well-defined nucleus (N), euchromatin clumps (C) and non-apparent nucleolus. The cytoplasm contains multiple mitochondria (M). (B) Group II showing PC (P) that appears shrunken and degenerated with irregular outline. The nucleus (N) appears ill-defined. The cytoplasm contains multiple vacuolations (V). Numerous dilated and swollen Mitochondria (M) are also found, dilated RER cisternae are also detected (arrow). (C) Group III showing PC (P) is normal with indented nucleus (N) and clumped chromatin(C). The cytoplasm contains some vacuoles (V) and mitochondria (M). (D) Group IV showing PC is normal with indented nucleus (N) and apparent nucleolus (Nu). The cytoplasm has mitochondria (M). Cytoplasmic vacuoles (V) also found in the cytoplasm (TEM×20000).

Morphometric results

At the Department of Histology, Faculty of Medicine, South Valley University, the morphometric measurements were carried out using the touch count method with a computer assisted image analyzer (soft imaging system - An Olympus Company). In ten randomly selected parts from six separate animals for each group, five non-overlapping fields, and a 10 objective lens were used for measurements. The following variables were looked at: The average thickness of the GCL and the average number of PCs (**Table.1**). Group II had considerably fewer PCs on average and a thinner GCL on average than control group. They were non-significantly lower than the control group and considerably higher in group III than groups II. They were much higher than group IV in comparison (**Table.1 & Histogram 1&2).**

Table 1. Morphometric and statistical results

Groups	Mean ±SD of PCs	Mean ±SD of thickness of GCL
Group I	10.1 ± 1.66	282.81 ± 49.98
Group II	3.9 ± 0.87*	125.13 ± 15.49*
Group III	8.5 ± 1.64$#	235.98 ± 48.89$#
Group IV	5.7 ± 1.41*$	178.22 ± 45.02*$

$ Significant to group I

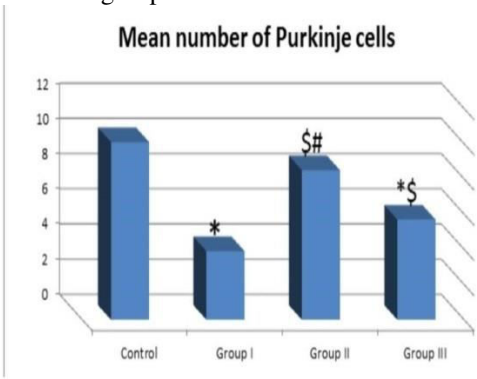
*Significant results at $p < 0.05$.

SD: Standard deviation

* : Significant to control group.

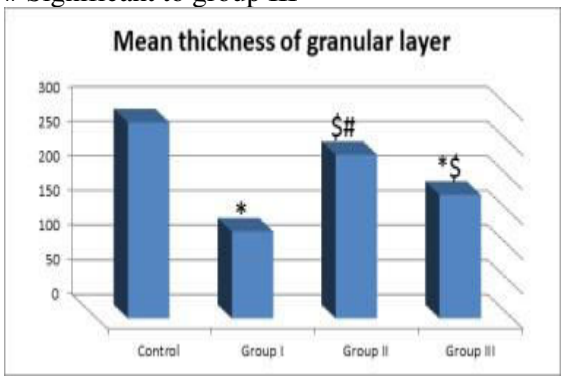
$: Significant to group I

Significant to group III



Histogram 1 :**The mean ±SD of number of PCs of control and experimental groups.**

Significant results at $p < 0.05$,* Significant to control group. $ Significant to group I. # Significant to group III



Histogram 2:The mean ±SD of thickness of granular layer (in micrometer) of control and experimental groups.

Significant results at $p<0.05$ *Significant to control group. $ Significant to group I. # Significant to group III.

Discussion

The results of this study supported acetamiprid's neurotoxic effects. According to **Singh et al.(2015**)'s study, reactive oxygen species may be responsible for the histological alterations in adult rats exposed to ACE in the cerebellar cortex. Also our results are confirmed by **phogat et al. (2022**) who showed that exposure to acetamiprid has altered the histo-architecture of the brain and lead to brain toxicity in the form of necrosis and hyperemia.

This study's observation of cytoplasmic vacuoles may be explained by the amino acid imbalance caused by acetamiprid in the area around the neurons. By interacting with the lipids and proteins of various organelles, they might release free radicals, which would then result in the formation of vacuoles in the cytoplasm. Additionally, the chromatin of the nuclei could be altered by these free radicals. The direct toxicity on neuronal cells was the primary cause of the increased cytoplasmic electron density, increased chromatin condensation, and deformation of the mitochondria. This toxicity causes significant intercellular biochemical events to go awry, resulting in aberrant protein synthesis, oxidative phosphorylation inhibition, and malfunction during detoxication **(Sobaniec et al**., **2001**).

According to **Yu et al.(2008)** the pyknotic nuclei and condensed nuclear chromatin in the GCL, along with dilated RER cisternae and disrupted swelling mitochondria, were observed to occur in the early stages of apoptosis. Additionally, acetamiprid free radicals may contribute to the degeneration of oligodendrocytes by impeding neuronal activity and sending suicide signals to the neurons (**Lin and Beal**, **2006**). Another potential mechanism is that oxidative stress may result in cell malfunction, membrane breakdown, and ultimately apoptosis. Another potential mechanism is that oxidative stress may result in cell malfunction, membrane breakdown, and ultimately apoptosis **(Catalá, 2007)**. It is well known that the antioxidant defence mechanism maintains a comparatively low level of the reactive and damaging OH (**Regoli and Principato**, **1995**). Vitamin C, for example, is an exogenous antioxidant that can effectively preserve neural cells **(Sánchez-Moreno et al., 2003; Varshosaz et al., 2014).**

The present results of group III corroborate with those of **Lonare et al**. **(2014)**. Because curcumin has phenolic, methoxyl, and diketonic groups, it has the potential to pass the blood-brain barrier (**Yang et al**., **2005**).

Administration of vitamin C in the present study to the treated rats resulted in improvement of the pathological findings. However, slight alterations were still detected in the PCL. This denotes that vitamin C neuroprotective sensitivity of the PC is less than other cerebellar cells. This was in match with **Farombi and Onyema** .**(2006)** and **Pavlovic et al**. (**2007**). In the present work, improvement of the electron microscopic results was in accordance with **Varshosaz et al**. (**2014**) and **Afifi & Embaby.** (**2016**), who first discovered ascorbic acid's preventive effects against cadmium-induced brain neurotoxicity in rats. According to some research, vitamin C may have neuroprotective effects by scavenging oxygen-free radicals. (**Peng et al**., **2005** and **Jetti et al**., **2014**). Ascorbic acid also had an antiapoptotic impact. **(Han et al., 2007)** and made a neuroprotective impact **t**hrough decreasing lipid preoccupation and increasing the activity of catalase (**Han et al**., **2007**; **Santos et al**., **2008**). Vitamin C may have this neuroprotective effect because it can pass through the blood-brain barrier **(Peng et al., 2005**). Additionally, vitamin C helped creation of amino acids, while catecholamines and carnitine controlled how the nervous system worked **(Grosicki, 2004)**.

Conclusion

Because they are antioxidants, curcumin and vitamin C may offer some protection

against the neurotoxicity that acetamiprid causes.

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