STUDY OF CHRONIC TOXIC EFFECT OF DELTAMETHRIN AND DIMETHOATE ON BRAIN OF ADULT MALE ALBINO RATS

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ABSTRACT

organophosphate pesticides are being increasingly used in combination with Pyrethroids. The effects of single molecules are being studied, but the risk associated with pesticide mixtures is still to estimate. So, the purpose of the current study was to evaluate the individual and combined toxic effects of deltamethrin (DLM) and dimethoate (DM) on brain of adult male albino rats. For this purpose, 60 adult male albino rats were randomized into **5**equal groups: **Group I:** negative control, **Group II:** positive control received 1ml corn oil once daily. **Group III** received deltamethrin (5 mg/kg b.w./orally /daily), **Group IV** received dimethoate (20 mg/kg b.w./ orally /daily), **Group V** received deltamethrin (5 mg/kg b.w) and dimethoate(20 mg/kg b.w)orally /daily . At the end of the study (12 weeks); Body weight was evaluated then blood sample were obtained for estimation of oxidative stress markers malondialdehyde (MDA) glutathione (GSH), total antioxidant capacity TAC) and plasma cholinesterase (PCHE) level then rats were sacrificed. The brains were excised, prepared for histopathological, immunohistochemical examination and DNA damage estimation by comet assay. Results revealed that single DLM and DM exposure significantly had adverse effects on adult male Albino rats in the form of decrease in the body weight, increase in brain &serum MDA, decrease in TAC &GSH and decrease in PCHE level. Pathological changes in brain of rats included, pychnosis, neuronal degeneration, neuropil vacuolization, hemorrhage and congested blood vessels beside positive immuoreaction for bax. Also they caused DNA damage compared to control group. In conclusion, chronic exposure to DLM and DM singly and in combination induced significant oxidative damage in rat brain, associated with marked perturbations in antioxidant defense system in addition to genotoxicity detected by comet assay. Also, the mixture of the two pesticides caused greater damage on the brain of male albino rats, than the individual pesticides.

Key words: Pesticides, Organophosphorus, Pyrethroids, deltamethrin, dimethoate, brain, comet assay

INTRODUCTION

Pesticides are the substances intended to control, repel, mitigate, kill or regulate the growth of undesirable biological organisms. These pests not only compete with humans for food, but also transmit diseases. Now pesticides usage became an indispensible and integral part of world agriculture **(1).**

In recent years, environmental contamination with pesticides represents one of the problems of the region as well as world-wide importance. The presence of these toxic chemicals was recorded in air, water, house dust and in the tissues of nonoccupationally exposed people, especially in the adipose tissue, blood and urine **(2)**.

Pesticide self-poisoning is a major public health problem, according to the World Health Organization, about 3 million cases of pesticide poisonings occur annually killing at least 250–370,000 people **(3)** .

Dimethoate (DM) [O,O-dimethyl Smethylcarbamoylphosphorodithioate] is one of the most important organophosphorus insecticides. Dimethoate is used in agriculture against a wide range of insects and mites as both a systemic and contact pesticides. It is also used for indoor control of houseflies **(4)**. It is classified as a moderately hazardous, Class II insecticide. Like other OPs, the principal mechanism of toxicity of DM is due to its

inhibition and accumulation of acetylcholinesterase (AChE), at the nerve endings and the neuromuscular junctions **(5)**.

Deltamethrin (DLM) is one of the most important insecticides synthetic pyrethroids (Pyr) class II that is widely used in veterinary products, crop production and in public health programs. Therefore exposure originates mainly from the household application of insecticides, contaminated food, and water. It is also considered the most potent neurotoxic pyrethroid **(6) & (7)**.

The marketing of mixtures of organophosphate and pyrethroids insecticides has become very common in developing countries and has resulted in an increase in the prevalence of toxicity **(8)**.

The majority of toxicological studies of chemicals have been concerned with the evaluation of exposures to single compounds. In practice, humans are exposed to complex and variable mixtures of chemicals, which may act independently as in a single exposure, but may also interact to modulate the effects of the mixture as a whole and components **(9)**.

So, the purpose of the current study was to evaluate the individual and combined toxic effects of deltamethrin and dimethoate on brain of adult male albino rats.

MATERIAL AND METHODS
(A) Chemicals:
 1.Deltamethrin: (DLM)
 Deltamethrin: (99% purity, CAS Number: 52918-63-5, Dimethoate: (99.9% purity, CAS 1219794-81-6) purchased from Sigma Egypt. ,Corn oil(used as a vehicle). It was obtained from Sekem, Cairo.

 (B) Experimental animals :
Sixty adult male Wister Albino rats (10-12 weeks) weighing 190–210 g   They were obtained from the breading animals house of the Faculty of Veterinary Medicine, Zagazig University and acclimatized to laboratory conditions for two weeks. The diet of the animals was the basic laboratory diet. The experiment was carried out in compliance with the "Guide of the Care and Use of Laboratory Animals" **(10)** and the institutional guidelines

for the care and use of experimental animals approved by the Medical Research Ethics Committee of Zagazig University, Egypt. Rats were randomly divided into 5 groups, 12 Rats in each group. The rout of administration was oral and the duration of the experiment was 12 weeks.

 (c) Experimental design :
Group I (negative control):- received regular diet and tap water to measure the basic parameters.

Group II (positive control):-Each rat received 1mL corn oil once daily. **Group III (deltamethrin group):-** received a daily dose of 5 mg/kg body weight deltamethrin dissolved in1 ml corn oil (1/20 of deltamethrin LD50) **(11).**

Group IV (dimethoate group): received a daily dose of 20mg/kg body weight dimethoate dissolved in1 ml corn oil (1/20 of dimethoate LD50) **(12).**

Group V (deltamethrin and dimethoate group): received a daily dose of (5mg/kg body weight deltamethrin + 20mg/kg body weight dimethoate). At the end of the study, the rats of all groups were anesthetized with light ether anesthesia and venous blood samples were obtained from them by means of capillary glass tubes from the retro-orbital venous plexus as described by **(13)** for estimation of level of pan antioxidant "Total antioxidant capacity", reduced glutathione (GSH), malondialdehyde (MDA) and plasma cholinesterase enzyme (PChE) level. Rats were sacrificed by decapitation, and the brains were obtained to assess the histopathological changes by light microscope and immunohistochemistry. Brain homogenate were used for measuring malondialdehyde (MDA) levels and determination of the extent of DNA damage by Comet assay. The comet assay was performed in Animal. Reproductive Research Institute (ARRI) of Agricultural Research Centre of Ministry of Agriculture and Land Reclamation in El Doky, Gizza.

METHODS
 (I) Biochemical studies:
1. Estimation of oxidative stress parameters:

Two ml of blood were collected from each rat in clean centrifuge tube and incubated at 37°C until blood clotted and then centrifuged at 3000 r.p.m for 10 minutes. The supernatant sera were pipette off using fine tipped automatic pipettes and stored in deep frozen at -20oC until assayed. Those samples were maintained at(-20 ^{o}C) to be used for estimating of oxidative stress parameters :Total antioxidant capacity (TAC), reduced glutathione (GSH) and malondialdehyde (MDA).

A. Total antioxidant capacity:-
Serum TAC was assayed Colorimetrically according to the method of (**14**). The determination of the antioxidative capacity is performed by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide (H2O2). The antioxidants in the sample eliminate a certain amount of the provided hydrogen peroxide. The residual H2O2 is determined colrometrically by enzymatic reaction which involves the conversion of 3,5,dichloro-2-hydroxyl benzensulphonate to a colored product.

B.Reduced Glutathione:-
Reduced Glutathione (GSH) in serum was assayed according to method proposed by (**15**). The method based on the reduction of 5,5 dithiobis (2- nit robenzoic acid) (DTNB) with Glutathione (GSH) producing a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm by spectrophotomer.

C. Serum & brain Malondialdehyde:-
Serum MDA was assayed colorimetrically according to the method of (**16**). While brain samples were homogenized in 0.1 M cold phosphate buffer saline (pH 7.4) using Teflon pestle. The homogenates were centrifuged at 14,000 $\times$g for 15 min at 4°C. The supernatant was used for measurement of neural LPO expressed as malondialdehyde (MDA) content according to method described by (**17**) Thiobarbituric acid (TBA) reacts with MAD in acidic medium at temperature of 95 C for 30 min forming thiobarbituric acid reactive product the absorbance of the resultant pink product. Individual data (MDA content in the serum) were gained from the absorbance on spectrophotometer at 534 nm.

2. Estimation of plasma cholinesterase enzyme (PChE) by Enzymatic colorimetric assay (18). The samples were added to phosphate buffer (60.7 mmol/l, pH 7.7) containing 5,5 dithiobisnitrobenzoic acid as chromogen and the substrate used to assay PChE is butyrylthiocholine iodide (43.5 mmol/l). Cholinesterase catalyzes the hydrolysis of butyrylthiocholine iodide to thiocholine iodide and butyrate. Thiocholine iodide reacts with 5,5-dithiobisnitrobenzoic acid forming the yellow product 2- nitro-5mercaptobenzoate. The rate of formation of 2-nitro-5-mercaptobenzoate is proportional to the PChE activity and is measured photometrically. Estimation has been carried out by measuring the increase in absorbance at 412 nm

II) Histopathological studies: (A) Light microscope technique:
The brain was fixed in 10% formalin saline. After fixation, brain was embedded in paraffin blocks and processed for the preparation of 5µ thickness sections. These sections were stains with Hematoxylin and Eosin stains and examine by light microscope (**19**).

(B) Immuno-histochemical study:
Immediately after dissection, immunohistochemical reactions were carried out on sections of brain of adult male albino rats using Bax. According to (**20**) & (**21**) Sections were fixed in paraformaldehyde in 0.1 M phosphate buffer for 20 min, and then treated with 0.3% Triton X-100 for 30 min. After 30 min of 0.3% H2O2 treatment for blocking endogenous peroxidase activity, sections were incubated in 2% normal goat serum at 37°C for 1 h, followed by a 48 h incubation at 4oC with primary antibody (anti-Bax) at a dilution of 1:100, respectively. The sections were then incubated with biotinylated link antibody (1:100) and in streptavidin-horseradish peroxidase solution (1:100) at 37°C for 2 h, respectively. After being stained with DAB,

sections were dehydrated, and examined under light microscopy. The specifcity of the immunoreactivity was confrmed by omission of the primary antibody.The site where antigen was present was clearly revealed by brown coloration in the cytoplasm.

(C)Histopathological analysis: Changes in the experimental histopathologic parameters were grded according to **(3)** as following, Perineuronal vacuolation and neuronal cell degeneration (cytoplasmic eosinophilia and pyknotic alteration) were graded as follows: (0) showing no change, (1) change in some areas, (2) change in many areas, and (3) extensive change. For neuropil vacuolization, grading was as follows: (0) showing no change, (1) change in some areas, and (2) extensive change. Congested blood vessels and hemorrhage were calculated from 12 different fields of x40 magnification photomicrographs using Digimizer 4.3.2. image analysis software (MedCalc Software bvba, Belgium).

Image Analysis and Quantitative Morphometric Measurements:

Image analysis and quantitative morphometric measurements were done by a method described by **(22).** Twelve non– overlapping fields for each specimen were selected randomly and analyzed. The optical density (OD) of Bax immunostaining was measured by using the NIH ImageJ (v1.50) program. Optical density (OD) was calculated by the following formula: Optical density (OD) = log (max intensity/mean intensity), where max intensity = 255 for 8–bit images. The degree of immune reaction is indicated by optical density value; the darker it is, the larger the value is **(23).** Image J analysis of prepared histology microscopic slides provides researchers with a rapid, cheap assessment tool when compared with advanced/ultrastructural methodologies. Image J analysis can convert structure into numerical data and easily mastered **(24).**

(III) Comet assay (alkaline singlecell microgel electrophoresis):

Comet assay was performed according to **(25).** Briefly, 100 mg of crushed brain samples was suspended in 1ml ice cold PBS, stirred for 5 min and filtered. 100µl of cell suspension was thoroughly mixed with 600µl of low-melting agarose, followed by spreading of 100µl of the mixture on agarose pre-coated slides. Theslides were left to solidify at 4 °C, and then they were immersed inchilled lysing solution for 1 h at 4 °C. The slides were removed andplaced in a horizontal electrophoresis chamber, filled with freshly prepared electrophoretic alkaline buffer for 20 min. After electrophoresis,the slides were washed gently in 0.4 M Tris–HCl buffer and stainedwith ethidium bromide. The DNA migration patterns of 100 cells foreach sample were observed using fluorescence microscope, and images were captured by a Nikon CCD camera. The qualitative and quantitative extent of DNA damage in the cells was estimated using the Comet 5image analysis software developed by Kinetic Imaging Ltd. (Liverpool, UK).linked to a CCD camera was used to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration [tail length] and the percentage of migrated DNA in the tail [tail DNA %]. Finally, the program calculates tail moment [correlation between tail length and tail DNA %]. Generally, images of 100 (50 X 2) randomly selected cells are analyzed per sample. The mean value (for 100 cells) was calculated.

(IV) STATISTICAL ANALYSIS

Data for all groups were expressed as mean± standard deviation (X±SD). The collected data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 20.0 (SPSS Inc, 2007). Statistically significant difference was determined by one-way analysis of variance (ANOVA), followed by the LSD test for multiple comparisons between different groups. The test results were considered significant when p-value <0.05, p-value <0.01 and <0.001 were considered highly significant.

RESULTS

All the parameters of both control negative & positive (I &II) were within normal and there were no statistically significant differences (p<0.05) between them (Table 1)

I-Biochemicals results:
Oxidative stress parameters:
The mean values of serum TAC (mM/l),GSH (mmol/l) levels in DLM group (III) and DM group (IV) showed a highly significant decreases (P<0.001) as compared with those of the control group. While, rats of DLM+DM group (V) showed highly significant decreases (P<0.001) when compared with other groups (I & II& III&IV) (Table-2).

The mean values of serum MDA (nmol/ml)) and brain MDAnmol/g.tissue) levels in DLM group (III) and DM group (IV) showed a highly significant increases (P<0.001) as compared with those of the control group. While, rats of DLM+DM group (V) showed highly significant increases (P<0.001) when compared with other groups (I & II& III&IV) (Table-2).

Plasma cholinesterase enzyme (PChE):
The mean values of PChE(U/L)level in DLM group (III) and DM group (IV) showed a highly significant decreases (P<0.001) as compared with those of the control group. There was significant decrease in mean values of PChE (p < 0.05) when compared DLM group with DM group.While, rats of DLM+DM group (V) showed highly significant decreases (P<0.001) when compared with other groups (I & II& III&IV) (Table-2).

II-Histopathological results:
1-The light microscopic examinations:
The light microscopic examinations of both control groups negative & positive (I&II) revealed similar results. Thus, we choose negative group I as the control group in the figures .

In group I (control group): The cortex of the cerebrum showed normal granular and pyramidal layer with large and small pyramidal cells with vesicular nuclei and darkly stained cytoplasm and processes and neurofibrillar network. The surrounding neuropil contained nerve fibers, glial cellsand blood vessselsby light microscopical examination with hematoxylin and eosin (H&E) stain (Fig. 1). There was no difference between control groups regarding neuropil vacuolization, neuronal cell degeneration, hemorrhage and the

number of congested blood vessels (Table 3).

Group (III) Deltamethrin treated group:
Histopathological examination of stained section of the brain of the deltamethrin treated rats showed; Glial cells were distorted with deeply stained shrunked nuclei and scanty eosinophilic cytoplasm (pyknosis of nuclei and shrinkage of cytoplasm), neuronal degeneration, neuropil vacuolization and degenerated glial cell with small nuclei were present. Dilated blood vessels were present also (fig. 2). Deltamethrin treated rats showed a highly significant increase (p< 0.001) in neuropil vacuolization, neuronal cell degeneration, and a significant increase(p< 0.05) in hemorrhage and the number of congested blood vessels mean values when compared to corresponding control group values (table 4, fig. 17).

Group (IV) Dimethoate treated group:
Histopathological examination of section from the brain of the dimethoate treated rats showed that some nerve cells in the cortical layer appeared normally while other cells were distorted with deeply stained shrunked nuclei and cytoplasm, dilated blood vessel, hemorrhage, degenerated neurons , neuropil vacuolization also loss of neuronal processes and lossof architectural details were observed (fig.3). Dimethoate treated rats showed a highly significant increases (p< 0.001) in neuropil vacuolization , neuronal cell degeneration, hemorrhage and the number of congested blood vessels mean values when compared to corresponding control group values (table 4).

Group (V) Deltamethrin & Dimethoate treated group:
Histopathological examination of stained section from the cerebral cortex of the rats of (deltamethrin &dimethoate) treated group showed more remarkable pathological changes as more cells were distorted with deeply stained shrunked nuclei and cytoplasm; most nuclei were apoptotic and destructed .Also the cytoarchitectural changes were remarkably more distinct, which include dilated blood vessel, remarkable hemorrhage, degenerated neurons, vacuoliztion also loss of neuronal

processes and loss of architectural details were also more remarkably observed (fig. 4). Deltamethrin & dimethoate treated rats showed a highly significant increases ($p < 0.001$) in neurophil vacuolization and neuronalcell degeneration with significant increases ($p < 0.05$) in the number of congested blood vessels and hemorrhage in mean values when compared to corresponding control group values while (deltamethrin &dimethoate) group showed highly significant increases ($p < 0.001$) in the number of congested blood vessels and hemorrhage mean values when compared with deltamethrin and dimethoate groups (table 4).

2-Immuno-histochemical examination:
According to intensity of positive staining of cells were evaluated. Intensity of the staining was scoredas follows; negative reaction, positive and strong positive reaction.

Group I (negative control), group II) positive control):
The Immuno-histochemical examination of the brain of these groups showed negative immunoractivity for Bax. Figure for the negative control group was representative for other control group (fig. 5). One way ANOVA test showed non-significant differences ($P > 0.05$) in optical density (OD) OF Bax immunoreaction in the brain tissue among the control groups (table 3). So, we used negative control group as a control group to be compared with other treated groups.

Group (III) Deltamethrin group:
The Immuno-histochemical examination of the brain of deltamethrin group showed many nerouns with strong positive immune reaction to Bax antibody in their cytoplasm (fig. 6). One way ANOVA test showed a highly significant increase ($p < 0.001$) in optical density (OD) of Bax immunoreaction in the brain tissue in deltamethrin treated rats when compared to corresponding control group values (table 4).

Group (IV) Dimethoate group:
The Immuno-histochemicalexamination of the brain of dimethoate group showed many neurons with strong positive immune reaction to Bax antibody in their cytoplasm (fig. 7). Dimethoate group showed highly significant increases ($p < 0.001$) in optical density (OD) of Bax immunoreaction in the brain tissue when compared with corresponding control group values (table 4)

Group (V) Deltamethrin & Dimethoate group:
The examination of Bax stained section from the brain of (deltamethrin and dimethoate) group showed strong positive immune reaction to Bax antibody in the cytoplasm of most nerouns (fig. 8). Deltamethrin & dimethoate group showed highly significant increases ($p < 0.001$) in optical density (OD) of Bax immunoreaction in the brain tissue when compared with control, deltamethrin and dimethoate groups (table 4)

III- Single cell gel electrophoresis (Comet assay):
Comet assay showed that the majority of brain cells in both negative and positive control groups, displayed a complete, spherical in shape head, and the comet trail was minimal. (Fig. 9). There were a nonsignificant difference among negative control, positive control groups ($P > 0.05$) as regard mean values of comet tail length, percentage of tail DNA (tail DNA%) and tail moment by ANOVA test as shown in table (5), so we used negative control group as a standard reference for comparison with other treated groups. The mean values of unit tail moment level in DLM group (III) and DM group (IV) showed highly significant increase ($p < 0.001$) when compared to control group values. While, rats of DLM+DM group (V) showed highly significant increases ($P < 0.001$) when compared with other groups (I & II& III&IV) (Table-6).

Table (1): Comparisons between mean values of of weight gain serum, brain Malondialdehyde (MDA), TAC, Guluathione and plasma cholinesterase enzyme (PChE) between the two control groups using independent t test:

	Control(-ve) (N= 12)	Control(+ve) (N= 12)	t	p
Weight gain: (gm) *Mean ± SD*	233.5 ± 22.71	219.83 ± 22.64	1.41	0.17
Malondialdahyle in serum (nmol/ml): *Mean ± SD*	1.36 ± 0.45	1.5 ± 0.5	0.72	0.48
Malondialdahyle in brain (nmol/g.tissue) *Mean ± SD*	2.02 ± 0.67	2.32 ± 0.77	0.83	0.42
TAC (mM/L) : *Mean ± SD*	1.74 ± 0.18	1.79 ± 0.1	0.14	0.45
Glutathione (nmol/ml) *Mean ± SD*	1.19 ± 0.39	0.94 ± 0.31	1.74	0.10
Choline esterase U/L: *Mean ± SD*	8333.3 ± 1207.05	8950 ± 685.57	0.18	0.14

SD: standard deviation. N: number   (Number of rats in each group=12 rats)   p >0.05 =non-Significant difference

Table (3): Comparisons between mean values of different histopathological changes and optical density of Bax immunoreaction in brain tissue between the two control groups using independent t test:

	Control(-ve) (N= 12) *Mean ± SD*	Control(+ve) (N= 12) *Mean ± SD*	t	p
Neuropil vacuolization	0.20±0.45	0.20±0.55	0.04	0.18
Neuronal cell degeneration (pyknosis)	0.010±0.44	0.012±0.17	0.87	0.66
Congested blood vessels	13.2 ±7.7	12.4±5.4	0.26	0.38
hemorrhage	15.80±3.27	16.16±1.00	0.14	0.45
Optical density (OD) of brain Bax	0.0024 ± 0.04	0.0082 ±0.06	1.7	0.96

SD: standard deviation. N: number   (Number of rats in each group=12 rats).   p >0.05 =non-Significant difference

Table (2): Statistical comparison mean values of weight gain serum, brain Malondialdehyde (MDA), TAC, Guluathione and plasma cholinesterase enzyme (PChE) in different studied groups (ANOVA test).

	Control (N= 12)	Deltametherin group (N= 12)	Dimethoate group (N= 12)	(deltamethrin & dimethoate group (N= 12)	F	p
Weight gain(gm): *Mean ± SD*	219.83 ± 22.64	183.5 ± 26.81 **a**	164.83 ± 27.77 **a**	133.5 ± 43.54 **a,b,c**	**58.25**	**<0.001**
Malondialdhyde in serum (nmol/ml) *Mean ± SD*	1.5 ± 0.5	5.07 ± 1.68 **a**	4.81 ± 1.68 **a**	10.97 ± 2.15 **a,b,c**	**70.82**	**<0.001**
Malondialdhyde in Brain (nmol/g.tissue) *Mean ± SD*	2.32 ± 0.77	4.66 ± 0.62 **a**	4.45 ± 0.13 **a**	9.86 ± 2.16 **a,b,c**	**84.38**	**<0.001**
TAC:(mM/l) *Mean ± SD*	1.79 ± 0.1	0.21 ± 0.07 **a**	0.18 ± 0.06 **a**	0.06 ± 0.02 **a,b,c**	**1718**	**<0.001**
Glutathione: (mmol/l) *Mean ± SD*	0.94 ± 0.31	0.02 ± 0.007 **a**	0.04 ± 0.01 **a**	0.006 ± 0.001 **a,b,c**	**105.2**	**<0.001**
Choline esterase: (U/L) *Mean ± SD*	8950±685.57	843.92 ± 209.76 **a,c**	679.17±74.42 **a,b**	525.67±53.89 **a,b,c**	**157.1**	**<0.001**

SD: standard deviation.   N: number (Number of rats in each group=12 rats).
p < 0.05 = Significant difference
p < 0.001 =highly Significant difference
a ;Significantly different from the controls (LSD test; p < 0.001)
b; Significantly different from the DLM (LSD test; p < 0.001)
c; Significantly different from the DM (LSD test; p < 0.001)

Table (4): Comparisons between mean values of different histopathologicls changes and optical density of bax immunoreaction in brain tissue in the different studied group using ANOVA (analysis of variance) test

	Control (N= 12) *Mean ± SD*	Deltamethrin group (N= 12) *Mean ± SD*	Dimethoate group (N= 12) *Mean ± SD*	DM & DLM group (N= 12) *Mean ± SD*	F	p
Neuropil vacuolization	0.20±0.45	1.90±0.90 **a,b**	1.60±0.90 **a,b**	2.20±1.09**a**	**70.82**	**<0.001**
Neuronal cell degeneration (pyknosis)	0.010±0.44	4.66 ± 0.62 **a,b**	4.45± 0.13 **a,b**	9.86 ± 2.16**a**	**84.38**	**<0.001**
Congested blood vessels	13.2 ±7.7	28.23 ± 0.11 **a,b**	33.68±0.28**a,b**	51.72 ± 0.09**a**	**105.6**	**<0.05**
Hemorrhage	15.80±3.27	32.87±6.17 **a,b**	31.40±3.05**a,b**	39.00±11.16**a**	**171.8**	**<0.05**
Optical density (OD) of brain Bax	0.0024±0.04	0.324 ± 0.070 **a,b**	0.293±0.029**a,b**	0.404 ±0.036**a**	**363**	**<0.001**

**SD: standard deviation.**

N: number (Number of rats in each group=12 rats).

a ;Significantly different from the controls (LSD test; p < 0.001)

**b; Significantly different from the DM & DLM (LSD test ; p < 0.05)**
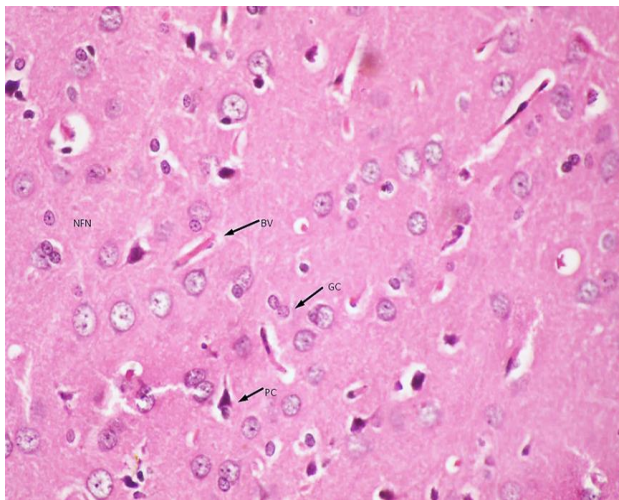
Fig. (1): A photomicrograph of a section in the brain of a (-ve)control rat showing granular cells (Gc) (astrocyte), pyramidal cells (pc) with vesicular nuclei, basophilic cytoplasm and processes and neurofibrillar network are present and normal blood vessels (BV). (H& E X 400 in original magnification).
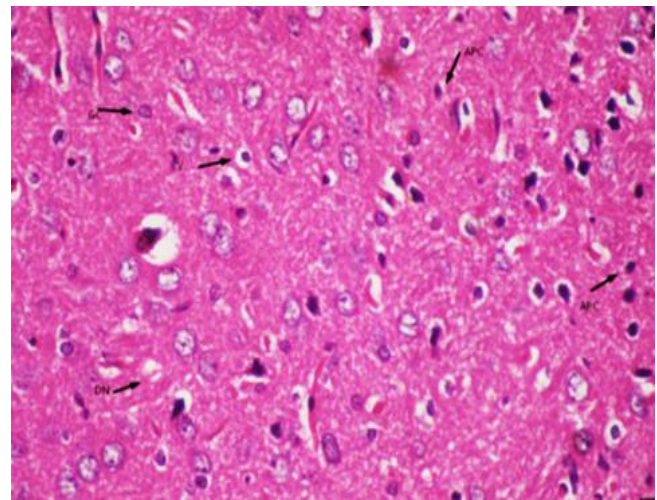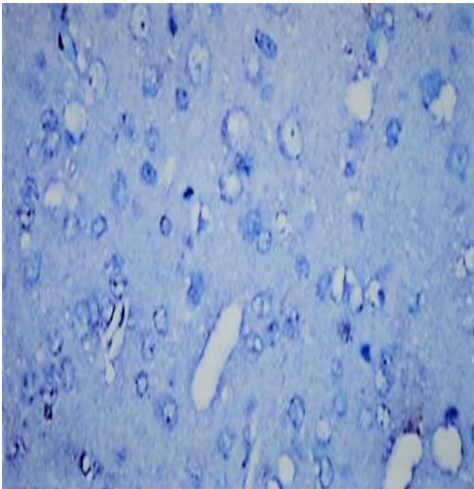

Fig. (2):A photomicrograph of a section from the brain of deltamethrin treated showing; distorted cells with deeply stained shrunken nuclei and cytoplasm (APC), degenerated neurons (DN) and vacuoliztion (V)((H& E X 400 in original magnification).
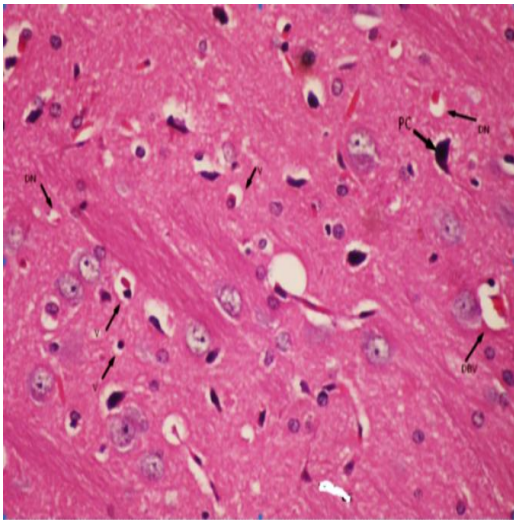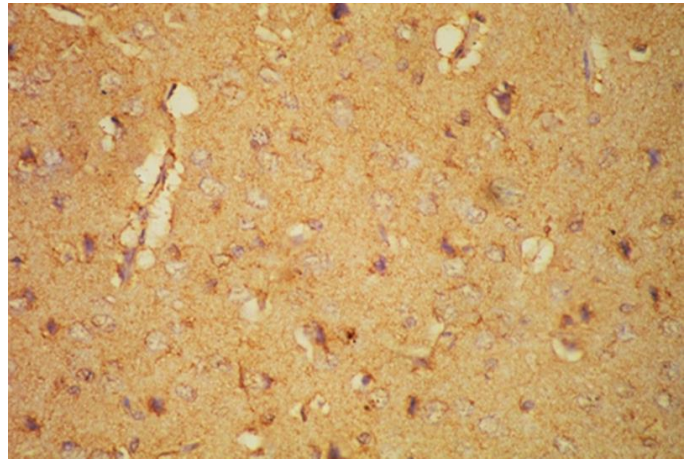
Fig. (3) A photomicrograph of a section from brain of dimethoate treated rat showing; distorted cells with deeply stained shrunken nuclei and cytoplasm, dilated blood vessel (DBV), hemorrhage (Hge), degenerated neurons (DN) and vacuolation (V), loss of architectural details. (H& E X 400 in original magnification).
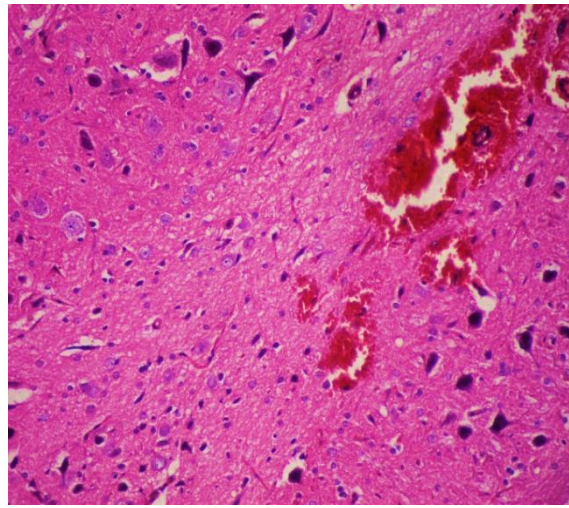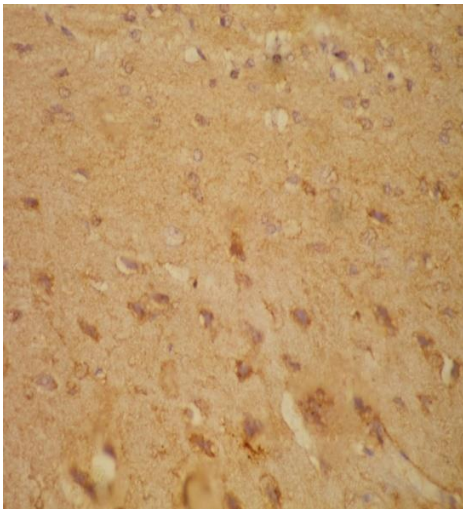

Fig.(4): A photomicrograph of a section from cerebral cortex of (deltamethrin &dimethoate) treated rat showing; distorted cells with deeply stained shrunked nuclei and cytoplasm(APC), dilated blood vessel(DBV), areas of hemorrhage (Hge), degenerated neurons (DN) vacuolation (V), loss of architectural details.(E X (200) in original magnification).
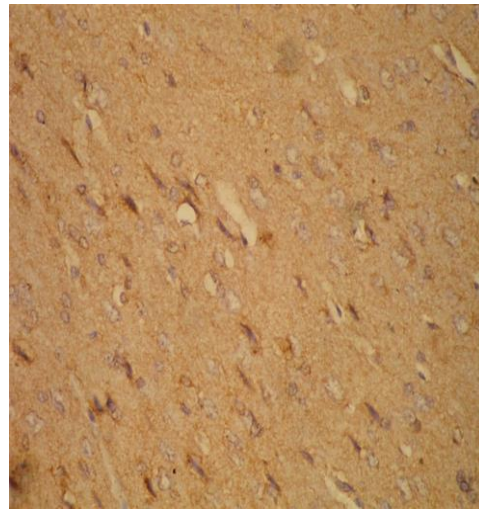

Fig. (5): A photomicrograph of a section from brain of adult male albino rat of Negative control groups (group I) showing negative immune reaction for Bax (immunostain x 400).


Fig. (6): A photomicrograph of a section from brain of adult male albino rat of Deltamethrin treated group (group III) showing: positive immune reaction for Bax (immunostain x 400).

Fig. (7): A photomicrograph of a section from brain of adult male albino rat of Dimethoate treated group (group IV) showing: Positive Bax immunostain in nerve cells (immunostain x 400).
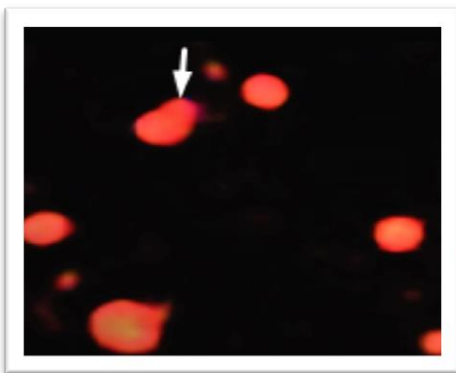

Fig. (8): A photomicrograph of section from brain of adult male albino rat of (deltamethrin & dimethoate) treated group (group V) showing: Astocytes with cytoplasmic strong immunostaining of Bax (immunostain x 400).
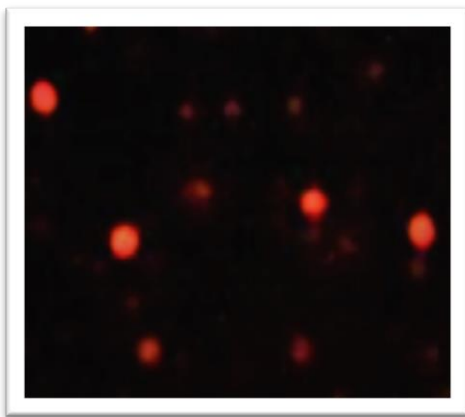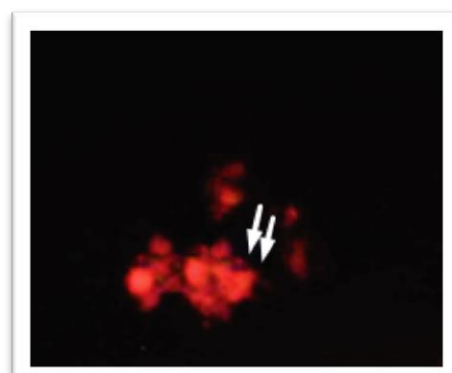

1
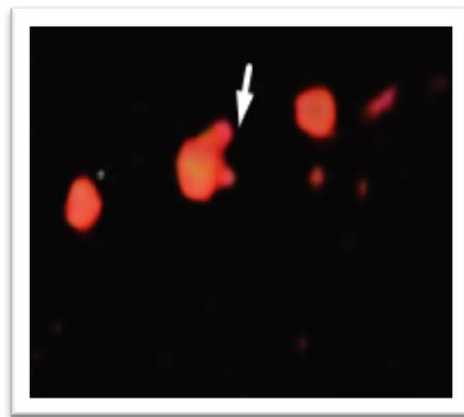
2

3

4

Figure.9: Comet figuresshowing (1) Nuclei of brain cells of male rats of control group showing almost normal condensed type nuclei & undamaged cells.(2) Nuclei of brain cells of male rats of deltamethrine treated group showing damaged cells with abnormal tailed nuclei(short tail)(arrow) .(3) Nuclei of braincells of male rats of dimethoate groupshowing damaged cells with abnormal tailed nuclei(short tail)(arrow).(4)Nuclei of brain cells of male rats of (deltamethrine&dimethoate) group showing damaged cells with abnormal tailed nuclei (long tail) (double arrow)

DISCUSSION

Many pesticides are used together or in away that results in exposure to multiple pesticides over time and that may result in unpredicted adverse health consequences to the exposed population **(Moser et al., (26).** Organophosphates (OPs) and pyrethroids act mainly on the nervous system and are used broadly in agricultural and veterinary applications **(Flaskos, et al., (27).**

Dimethoate (DM) is one of the most imperative organophosphorus insecticides that used in cultivation and also indoor for control of houseflies **(Hassanin and El Asely (4).** Deltamethrin (DLM) is a type II pyrethroids insecticide, one of extensively used pyrethroids. It is considered as the most potent neurotoxic pyrethroids. It is broadly used in veterinary products as well as in agricultural and in public health programs **(Romero et al., (28).** When pesticides are ingested together, toxic effects may be observed, which differ quantitatively and/or qualitatively from those observed following exposure to single pesticides **(Groten et al. (9).** Accordingly, the present study was designed to evaluate the adverse effects of single and combined effect of oral administrations of deltamethrin, dimethoate on the brain of adult male albino rat.

Regarding the biochemical results:

Oxidative stress parameters

. In the present study, there was a highly significant increases brain &serum MDA and a significant decrease in (TAC &GSH), mean values in DLM group (III) and DM group (IV) as compared with those of the control group. While rats of DLM+DM group (V) showed highly significant increases brain &serum MDA and a highly significant decrease in mean values of serum TAC, GSH) levels when compared with other groups (I & II& III&IV).

The results of present work were in line with those of **Manna et al. (29)** who reported that DLM administered to female rats provoked a significant increase of

plasma MDA concentration after 45 days of treatment. Also **Varol et al. (3)** reported that DLM (35 mg/kg, per oral one dose)

intoxication significantly increased brain TOS and decrease the activities of the antioxidant enzymes superoxide dismutase and GSH reductase in rat brains The increase in mean values of serum and brain malondialdehyde in DLM administered rats may be attributed to its lipophilic molecule that can easily pass through the cell lipid bilayer and damage its integrity resulting in increased oxidant species with irreversible oxidation reaction which lead to chemical modification of biological processes **(Sharma et al., (30).** While the reduction of TAC might be due to utilization of antioxidant enzymes to challenge the prevailing oxidative stress under the influence of free radicals generated from DLM and/or inhibition of enzyme synthesis by DLM **(Slaninova et al., (31) & Zhang et al., (32).**

As showed in this study MDA was significantly elevated and serum TAC, GSH levels were significantly decreased in dimethoate treated rats; This finding is in agreement with that obtained by **Ben Amara et al. (33)** who reported that female wistar rats showed a significant elevation of MAD in their erythrocytes after randomly receiving dimethoate (2 g/ l) in their drinking water) for 30 days. Also Studies by **Prakasam et al. (1)** and **Singh et al. (34)** revealed a significant rise in plasma MDA levels and a decrease in the biological activities of some antioxidant enzymes, such as CAT and GST in dimethoate exposed group. The oxidative stress following exposure to OP has been reported by **(Budin et al., (35); Novais et al., (36) &Jallouli et al., (37).** The basis of oxidative stress caused by OPs may be due to either their „redoxcycling" activity, where they generate superoxide anion and hence hydrogen peroxide, or via changes in normal antioxidant homeostasis **(Jokanovic, (38).**

In this study, induction of lipid peroxidation in co exposure group was larger than the sum of the peroxidizing effects produced by individual treatment these findings are supported partially by those found by **Tuzmen et al., (39)** who reported Increase in lipid peroxidation in brain of oral chlorpyrifos and Deltamethrin

adminstrated rats   in different doses of 1.0, 15.0 and 5.0, 35.0 mg/kg/day, respectively for 16 weeks. Also **Elhalwagya and Zaki, (8)** reported enhanced lipid peroxidation and increased MDA level in male albino rats intoxicated with organophosphorus (Diazinon) and pyrethroid (deltamethrin) in commercial formulation.

 As regard plasma choline esterase.
Inhibition of AChE activity on OP exposure is an indicator of OP poisoning. OP compounds generally elicit their effects by inhibition of AChE. Recently, evidence has emerged that the reduction of AChE activity is not due exclusively to OP and carbamates, but that other environmental contaminants, such as pyrethroid insecticides, can induce AChE inhibition (**Badiou and Belzunces, (40).** The present study revealed that DM and DLM caused an inhibition of AChE in the blood of exposed rats. The degree of inhibition was notably higher in rats exposed toco-exposed to DM and DLM than those exposed to either DM or DLM. Similar inhibition of AChE activity in plasma was also reported by **El-Halwagy and Zaki (8).** in rats exposed to the combination of an OP, diazinon and DLT, and they attributed it to the antagonistic effect of the OP and pyrethroid. The decrease in AChE activity observed in the serum of DLM exposed rats agrees with the results of **Kale et al. (41), Yousef et al. (42) and El-Demerdash (43)** who demonstrated that pyrethroids cause a decrease of AChE activity in the erythrocytes, plasma and brain of exposed rats. The decrease in AChE activity following DM and DLM and DLM exposure may also be due to increased LPO, as LPO induced by pesticides other than OPs have been reported to indirectly affect membrane bound enzymes such as AChE leading to a decrease in its activity (**Lo´pez et al., (44).**
 Regarding Histological results: A. Histopathological results of the brain:
The brain is vital part of the organism functioning as coordinating and regulating system for body parts. Any damage due to physical, physiological and chemical stress may have serious impact on the entire organism (**Sharma et al., (45).** The brain of the deltamethrin or dimethoate treated rats showed that cells were distorted with deeply stained shrunken nuclei and scanty eosinophilic cytoplasm (pyknosis of nuclei and shrinkage of cytoplasm), neuronal degeneration, neuropil vacuolization and degenerated glial cells with small nuclei were present. Dilated blood vessels, hemorrhage, loss of neuronal processes and loss of architectural details were detected .
More remarkable pathological changes were found as more cells were distorted with deeply stained shrunken nuclei and cytoplasm; most nuclei were apoptotic and destructed. The cytoarchitectural changes which include dilated blood vessel, remarkable hemorrhage, degenerated neurons, vacuolization, loss of neuronal processes and loss of architectural details were more remarkably observed in DLM & DM group. These changes were significantly different from that of control and from the changes occurred in single pesticide treated rats.
The results of the present study were supported by **Ogaly et al. (46), Sharma et al. (47) and Wu and Liu (48)** who revealed that repeated oral administration of different doses of Deltamethrin showed neuronal degeneration with vacuolization, degenerated glial cells and necrosis. Due to the lipophilic nature of brain tissue, DLM may have accumulated; causing excessive production of ROS and tissue damage (**Oikawa et al., (49)& Abdel-Daim et al., (50).**
 As shown in this study the morphological changes in brain cells observed in the coexposed group was significant when compared to those of single pesticide exposure treatment these results coincide **with Latuszynsks et al., (51)** who reported that the dermal exposure to chlorpyrifos and cypermethrin leads to the several histopathological changes as well as increased density of the cytoplasm and focal pyknosis of the cytoplasm in the cerebral cortex, cerebri and the cerebellum.
 B. Immunohistochemical staining and quantitative morphometric measureements:

Neurotoxins are well known risk factors for chronic neurodegenerative diseases, even though molecular mechanisms involved in the pathogenesis of diseases remain unclear, oxidative stress, excitotoxicity, inflammation and apoptosis have been involved as possible causes on neurodegeneration **(Khalatbary et al., (52).**

Apoptosis or programmed cell death is an active process of normal cell death development distinct from necrosis, and also, occurs as a result of the cytotoxic effect of various neurotoxins **(Sharifipour et al., (53).**Apoptosis is a key molecular mechanism of neurodegenerative diseases that is regulated by the Bcl-2 family proteins **(Cavallucci and D'Amelio, (54).**

Bax is a pro-apoptotic Bcl-2-family protein that resides in the cytosol and translocats to mitochondria upon induction of apoptosis. This process is likely to be a major regulatory checkpoint for apoptosis. Bax blocks the anti-apoptotic effect of Bcl-2 and expected to be a major pharmacological target in numerous diseases **(Cartron et al., (55) & Xu et al., (56).**

 Immunohistochemical staining of brain of deltamethrin, dimethoate treated rats showed many neurons with strong positive immune reaction to Bax antibody in their cytoplasm indicated by increased optical density while the brain of rats in (deltamethrin& dimethoate) group showed strong positive immune reaction to Bax antibody in the cytoplasm of most neurons with highest optical density, these results reflected increased apoptosis in the neuronal cells of cerebral cortex of deltamethrin& dimethoate treated rats . The results of the present study are confirmed by the results obtained by **Wu et al. (20)** who reported that immunohistochemical staining of brain of deltamethrin treated rats show increase of P53 and Bax expression and decrease of Bcl-2 expression in cortex and hippocampus comparing to control rats, resulting in an increased ratio of Bax to Bcl-2, which may contribute to apoptotic cell death in rat brain following deltamethrin treatment.

The result of the present study are confirmed by the study conducted by **Wang et al. (57)** who observed an increase in the expression of proapoptotic protein (Bax) and anti-apoptotic protein (Bcl2)in testes in rats treated with dimethoate. Also **Kashyap et al. (58)** Similar kind of associations has also been reported using cultured cells of neural origin and rat brain slices **(Slotkin and Seidler, (59)**.The activation of cytochrome P450s and their interaction with mitochondrial chain complexes have been suggested in chemical induced apoptosis. The involvement of CYPs in organophosphates-induced apoptosis in neuronal cells has also been indicated **(Kaur et al., (60) & Galluzzi et al., (61).**

The similar report of **Raszewski et al. (62)** regarding the effect of pesticide mixture supported the present study.When cells exposed to a significantly greater than additive interaction was observed in both apoptotic and necrotic populations of cells. The induction of drug metabolizing mixed function oxidase system with lindane may, in part, be responsible for the potentiation of cytotoxicity in the combined exposures **(Olgun et al., (63).**

 Regarding the alkaline single-cell gel electrophoresis (SCGE or comet assay):
The alkaline single-cell gel electrophoresis (SCGE or comet assay) is one of the most sensitive and rapid techniques for quantitating DNA lesions in mammalian cells. It having wide ranging applications in the assessment of different types of DNA damage and repairand has been widely used in the field of genetic toxicology and environmental biomonitoring, dietary intervention studies and cancer **(Singh et al., (25)& Fairbairn et al., (64).**

 The current study showed that oral exposure of pesticides, deltamethrin and dimethoate, alone or in combination caused significantly marked DNA damage in examined rat tissues; as evidenced by significant increase in the mean values of comet tail length, tail DNA% and tail moment among the deltamethrin, dimethoate groups and the combined exposure of these pesticides also caused significantly marked increase in the DNA damage in rat tissues.

These results of the present study are supported

by **Ogaly et al. (46)** who reported that DLM significantly induced DNA damage in rat's brain manifested by the elevated tail moment and extensive bright comet tail compared to control group bright comet tail compared to control group (**Hossain and Richardson, (65)& Ismail and Mohamed, 66). Ibrahim et al. (67)** considered the possible mechanism of DLM genotoxicity is either due to its reaction with DNA or by the generation of ROS which caused DNA damage as the higher level of NO produced through DLM intoxication inhibits cellular respiration and triggers apoptosis causing DNA damage.

These results also consistent with data reported by **Heikal et al. (68)** who stated that DM (38.7 mg /kg b. wt.) daily for 28 days induced brain DNA damage monitored by damage index (DI) and damage frequency % (DF) using the comet assay. DNA damage documented In this study in Dimethoate treated rats could be attributed to enhancement of lipid peroxidation and induction of ROS, which could also cause formation of DNA breaks (**Banerjee et al., (69).**

The DNA damage that recorded in this study in the co-exposure group were in harmony with previous data about the correlation between toxicant-induced oxidative stress and DNA d. They stated that despite differences in their pharmacokinetic profiles, most of pesticides have at least one characteristic in common: they induce oxidative stresses which subsequently results in DNA damage (**Mehta et al., (70); Sarabia et al., (71); Moore et al., (72) &Yaduvanshi et al., (73). Concoulsion**

From the above result it can be concluded that chronic exposure to deltamethrin and dimethoate singly and in combination cause significant oxidative damage in rat brain, which is associated with marked perturbations in antioxidant defense system in addition to genotoxicity detected by commet assay. Also, the combination of the two pesticides caused greater damage on the brain of male albino rats, than the individual pesticides .

Acknowledgment

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