

Histological and Immunohistochemical Study on the Effect of Methotrexate on the Cerebellum of Adult Male Albino Rats and The Possible Protective Role of Lepidium Sativum

Original
Article

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ABSTRACT

Introduction: Methotrexate is a cytotoxic chemotherapeutic agent, vastly utilized not only for malignancies but also for various inflammatory diseases, but it had hazardous effects on cerebellum. Lepidium sativum aherbal medication seemed to have neuroprotective effects.

Objectives: This study was done to assess the effects of methotrexate on the cerebellum of adult male albino rats and the possible neuroprotective effect of lepidium sativum.

Material and Methods: Sixty (60) adult male albino rats, weighing nearly 170-200gms were used in the current study. The first group (n=10) was the control group. Rats of second group (n=10) were given lepidium sativum aqueous extract (150mg/kg/day) by gastric tube daily for 4 weeks. Rats of third group (n=20) were given methotrexate (20mg/kg) on the 14th day then left without treatment for 2 weeks. Rats of fourth group (n=20) were given both Methotrexate and Lepidium Sativum as groups II and III. After 4 weeks, animals were sacrificed then the cerebellum was excised and processed for histological, immunohistochemical and quantitative studies.

Results: Rats given lepidium sativum aqueous extract was nearly similar to control group. Methotrexate treated rats showed significant decrease in number of Purkinje cells, significant increase in mean area % of GFAP positive immuno-staining and destructive changes of Purkinje cells with marked reduction of Nissl's granules. This was confirmed by electron microscopic examination and strong positive reaction for GFAP and caspase -3 reactions. Rats treated by Methotrexate and Lepidium Sativum showed nearly normal histological appearance of Purkinje cells with less vacuolated cytoplasm. This was confirmed by a significant rise in the number of Purkinje cells, significant diminution in caspase-3 positive cells and in GFAP immunostaining.

Conclusion: Lepidium Sativum aqueous extract ameliorates the neurotoxic effects of methotrexate. So, it is recommended to use Lepidium Sativum, whenever methotrexate treatment is needed.

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Key Words: GFAP, lepidium sativum, methotrexate.

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INTRODUCTION

Methotrexate (MTX; amethopterin; 4-amino-10-methylfolic acid), a structural analogue of folic acid, is one of the most widely used chemotherapeutic drugs^[1]. MTX as folate antagonist is used to treat autoimmune diseases as rheumatoid arthritis, haematological malignancies and various types of solid tumors^[2]. However, the efficacy of this agent is often restricted by intense toxic side-effects such as mucositis, vomiting and diarrhea. Also, neurotoxicity is one of the most critical side effects of the MTX. Although neuronal symptom is also frequently observed, the mechanisms of MTX-induced neurotoxicity remain unclear yet as it has been less extensively investigated than other side effects of MTX. Oxidative stress has been suggested among several hypotheses as a possible cause of MTX-induced toxicity^[3].

Plants still remain major source for drug discovery regardless of the great evolution of synthetic molecules. Thus, there is a major increase in the use of traditional plants in treatment of different diseases^[4]. Lepidium Sativum (LS) is locally known as 'hub arachad', belonging to family Brassicaceae. The chemical composition of the plant seeds and leaves is flavonoids, isothiocyanates glycosides, carbohydrates, proteins, essential oils, fatty acids, β -carotene and vitamins like niacin, riboflavin and ascorbic acid^[5]. The plant seeds are used in salad and as a condiment. Different parts of this plant have been used in traditional medicine, as LS has been reported to have hypoglycemic, diuretic, antihypertensive, hemagglutinating, hepatoprotective, fracture healing, anti-inflammatory, anti-neuropathic, antioxidant and anti-carcinogenic activities^[6].

Since the mechanisms of MTX-induced toxicity have not been exactly detected yet, therefore the current work was done to evaluate the possible protective effect of *Lepidium Sativum* on methotrexate induced changes on cerebellum.

MATERIALS AND METHODS

Drugs

Methotrexate is available as methotrexate sodium solution for IM or IV injection (25 mg/ml). It is brought by Orion pharmaceutical Company Nasr city, Cairo, Egypt. It was given for animals by intraperitoneal injection. *Lepidium Sativum* seeds were purchased from super market in Shebin el Kom, Menoufiya Government. They were ground and dissolved in water and given orally by gastric oral tube.

Animals and treatment

Sixty (60) adult male albino rats, weighing nearly 170-200gms were used in the present study. They were dwelled in stainless steel cages at room temperature and allowed laboratory rat chow diet and water ad-libitum. Strict care and hygiene were given to the rats all time of the experiment to preserve a normal and healthy environment, the general condition and behavior of the animals were observed. Randomly, the animals were classified into four main groups:

Group I (control group) composed of 10 rats that were further divided into two equal subgroups, half of rats were kept without any treatment, while the other half was given the vehicle used to dissolve *Lepidium sativum* (distilled water) orally for four weeks.

Group II (*Lepidium Sativum* - treated group) composed of 10 rats, were treated with *Lepidium Sativum* aqueous extract at a dose of 150mg/kg/day^[7] (30 mg/rat/day = 1ml from *lepidium* solution / rat/day) given orally for four weeks. *Lepidium Sativum* suspension was prepared by dissolving 300mg LS powder in 10 ml distilled water, to get a solution containing 30 mg *Lepidium Sativum* / 1ml distilled water.

Group III (Methotrexate-treated group) composed of 20 rats, received single intraperitoneal injection of methotrexate at a dose of 20mg/kg^[8,17] (4mg/rat = 0.2 ml/solution/rat) on the 14th day of the experiment, then left without any remedy till the end of the experiment (4 weeks).

Group IV (Methotrexate and *Lepidium Sativum*-treated group) composed of 20 rats, received *Lepidium Sativum* aqueous extract and single intraperitoneal injection of methotrexate at the same dose as group II & III.

Methods

The animals from all groups after 4 weeks were scarified by decapitation. The cerebellum of each rat was carefully dissected out and divided into 2 parts. One part was fixed in 10% buffered formalin for light microscopic study. The

other part was cut into small pieces and rapidly fixed in 1% phosphate buffered glutaraldehyde, then processed for Electron Microscopic study at National Cancer institute (Cairo- Egypt).

Light microscopic study

Tissue samples were fixed in 10 % buffered formalin and processed to get the ordinary paraffin blocks. Sections of 5µm thick were cut and subjected to the following studies.

(1) Histological study

- Hematoxylin & Eosin (H&E) stain for routine histological examination.
- Toluidine blue for demonstration of Nissl's granules.

(2) Immunohistochemical study

- Glial fibrillary acidic protein (GFAP) for detection of neuroglial astrocytes.
- Caspase-3 for detection of apoptosis.

Immunohistochemical stain

For GFAP: The deparaffinized 5 µm thickness paraffin sections which were put on positively charged slides were incubated in 10% hydrogen peroxide in absolute methanol (10 min). Heating the sections in 0.01 mol/l citrate buffer (pH: 6) in a water bath in a microwave (30 min) was done to unmask performed antigen. The slides were rinsed for 5 min in PBS at pH 7.4 and the sections were incubated with primary antibody (1h)^[9]. The 1ry antibody was anti GFAP (Ab-1, clone GA-5 mouse monoclonal antibody, then secondary antibody was applied (1h) at room temperature. After that, the sections were incubated in preformed streptavidin peroxidase for 10 minutes. Then sections were rinsed in PBS (5 minutes). Staining was completed by incubation with substrate chromogen 3,3'-Diaminobenzidine (5-15 min) which resulted in brown-colored precipitate at the antigen sites. After that they were counterstained by Harris's hematoxylin (0.5-1 min) to stain nuclei, dehydrated, cleared and mounted. The primary antibody was replaced by non-immune rabbit serum innegative control sections^[10].

For caspase-3: the sections were treated the same as GFAP as mentioned above. After that, the sections were washed twice in PBS (5 min each). The sections were treated with caspase-3 antibody at appropriate dilution in antibody dilution buffer in a humidified chamber (1 h) at room temperature, then were washed twice with PBS-T (5 min), to remove excess chromogen. The sections were incubated with the secondary antibody (20 min), Then washed with PBS for 3times (5min). DAB was added (10 min). Finally, the slides were counterstained by Mayer's hematoxylin, dehydrated, cleared, and mounted by DPX^[11].

Electron microscopic study

Eight 1×1 mm sized small segments of cerebellar tissue were fixed in buffered glutaraldehyde solution

(pH 7.4) at 4°C for 4 h. Then, the specimens were then rinsed three times with phosphate buffer (two changes) and post fixed in 1% aqueous buffered osmium tetroxide at room temperature for 2h. After that, the specimens were rinsed in buffer, dehydrated in ascending grades of alcohol, and embedded at the apex of inverted polythene beam capsule filled with liquid resin. The sections were cut using ultramicrotome into semithin (0.5 µm thickness) and ultrathin sections. The semithin sections were stained with toluidine blue. Ultrathin sections (80–90 nm) were stained with uranyl acetate and lead citrate^[12] and examined by transmission electron microscope (TEM, JEOL 100 CX) at National Centre Institute (Cairo-Egypt).

Morphometric analysis

Five fields were captured from randomly selected light microscopic slides per group. Images were taken at 400× magnification and 2.6 zoom. Morphometric analysis was carried out on photomicrographs by use of Image J Software^[13]. Using light microscopy, all Purkinje cells were counted in 5 cerebellar lobules of each 400 magnification H&E stained sections. Then the average value for the five lobules was calculated for each section. The mean area percentage of positive GFAP immunostaining was estimated at 400× magnification in 5 non-overlapping fields of each section.

Statistical analysis

The collected data were analyzed using statistical package for the Social Science Software (SPSS) software version 20 (SPSS Inc., Chicago, Illinois, USA). *P* value was considered highly significant if *P* value <0.01, but *P* value <0.05 was significant and was non-significant if *P* > 0.05. All data were expressed as mean ± SD.

RESULTS

General appearance of animals

The general condition of all animals of the control group including subgroup IA (receiving no treatment) and subgroup IB (receiving distilled water) were good and revealed a normal behavior in activity, zest and development. There was no mortality among those control animals all through this study. Animals treated with *Lepidium Sativum* for four weeks and sacrificed 24 hour after the last dose were in a good general condition with normal appetite throughout the experiment. But, In Methotrexate-treated group, animals revealed diminished activity and progressive decrease in their appetites with loss of weight. Some animals became cachectic during the experiment. 6 animals died. The general condition of animals treated with both methotrexate and *Lepidium Sativum* for four weeks was nearly better than methotrexate treated group and they also had a moderate appetite.

Light microscopic results

Group I (control group)

The control rats showed the well-known histological and ultrastructural picture of the cerebellar cortex and medulla

(Figures 1,2). In toluidine blue stained sections, Purkinje cells appeared rounded or flask shaped, arranged in one row and their cytoplasm showed coarse darkly blue stained Nissl's granules (Figure 3). The immunohistochemical staining for GFAP of this group showed mild to moderate staining of cells in different layers of cerebellar cortex (Figure 4). Whereas, in the immunostained sections of caspase-3, the reaction was negative in all layers of the cerebellar cortex (Figure 5).

Group II (*Lepidium Sativum* -treated group)

Both H&E-stained and toluidine blue-stained sections were the same as the control group (Figures 6,7). The immune reaction for GFAP and caspase-3 in all layers were similar to the control group (Figures 8,9).

Group III (Methotrexate -treated group)

The cerebellar sections of Methotrexate treated rats showed destructive changes variable in severity and distribution. The purkinje cells appeared shrunken with darkly stained pyknotic nuclei. Few cells became markedly distorted having ghost shape appearance with ill-defined shape and contents. The granule cells appeared smaller in size with pyknotic darkly stained nuclei and clumped together in groups with wide spaces in between. Also, the cells of molecular layer became smaller in size with pyknotic darkly stained nuclei. Spaces and congested blood vessel were observed in the cerebellar cortex and medulla (Figures 10,11). Purkinje cells by Toluidine blue stain showed vacuolated cytoplasm with marked reduction in the Nissl's granules (Figure 12). As regards the Immunohistochemical study, GFAP showed intense staining of astroglial cells with increase in thickness of astrocytic branches than control (Figure 13). Caspase-3 immunostaining of this group revealed strong positive immunoreactivity in the three layers of cerebellar cortex indicating signs of apoptosis (Figure 14).

Group IV (Methotrexate and *Lepidium Sativum*-treated group)

Lepidium Sativum and methotrexate treated rats showed better histological picture compared to group III with H&E and toluidine blue (Figures 15,16,17). Immunohistochemical staining for GFAP of this group revealed strong staining intensity of astroglial cells (Figure 18) and for caspase-3 showed moderate intensity of different layers of the cerebellar cortex and medulla (Figure 19).

Transmission electron microscopic results

Group I & Group II

Electron microscopic examination of the cerebellar cortex of the control group showed the purkinje cells with nucleus and cytoplasm containing plenty of mitochondria. The granular layer showed small numerous closely packed granule cells with oval heterochromatic nuclei and thin rim of cytoplasm around nucleus. The glial cells were seen around the purkinje cells and appeared lightly stained

(Figures 20,21). Electron microscopic examination of cerebellum of LS treated rats showed a picture more or less similar to control (Figure 22).

Group III

Electron microscopic examination of cerebellum of MTX-treated group showed degenerative changes compared to control rats. The Purkinje cells showed different degrees of degeneration. Some Purkinje cells seemed shrunken with irregular outline, shrunken nuclei and increased density of their cytoplasm. Also, other Purkinje cell appeared degenerated shrunken with many cytoplasmic vacuoles. Numerous empty spaces were observed surrounding Purkinje cells reflecting degenerated glial cells (Figures 23,24). The granule cells appeared degenerated. Also, there were wide spaces filled with edema fluid in the granular layer (Figures 25,26). In cerebellar medulla, most of nerve fibers were degenerated and disrupted. Also, there were dilated congested blood capillaries, fluid and wide spaces (Figure 27).

Group IV

Electron microscopic examination of cerebellum of methotrexate and Lepidium Sativum treated group showed less degenerative changes of Purkinje cell which appeared

nearly normal and their cytoplasm had slightly dilated RER. Wide spaces were still seen around the Purkinje cells. Most of the granule cells seemed normal with oval heterochromatic nuclei and thin rim of cytoplasm. Few cells appeared degenerated. In medulla, most of nerve fibers appeared normal, others appeared degenerated and fluid were still present (Figure 28,29,30)

Morphometric and statistical results

Compared to control, there was a highly significant decrease in the number of Purkinje cells observed in MTX treated group ($p < 0.01$), while there was non-significant decrease in the number of Purkinje cells in both Lepidium Sativum treated (II) and MTX and Lepidium Sativum (IV) treated groups compared with control ($p > 0.05$), but group IV remained significantly ($P < 0.05$) increased compared with group III (Table 1, Diagram 1). The mean area \pm SD of GFAP positive staining of astrocytes of all groups were illustrated in (Table 2, Diagram 2). There was a highly significant rise ($P < 0.01$) of mean area % of GFAP positive immunostaining in group III in comparison with group I. There was a significant decrease ($P < 0.05$) of GFAP positive immunostaining in group IV compared with group III, but it remained non-significantly increased ($P > 0.05$) compared with group I.

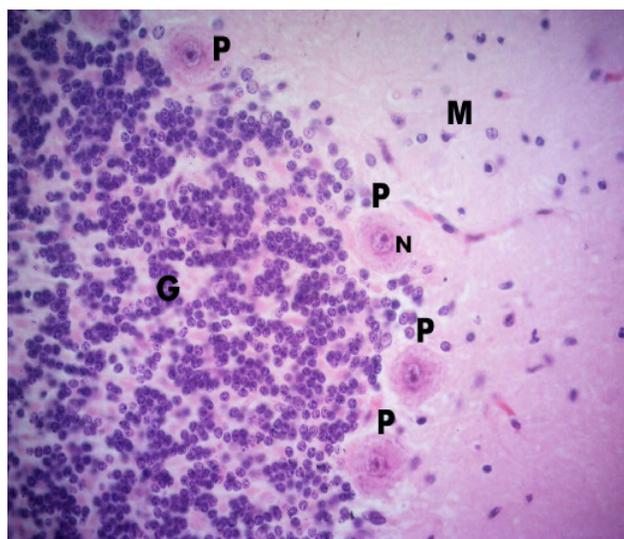


Fig. 1: A photomicrograph of cerebellar cortex of control adult rat (GI) showing the three layers of cerebellar cortex. The outer molecular layer (M), the middle Purkinje cell layer (P) with pale vesicular nucleus (N) and prominent nucleolus, and inner granular layer (G). (H&E \times 400)

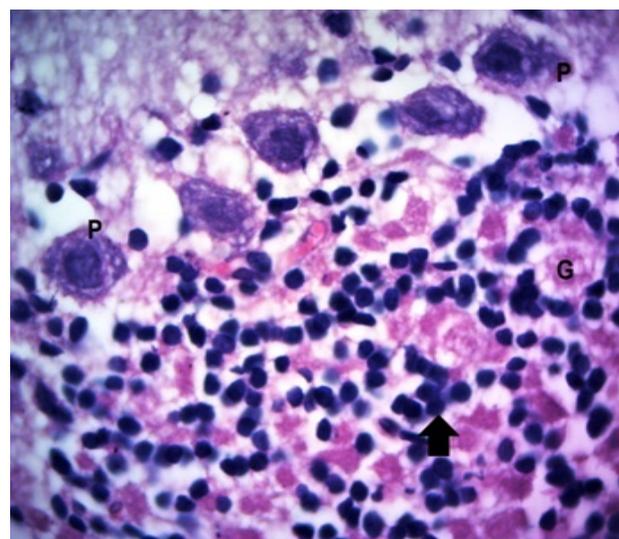


Fig. 2: A photomicrograph of a section of the cerebellum of control adult rat (GI) showing Purkinje cells (P) having big rounded vesicular nucleus, and part of granular layer consisting mainly of numerous small deeply stained closely packed granule cells with spherical deeply stained nuclei and scanty cytoplasm. Lightly stained acidophilic areas representing glomeruli are scattered in the granular layer (G). (H & E \times 1000).

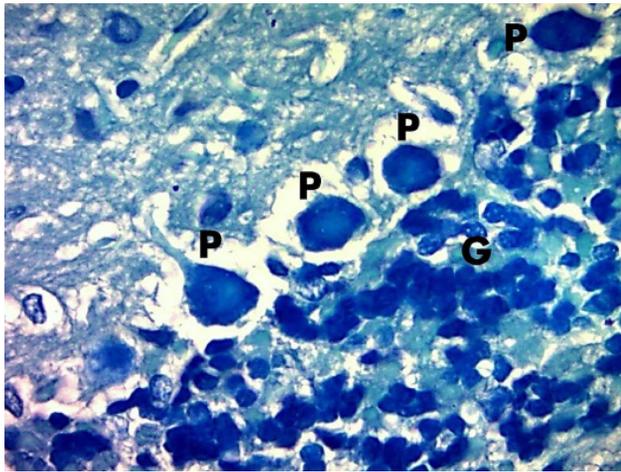


Fig. 3: A photomicrograph of a section of control adult rat cerebellar cortex (GI) showing flask shaped Purkinje cell (P) having big rounded vesicular nucleus and dark blue cytoplasm containing Nissl's granules. The granule cells (G) are more deeply stained. (Toulidine blue $\times 1000$)

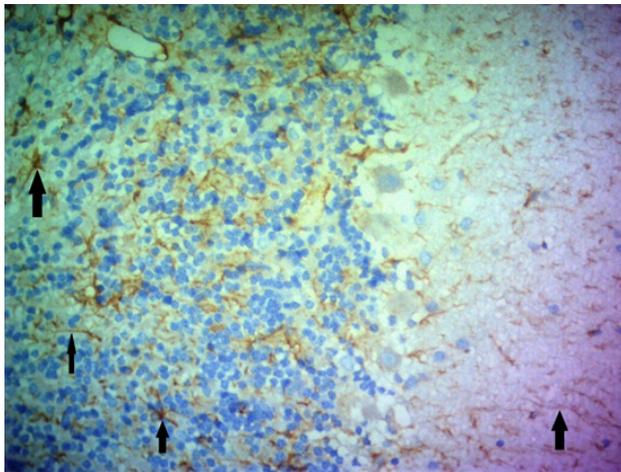


Fig. 4: A photomicrograph of a section of a control adult rat cerebellar cortex (GI) showing brownish astroglial cells with faintly stained processes (Arrow) dispersed between the different layers of cerebellar cortex, denoting a positive reaction for glial fibrillary acidic protein (GFAP). (Immunohistochemical stain for GFAP $\times 400$)

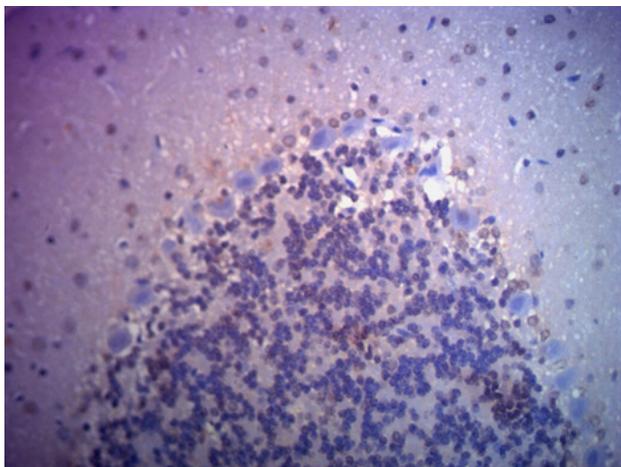


Fig. 5: A photomicrograph of a section of control adult rat cerebellar cortex (GI) showing negative immunoreactivity for Caspase 3 in different layers of cerebellar cortex. (Immunohistochemical stain for Caspase 3 $\times 400$)

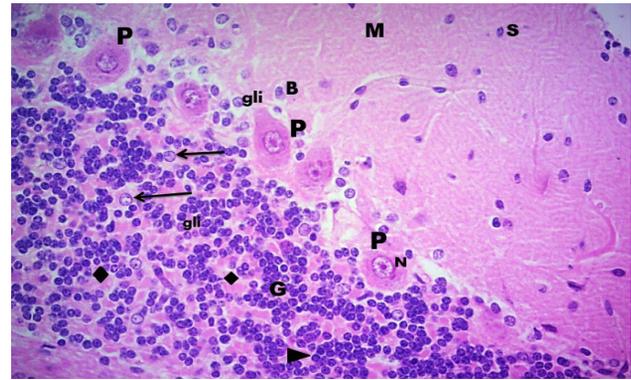


Fig. 6: A photomicrograph of a section of the cerebellum of Lepidium Sativum treated adult rat (GII) showing the three layers of cerebellar cortex and medulla (Med). Nerve cells found in the molecular layer are outer stellate cells (S) and basket cells (B). The middle Purkinje cell layer (P) consists of a single row of large pear shaped cells with pale vesicular nucleus (N) and prominent nucleolus. The granular layer (G) consists mainly of numerous small closely packed deeply stained granule cells (arrow head) & sporadic Golgi cells (arrow) which are large and have vesicular nuclei. Lightly stained glomerulus are scattered in the granular layer (diamond). The glial cells are scattered in all layers of cerebellar cortex (gli). (H & E $\times 400$).

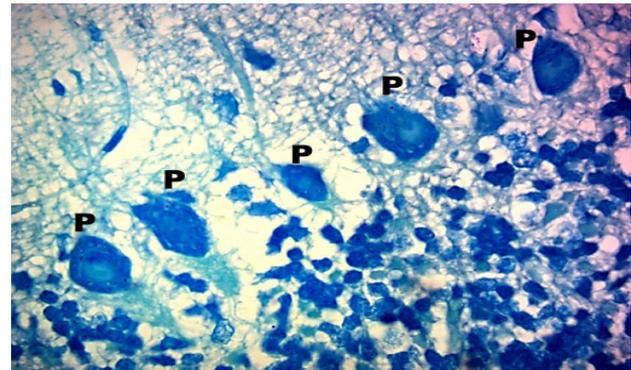


Fig. 7: A photomicrograph of a section of Lepidium Sativum-treated rat cerebellar cortex (GII) showing flask shaped to rounded Purkinje cells (P) having big rounded vesicular nucleus with prominent nucleolus and dark blue cytoplasm containing Nissl's granules. (Toulidine blue $\times 1000$)

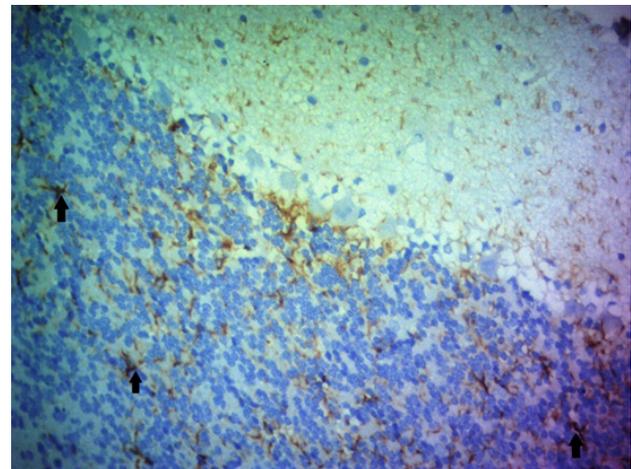


Fig. 8: A photomicrograph of section of Lepidium sativum-treated adult rat cerebellar cortex (GII) showing mild to moderate staining of astroglial cells (arrow) which are dispersed between the different layers of cerebellar cortex (brown colour). (Immunohistochemical stain for GFAP $\times 400$)

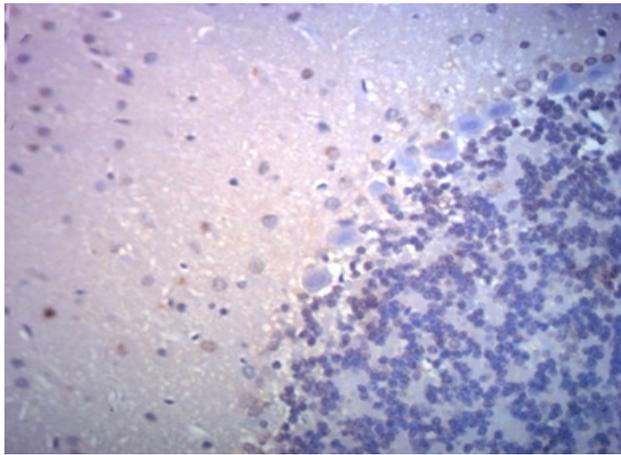


Fig. 9: A photomicrograph of a section of *Lepidium sativum*-treated adult rat cerebellar cortex (GII) showing negative immunoreactivity for caspase-3 in different layers of cerebellar cortex. (Immunohistochemical stain for Caspase 3 × 400)

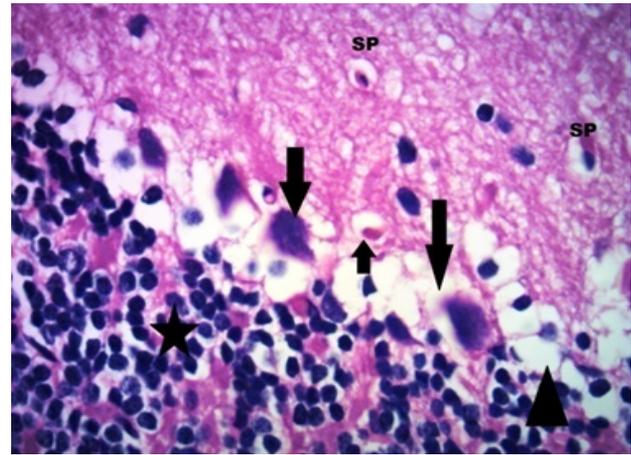


Fig. 11: A photomicrograph of a section of the cerebellum of methotrexate-treated adult rat (GIII) showing different degrees of purkinje cell degeneration. Cells appear shrunken with darkly stained pyknotic nuclei (arrow). Other one showed complete degeneration with vacuolated cytoplasm and disappearance of its nucleus with ghost shape appearance (up arrow). The granule cells appear shrunken and deeply stained and clumped in groups (star). Wide spaces are observed in the molecular layer (sp) and Purkinje cell layer (arrow head). (H& E × 1000)

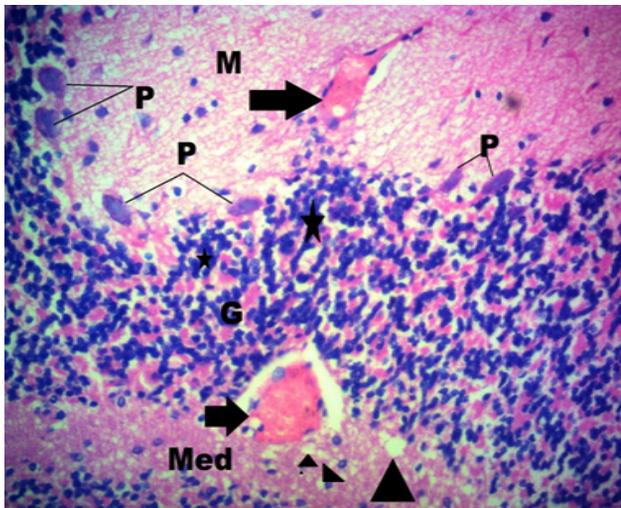


Fig. 10: A photomicrograph of a section of the cerebellum of methotrexate treated adult rat (GIII) showing congestion of cortical and medullary blood vessels (arrow) and different degrees of purkinje cell degeneration (P). The granule cells (G) appear shrunken and deeply stained (star). There are some vacuolated cells in the molecular and granular layers with presence of extra cellular spaces(circle). Also, wide spaces are observed (arrow head) in cerebellar medulla (Med). (H & E × 200).

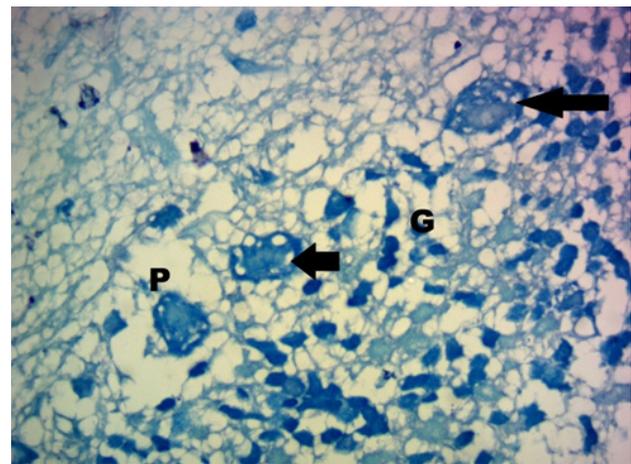


Fig. 12: A photomicrograph of a section of Methotrexate-treated rat cerebellar cortex (GIII) showing decreased intensity of stain for Nissl's granules in Purkinje (P) and granule cells (G). All the cells appear vacuolated (arrow). (Touildine blue × 1000)

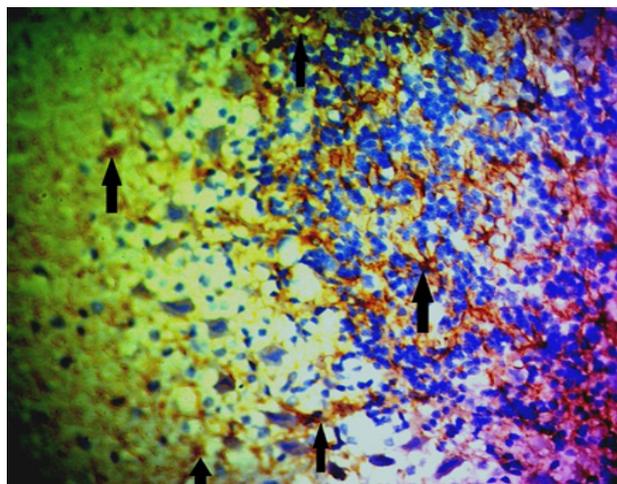


Fig. 13: A photomicrograph of a section of methotrexate-treated adult rat cerebellar cortex (GIII) showing intense staining of astroglial cells (arrow) dispersed between the different layers of cerebellar cortex. They show intense reaction in their soma and their processes (brown colour). (Immunohistochemical stain for GFAP × 400)

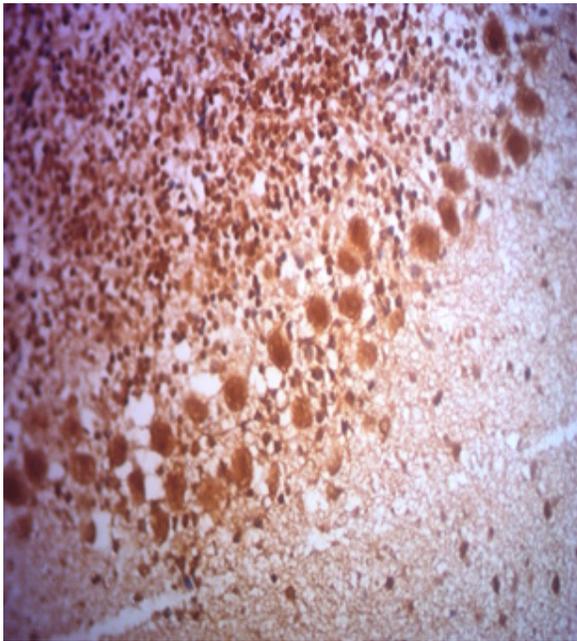


Fig. 14: A photomicrograph of a section of methotrexate-treated adult rat cerebellar cortex (GIII) showing strong positive immunoreactivity for caspase-3 in different neurons of different layers of cerebellar cortex. (Immunohistochemical stain for Caspase 3 \times 400)

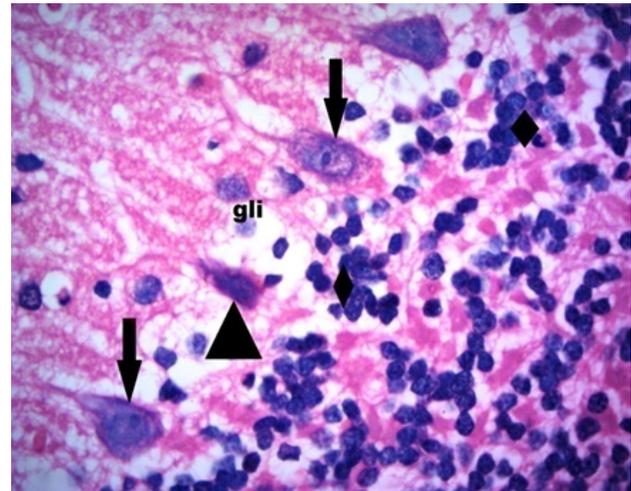


Fig. 16: A photomicrograph of a section of the cerebellum of methotrexate and lepidium Sativum-treated adult rat (GIV) showing normal Purkinje cells with pale vesicular nucleus and prominent deeply stained marginating nucleolus (arrow). Only one cell is seen shrunken with deeply stained pyknotic nucleus (arrow head). The granular layer appears nearly normal except for clumping of some cells (Diamond). The glial cells appear with lightly stained nucleus (gli). (H& E \times 1000)

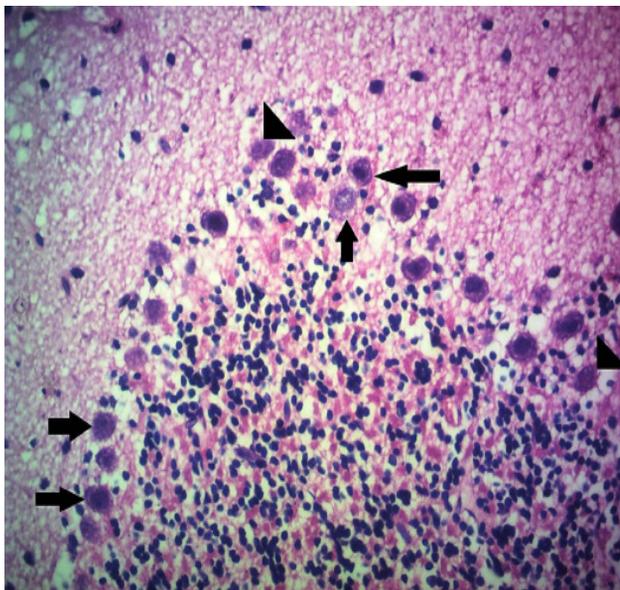


Fig. 15: A photomicrograph of a section of the cerebellum of methotrexate and Lepidium Sativum-treated adult rat (GIV) showing some nearly normal Purkinje cells in size and shape with vesicular nucleus and prominent nucleolus (arrow). Only few cells appear shrunken with deeply stained pyknotic nuclei (arrow head). The granular and molecular cell layers appear nearly normal. (H& E \times 400)

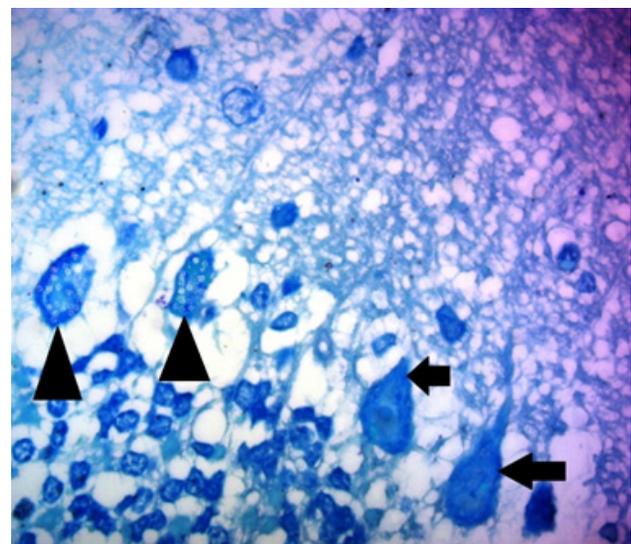


Fig. 17: A photomicrograph of a section of methotrexate and Lepidium Sativum-treated rat cerebellar cortex (GIV) showing two Purkinje cells (arrow) appear nearly normal and shows the characteristic flask shape appearance having vesicular nucleus and dark blue cytoplasm containing Nissl's granules. The other Purkinje cells (arrow head) appear shrunken with vacuolated cytoplasm and show decreased intensity of stain for Nissl's granules. (Touline blue \times 1000)

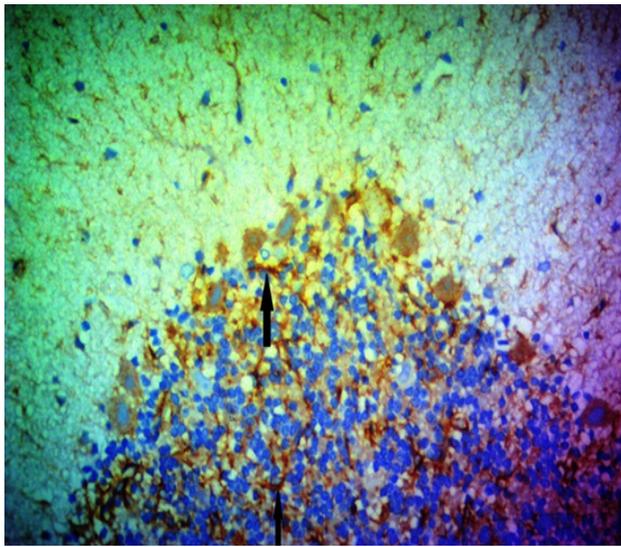


Fig. 18: A photomicrograph of a section of methotrexate and lepidium sativum treated adult rat cerebellar cortex (GIV) showing strong staining of astroglial cells (arrow) among different layers of cerebellar cortex. (Immunohistochemical stain for GFAP $\times 400$)

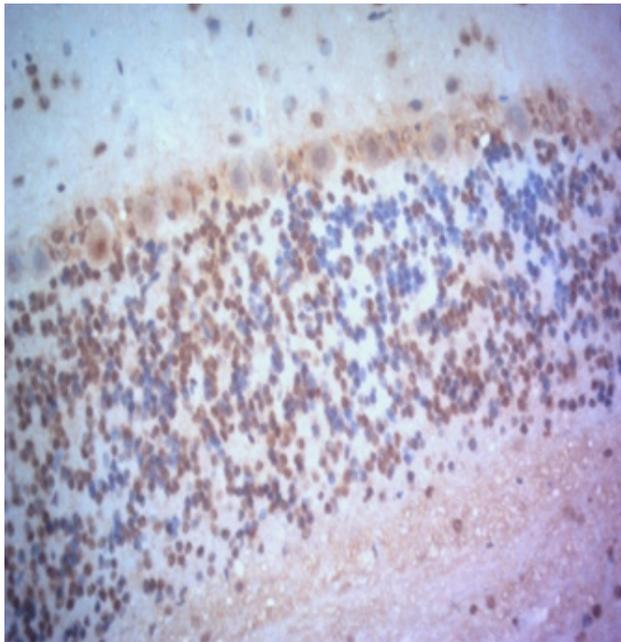


Fig. 19: A photomicrograph of a section of methotrexate and Lepidium Sativum-treated adult rat cerebellar cortex (GIV) showing moderate positive cytoplasmic immunoreactivity for caspase-3 in different neurons of different layers of cerebellar cortex. (Immunohistochemical stain for Caspase 3 $\times 400$)

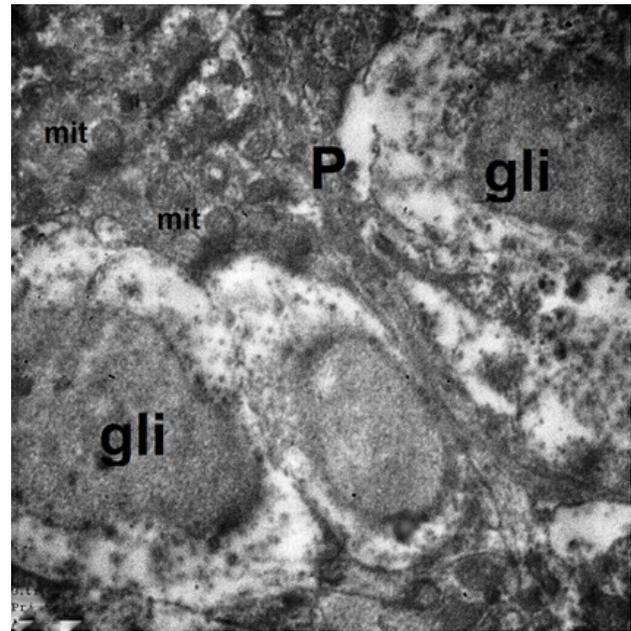


Fig. 20: Electron micrograph of a section of control rat cerebellar cortex (GI) showing part of large Purkinje cell (P) with long apical dendrite, cytoplasm contains numerous mitochondria (mit). Glial cells surround the Purkinje cell (gli). (TEM $\times 2000$)

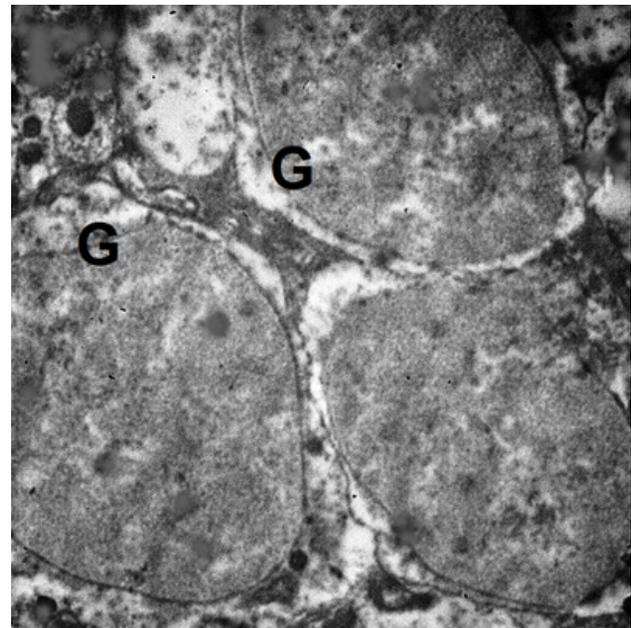


Fig. 21: Electron micrograph of a section of control rat cerebellar cortex (GI) showing three normal granule cells (G) with oval heterochromatic nuclei and thin rim of cytoplasm around the nucleus. (TEM $\times 2000$)

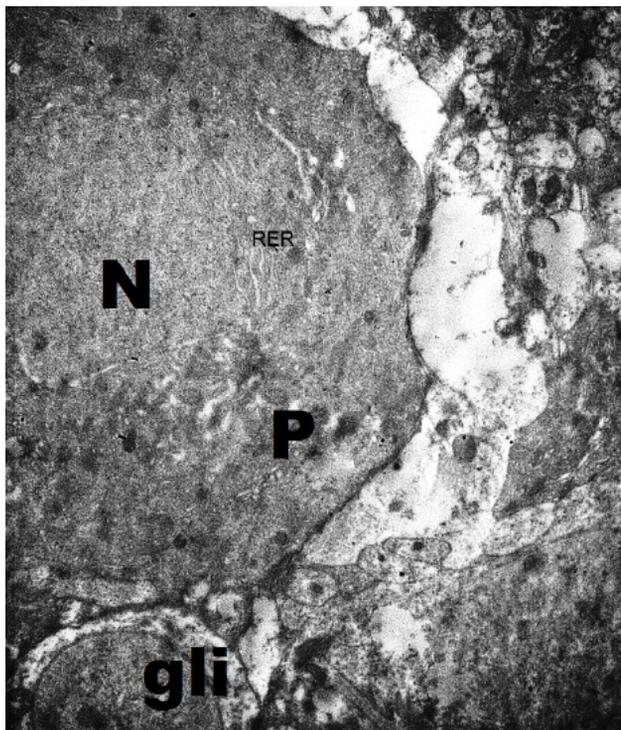


Fig. 22: Electron micrograph of *Lepidium Sativum*-treated adult rat cerebellar cortex (GII) showing Purkinje cell (P) having a large oval vesicular euchromatic nucleus. The nuclear membrane appears regular. A gli cell is observed around the Purkinje cell (gli). (TEM ×2000)

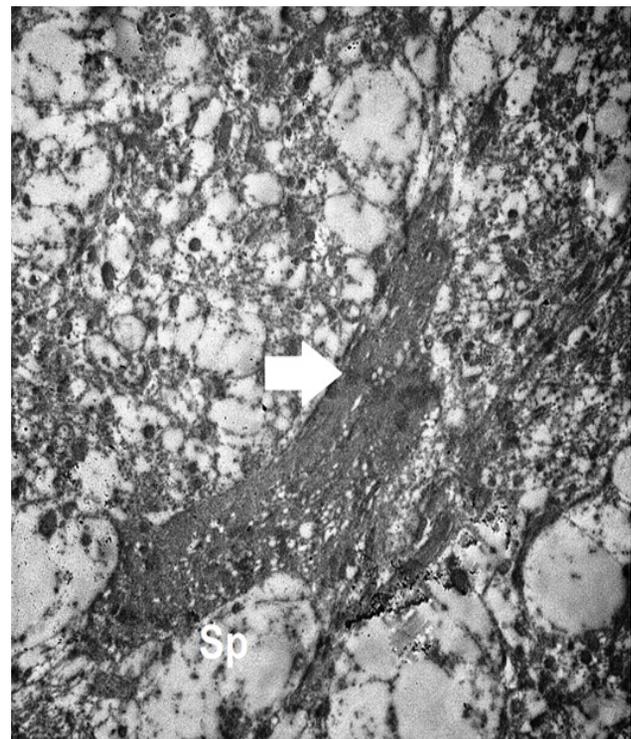


Fig. 24: Electron micrograph of the Purkinje cell layer of the cerebellar cortex of Methotrexate-treated rat (G III) showing degenerated Purkinje cell with numerous cytoplasmic vacuolation (arrow). Wide spaces are also present (Sp). (TEM×1000)

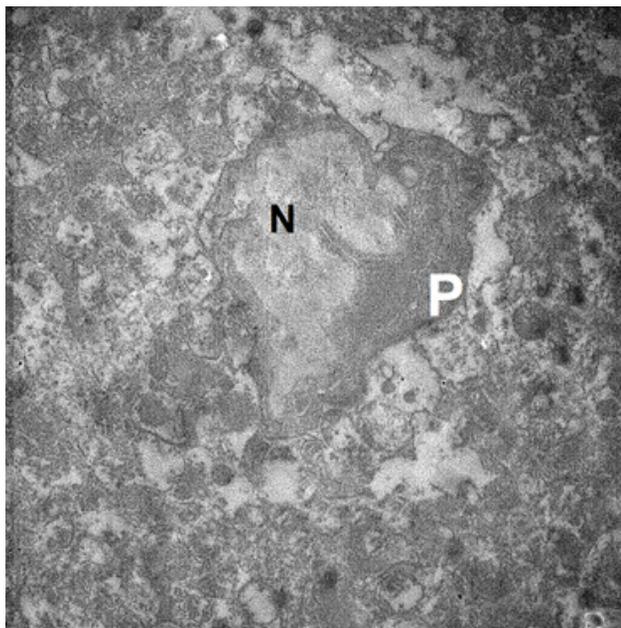


Fig. 23: Electron micrograph of methotrexate-treated adult rat cerebellar cortex (G III) showing shrunken degenerated Purkinje cell (P) with irregular outline. The nucleus appears irregular and pale (ghost shape appearance) (N). Increased electron density of cytoplasm is observed. (TEM ×2000)

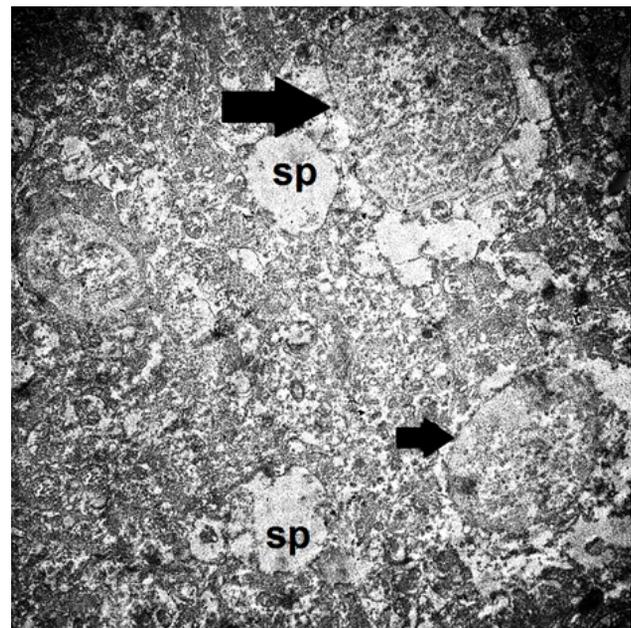


Fig. 25: Electron micrograph of cerebellar cortex of methotrexate-treated rat (GIII) showing two degenerated granule cells (arrow). Wide spaces filled with fluid is also observed (Sp). (TEM ×1000)

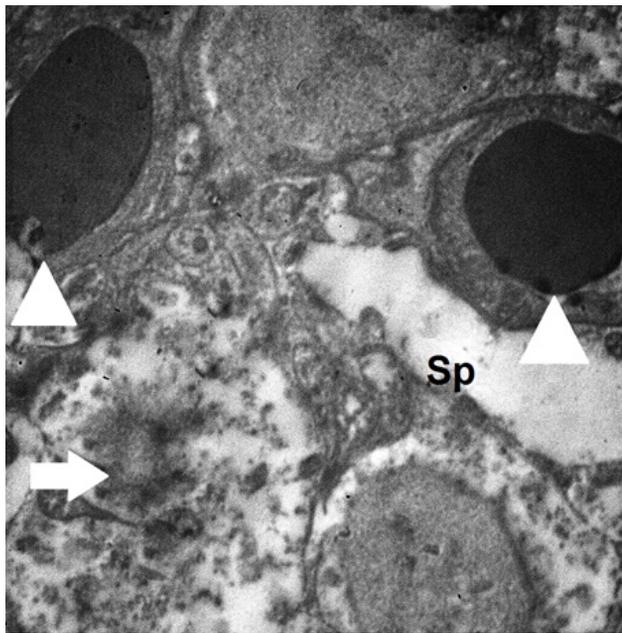


Fig. 26: Electron micrograph of a section of a Methotrexate-treated rat cerebellar cortex (GIII) showing that granule cells become shrunken and degenerated (arrow). Wide spaces are present (SP). Blood capillaries containing RBC are also observed in the granular layer (arrow head) (TEM×2000)

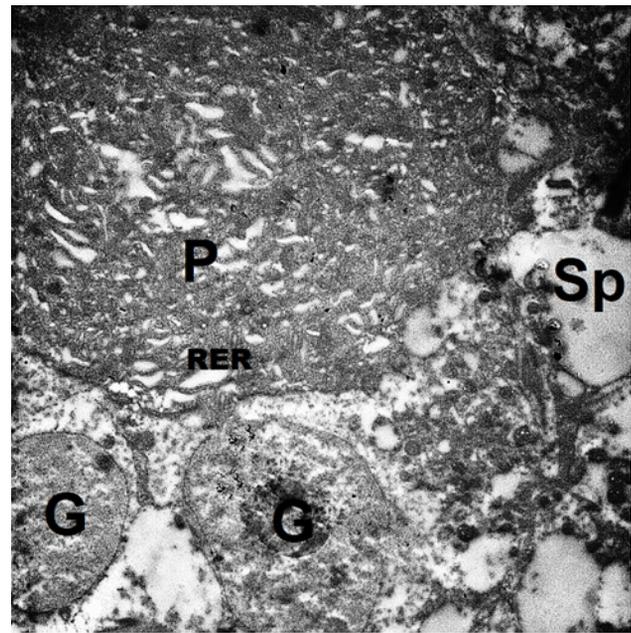


Fig. 28: Electron micrograph of a section of Methotrexate and Lepidium Sativum-treated rat cerebellar cortex (GIV) showing part of Purkinje cell with more or less regular contour (P). Its cytoplasm contains slightly dilated RER. Two Granule cells are seen (G). Wide spaces (Sp) are still seen. (TEM×1000)

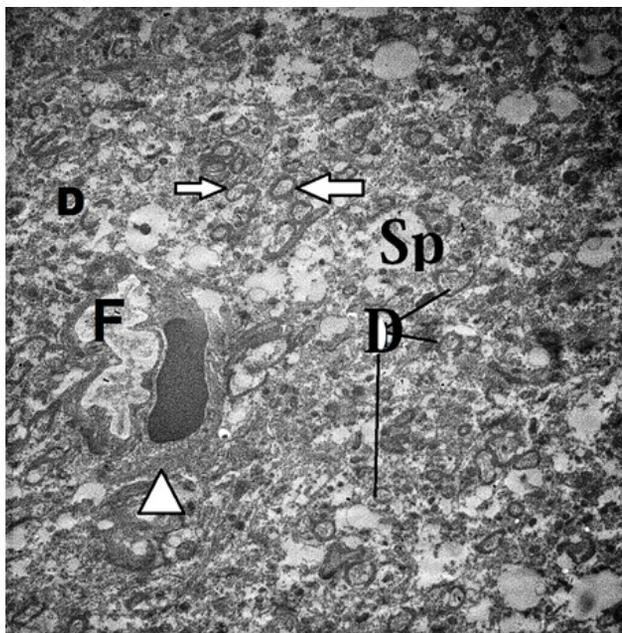


Fig. 27: Electron micrograph of the cerebellar Medulla of Methotrexate treated rat (G III) showing dilated congested blood capillary containing RBCs (arrow head), fluid (F), wide spaces (Sp). Most nerve fibers appear degenerated (D), while few nerve fibers appear normal (arrow). (TEM ×1000)

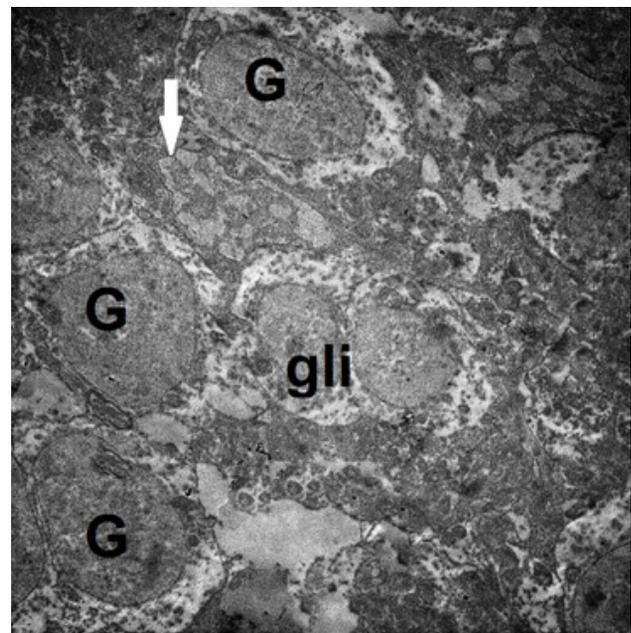


Fig. 29: Electron micrograph of methotrexate and Lepidium Sativum-treated adult rat cerebellar cortex (GIV) showing normal granule cells (G) with oval heterochromatic nuclei and thin rim of cytoplasm. Two normal glial cells are observed among other cells (gli). One degenerated granule cell is also observed (white arrow). (TEM ×1000)

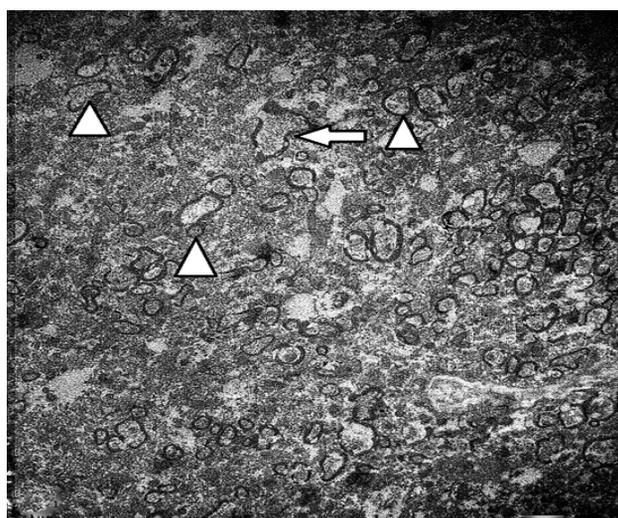


Fig. 30: Electron micrograph of methotrexate and *Lepidium sativum*-treated adult rat cerebellar medulla (GIV) showing most of the fibers appear normal (arrow head) while others appear degenerated (white arrow). (TEM ×1000)

Table 1: Mean number of Purkinje cells of various experimental groups

Group	Mean area ± SD	T-test	P value
Group I (Control)	12± 1.6		
Group II (<i>Lepidiumsativum</i>)	11.8 ± 1.5	0.203	P1:0.842*
Group III (MTX)	7.4 ± 1.7	3.712	P2:0.002***
Group IV (Protected)	10.3± 1.3	1.789	P3:0.094* P4:0.018**

- Group I: Control group (Number of animals is 10)

- Group III: MTX –treated group (Number of animals is 14)

-Non-significant * ($P > 0.05$)

-Highly significant*** ($P > 0.01$) t: Student test.

- Group II: LS – treated group (Number of animals is 10)

- Group IV: MTX and LS -treated group (Number of animals is 20)

-Significant** ($P > 0.05$)

-P1, P2, P3: compared to control. P4:groupIVcompared to group III.

Table 2: Mean area (%) of the of GFAP immunoreactivity among different studied groups

Group	Mean area ± SD	T-test	P value
Group I (Control)	18.4±.5		
Group II (<i>Lepidiumsativum</i>)	18.2 ±1	0.718	P1: 0.484*
Group III (MTX)	32.5± 7.7	3.466	P2: 0.003***
Group IV (Protected)	22.6± 4.2	2.014	P3: 0.062* P4: 0.035**

- Group I: Control group (Number of animals is 10)

- Group III: MTX –treated group (Number of animals is 14)

-Non-significant * ($P > 0.05$)

-Highly significant*** ($P > 0.01$) t: Student test.

- Group II: LS – treated group (Number of animals is 10)

- Group IV: MTX and LS -treated group (Number of animals is 20)

-Significant** ($P > 0.05$)

-P1, P2, P3: compared to control. P4:groupIVcompared to group III.

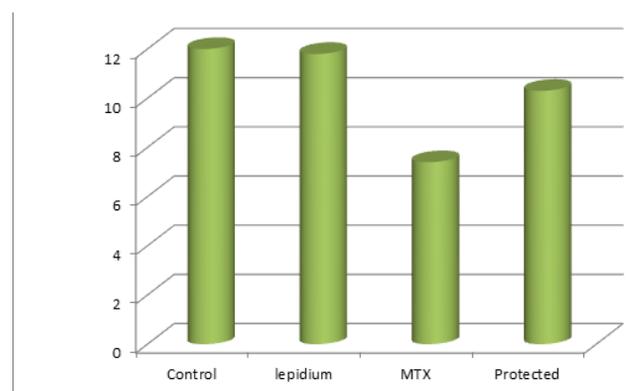


Diagram 1: Mean number of Purkinje cells among different studied groups

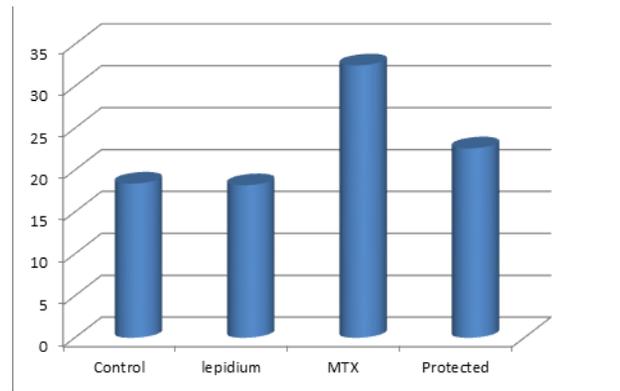


Diagram 2: Mean area (%) of the intensity of GFAP immunoreactivity among different studied groups

DISCUSSION

Methotrexate is a cytotoxic chemotherapeutic agent, widely used for malignancies and different inflammatory diseases such as psoriasis, sarcoidosis and rheumatoid arthritis^[14]. The present study was done to evaluate the effect of methotrexate on cerebellum of adult male albino rats and the possible protective role of *Lepidium Sativum* based on histological and immunohistochemical study.

The histological study of control rat using H& E stain showed the well known normal histological structure of the cerebellar cortex and medulla. The treatment with *Lepidium Sativum* for four weeks revealed histological findings more or less similar to those of control group and this was supported by Balgoon *et al.*^[15] who reported that LS water extract given alone did not induce any change in the normal structure of hippocampal neurons.

The dose of MTX was decided on the basis of previous study on methotrexate induced cerebellar Purkinje cell damage in rats by Vardi *et al.*^[16].

In the present study, the cerebelli obtained from rats treated with methotrexate were apparently much smaller in size than those of the control group I, this is in harmony with the results of Sugiyama *et al.*^[17] in their study on the effect of methotrexate on cerebellar development in infant rats. They reported a decrease in length and width of cerebrum and cerebellum of rats. They attributed these changes to inhibition of cell proliferation activity and apoptosis of the cells.

The histological examination of methotrexate treated group revealed marked destructive changes in Purkinje cells and most of cells of the molecular layer. These results were in accordance with the results of Sugiyama *et al.*^[17] who reported that methotrexate caused pyknotic changes in external granular cells of cerebellum of developing infants. Also, Vardi *et al.*^[16] detected severely Purkinje cell loss and damage in the cerebellum by the effect of methotrexate. These structural changes could be explained by increased oxidative stress as had been reported by Uzar *et al.*^[18] who stated that the administration of MTX increases the level of lipid peroxidation in the cerebellum and decreases the antioxidant enzyme activity. Celik *et al.*^[19] stated that MTX causes oxidative stress and an inflammatory response.

The toluidine blue stained sections of methotrexate-treated group (III) showed marked reduction in the Nissl's granules of Purkinje cells and cytoplasmic vacuolations. This could be explained by fragmentations and dilation of rough endoplasmic reticulum as had been previously reported^[20].

The Electron Microscopic pictures of methotrexate-treated group (III) revealed neuronal structural changes and dilated congested blood capillary containing RBCs and degenerated nerve fibers in medulla. Such findings were in agreement with the results of Gilbert *et al.*^[21] who showed degeneration of axons and myelin sheath. Moreover, El Badawi *et al.*^[22] reported various ultrastructural degenerative changes in their study on the cerebellum of guinea pigs

following low-dose methotrexate. In present study, immunohistochemical stains for GFAP of MTX-treated group revealed increasing staining intensity of GFAP and this was in agreement with the results of Bruce-Gregorios *et al.*^[23] and Moore *et al.*^[24] who attributed this increase of GFAP intensity to the excessive proliferation of astrocyte (gliosis) adjacent to degenerating Purkinje cells. This gliosis that occurred in response to drug injury was the result of activation of astrocytes as a compensatory mechanism after neuronal degeneration^[25]. Immunohistochemical stains for Caspase-3 in Methotrexate-treated rat revealed strong positive immunoreactivity in different layers of cerebellar cortex and this was in agreement with Vardi *et al.*^[16] who reported morphologic signs of apoptosis in great number of cells that was determined by caspase3 staining.

Mild histological changes were detected in group treated with *Lepidium Sativum* and methotrexate with decrease in intensity of staining for GFAP and Caspase-3. These results were in agreement with Balgoon *et al.*^[15] who showed that the number of degenerated hippocampal cells was significantly decreased in LS treated rats of Alzheimer model indicating that L.S reduces the signs of oxidative stress. This was supported by the findings of Abuelgasim *et al.*^[26] who reported the ability of L.S to inhibit lipid peroxidation. The decrease in intensity of GFAP indicates the protective effect of LS and its ability to inhibit lipid peroxidation as reported by Abuelgasim *et al.*^[26]. Also, decreased Caspase-3 immunoreaction in group(IV) indicating neuroprotective effect of L.S as previously detected by Abuelgasim *et al.*^[26] who found that LS inhibits free radical mediated damage to cells. Furthermore, Chenna-Kesava *et al.*^[27] stated that *Lepidium Sativum* seeds extract has protective effect on neuropathy induced by chemotherapy.

Accordingly, it was concluded that methotrexate caused marked histological, ultrastructural and immunohistochemical changes in the cerebellum of adult male albino rat indicating its neurotoxicity, however co-administration of *Lepidium Sativum* attenuates these changes indicating its neuroprotective effect. So, *Lepidium Sativum* should be advised as a protective agent for attenuation of neurodegeneration induced in MTX treatment.

CONFLICT OF INTERESTS

There are no conflicts of interest.

REFERENCES

1. Neradil J, Pavlasova G and Veselska R (2012): New Mechanisms for an Old Drug; DHFR- and non-DHFR-mediated Effects of Methotrexate in Cancer Cells 25; *Klin Onkol.* (2012) (Suppl 2): 2S87– 2S92.
2. Abolmaali SS, Mohammad A and Dinarvand T R: A review of therapeutic challenges and achievements of methotrexate delivery systems for treatment of cancer and rheumatoid arthritis; *Cancer ChemotherPharmacol.* (2013) 71(5):1115-30.

3. Cruz-Carreras MT, Chaftari P, Shamsnia A, Guha-Thakurta N and Gonzalez C: Methotrexate-induced leukoencephalopathy presenting as stroke in the emergency department; *Clinical Case Rep.* (2017)5(10):1644-1648.
4. Al-Asmari AK, Athar T, Al-Shahrani H M, Al-Dakheel S and Al-Ghamdi M: Efficacy of *Lepidium sativum* against carbon tetra chloride induced hepatotoxicity and determination of its bioactive compounds by GC-MS; *Toxicol Rep.* 2(2015):1319-1326.
5. Al-Hamedan W A: Protective Effect of *Lepidium sativum* L. Seeds Powder and Extract on Hypercholesterolemic Rats. *Journal of American Science.* (2010) 6(11):873-879.
6. Mohamed ET and Safwat GM: Evaluation of cardioprotective activity of *Lepidium sativum* seed powder in albino rats treated with 5-fluorouracil. *Beni-Suef University Journal of Basic and Applied Sciences.* (2016) 5(2): 208-215.
7. Al-khazraji SM: Biopharmacological studies of the aqueous extract of *Lepidium sativum* seeds in alloxan - induced diabetes in rats, *Proceeding of the Eleventh Veterinary Scientific Conference.* (2012) 36: 158-163.
8. Ayromlou H, Hajipour B, Hossenian M M, Khodadadi A and Vatankhah AM: Oxidative effect of methotrexate administration in spinal cord of rabbits, *J Pak Med Assoc.* (2011) 61(11):1096-1098
9. Mohamed NA: Chronic effect of aspartame versus stevioside on the cerebellar cortex of the adult albino rat: a histological and immunohistochemical study, *the Egyptian Journal of Histology* (2013), 36:213-232.
10. Buchwalow I and Böcker W (2010) (editor): *Immunohistochemistry: Basics and Methods*; Springer-Verlag Berlin Heidelberg 2;14(3)pp:19-29.
11. Kaushal V, Herzog C, Haun R and Kausha GP: Caspase Protocols in Mice; *Methods Mol Biol.*; (2014)1133: 141–154.
12. Kuo J (2007) (editor): *Electron Microscopy: Methods and Protocols.: Conventional Specimen Preparation Techniques for Transmission Electron Microscopy of Cultured Cells & Processing Biological Tissues for Ultrastructural Study*; Second Edition; ELSEVIER; pp:1-35.
13. Schneider CA, Rasbandws and Eliceiri KW: NIH Image to ImageJ: 25 years of Image Analysis; *Nat Methods.* (2012) 9(7): 671–675.
14. Cetinkaya A., Bulbuloglu E., Kurutas E.B. and Kantarceken B.: N-acetylcysteine ameliorates methotrexate-induced oxidative liver damage in rats, *Med Sci Monit*; (2006) 12(8):BR274-8.
15. Balgoon MJ, Raouf GA, Qusti SY and Ali SS :ATR-IR Study of the Mechanism of Aluminum Chloride Induced Alzheimer's Disease; Curative and Protective Effect of *Lepidium sativum* Water Extract on Hippocampus Rats Brain Tissue, (2015):9(11):786-796.
16. Vardi N, Pinar HP and Ates B: Beneficial effects of chlorogenic acid on methotrexate-induced cerebellar Purkinje cell damage in rats, *Journal of Chemical Neuroanatomy* (2012)43: 43–47.
17. Sugiyama A, Sun J, Ueda K, Furukawa S and Takeuchi T: Effect of methotrexate on cerebellar development in infant rats; *J. Vet. Med. Sci.* (2015) 77(7): 789–797.
18. Uzar E, Koyuncuoglu HR, Efkani U Z, Yilmaz HR, Kutluhan S, Kilbas and Gultekin F: The Activities of Antioxidant Enzymes and the Level of Malondialdehyde in Cerebellum of Rats Subjected to Methotrexate: Protective Effect of Caffeic Acid Phenethyl Ester October, *Molecular and cellular biochemistry*; (2006) 291: 63–68.
19. Celik F, Gocmez C, Bozkurt M, Kaplan I, Kamasak K, Akil E, Dogan E and Guzel AE: Neuroprotective effects of carvacrol and pomegranate against methotrexate induced toxicity in rats, *Eur Rev Med Pharmacol Sci.* (2013)17(22):2988-93.
20. Hernández-Fonseca J P, Rincón J, Pedrañez A, Viera N, Arcaya J L, Carrizo E, and Mosquera J: Structural and ultrastructural analysis of cerebral cortex, cerebellum, and hypothalamus from diabetic rats, *Experimental diabetes research*, 12 pages; 2009 Article ID :329632.
21. Gilbert M. R., Harding B. L. and Stuart A.: Methotrexate Neurotoxicity: In *Vitro* Studies Using Cerebellar Expants from Rats, *Cancer Research* , (1989)1;49: 2502-5.
22. ElBadawi MG, Fatani JA, Bahakim Hand Addalla MA): Light and electron microscopic observations on the cerebellum of guinea pigs following low-dose methotrexate. *Exp. Mol. Pathol.* (1990)53(3): 211–222.
23. Bruce-Gregorios J. H. , Soucy D.M., Chen M.G. and Norenberg: Effect of methotrexate on glial fibrillary acidic protein content of astrocyte in primary culture *J Neuropathol Exp Neurol.* Mar(1991) 50(2):118-25.
24. Moore BE, Somers NP and Smith TW: Methotrexate-Related Nonnecrotizing Multifocal Axonopathy Detected by b-Amyloid Precursor Protein Immunohistochemistry, *Arch Pathol Lab Med.* (2002) Jan; 126(1):79-81

25. Laag EM and Elaziz HOA: Effect of aflatoxin-B1 on rat cerebellar cortex: light and electron microscopic study. *Egyptian Journal of Histology*, (2013): 36(3), 601-610.
26. AbuelgasimAI, Nuha HS and Mohammed AH: Hepatoprotective Effect of *Lepidium sativum* Against Carbon Tetrachloride Induced Damage in Rats, *Research Journal of Animal and Veterinary Sciences*, (2008)3: 20-23
27. Chenna-Kesava RAT, Priyanka B., Maheswari K., Rambabu G. and SagarJ.S. (2016): Protective effect of ultra sonic bath assisted methanol extract of *lepidiumsativum* linn. seeds by using chemotherapy induced neuropathy in rats, *World Journal of Pharmaceutical Research*; 5(12): 205-233.

الملخص العربي

دراسة هستولوجية و هستوكيميائية مناعية عن تأثير عقار الميثوتريكسيت علي مخيخ ذكور الجرذان البيضاء البالغة والدور الوقائي المحتمل لحب الرشاد

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المقدمة: الميثوتريكسيت هو عامل علاج كيميائي سام للخلايا، يستخدم على نطاق واسع ليس فقط للأورام الخبيثة ولكن أيضاً للأمراض الالتهابية المختلفة، ولكن له آثار خطيرة على المخيخ. حب الرشاد هو دواء عشبي يبدو أن له تأثيرات واقية للأعصاب.

الأهداف: أجريت هذه الدراسة لتقييم آثار عقار الميثوتريكسيت على المخيخ عند ذكور الجرذان البيضاء البالغة والتأثير الوقائي المحتمل لحب الرشاد.

مواد وطرق البحث: تم استخدام ستين (٦٠) من ذكور الجرذان البيضاء البالغة، تزن ما يقرب من ١٧٠-٢٠٠ جرام في الدراسة الحالية. المجموعة الأولى (ن = ١٠) كانت المجموعة الضابطة. أعطيت فئران المجموعة الثانية (ن = ١٠) المستخلص المائي لحب الرشاد بجرعة مقدارها ١٥٠ مجم/كجم/يوم بواسطة أنبوب معدي يومياً لمدة أربعة أسابيع. تم إعطاء جرذان المجموعة الثالثة (ن = ٢٠) ميثوتريكسيت بجرعة واحدة مقدارها ٢٠ مجم/كجم في اليوم الرابع عشر من التجربة ثم تركت دون علاج لمدة أسبوعين. أعطيت جرذان المجموعة الرابعة (ن = ٢٠) كلا من الميثوتريكسيت وحب الرشاد مثلما تم في المجموعة الثانية والثالثة. بعد ٤ أسابيع، تم ذبح الحيوانات ثم استئصال المخيخ ومعالجته لإجراء دراسات هستولوجية و هستوكيميائية مناعية وكمية.

النتائج: المجموعة المعطاة المستخلص المائي لحب الرشاد كانت مماثلة تقريباً للمجموعة الضابطة. أظهرت الفئران المعالجة بالميثوتريكسيت انخفاضاً ذو دلالة إحصائية في عدد خلايا بركنجي، وزيادة ذات دلالة إحصائية في متوسط النسبة المئوية لمساحة المنطقة ذات التفاعل المناعي الايجابي للبروتين الليفى الحمضى للخلية الدبقية وتغيرات مدمرة لخلايا بركنجي مع انخفاض ملحوظ في حبيبات نيسل. تم تأكيد ذلك من خلال الفحص المجهرى الإلكتروني ورد الفعل الإيجابي القوي لتفاعلات البروتين الليفى الحمضى للخلية الدبقية و كاسبس-٣. أظهرت الفئران المعالجة بالميثوتريكسات و حب الرشاد مظهرًا هستولوجيا طبيعياً تقريباً لخلايا بركنجي مع سيتوبلازم أقل تفرغاً. تم تأكيد ذلك من خلال زيادة ذات دلالة إحصائية في عدد خلايا بركنجي، انخفاض ذو دلالة إحصائية في الخلايا الإيجابية لكاسبس-٣ و البروتين الليفى الحمضى للخلية الدبقية.

الخلاصة: يعمل المستخلص المائي لحب الرشاد على تحسين التأثيرات السامة للميثوتريكسيت علي الاعصاب. لذلك، يوصى باستخدام حب الرشاد عند الحاجة إلى العلاج بالميثوتريكسيت.