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Histological and Immunohistochemical Study of the Possible Protective Effect of Bee Pollen Extract on the Cerebellar Cortex of Valproic Acid-Induced Experimental Model of Autism in Albino Rat Pups

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Abstract:

Background: Valproic acid-induced autism represents a well-recognized model to induce autism in rats. Bee pollen has potent anti-inflammatory, antioxidant and anti-apoptotic effects. **Aim:** This work was performed to study the protective effect of bee pollen extract on valproic acid-induced model of autism in rat pups. **Materials and methods:** The study was performed on 50 male albino rat pups 10-15 gm that were classified into four groups; group 1:control group, group II: rat pups received oral aqueous extract of bee pollen 250 mg/kg once daily from PND 14 to 40, group III: autism was induced by subcutaneous injection of a single dose of valproic acid 400mg/kg on PND 14, and group IV: after induction of autism as in group III, rat pups were treated with an aqueous extract of bee pollen from PND 14 to 40. All rats were sacrificed at PND 41. Cerebellar specimens were processed for histological and immunohistochemical study in addition to morphometric, behavioral and statistical studies. **Results:** Valproic acid-induced autism group depicted

behavioral changes and marked alteration of the histological picture of the cerebellar cortex by light and electron microscopes. A significant decrease in the granular cell layer thickness and a significant increase in the mean area percentage of GFAP immunopositive reaction were observed in group III as compared to the control group. Treatment with Bee pollen showed marked improvement of the previously mentioned pathological changes. **Conclusion:** Bee pollen could improve the behavioral and histological alterations in the cerebellar cortex of valproic acid-induced autism in rat pups.

Running title: Bee pollen could improve autism.

Keywords: Valproic acid, autism, Bee pollen, Cerebellum, GFAP.

1. Introduction:

Autism spectrum disorder (ASD) is a complex childhood neurodevelopmental disorder that is characterized by a decrease in social interaction and communication, as well as monotonous and stereotyped performance [1&2]. Many studies have revealed that, different factors may play a role in the pathogenesis of autism [3]. Numerous studies have established that early exposure to VPA either prenatal or postnatal is used to evoke animal model for autism [4 & 5]

Bee pollen (BP) is a combination of nectar, wax, flower pollen, enzymes, honey, and bee secretions containing large amounts of proteins, folic acid, active enzymes, microelements and significant quantities of polyphenols such as flavonoids [6,7]. Furthermore, BP was proved to possess many therapeutic effects with strong anti-inflammatory, anti-oxidant, anti-allergenic, anti-toxic, anti-carcinogenic, anti-microbial, and anti-radiation activities [8&9]

2. Materials and Methods:

Experimental Animals:

This study was done on 50 male albino rat pups at the age of 14 days with an average weight 10-15gm each. They were left with their mothers in the cages until postnatal day 13 (PND13). On that day, the rat pups were haphazardly grouped into four groups to be ready to start the experiment at PND14. The local ethical committee of the Faculty of Medicine, Tanta University, Egypt approved this protocol with approval code (No. 36264MS29/1/23).

Chemical Reagents:

1- Valproic acid (VPA):

Valproic acid was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) as

valproic acid sodium salt 98% (Cat.no. P4543) in the form of white powder for dissolution in normal saline. It was used to induce autism in rat pups.

2- Bee Pollen (BP):

Bee pollen was obtained from the Egyptian Ministry of Agriculture, El Dokki as a natural BP 100%, and was prepared as aqueous extract solution in the department of Chemistry, Faculty of Science, Tanta University.

Study design:

The rat pups were randomly classified into four groups:

1- **Group I (Control group):** Included 10 rat pups that did not receive treatment at all.

2- **Group II (BP extract-treated group):** Included 10 rat pups that were given aqueous extract solution of BP 250mg/kg/day orally one time daily starting from PND14 to PND 40 [10]

3- **Group III (VPA- induced autism group):** Included 15 rat pups that were given VPA 400mg/kg by subcutaneous injection once on PND 14 [3,11]

4- **Group IV: (VPA and BP extract-treated group):** Included 15 rat pups that were given VPA (by a similar dose and administration route as group III) once on PND14, then they were given BP extract (by a similar dose and administration route as group II) once daily from PND14 to PND 40.

Behavioral tests:

The rat pups were subjected to various behavioral testing:

1- Open field test:

It was performed to assess the general locomotion, exploration and stereotyped actions. This test was done at PND 40 by using open field like apparatus in the form of cartoon box measuring 40 x 50 cm with 30cm walls. The floor of the box was divided by marker lines into 20 identical squares. The rats were put individually in the box and allowed to discover the environment for 1 minute, and then their behavior started to be recorded [3,11]. Five parameters were quantified: *line crossings* and *freezing time* for assessment of spontaneous motor activity. *Grooming* for stereotyped behaviors. *Rearing* and *sniffing* for exploratory activity [4,12]

2- Social interaction test:

This test was performed at PND 40. Two rats from the same group were isolated in different cages the day before the experiment. Then, they were placed into a plastic cage and exposed to the red light for 20 minutes. The paired rats were tested for two social parameters, the frequency of

sniffing body parts and the duration of following each other [11,13]

Sample collection and tissue preparation:

At PND 41, rats from different experimental groups were anaesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg) according to ***Gaertner et al*** [14] to be able to do perfusion fixation. Perfusion fixation was done by cannulation to the heart and irrigation with saline followed by 4% paraformaldehyde solution according to ***Zheng et al*** [15] and then, rats were sacrificed by decapitation to the head with part of the neck and the cerebellum was carefully dissected, immediately isolated and divided into two parts. The first one was post-fixed in 10% formal saline for 24h at room temperature and prepared for study under the light microscope, while the second part was fixed in 4% glutaraldehyde and prepared for study under the electron microscope.

Processing for light microscopic study:

Cerebellar specimens were immediately put in 10% formal saline solution for 24 hours, dehydrated by using ascending concentrations of ethyl alcohol, cleared by using xylene, impregnated and paraffinized. Next, paraffinized sections were cut (5 µm thickness) and mounted on clean slides. Then, slides were stained with H&E stain [16]

For immunohistochemistry, 5-µm-thick sections were dewaxed, rehydrated, and washed using phosphate buffered saline (PBS). After that, incubation of the sections with rabbit polyclonal anti-GFAP primary antibody (Cat no. PDR028 obtained from Diagnostic Bio-Systems, USA) at a dilution of (1:50-1:100). Then, the sections were immersed in PBS and brood with Goat anti-rabbit secondary antibodies (Catalog # 65-6140 obtained from Invitrogen, Thermofisher, India) for 30 minutes at 37^{o} [17]

Incubation of the sections with diaminobenzidine (DAB) was done and they were treated with hematoxylin as a counterstain. The negative control was done by similar preceding steps without adding the primary antibody. Human brain tissue was used as a positive control according to the product data sheet (**https://dbiosys.com/product/gfap**). The positive immunoreactivity appeared as cytoplasmic brown particles of astrocytes for GFAP [18]

A light microscope (Olympus, Japan) with a built-in camera was used to examine and photograph the slides in the department of Histology and Cell Biology, Faculty of Medicine, Tanta University.

Processing for electron microscopic study:

Specimens of cerebellum were finely cut and immersed in 4% phosphate-buffered glutaraldehyde (0.1 mol/L, pH 7.4) as a primary fixative, then post-fixed using 1% osmium tetroxide. Subsequently, specimens were dehydrated by using ascending grades of alcohol. Then, sections were put in propylene oxide then were embedded in a mixture of epoxy resin. By using ultramicrotome **(**LKB. Leica, Australia**),** semi-thin and ultrathin sections were cut. Ultrathin sections were double stained using lead citrate and uranyl acetate [19,20] to be studied and photographed by the transmission electron microscope (JEOL-JEM-100, Tokyo, Japan) at the Electron Microscopy Unit, Faculty of Medicine, Tanta, Egypt.

Morphometric study:

For morphometry, the Image J software program (1.48v, National Institute of Health, Bethesda, Maryland, USA) was used. Ten non-overlapping fields (images) for each group were examined to make quantitative measurement to the following parameters:

a- The mean thickness of the granular cell layer (µ) in sections of cerebellar cortex stained with H&E (magnification power of 200x).

b- The mean area percentage of anti-GFAP positive cells (magnification power of 400x).

Statistical analysis:

Six rat pups from each group were subjected for various behavioral tests. Data from the behavioral parameters were displayed as means ± SEM (standard error of mean), while the morphometric results from histological slides were displayed as means ± SD (standard deviation). All these data were analyzed by using the one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparisons post hoc test to compare other groups with the control. If the probability value $P \leq 0.05$, differences were considered as significant. Additionally, $P < 0.001$ was considered highly significant while non-significant if $P > 0.05$ [11,18]

3- Results:

Throughout the experimental period, no mortality was documented. The behavioral, histological, immunohistochemical and electron microscopic results revealed similar findings in group I (control) and group II (BP extract-treated group) with a non-significant difference in the morphometric results in-between them.

Results of light microscopic examination:

Group I (Control group) & Group II (BP extract-treated group):

Examination of cerebellar sections stained with *H&E* of rats in group I & group

II showed similar findings. They revealed the normal histological picture of the cerebellar cortex that consisted of three layers arranged as: outer molecular, middle Purkinje, and inner granular cell layers **(Fig. 1-A)**

The outer molecular layer comprised of numerous acidophilic cell processes (axons and dendrites) representing the neuropil with multiple dark-stained neuroglial cells, few scattered cells with pale vesicular nuclei and blood vessels **(Fig. 1-B).** The Purkinje cell layer consisted of large cells that have pyriform shapes arranged in one row intervening between the outer molecular and the inner granular cell layers. Purkinje cells (PCs) appeared with large central rounded vesicular nuclei with prominent nucleoli, basophilic granular cytoplasm and apical arborizing dendrites extending into the molecular layer **(Fig. 1-B)**. As regards the inner granular cell layer, it appeared as a thick layer of small numerous closely packed granule cells. These granule cells had small rounded deeply stained nuclei. Cerebellar islands representing glomeruli appeared as acidophilic areas in-between the granule cells **(Fig. 1-B).**

Group III (Valproic acid (VPA)-induced autism group): Examination of cerebellar sections stained with *H&E* of rats in group III revealed marked alterations and disorganization of the normal histological construction of the cerebellar cortex **(Fig. 2-A).** The molecular layer exhibited small vacuoles in the neuropil and dilated thick-walled blood vessels **(Fig. 2-B).** External migration of Purkinje- like cells to molecular layer was observed **(Fig. 2-C).** Purkinje cell layer showed an apparent decline in the number of PCs, and many of PCs appeared shrunken, irregular in shape, with deeply stained nuclei or even with karyolytic nuclei, and surrounded by empty neuropil **(Fig. 2-D&E).** Furthermore, wide spaces were detected in between the granule cells **(Fig. 2-E)** and internal migration of Purkinje like cells to the granular layer and arranged in the form of multiple layers **(Fig. 2-F)**. A decrease in the thickness of the granular cell layer was apparent in addition to a decline in the number of the granule cells **(Fig. 2-G)**. Moreover, congested blood vessels were detected in between the granule cells **(Fig. 2-H)**

Group IV: (VPA and BP extract-treated group): Examination of cerebellar sections stained with *H&E* of rats in group IV revealed marked amelioration in the histological construction of the cerebellar cortex to be similar to the control group **(Fig. 3-A).** The outer molecular layer consisted of acidophilic neuropil, small scattered pale vesicular nuclei,

and multiple neuroglial cells. Most of PCs restored the linear arrangement and appeared as a single row of large pyriform cells having rounded vesicular nuclei with prominent nucleoli. Some Purkinje cells appeared with cytoplasmic homogenization. The inner granular cell layer contained small rounded and closely packed granule cells **(Fig. 3-B).**

Results of anti-GFAP immunohistochemistry:

Human brain tissue stained with anti-GFAP antibodies was used as a Positive control section **(Fig. 4)** and a section from rat cerebellar cortex was used as a negative control for anti-GFAP antibodies **(Fig. 5).** Examination of cerebellar sections stained immunohistochemically with anti-GFAP of rats in group I (control group) and group II (BP extract-treated group) revealed positive anti-GFAP immunoreaction in the cytoplasm and cell processes of astrocytes that appeared few and small in the cortical three layers **(Fig. 6-A).** As regards group III (VPA-induced autism group), its examination revealed apparent positive anti-GFAP immunoreaction in the cytoplasm and cell processes of astrocytes that appeared numerous with linear processes in the molecular layer and numerous with star shaped processes in both Purkinje and granular cell layers **(Fig. 6-B&C).** Noteworthy, examination of VPA and BP extract-treated group IV revealed anti-GFAP immunoreaction in the cytoplasm and cell processes of astrocytes to be greatly similar to the control group **(Fig. 6-D).**

Morphometric and statistical analysis results:

Results of behavioral tests:

Open field test:

There was non-significant difference in ***group II (BP extract treated group)*** as compared to control group. ***Group III (VPA-induced autism group)*** revealed a highly significant decrease ($P < 0.001$) in the line crossing, rearing and sniffing in addition to a highly significant increase ($P < 0.001$) in the freezing time and grooming as compared to the control group. On the other hand, ***group IV (VPA and BP extract-treated group)*** revealed a significant increase ($P<0.05$) in the freezing time and grooming in addition to a non-significant change ($P>0.05$) in the sniffing as compared to the control rats. Also, there was significant ($P<0.05$) decrease in the rearing and significant ($P<0.01$) decrease in line crossing in group IV as compared to the control rats.

Furthermore, examination of this group revealed a highly significant ($P <$

0.001) increase in the line crossing, rearing and sniffing in addition to a significant (P < 0.01) decrease in the freezing time and a highly significant decrease the in grooming as compared to VPA induced- autism group III **(Table1- Fig.7)**

Social interaction test:

There was non-significant difference in ***group II (BP extract treated group)*** as compared to control group. ***Group III (VPA-induced autism group)*** revealed a highly significant (P<0.001) decrease in the duration of following as well as the frequency of sniffing as compared to the control rats. On the other hand, ***group IV (VPA and BP-treated group)*** revealed a highly significant increase (P<0.001) in both following and sniffing body parts as compared to group III (VPA-induced autism group) and also expressed a non-significant difference (P> 0.05) as compared to the control rats **(Table 1-Fig. 8)**

The mean thickness of the granular cell layer:

There was non-significant difference in ***group II (BP extract treated group)*** as compared to control group. Group III (VPA-induced autism group) revealed a highly significant (P<0.001) decrease in the mean thickness of the granular cell layer in as compared to the control group. On the other hand, group IV (VPA and BP-treated group) expressed a non-significant difference (P > 0.05) from the control. Moreover, there was highly significant (P<0.001) increase in the mean thickness of the granular layer in ***group IV (VPA and BP- treated group)*** as compared to group III (VPA- induced autism group) **(Table 2 – Fig. 9)**

The mean area percentage of anti-GFAP positive cells:

There was non-significant difference in ***group II (BP extract treated group)*** as compared to control group. ***Group III (VPA-induced autism group)*** revealed a highly significant increase (P < 0.001) in the mean area percentage of anti-GFAP positive immunoreaction as compared to the control group. On the other hand, ***group IV (VPA and BP- treated group)*** expressed a non-significant difference (P >0.05) from the control group. Moreover, there was a highly significant decrease (P < 0.001) in the mean area percentage of anti-GFAP positive immunoreaction as compared to group III (VPA- induced autism group) **(Table 2- Fig. 10)**

Results of electron microscopic examination:

Group I (Control group) & Group II (BP extract-treated group):

Examination of ultra-thin sections of cerebellar cortex from rats of group I (control group) and group II under the transmission electron microscope showed similar results. They revealed the typical ultra-structural features of the cerebellar cortex. Basket cell appeared in the lower part of the molecular layer having an oval euchromatic nucleus with heterochromatin clumps attached to the nuclear envelope and scanty cytoplasm containing mitochondria, rough endoplasmic reticulum and free ribosome. Normal mitochondria were seen in the surrounding neuropil **(Fig. 11-A)**. Purkinje cells with part of their apical dendrites appeared containing large euchromatic rounded nuclei with regular demarcated nuclear envelopes. Their cytoplasm containing many mitochondria with prominent cristae, multiple Golgi apparatus, parallel cisternae of RER, free ribosomes and polysomes **(Fig. 11-B&C).** Neuropil in-between PCs was observed containing multiple processes mainly myelinated axons with mitochondria in addition to multiple axoaxonic synapses **(Fig. 11-D)**

Granule cells appeared as rounded cells having large rounded eccentric nuclei with peripheral heterochromatin clumps surrounded by thin cytoplasmic rim containing mitochondria, small Golgi, and free ribosomes **(Fig. 11-E)**. Mossy rosettes were observed in between the granular cells containing multiple mitochondria **(Fig. 11-F)**

Group III (VPA-induced autism group): Electron microscopic examination revealed disturbance of the normal ultrastructure of the cerebellar cortex as compared to the control group. Basket cell appeared in the deep molecular layer with increased clumps of heterochromatin in its nucleus and mitochondria with destroyed cristae were observed in the surrounding neuropil **(Fig. 12-A).** Marked alterations were observed in the structure of the Purkinje cells. They appeared shrunken and irregular having shrunken irregular nuclei. Their cytoplasm exhibited vacuoles, dilated RER cisternae, electron dense bodies and mitochondria with destroyed cristae. Vacuoles in the surrounding neuropil were also present **(Fig. 12-B&C).** In addition, neuropil in-between the PCs appeared containing myelinated axons. Some axons contained mitochondria with destroyed cristae **(Fig. 12-D)**

Granule cells appeared having nuclei with irregular nuclear membranes and cytoplasmic vacuolations **(Fig. 12-E).** In addition, mossy rosettes were observed in between the granular cells with few widely separated mitochondria, some of them appeared with destroyed cristae. Some granule cells had nuclei with peripheral chromatin condensation and contained mitochondria with destroyed cristae **(Fig. 12-F).**

Group IV: (VPA and BP extract-treated group): Electron microscopic examination revealed marked improvement in the cerebellar cortex ultrastructure as compared to the VPA-induced autism group. Basket cell in the deep molecular layer appeared having large oval nucleus with prominent nucleolus and surrounded by a thin rim of cytoplasm containing some mitochondria with destroyed cristae. The surrounding neuropil appeared with normal appearance and contained mitochondria **(Fig. 13-A).** Purkinje cells greatly appeared similar to the control. They had large euchromatic nuclei with prominent nucleoli and their cytoplasm contained RER, Golgi apparatus, ribosomes and polysomes, and normal mitochondria except some of them still appeared with destroyed cristae **(Fig. 13-B&C).** Examination of the granular cell layer revealed granule cells having rounded nuclei with peripheral heterochromatin clumps surrounded by thin cytoplasmic rim containing free ribosomes, mitochondria and Golgi apparatus **(Fig. 13-D).** Mossy rosettes appeared greatly similar to the control containing multiple mitochondria with regular cristae **(Fig. 13-E).**

Table (1): Comparison between the studied groups as regard means ± SEM of open field test and social parameters

Parameter	Group I (Control)	Group II (BP)	Group III (VPA)	Group IV (VPA+BP)	P value
Line Crossing	58.667 ± 2.171	59 ± 1.77 **ns**	5.667 ± 3.007 *******	40.5 ± 4.972 ****, ###**	**P*:** significant <0.05 **P##, P **** significant <0.01 **P***, P###:** highly significant <0.001
Freezing Time	2.5 ±0.4282	3.167 ± 0.8724 **ns**	145.33 ±23.61 *******	60.833 ±12.937 *** ,##**	
Rearing	28.333± 1.308	27.833±1.138 **ns**	3.333± 0.8433 *******	21.5±1.9 *** ,###**	
Grooming	7.5 ±0.9574	8.167± 0.7491 **ns**	21± 0.5774 *******	10.667±0.6146 *** ,###**	
Sniffing	52.333± 5.011	49.333± 4.674 **ns**	10.667± 1.256 *******	48.833± 3.754 **ns, ###**	
Duration of following	131.67±10.14	125±14.78 **ns**	13.67± 3.88 *******	118.33±7.49 **ns, ###**	
Frequency of sniffing	14.67±1.52	17 ± 1.77 **ns**	2.33± 0.56 *******	17.17±1.68 **ns,###**	

SEM: standard error of mean **ns:** non-significant change with control group.

- ***** indicates significance when compared to control group
- **#** indicates significance when compared to group III

Table (2): Comparison between the studied groups as regard means ± SD

Group	Group I (Control)	Group II (BP)	Group III (VPA)	Group IV (VPA+BP)	P value
Thickness of the granular cell layer	203.94 ± 29.135	188.07± 25.87 **ns**	39.264± 14.15 *******	193.72 ± 31.944 **ns , ###**	**P***, P###** highly significant <0.001
Area percentage of anti-GFAP	8.079± 0.9694	9.812 ± 1.547 **ns**	21.12 ± 2.801 *******	10.092 ± 1.642 **ns , ###**	

SD: standard deviation **ns:** non-significant change with control group

*indicates significance when compared to control group

# indicates significance when compared to group III

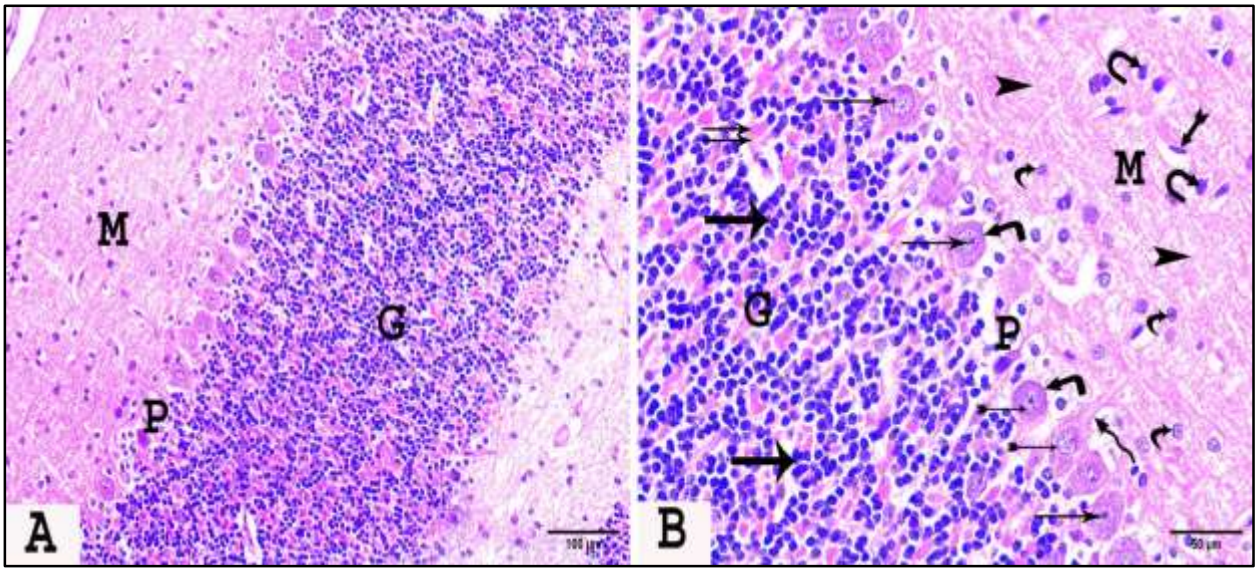

Fig. (1): Photomicrographs of sections in a rat cerebellar cortex from the control group. ***(A)*** Showing layers of the cerebellar cortex arranged as outer molecular layer (M), middle Purkinje cell layer (P) and inner granular layer (G). ***(B)*** Showing the outer molecular layer (M) consists of numerous acidophilic cell processes (axons and dendrites) representing the neuropil (arrowheads) with multiple dark-stained neuroglial cells (arched arrows) and few cells with pale nuclei (curved arrow) in addition to blood vessels (thick bifid arrow). Middle Purkinje cell layer (P) consists of a single row of large pyriform-shaped cells (angled arrows) having large central rounded vesicular nuclei (bifid arrows), prominent nucleoli (thin arrows), basophilic granular cytoplasm and apical arborizing dendrites (wavy arrow) extending into the molecular layer. Inner granular cell layer (G) appears as a thick layer of small numerous closely packed granule cells. These granule cells have small rounded deeply stained nuclei (thick arrows). Notice: the acidophilic areas between the granule cell groups (double arrow) representing the cerebellar glomeruli **(H&E, A x 200 scale bar = 100µ, B x400 scale bar).**

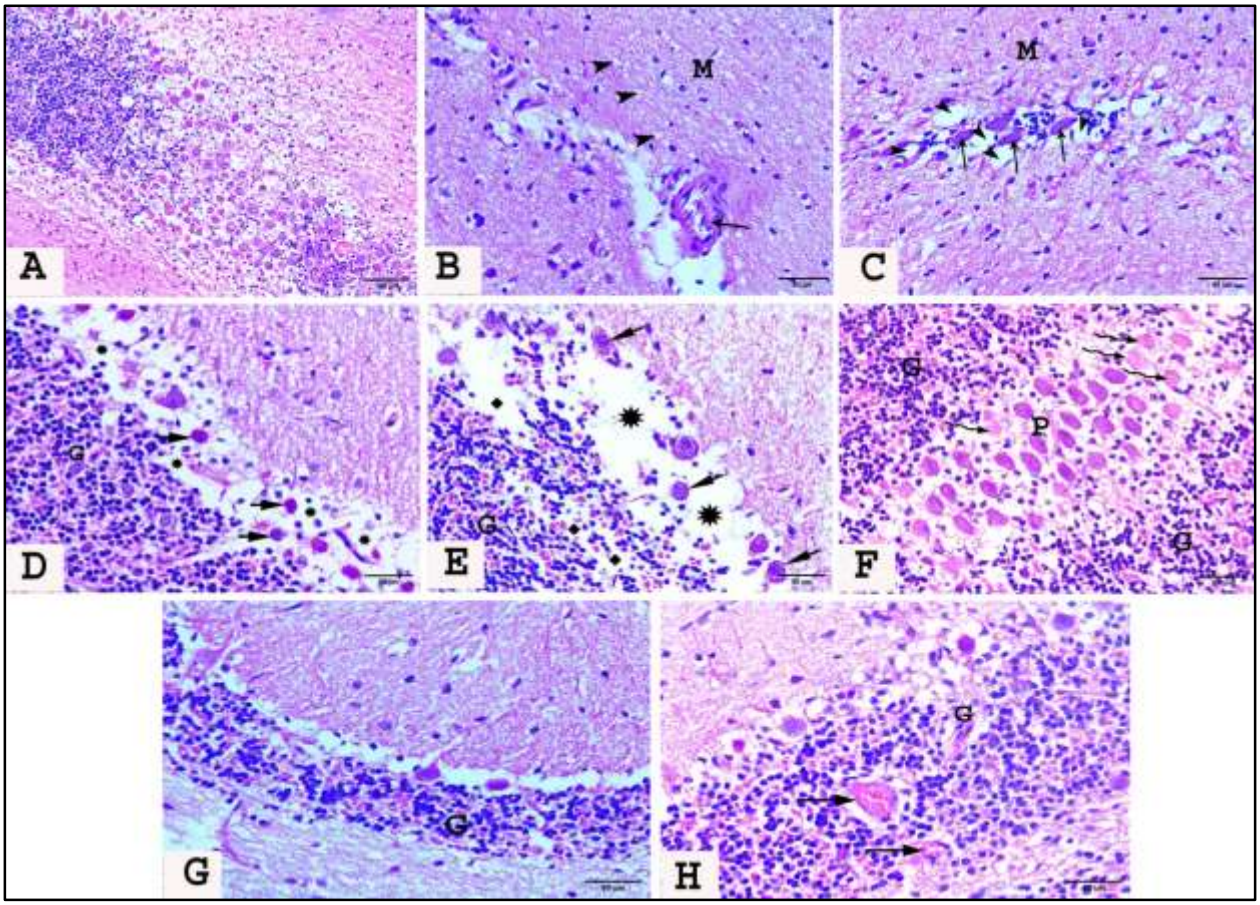

Fig. (2): Photomicrographs of sections in a rat cerebellar cortex from group III (VPA- induced autism group). ***(A)*** Showing marked alterations and disorganization of the normal histological structure of the cerebellar cortex. ***(B)*** Showing dilated thick-walled blood vessel (arrow) and small vacuoles (arrowheads) within the molecular layer (M). ***(C)*** Showing external migration of some Purkinje like cells (arrows) to the molecular layer (M) surrounded by vacuolated neuropil (arrowheads). ***(D&E)*** Showing marked decrease in the number of PCs, and many of PCs appeared shrunken, irregular in shape, with deeply stained nuclei (arrows) and surrounded by empty neuropil (stars). Notice the wide spaces (diamonds) in between the granule cells. ***(F)*** Showing internal migration of multiple layers of Purkinje like cells (P) to the granular cell layer (G) and appear with karyolytic nuclei and cytoplasmic homogenization (wavy arrows). ***(G&H)*** Showing a decrease in the thickness of the granular cell layer (G) in addition to a decrease in the density of the granule cells as well as dilated and congested blood vessels (arrows) within the granular cell layer (G). **(H&E, Ax200 scale bar=100µ, B,C,D,E,F,G and H x400 scale bar =50µ)**

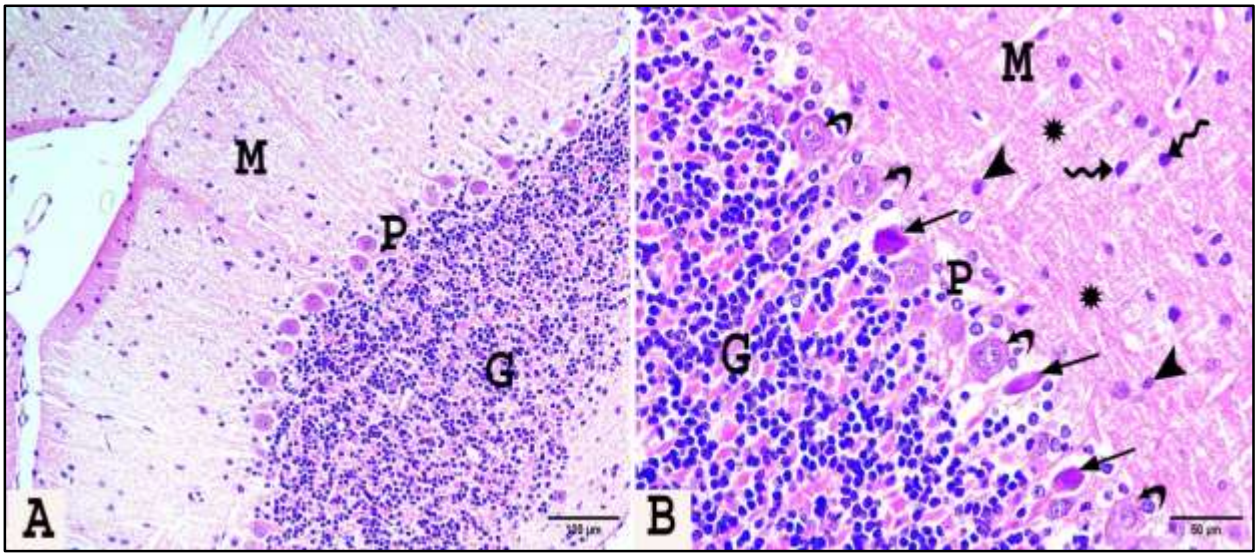

Fig. (3): Photomicrographs of sections in a rat cerebellar cortex from group IV (VPA & BP-treated group). ***(A)*** Showing the three layers of cerebellar cortex normally arranged as: outer molecular layer (M), middle Purkinje cell layer (P) and inner granular cell layer (G). ***(B)*** Showing the outer molecular layer (M) consists of acidophilic neuropil (stars), small scattered vesicular nuclei (arrowheads) and multiple dark stained neuroglial cells (wavy arrows). The middle Purkinje cell layer (P) consists of a single row of large pyriform cells (curved arrows) having large rounded vesicular nuclei with prominent nucleoli. Few Purkinje cells appear with cytoplasmic homogenization (arrows). The inner granular cell layer (G) contains small rounded and closely packed cells. **(H&E, A x200 scale bar=100µ, B x400 scale bar=50µ)**

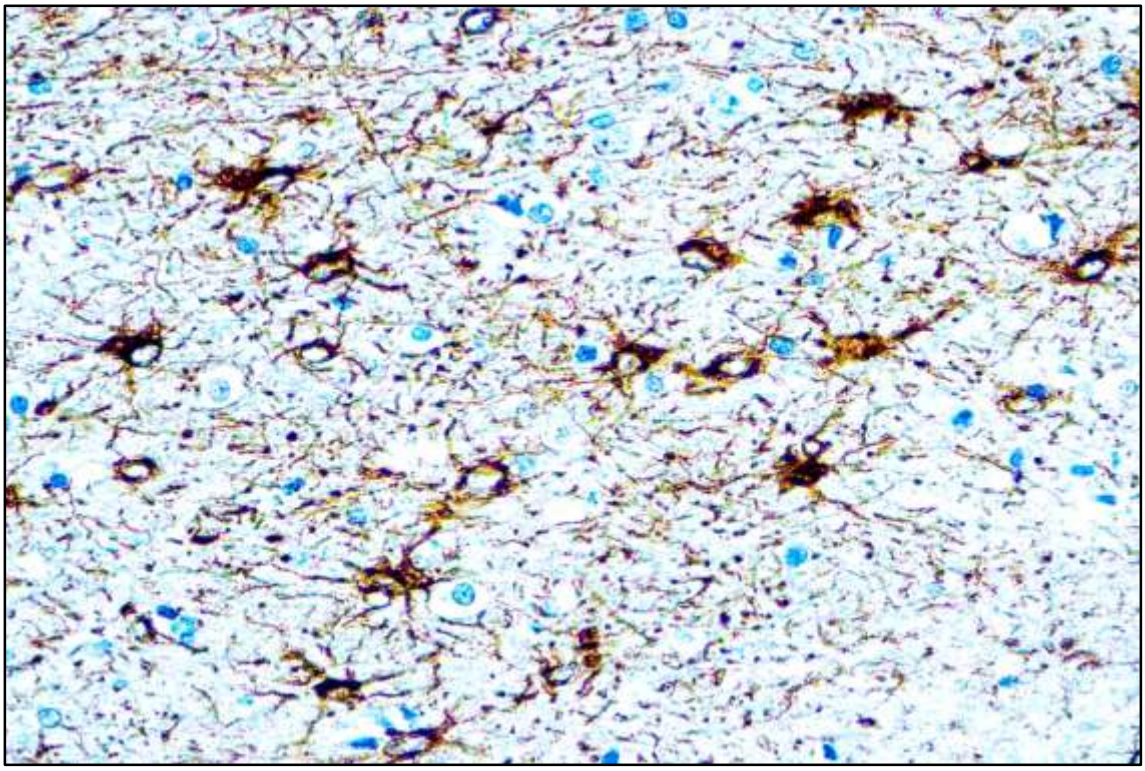

Fig. 4: A photomicrograph of a section in human brain stained with anti-GFAP antibodies (**Positive control, https://dbiosys.com/product/gfap)**

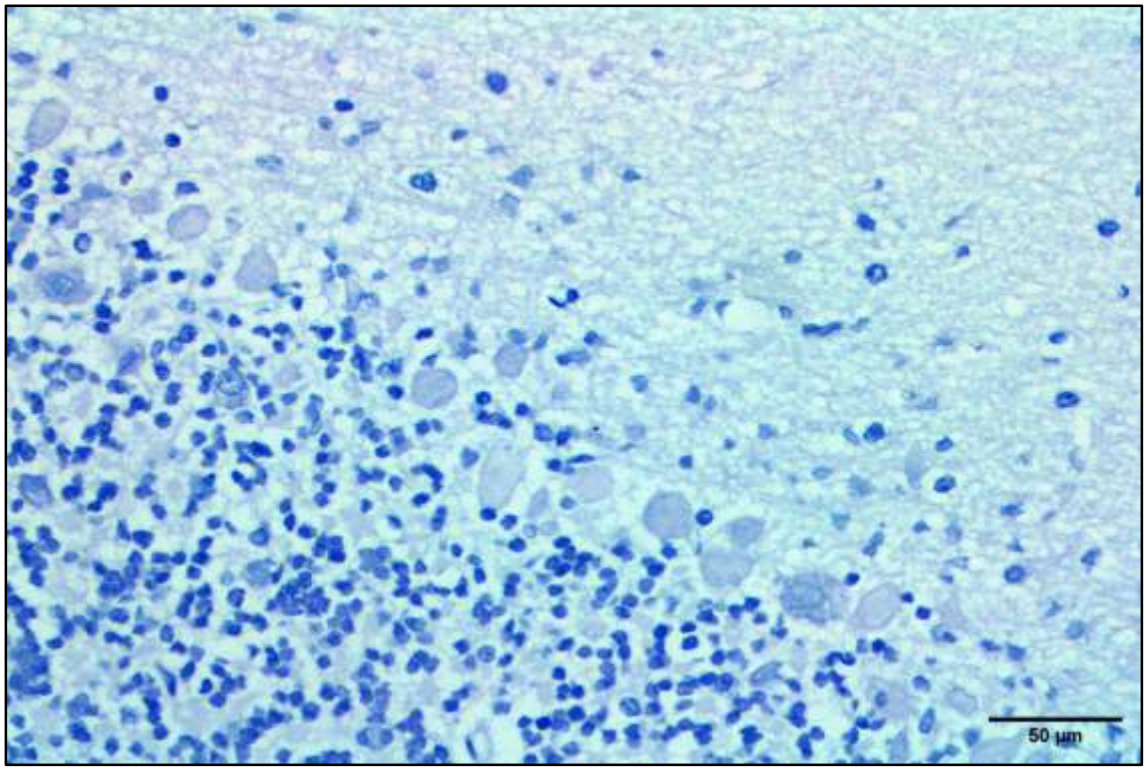

Fig. (5): A photomicrograph of a negative control section from rat cerebellar cortex showing negative reaction for anti-GFAP antibodies **(Hematoxylin x400, scale bar=50 µ)**

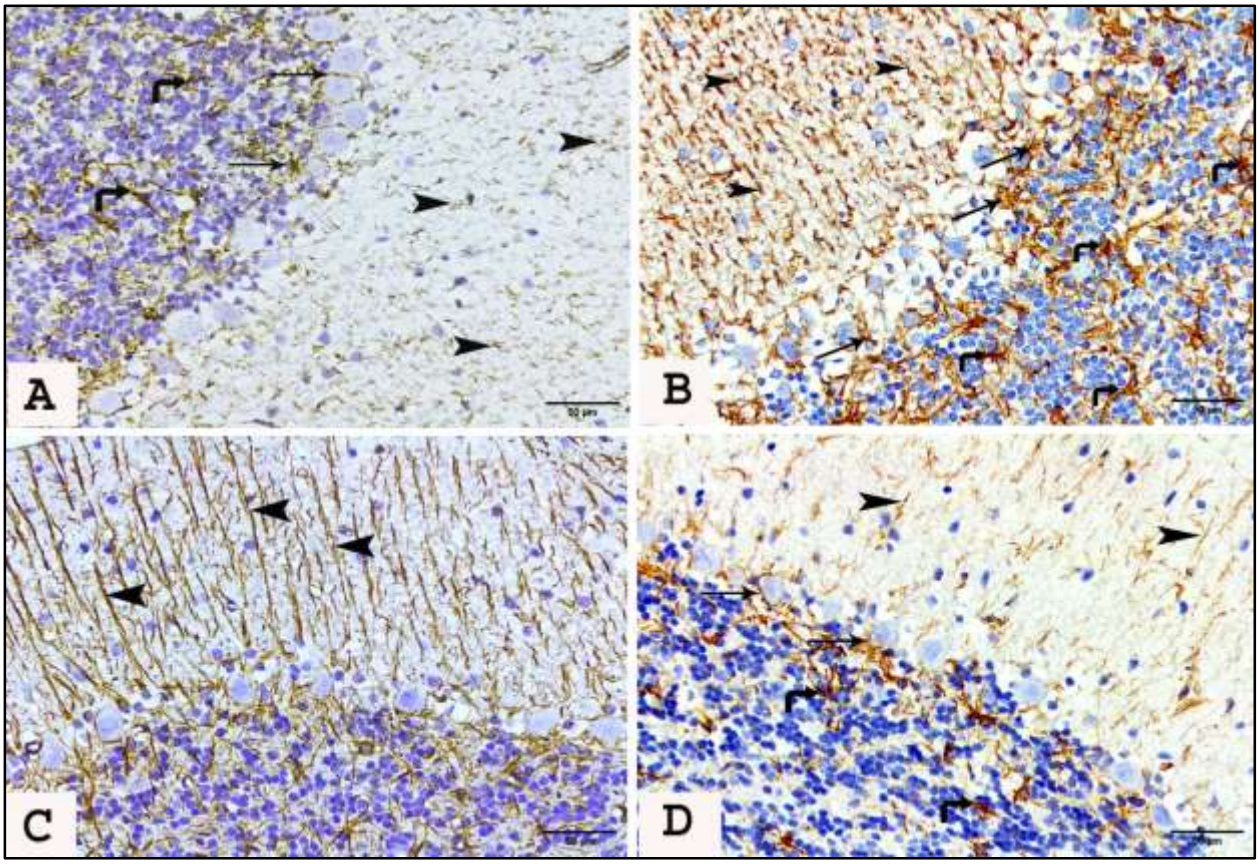

Fig. (6): Photomicrographs of sections in a rat cerebellar cortex immunostained with anti-GFAP. ***(A)*** Control group showing positive anti-GFAP immunoreaction in the cytoplasm and processes of few astrocytes in the molecular layer (arrowheads), the Purkinje cell layer (arrows) and granular cell layer (angled arrows). ***(B&C)*** VPA-induced autism group showing apparent positive anti-GFAP immunoreaction in the cytoplasm and processes of astrocytes which appear numerous with linear processes (arrowheads) in the molecular layer and numerous with star-shaped processes in both Purkinje (arrows) and granular cell layers (angled arrows). ***(D)*** VPA and BP extract-treated group showing anti- GFAP immunoreaction in the cytoplasm and processes of astrocytes within the molecular layer (arrowheads), the Purkinje cell layer (arrows) and the granular cell layer (angled arrows) to be more or less similar to the control group **(GFAP immunostaining A,B,C and D x400, scale bar =50µ)**

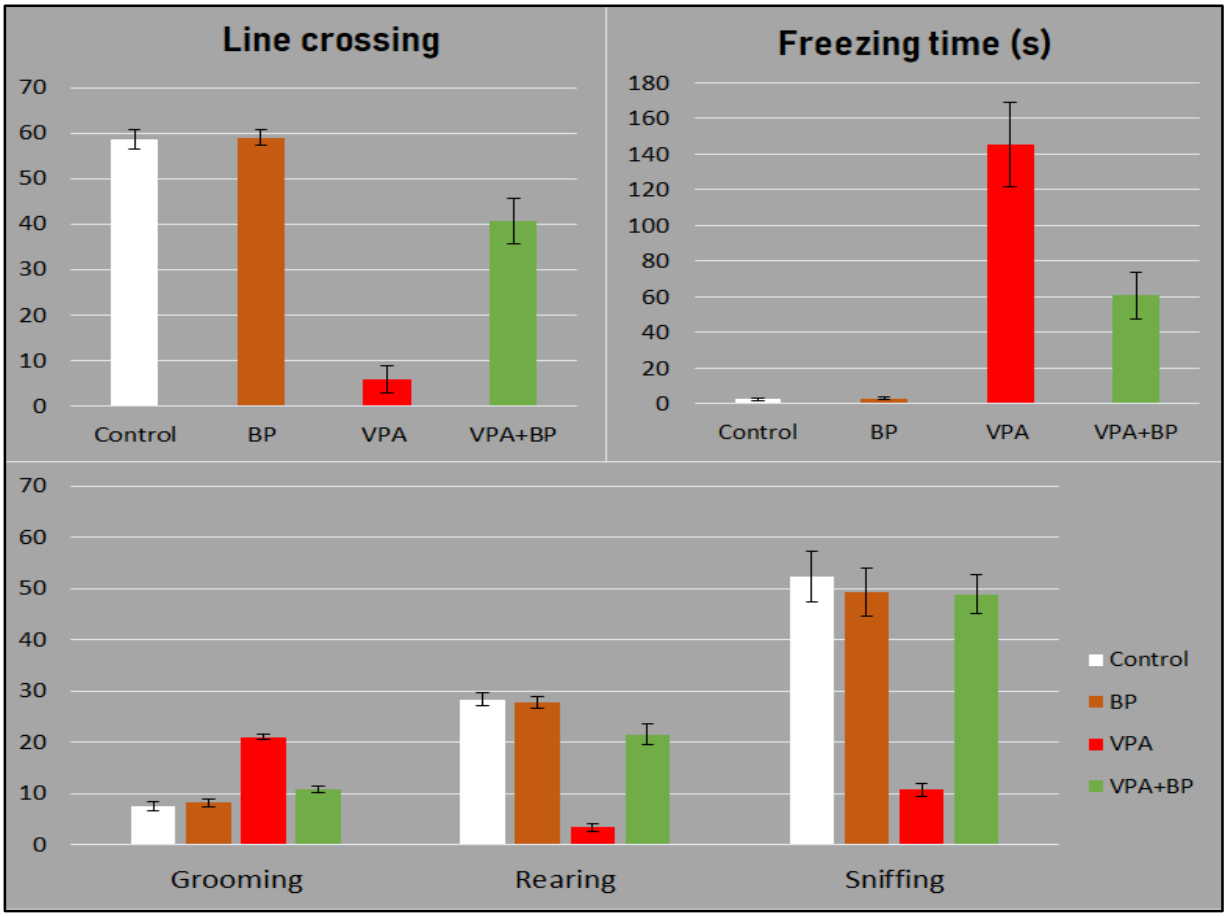

Fig. (7): Statistical results of the different experimental groups. Values presented as mean ± SEM of the open field test. BP (bee pollen extract-treated group); ***VPA*** (valproic acid-induced autism group) and ***VPA+BP*** (valproic acid induced autism group and treated by bee pollen extract) as compared to the control group respectively.

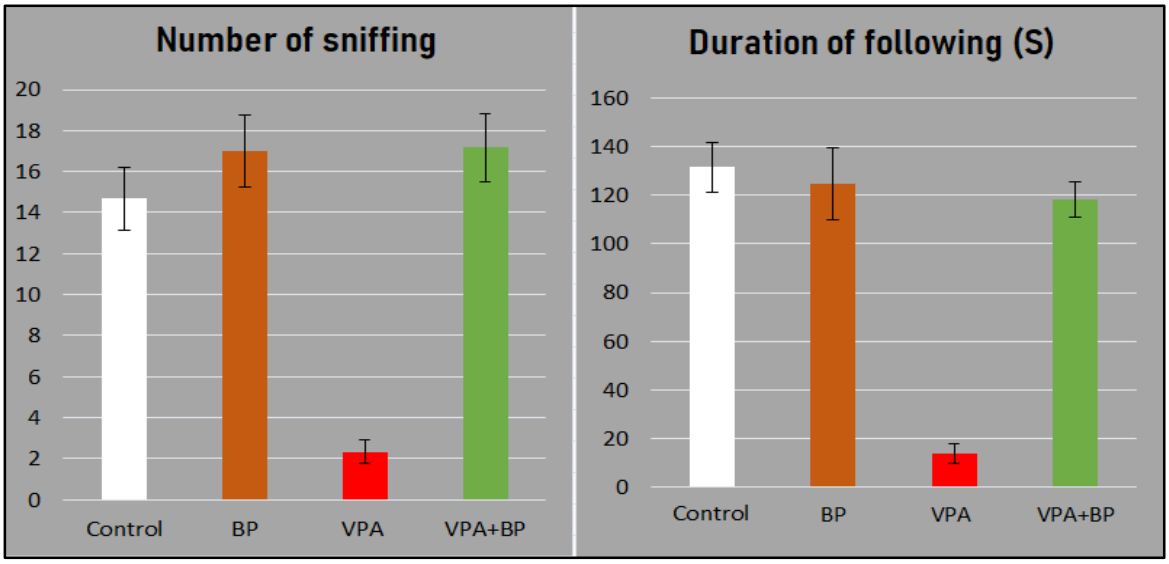

Fig. (8): Statistical results of the different experimental groups. Values presented as mean ± SEM of the social interaction test. BP (bee pollen extract-treated group); ***VPA (***valproic acid- induced autism group) and ***VPA+BP*** (valproic acid-induced autism group and treated by bee pollen extract) as compared to the control group respectively.

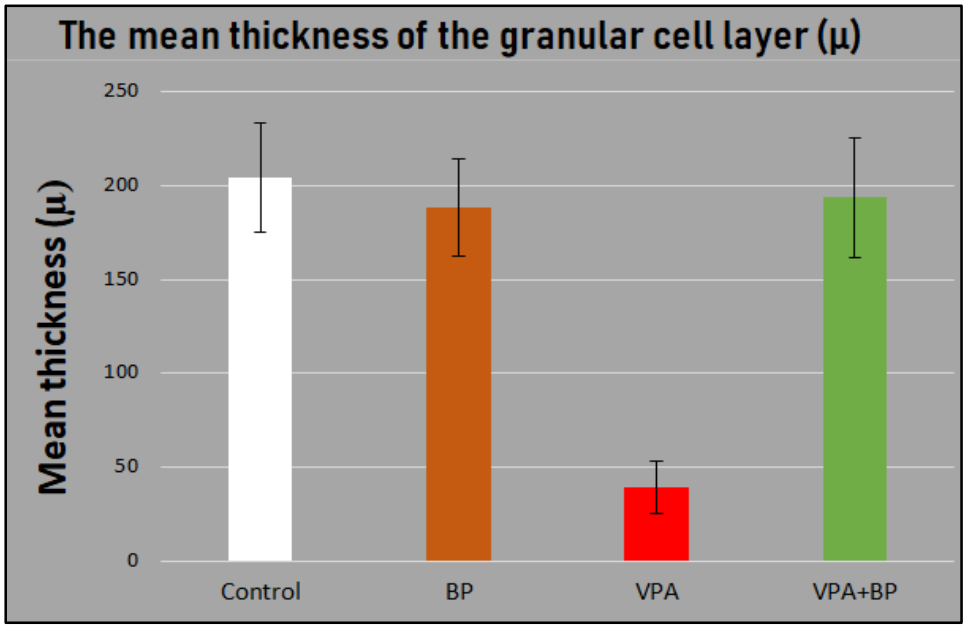

Fig. (9): Statistical results of the different experimental groups. Values presented as means ± standard deviation (SD) of the thickness of the granular cell layer (µ). BP (bee pollen extract-treated group); ***VPA*** (valproic acid-induced autism group) and ***VPA+BP*** (valproic acid-induced autism group and treated by bee pollen extract) as compared to the control group respectively.

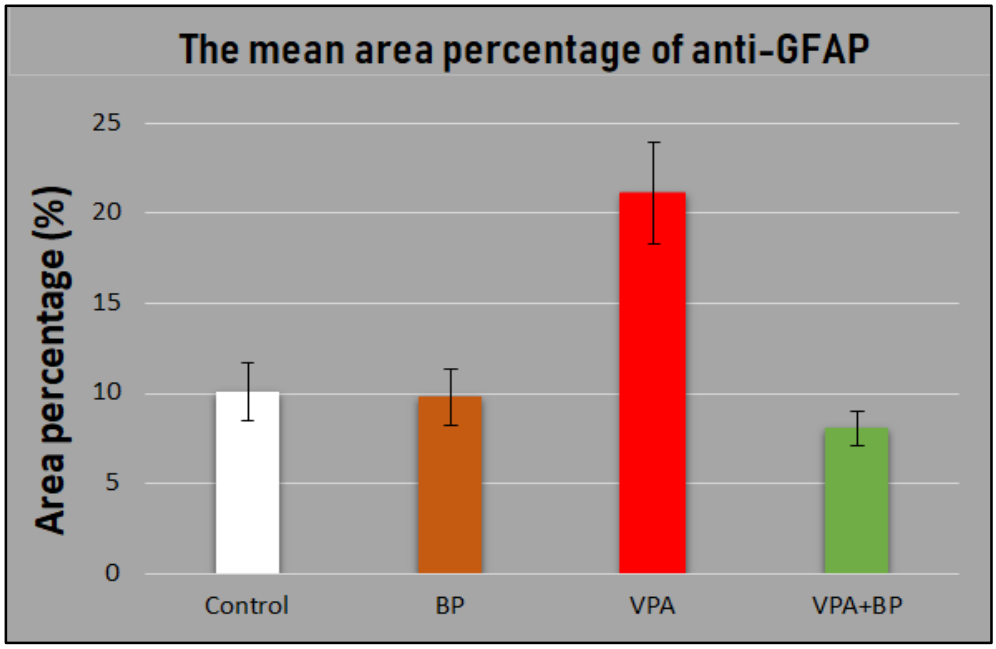

Fig. (10): Statistical results of the different experimental groups. Values presented as means ± SD of the area percentage of anti-GFAP positive immunoreaction. BP (bee pollen extract-treated group); ***VPA*** (valproic acid-induced autism group) and ***VPA+BP*** (valproic acid-induced autism group and treated by bee pollen extract) as compared to the control group respectively.

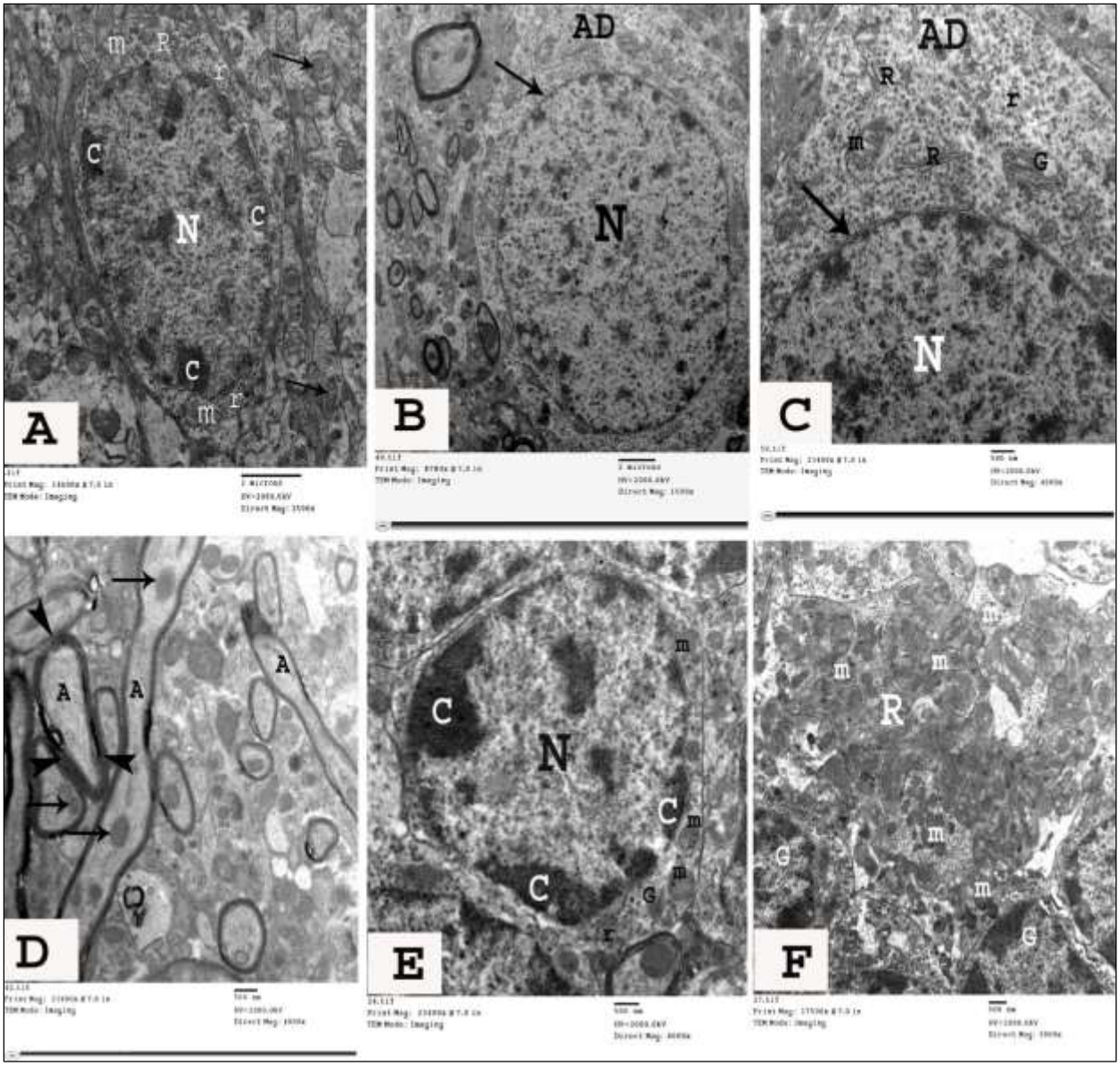

Fig. (11): Electron micrographs of the cerebellar cortex of control rats: ***(A)*** Showing basket cell in the lower part of molecular layer having an oval euchromatic nucleus (N) and scanty cytoplasm containing mitochondria (m), rough endoplasmic reticulum (R) and free ribosome (r). Notice clumps of heterochromatin (C) are attached to the nuclear envelope and the surrounding neuropil containing mitochondria (arrows). ***(B&C)*** Showing a Purkinje cell and part of its apical dendrite (AD) having a large rounded euchromatic nucleus (N) with regular demarcated nuclear membrane (arrow), cytoplasm containing many mitochondria with prominent cristae (m), Golgi apparatus (G), parallel cisternae of RER (R), free ribosomes and polysomes (r). ***(D)*** Showing neuropil in-between PCs containing multiple processes mainly myelinated axons (A) containing

mitochondria (arrows). Notice: the multiple axoaxonic synapses (arrowheads). ***(E)*** Showing granule cell having large rounded eccentric nucleus (N) with peripheral clumps of heterochromatin (C) surrounded by a thin rim of cytoplasm containing mitochondria (m), small Golgi (G) and free ribosomes (r). ***(F)*** Showing mossy rosette (R) in between the granule cells (G) containing multiple mitochondria (m) **(Mic. Mag. A x 2500, B x 1500**, **C x4000, D x 4000, E x 4000**, **F x3000).**

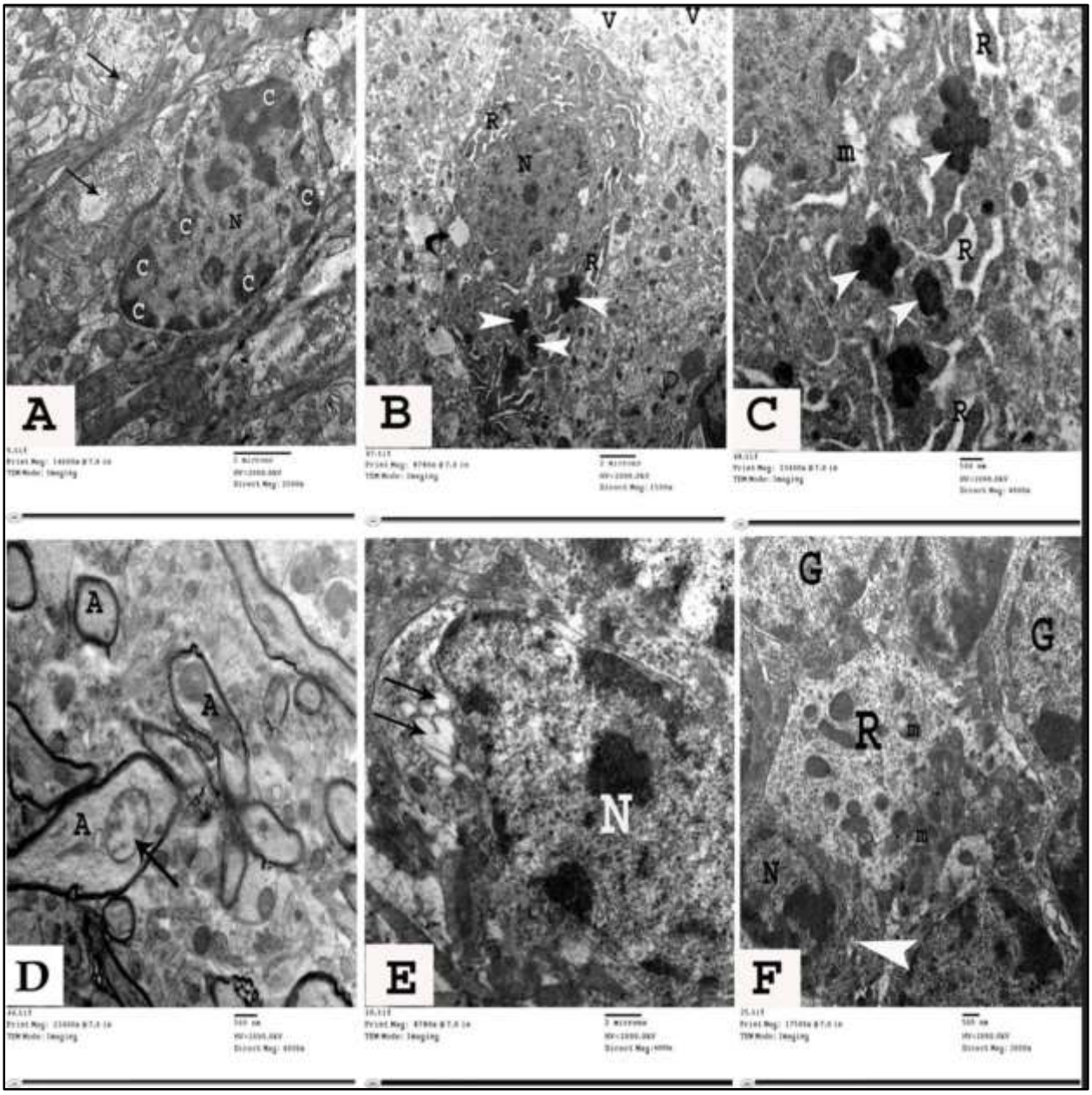

Fig. (12): Electron micrographs of the cerebellar cortex from rats of group III (VPA-induced autism group). ***(A)*** Showing basket cell in the deep molecular layer with increased clumps of heterochromatin (C) in its nucleus (N). Notice mitochondria with destroyed cristae (arrow) in the surrounding neuropil. ***(B)*** Showing a shrunken Purkinje cell with irregular shrunken nucleus (N) and cytoplasm contains vacuoles (curved arrow), dilated RER (R), electron dense bodies (arrowheads) and surrounded by vacuolated neuropil (V). ***(C)*** Higher magnification of the previous PC showing dilated RER (R), mitochondria with destroyed cristae (m) and electron dense bodies (arrowheads). ***(D*** Showing neuropil in-between PCs containing myelinated axons some of them

containing mitochondria with destroyed cristae (arrow). ***(E)*** Showing a granule cell having a nucleus (N) with an irregular nuclear membrane and cytoplasmic vacuolations (arrows). ***(F)*** Showing mossy rosette (R) in between the granule cells (G) having few widely separated mitochondria, some of them with destroyed cristae (m). Notice: a granule cell appears with peripheral chromatin condensation in its nucleus (N) and contains mitochondria with destroyed cristae (arrowhead). **(Mic. Mag. A x2500**, **B x1500, C x 4000, D x4000, E x4000, F x3000).**

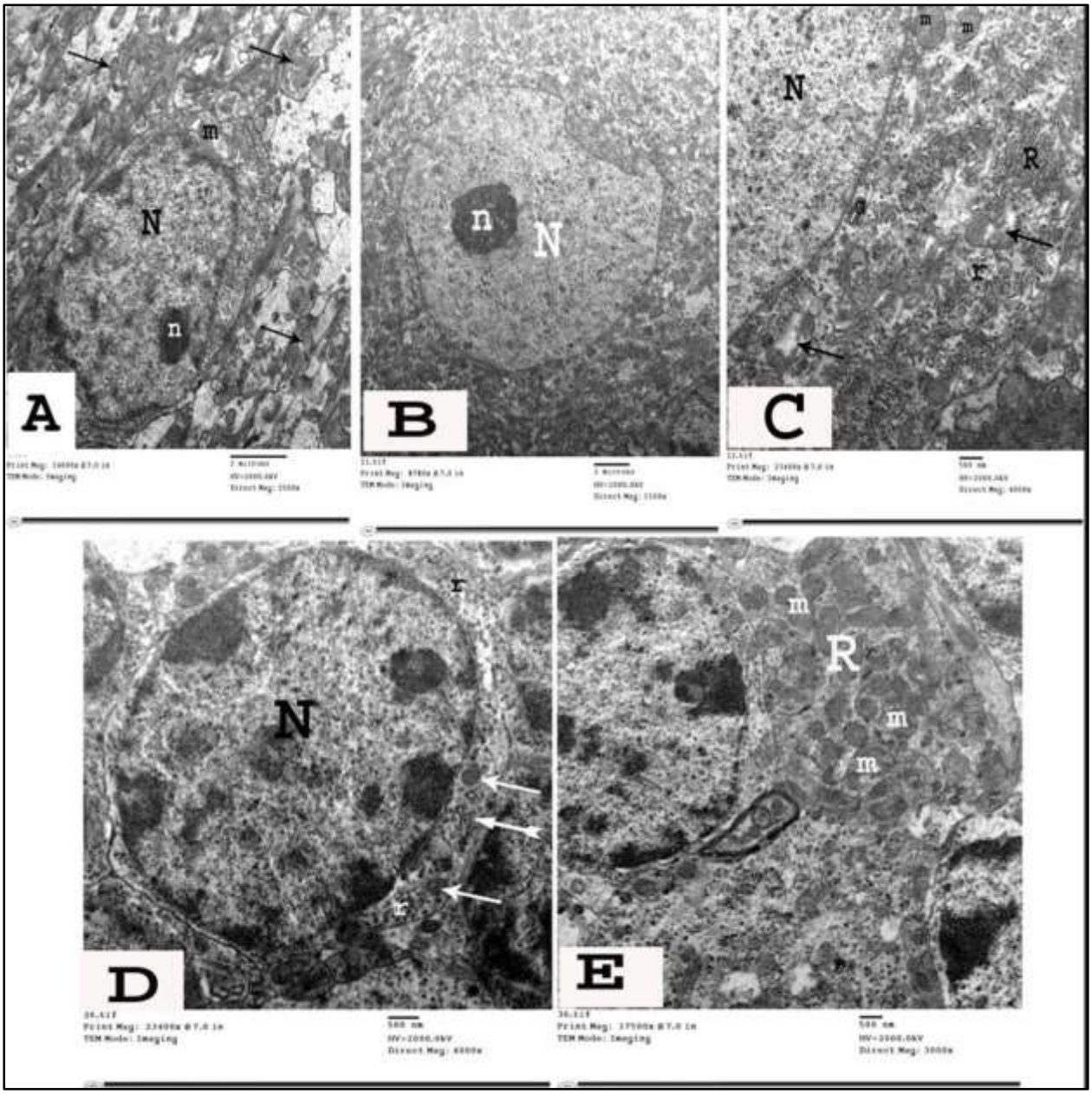

Fig. (13): Electron micrographs of the rat cerebellar cortex of group IV (VPA and BP extract-treated group): ***(A)*** Showing basket cell in the deep molecular layer that has large oval euchromatic nucleus (N) with prominent nucleolus (n) surrounded by a thin rim of cytoplasm containing mitochondria (m) with destroyed cristae. Notice the surrounding neuropil containing mitochondria (arrows). ***(B&C)*** Showing Purkinje cell having large rounded euchromatic nucleus (N) with prominent nucleolus (n) and the cytoplasm contained RER (R), ribosomes and polysomes (r), Golgi apparatus (G) and mitochondria (m) except some of them still with destroyed cristae (arrows). ***(D)*** Showing a granule cell having rounded nucleus (N) with

peripheral clumps of heterochromatin surrounded by a thin rim of cytoplasm containing free ribosomes (r), mitochondria (arrows) and Golgi apparatus (bifid arrow). ***(E)*** Showing mossy rosette (R) in between the granule cells (G) containing multiple mitochondria with regular cristae (m) **(Mic. Mag. A x 2500, B x1500, C x4000, D x4000 and E x3000).**

4. Discussion:

Autism is considered a chronic neurodevelopmental disorder that lies under the broad diagnosis of autism spectrum disorder (ASD). In spite of the increasing incidence of ASD, its accurate pathophysiology and treatment are still unknown. So, the use of valid animal models of autism is highly recommended to understand a lot about this disorder [3,5]

Induction of postnatal autism model with VPA was achieved by administration of this neurotoxic agent to the rat pups during the first 14 days of postnatal life. This is a critical period in neuronal development because the granule cells migrate and differentiate in this period that is roughly corresponds to the human third trimester of pregnancy [5,21]

Bee pollen (BP) is a natural food that contains high amounts of flavonoids, trace elements and other healthy contents. They have been established to possess several marvelous health effects [6]. Therefore, this work was carried out to evaluate the conceivable protective effect of BP on cerebellar cortex in VPA- induced postnatal autism model.

In our study, a significant decrease in the exploratory activities such as rearing and sniffing were observed in group III (VPA-induced autism group) as compared to the control. These results were in agreement with ***Sunand et al.*** [3]. ***Jiang et al.*** [22] who explained this finding by decreasing the motivation to explore a new environment or by fear related inhibition of exploratory activity. Also, group III (VPA-induced autism group) showed a significant decrease in spontaneous motor activity as compared to the control. The same results were previously reported by ***Ishola et al.*** [12] and explained by ***Mitsuhashi et al.*** [23] who referred it to the decreased strength in muscle and abnormal motor function.

In the current study, the stereotyped behaviors such as grooming showed a significant increase in the VPA-autism group as compared to the control. This finding was in line with ***Chaliha et al.*** [24] ***and Atia et al.*** [25]. This finding was clarified by ***Ishola et al.***

[12] who assigned the repetitive behavior to stress, fear, anxiety or to the difficulties in communication.

In the present study, as regards the social interaction test, there was an obvious decrease in the social activity represented by following and sniffing body parts of each other in the VPA-induced autism group as compared to the control group. Similar results were obtained by ***Chaliha et al.*** [24]**.** ***Schiavi et al.*** [26] who explained these findings by impairments in the cognitive functions required for proper perception and understanding the social environment leading to failure to respond to the fluctuating social conditions. ***Elgamal et al.*** [27] also added that, these findings might be due to changes in the acetylcholine level, which can trigger anomalous social, repetitive, anxiety-like, and hyperactive behaviors.

In the current study, light microscopic examination of cerebellar sections stained with *H&E* of rats in group III (VPA-induced autism group) revealed marked alterations and disorganization of the normal histological orientation of the cerebellar cortex. Similar findings were also observed by ***Elgamal et al.*** [27] ***and Alpay & Yucel*** [28]**.** These findings could be attributed collectively to neuronal oxidative stress, neuroinflammation and neurotransmitter disturbances resulting in neurodegenerative features and cell death as explained by ***Morakotsriwan et al.*** [11]**.** ***Sunand et al.*** [3] referred the decrease in PC number to high vulnerability of PCs to the effect of reactive oxygen species (ROS), which cause destruction to proteins and lipids in cellular membranes and also DNA thus interfere with the neurodevelopmental process.

As regards ***Al-Gholam & Ameen*** [29]**,** elevation of inflammatory cytokines in VPA treated rats resulted in neurotoxicity and neuronal death. ***Abdel Mohsen et al.*** [30] also referred the PCs degeneration and loss to the liberation of cytochrome c from the stressed mitochondria into the cytoplasm activating apoptosis and subsequently inducing cell death.

Disturbance of the normal linear organization of PCs and their multi-layered displacement into the molecular layer (ML) and granular cell layer (GCL) respectively could be explained by their delayed migration as pronounced by ***Abdel Mohsen et al.*** [30]. ***Galal et al.*** [31] ***and Zedan*** [32] attributed the disturbed PCs linear organization to the sustained neuronal insult that causes the PCs to crowd themselves in other areas trying to adapt and reestablish synapsis with other neurons achieving their

normal functions. Moreover, heterotopic PCs could arise due to subnormal migration of external granular layer cells during histogenesis as explained by ***Viswasom & jobby*** [33]

Irregularity in the shape of PCs and loss of their normal pyriform appearance was explained by ***Eid et al.*** [34] who referred it to the ability of VPA to interact with components of cell cytoskeleton. ***Soliman & Ali.*** [35] clarified that dark cells can be caused by apoptosis due to DNA damage as a result of production of ROS or a sign for chromatolysis and gliosis as mentioned by ***Zedan*** [32]**.** Spaces in the neuropil surrounding the PCs were attributed to the degeneration of the cell cytoskeletal elements leading to shrinkage and withdrawal of the protoplasmic processes of PCs as mentioned by ***Abdel Mohsen et al.*** [30] ***and Ibrahim et al.*** [36]

In this study, vacuolations in the neuropil of molecular layer of VPA-induced -autism group and presence of dilated blood vessels were evident. Similar findings were observed by ***Elgamal et al.*** [27] ***and Eid et al.*** [34]**.** ***Shona et al.*** [37] described the ML vacuolations as spongiosis in the cerebellum and referred it to the mitochondrial dysfunction and increased ROS production. ***Abdel-Aziz et al.*** [38] referred the ML vacuolations to the degeneration of their cytoskeletal elements leading to shrinkage of cells and the withdrawal of the cell processes. The presence of dilated blood vessels was in line with ***Al-Amoudi*** [39] and could be explained by ***Ali et al.*** [40] due to the release of vasodilator nitric oxide (NO) from the activated astrocytes.

In our study, decrease in the density of cells, appearance of spaces, dilated congested blood vessels and significant reduction in mean thickness of the granular cell layer in VPA-induced autism group were detected. Similar findings were observed by ***Elgamal et al.*** [27]**;** ***Alpay & Yucel*** [28]**;** ***Zedan*** [32] ***and Ali et al.*** [40]

Eid et al. [34] ***and Khair & Abdel-Aziz*** [41] explained these degenerative changes in the granular cell layer as they might be secondary to degeneration of PCs as the degenerated PCs failed to make normal connection with granule cells resulted in loss of the regulatory synchronization between them. ***Magar et al.*** [42] previously explained the reduction in the GCL thickness by degeneration to cells due to disturbed system of cyclin-dependent kinase that controls cellular division, differentiation and cellular function.

In the present work, anti-GFAP immunohistochemically stained sections of

group III (VPA-induced autism group) showed apparent strong positive reaction in the cytoplasm and cell processes of astrocytes. In addition to, a highly significant increase in the mean area percentage of anti-GFAP positive immunoreaction as compared to the control group. These findings are in line with ***Galal et al.*** [31]

Abdel Mohsen et al. [30]***; Eid et al.*** [34] ***and Dossi et al.*** [43] explained these changes as the exposure to VPA injury causes morphological and molecular changes as well as functional deviations in astrocytes and activate them, what is so-called reactive astrogliosis. Oxidative stress and increased ROS levels are the possible mechanisms of this gliosis.

Ultrastrucurally, sections obtained from group III (VPA-induced autism group) showed marked disturbances in the cerebellar cortical ultrastructure especially on Purkinje cells. Parallel results were obtained by ***Alpay &Yucel*** [28]***; Abdel Mohsen et al.*** [30]***; Eid et al.*** [34]***; Shona et al.*** [37] ***and Ali et al.*** [40]

Hafez et al. [21] ***and Ibrahim et al.*** [36] ***and Arafat & Shabaan*** [44] previously mentioned that, shrunken of Purkinje cell nuclei and dense PCs cytoplasm could be attributed to catastrophe of the antioxidant system leading to occurrence of oxidative stress followed by gathering of destructed proteins. Similarly, dense cytoplasm could be credited to damage to the cytoplasmic organelles that play a role in the biosynthesis of cell proteins as mentioned by ***Abdel-Aaziz et al.*** [38]. ***Zaghloul et al.*** [45] referred them to inhibition of oxidative phosphorylation processes within the PCs mitochondria leading to decreased phospholipid synthesis and disturbed membrane integrity.

Sarhan & Taalab [46] reported that, various stimuli could disrupt the function endoplasmic reticulum (ER) with subsequent accumulation of misfolded proteins in the lumen resulted in ER stress. This might be the cause of the dilated RER.

In our study, mitochondria with destroyed cristae was a prominent finding in PCs cytoplasm and also in the neuropil of all layers in the VPA-induced autism group. This finding was in line with ***Eid et al.*** [34] ***and Arafat & Shabaan*** [44]***. Ibrahim et al*** [36] explained this by the oxidative stress and ***Finsterer*** [47] referred the disturbed morphology of mitochondria to the conformational alterations of respiratory chain proteins and fundamental defects in the structure inner mitochondrial membrane. ***Khair & Abdel-Aziz.*** [41] stated that the electron dense bodies appeared in the

cytoplasm might be disintegrated mitochondria as a product of auto-phagocytosis along with lysosomal processing.

As regards the molecular layer in VPA- induced autism group, it revealed disturbances in the ultrastructure of basket cells and the surrounding neuropil. They appeared with increased nuclear clumps of heterochromatin in addition to mitochondria with destroyed cristae in the surrounding neuropil. These findings were in agreement with ***Eid et al.,*** [34] ***and Ibrahim et al.,*** [36] who referred those changes to oxidative stress. ***Arafat & Shabaan,*** [44] explained the increased nuclear clumps of heterochromatin by condensation of chromatin during neuronal cell death or may be due to apoptosis as explained by ***Taleb et al.*** [48] ***and Teleanu et al.*** [49]**.** They clarified that, VPA induced a significant elevation of apoptotic markers due to the oxidative stress and neuroinflammation, which in turn causes damage to the cellular components such as protein, lipids and DNA leading to cell death.

As regards the granular cell layer, electron microscopic examination of VPA-induced autism group revealed granule cells having nuclei with peripheral chromatin condensation, irregular nuclear membranes and cytoplasmic vacuolations. In addition, mossy rosettes containing few widely separated mitochondria with destroyed cristae were also present. These findings were in line with ***Eid et al.*** [34] ***and Abdel-Aziz et al.*** [38]**.** ***Hafez et al.*** [21] and ***Shona et al.*** [37] explained that by VPA-induced damage of granule cells with subsequent loss of a great part of the cortical building.

Nuclei with peripheral chromatin condensation might be attributed to apoptosis as explained by ***Hafez et al.,*** [21] ***and Galal et al.,*** [31]**.** ***Savran et al.,*** [50] reported that, VPA might make changes the mitochondrial membrane potential and activates the intrinsic apoptotic pathway.

Kassab et al., [51] added that, ultrastrucural changes might be due to VPA cytotoxicity because it enhances the clearance of copper, selenium and zinc, subsequently resulting in decreased synthesis of free radicals scavenging enzymes as glutathione peroxidase and glutathione reductase. Furthermore, the cytotoxicity of VPA may be due to lysosomal membrane leakage along with ROS production.

Laag & Abd Elaziz, [52] explained the changes in neuropil of all layers of the cerebellar cortex as the injury of Purkinje cells and granular cells made them incapable of maintaining their distal processes. Therefore, axon and dendrites degeneration in the

cerebellum are components of the neuronal injury process.

Cytoplasmic vacuolations observed within PCs and granule cells might be due to increased lipid peroxidation and production of ROS that attack the cell membrane altering the permeability as explained by ***Hamouda et al.*** [53]

Behavioral tests exhibited marked improvement in group IV (VPA and BP extract-treated rats) as compared to group III (VPA-induced autism group). Besides, light, and electron microscopic examination of cerebellar sections in group IV revealed noticeable improvement which was proved statistically.

Such behavioral, histological, immunohistochemical and statistical improvement in group IV could be attributed to the neuroprotective effects of BP as mentioned by ***Al-Salem et al.*** [6] ***and El-Ansary et al.*** [54] who reported that, BP was effective in improving the neurotoxicity due to their potent anti-oxidant and anti-inflammatory effects. They also added that, BP could be safely used to enhance the chronic degenerative diseases as the mechanisms that participate in the pathogenesis of autism.

In our study, the ameliorating effect of bee pollen was in line with ***Klaric et al.*** [55] who reported improved hepatotoxicity by bee pollen and attributed it to its polyphenolic components such as flavonoids that are strong antioxidants prevent against lipid peroxidation occurring in the cell membranes. ***Umesh et al.*** [56] previously stated that, BP was very effective in ameliorating oxidative stress induced by drugs. Also, ***Saral et al.*** [57] reported that, BP suppresses neuroinflammation, oxidative stress and peroxidation of lipids in the hippocampus and also increases the neurotrophic factors in the adult rat's hippocampus.

Bee pollen was verified to do anti-inflammatory effects via different mechanisms as mentioned by ***Chelucci et al.*** [58]***. Li et al.*** [59] stated that, BP upregulates the gene expression of transforming growth factor beta 1 (TGF-β1) and downregulates gene expressions of TNF-α and IL-6, and inhibits the signaling of mitogen activated protein kinase. Furthermore, BP downregulates the pro-inflammatory cytokines like interleukins 1A and 6 and (IFN-γ) in cases of neuroinflammation and inhibits nitric oxide production as mentioned by ***El-Sayed*** [60] ***and Li*** [61]***. Zhang et al.*** [62] also mentioned that, BP inhibits

Cyclooxygenase-2 expression in the macrophage.

Aabed et al. [10] previously reported that, pre-treatment with α-tocopherol anti-oxidant, a component of BP, prevented the IL-1 induced neuronal cell death. ***Bacha et al.*** [63] stated also that, BP improves defects in neurotransmitter levels, apoptosis, inflammation, and glutamate excitotoxicity. Similarly, ***Al-Salem et al.*** [6] reported that, BP caused ameliorative effects on markers of oxidative stress such as ascorbic acid (vitamin C) and glutathione (GSH) in autistic rats.

Bee pollen was reported to restore complex-I, -II, -III and -IV enzyme activity of mitochondria to normal, increase glutathione and SOD and reduce NO and MDA as reported by ***Ali & Konugi*** [64]**.** The antioxidant activity of bee products could be related to their strong ability to activate Nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathway, which stimulates the liberation of heme oxygenase-1 (HO-1) as an internal antioxidant. NRF2 and HO-1 indirectly block production of ROS through suppression of inflammatory reactions as stated by ***Ali & Konugi*** [64]

BP has a positive effect in reducing anxiety like behaviors due to reduced oxidative damage and neuroinflammation as mentioned by ***Saral et al.*** [57] and due to reduced glutamate excitotoxicity as mentioned by ***Bacha et al.*** [63]. ***Omnia et al.*** [65] clarified also that, BP significantly decreases GABA, cholinesterase and nitric oxide, which contribute in VPA induced toxic changes. Also, they reported that, BP caused inhibition of xanthine oxidase, which is known to generate free radicals.

According to ***Al-Salem et al.*** [6]**,** BP fractions can protect brain tissue due to their huge amounts of polyphenolic compounds, cholinergic acids and flavonoids, which have been informed to possess high antioxidant effects due to their capacity to attack ROS leading to inhibition of lipid peroxidation.

El-Seedi et al. [66] reported another mechanism of neurodegeneration is the elevation of extracellular glutamate levels. A study performed by ***El-Ansary et al.*** [54] showed that, BP caused impairment in glutamate excitotoxicity. The antioxidants present in large amounts in BP such are carotenoids, vitamins and polyphenols play an essential role in ameliorating the disruption in the blood brain barrier as reported by ***Kim et al.*** [67]

Nowadays, there is an increasing tendency in studying the natural components

capable of lessening the effects of oxidative stress which is a common factor in the pathogenesis of various diseases. Our study confirmed that BP as a natural product made significant improvement in VPA induced oxidative stress and inflammatory changes. Based on these results, BP could be advisable as a food plan for autistic children.

5. Conclusions:

From the previously mentioned data. It could be concluded that, bee pollen could ameliorate valproic acid induced-autism. Therefore, it could be useful for the treatment of social, behavioral and histological features of autism.

Conflicts of Interest:

No conflict of interest has declared by authors.

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