Original Article

Neuroprotective Effect of Nigella Sativa Extract on Cerebral Frontal Cortex and Hippocampus in Experimentally Induced Diabetes Mellitus in Adult Male Albino Rat

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ABSTRACT

Introduction: Diabetes mellitus is a contributor to neurological disorders which affect the central nervous system. It is acknowledged that oxidative stress plays a key mediating work in the etiology of diabetes complications. Strong antioxidant properties of Nigella Sativa (NS) have been linked to eliminating reactive oxygen species to prevent lipid peroxidation, and to increase antioxidant enzymes.

Aim of the Work: To study and assess the neuroprotective effect of nigella sativa (NS) extract on the frontal cortex and hippocampus in experimentally induced diabetes mellitus in adult male albino rat. This was based on histological, immunohistochemical, biochemical and morphometric analyses.

Materials and Methods: Thirty-six male albino rats were divided into five groups: Group I included control animals, Group II (NS group), Group III (Diabetic group), Group IV (Experimentally protected), Group V (Experimentally treated). All animals were sacrificed after 6 weeks. Blood samples were collected for biochemical assay and the frontal cortex and hippocampus sections were prepared for histological and immunohistochemical studies. Morphometric and statistical analysis were carried out.

Results: In contrast to the control group, the diabetic group demonstrated shrunken pyknotic pyramidal cells of the frontal cortex and hippocampus, decrease in Nissl granule density, a significant up regulation for glial fibrillary acidic protein (GFAP) immunoreaction, iNOS immunoreaction, caspase3 immunoreaction and decrease in Ki67 positive cells also significant increase in MDA level but protected and treated groups with NS extract showed amelioration with all parameters in the study.

Conclusion: Antioxidant components of NS extract make NS specialized with neuroprotective effect on frontal cortex and hippocampus.

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Key Words: Caspase-3, diabetes, frontal cortex, hippocampus, nigella sativa extract.

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INTRODUCTION

Hyperglycemia is a defining feature of Diabetes Mellitus (DM), a disorder of metabolism with severe morbidity and mortality[1]. Diabetic encephalopathy is a pathological state characterized by progressive damage to the end organs comprising the central nervous system (CNS)[2]. Damage to multiple brain regions, including the frontal cortex and hippocampus, manifesting as neurodegenerative, structural, and neurochemical changes, may have been induced by a persistently elevated intracellular glucose concentration[3,4]. The hippocampus, a susceptible region to changes in cerebral glucose supply, is implicated in learning and memory. Diabetic rats exhibit deficits in hippocampal neurogenesis, synaptic plasticity, and learning[5]. Additionally, changes to the frontal cortex, which regulates memory, planning, and decision-making, are particularly susceptible to those brought about by diabetes. There is proof that poorly controlled diabetes contributes to a decline in prefrontal cortex cortical thickness[6].

Diabetes induces neuronal damage, which manifests as an elevation in apoptotic cell count and cognitive decline, ultimately culminating in a significant surge in oxidative stress within the brain[7]. The initiation of dysfunctional coupled electron transport systems in neurons is attributed to reactive oxygen species (ROS), which are predominantly formed within damaged mitochondria, during the pathogenesis of brain damage associated with diabetes. As a result, oxidative stress is known as a principal element in the pathogenesis of diabetic problems, due to aberrant antioxidant defense mechanisms and heightened free radical production[8]. Moreover, pro-inflammatory cytokines have higher plasma concentrations in diabetic individuals, which may exacerbate inflammation[9].

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Glucose metabolism dysfunctions within the nervous system affect not only the function of neurons but also normal astrocyte activity. Astrocytes perform numerous crucial functions in the CNS, including glucose uptake. An intermediate filament cytoskeletal protein called glial fibrillary acidic protein (GFAP) is thought to be the main marker of astroglial activation following CNS damage. An animal diabetic model's hippocampal (GFAP) staining has been shown to be noticeably elevated[10].

Nigella Sativa (NS) is classified as a member of the Ranunculaceae family. Its seed contains over a hundred unique chemical constituents and is an abundant source of all the essential fatty acids[11].

NS has been shown to possess antidiabetic and glucose-lowering properties in numerous animal models of diabetes[12]. The remarkable antioxidant qualities of NS have been linked to its protective effects. These qualities originated from its capacity to eliminate a range of reactive oxygen species, such as hydroxyl free radicals and anions of superoxide radicals[13], activate antioxidant enzymes, and prevent lipid peroxidation[14]. Given these circumstances, the current study assessed the neuroprotective effects of NS extract using a rat model of diabetes. The objective of the quantitative immunohistochemical analyses was to identify the protective mechanism or mechanisms NS extract may employ.

MATERIALS AND METHODS

Chemicals

Streptozotocin (STZ) Was purchased from Sigma-Aldrich (St Louis, MO, USA) in powder form and subsequently dissolved in citrate buffer.

Nigella Sativa extract: NS seeds were procured from a nearby market in the governorate of Menoufia's Shebin El-koum. Following shade-drying at 35–40°C, the seeds were ground into a coarse powder. In order to generate the hydroalcoholic extract, 200 grams of the seed powder was soaked into 800 mL of 70% ethanol. Following three days of shaking the solution every twenty-four hours, it was filtered through filter paper. The alcohol content of the extract solution was extracted via rotary evaporation under vacuum conditions until a liquid gel was produced. 200 mg/kg of the extract was dissolved in distilled water for the purpose of preparing the dosage[15].

Animals

Menoufia University's Faculty of Medicine animal house accommodated 36 adult male albino rats, with weights varying between 200 and 250g. The food and water supplies of these rats were not limited, and every day, they experienced a 12-hour cycle of light and dark. Approval for the procedure was obtained from the animal experiment ethics committee of the Faculty of Medicine, Menoufia University, Egypt, 92021 ANAT25.

Experimental design

Induction of diabetes mellitus: By a single injection of STZ intraperitoneally at a dosage of 50 mg/kg, a DM was induced. STZ was dissolved in a freshly prepared 0.1 M citrate buffer with a pH of 4.5[16]. Blood glucose levels were assessed 72 hours after STZ induction in conscious rats via tail plexus blood collection. Blood glucose concentrations were determined through the utilization of a glucometer. Diabetic classification was employed for rats exhibiting blood glucose levels surpassing 250 mg/dL.

The experimental groups: (All were observed for the entire six-week which was the duration of the experiment.) The rats were split up into five groups:

Group I (Control group): In this group, twelve animals in total were used. They were divided into two more subgroups:

- Subgroup Ia: (Plain control): Included six animals that were maintained without any treatment throughout the duration of the experiment.
- Subgroup Ib: (Sham control): Included six animals that were administered a solitary dose via intraperitoneal injection of the solvent of STZ (0.1M sodium citrate buffer pH 4.5).

Group II (NS group): At the commencement of the study, a total of six animals were administered NS extract (200 mg/kg) dissolved in distilled water orally via gavage tube daily for six weeks[17].

Group III (Diabetic group): Six animals were administered a single intraperitoneal (I/P) injection of STZ at a dose of 50 mg/kg each until DM was diagnosed; the animals were then monitored throughout the duration of the experiment.

Group IV (Protected group): Included 6 animals each received NS extract 200 mg/kg/day orally by a gavage tube for three weeks before induction and diagnosis of DM and continue receiving the same dose for another 3 weeks.

Group V (Treated group): Included six animals. A single I/P injection of 50 mg/kg of STZ was used to induce diabetes mellitus in each animal until DM was diagnosed; thereafter, they were gavage-tubed daily for six weeks with an oral solution of NS extract containing 200 mg/kg.

Biochemical analysis

Rats underwent anesthesia by the time our experiment was over. A blood sample was produced from the venous plexus located retro-orbitally in the rats then rats were sacrificed. The serum concentrations of glucose and malondialdehyde (MDA) were determined by centrifuging the blood sample that was collected.

Histological study

Animals were sacrificed via rapid dislocation of the cervical region. Following twenty-four hours of fixation in 10% neutral formaldehyde, the brain was dehydrated using

ethanol of increasing grades, cleaned with xylene, and then encased in paraffin. A microtome was employed to section coronal portions of the frontal cortex and hippocampus to a thickness of 5 µm. Following this, the sections underwent histological evaluation using:

1. Haematoxylin and eosin staining: for routine histological examination of the rat frontal cortex and hippocampal CA1 region.
2. Toulidine blue stain.

Immunohistochemical study

Glial fibrillary acidic protein (GFAP): for detection of astrocytes activation.

Results: GFAP sites appeared brown cytoplasm, while the nuclei appeared blue. Astrocytes and their processes appeared like stars[18].

Ki -67: for detection of neurogenesis.

Result: positive reaction was seen as brown nucleus[18].

Caspase-3 Antibody: for detection of apoptosis.

Results: neurons are stained brown in the nuclei and / or cytoplasm[18].

Inducible nitric oxide synthase (iNOS) expressed in the cytoplasm of the pyramidal cells (marker for oxidative stress).

Results: positive reaction was seen as brown cytoplasm[19].

Sections from a minimum of five animals per experimental group were analyzed for quantitative assessment using histology and immunohistochemistry. For morphometric measures, five fields per section were chosen at random utilizing the ImageJ analyzer version 1.43 program from the National Institutes of Health in the USA. The color intensity of the Toluidine blue slices from each of the experimental groups was measured at a magnification of 400 (scale bar 20).

From immunohistochemical sections of several experimental groups (GFAP, ki 67, Caspase-3, iNOS): the number of cells that are positive for GFAP, Ki-67, Caspase-3, and iNOS. Magnification 400 was used for all measurements (scale bar 20).

Statistical analysis

The statistical package for social science was used to tabulate and assess the gathered data.

Forms of statistics which was done:

Descriptive statistics: The outcomes stated as mean (x) ± standard deviation (SD).

Analytic statistics: (one way- ANOVA) test: is a test of significance used for comparison between the different groups. *P value* ≤0.05 was considered statistically significant.

RESULTS

The results of subgroups Ia and Ib did not differ in a statistically significant way. Consequently, both were combined into one group that was referred to as the control. In contrast to the control group, the NS extract group did not show a statistically significant difference ($p > 0.05$). Groups I and II were thus designated as the control group for histological and immunohistochemical analyses.

Biochemical results

Compared to the control group, the rats of the diabetic group revealed a significant increase ($p < 0.001$) in their blood glucose and MDA levels at the end of the experiment. Using NS extract as a protective and therapeutic agent, there was an improvement in these levels and hence these groups demonstrated a statistically significant decline ($p < 0.001$) in these levels in contrast to the diabetic group. There was significant decrease ($P < 0.05$) in blood glucose and MDA levels in protected group in relative to treated group (Figures 1,2).

Impact of NS extract on the diabetic brain's frontal cerebral cortex

A thin layer of pia mater covered the control group's frontal cerebral cortex. It was possible to identify six layers that comprise the frontal cortex (Figure 3A). The pyramidal cells comprising the third layer, also known as the external pyramidal layer, exhibited processes, basophilic cytoplasm, and large rounded vesicular nuclei. In addition, tiny, dense neuroglia cell nuclei were discernible in the eosinophilic neuropil that serves as the cells' background (Figures 4A,4A#). while the structure of the frontal cortex of diabetic group revealed disruption of cortical layers (Figure 3B) distorted pyramidal cells with heavily stained nuclei. There were obvious halos around some of the cells. Some cells were vacuolated. Moreover, dilated blood vessels were observed (Figures 3B,4B,4B#). In the protected group, the structure of the frontal cortex shows nearly normal appearance with maintenance of most of the cells of their normal architecture (Figure 3C). The pyramidal cells appeared normal with apical dendrites and open face vesicular nucleus while few cells were distorted and pyknotic. (Figures 4C,4C#). The structure of the cortex in the treated group exhibited disruption of the cortical layers (Figure 3D). An admixture of darkly stained and normal pyramidal cells was observed (Figures 4D,4D#). The frontal cortex of diabetic group exhibited a reduction in Nissl granule density in contrast to the control group ($P < 0.001$). Conversely, the protected and treated groups demonstrated an increase in Nissl granule density ($P < 0.001$) (Figures 5,6).

The significant rise in the number of GFAP-positive cells was detected in the diabetic group in comparison to the control group ($P < 0.001$). Nevertheless, the rise was significantly reduced in both the treated and protected groups ($P < 0.001$) (Figures 7,8).

In comparison to the control group, the diabetic group showed a substantial drop ($P2 < 0.001$) in the number of Ki-67 positive cells (Figure 9). In contrast to the group with diabetes, sections obtained from the protected group exclusively contained Ki67-positive cells, except for a limited number of cells. Brain sections from the treated group demonstrated that specific cells exhibited Ki67 positivity while others demonstrated the opposite (Figure 10).

Indicator of oxidative stress, iNOS expression, was found to be significantly increased in the diabetic group. The expression of iNOS was considerably declined in the treated and protected group compared to the diabetic group ($P < 0.001$) (Figures 11,12). The up-regulation of iNOS expression was associated with a significant increase in the number of caspase-3 positive cells in the diabetic group ($p< 0.001$), which was dramatically reduced in the protected and treated groups ($p< 0.001$) (Figures 13,14).

Impact of NS extract on the diabetic brain's hippocampus

The hippocampus of the control group was partitioned into four distinct regions denoted CA1 through CA4, with each region consisting of three distinct layers: molecular, pyramidal, and polymorphic (Figure 15). Multiple rows of pyramidal cells arranged in a regular pattern comprised the pyramidal layer. Triangular in shape, the pyramidal cells featured prominent nucleoli, large vesicular nuclei, and basophilic cytoplasm. A dense network of nerve fibers and eosinophilic neuropil matrix were the primary constituents of both the molecular and polymorphic layers. Different neuroglial cells were embedded in the neuropil and included: oligodendroglia with dark small nuclei, few astrocytes with oval vesicular nuclei and microglia with rod shaped nuclei were observed (Figures 16A,17A,17A#).

In the diabetic group, hippocampal tissue integrity was lost. The CA1 region exhibited a major decrease in both the cell count and thickness of the pyramidal layer (Figure 16B). Both molecular and polymorphic layers exhibited numerous vacuolations and dilated blood vessels in their neuropil. Degenerated pyramidal cells exhibited perinuclear halos and shrunken hyperchromatic nuclei. The number of astrocytes increased in both the molecular and polymorphic layers (Figures 17B,17B#). Preservation of hippocampal tissue integrity was observed in brain sections from the protected group. The thickness and number of cells comprising the pyramidal layer in the CA1 region exhibited a discernible increase (Figure 16C). Most pyramidal cells appeared more or less normal with basophilic cytoplasm, large vesicular nuclei and prominent nucleoli but few cells appeared degenerated with hyperchromatic nuclei and perinuclear halos. Other cells were swollen and lost their nuclei (ghost like cells). (Figures 17C,17C#). As shown in (Figure 16D), both the thickness and number of pyramidal layer cells in the CA1 region increased in the treated group. Certain pyramidal cells harbored hyperchromatic nuclei and perinuclear halos, while appeared normal with prominent nucleoli, basophilic cytoplasm, and large vesicular nuclei (Figures 17D,17D#).

Nissl granule density was decreased in the cytoplasm of pyramidal cells in the hippocampus of diabetic group relative to the control group ($P < 0.001$), whereas it was increased in the protected and treated groups ($P < 0.001$) (Figures 18,19).

The number of GFAP-positive cells in the hippocampus was significantly higher in the diabetic group ($P < 0.001$). The protected and treated group exhibited a major decline in this value ($P < 0.001$) (Figures 20,21). In diabetic group, application of Ki67 immunostaining on pyramidal cells exhibited a major decrease in the number of Ki67-positive cells, which may indicate inhibition of neurogenesis ($P < 0.001$). The observed decrease in Ki67 expression was significantly restored in the protected and treated groups, where Ki67 levels increased significantly ($P < 0.001$) (Figures 22,23).

The results imply that the histological alterations seen in the diabetic group's hippocampal CA1 area were mitigated by NS extract. The immunoreactivity of iNOS and caspase-3, which are indicators of oxidative stress and apoptosis, was found to be elevated in the pyramidal layer of the hippocampus of diabetics ($P < 0.001$, respectively). The observed increase in the protected and treated group was found to be significantly diminished ($P < 0.001$) (Figures 24,25,26,27).

group	Blood glucose level *x*+SD	F	P value
Control	77.18±4.35	275.16	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa (NS)	82.8±7.12		
Diabetic	354±32.8		
Protected	134.4±11.57		
Treated	153.43±10.51		

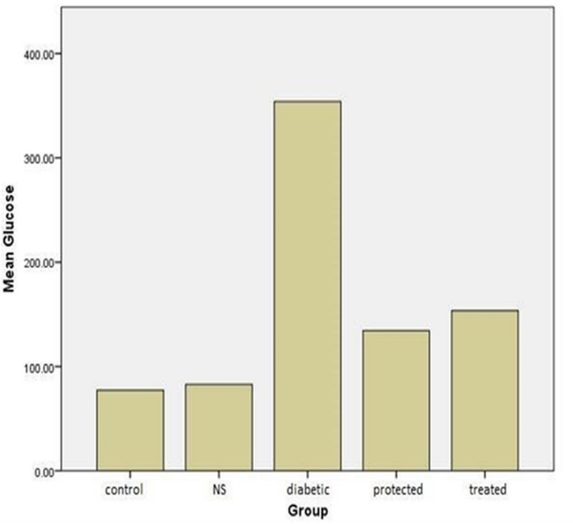


Fig. 1: Blood glucose levels in the experimental groups (mg/dl) and Histogram of blood glucose level in experimental groups.
χ= Mean, SD = Standard deviation. *P value* > 0.05 non-significant, ≤ 0.05 significant Mg/dl=milligram/deciliter.
P1: Comparison between nigella sativa extract group & control group.
P2: Comparison between diabetic group & control group.
P3: Comparison between diabetic group & protected group.
P4: Comparison between diabetic group & treated group.
P5: Comparison between protected group & treated group.

group	MDA level *x*+SD	F	P value
Control	5.85±.65	105.45	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa	5.9±.62		
Diabetic	12.46±.57		
Protected	6.49±.59		
Treated	7.11±.83		

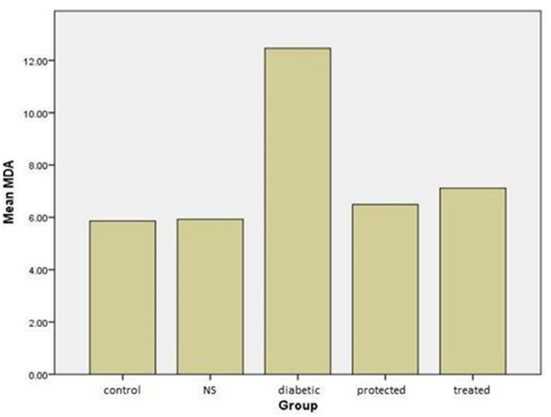


Fig. 2: MDA level in the experimental groups and Histogram of its level in experimental groups.

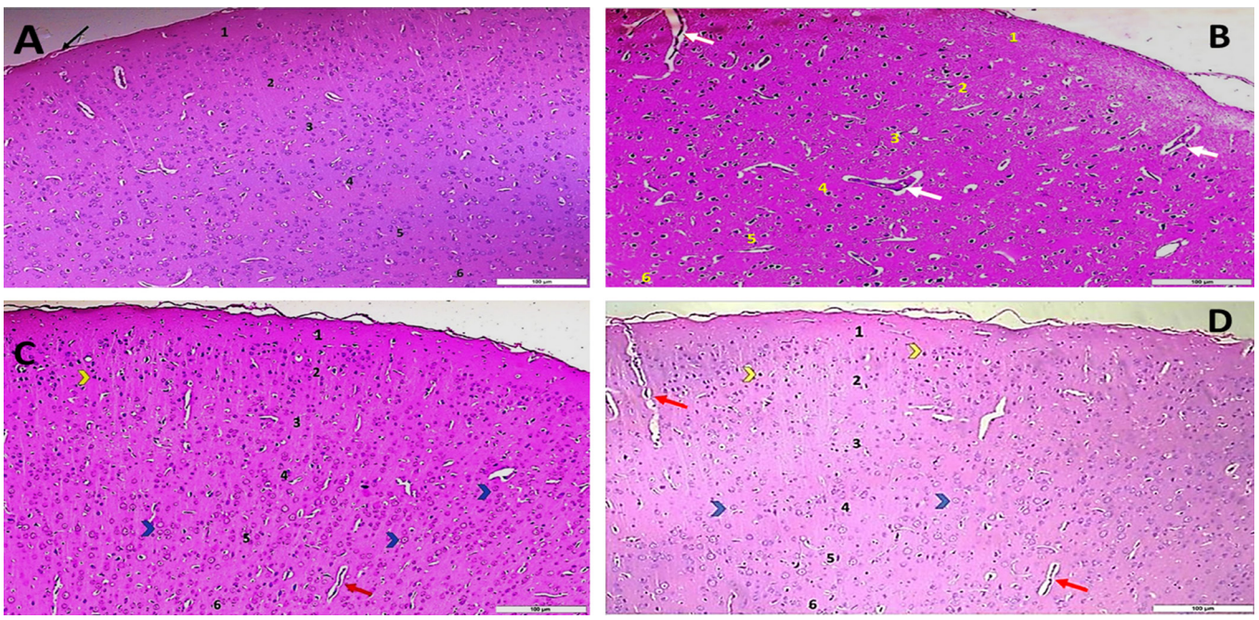


Fig. 3: H&E staining of brain sections of frontal cortex (A) control group showing the normal organization of the cerebral cortex into six layers; the molecular layer (1) the external granular cells (2), the external pyramidal layer (3), the internal granular layer (4), the internal pyramidal layer (5) and the multiform layer (6). Note the pia matter (black arrow). (B) diabetic group showing disruption of cortical layers Dilated blood vessels could be seen (white arrows). (C) protected group showing nearly normal appearance of the cortical layers. most of pyramidal cells appear nearly normal (blue arrowhead) while other appear with dark stained nucleus (yellow arrow). Mild dilation of blood vessel is detected (red arrow). (D) treated group showing disruption of the cortical layers. Some pyramidal cells appeared darkly stained (yellow arrowhead), while others appeared normal (blue arrowhead). Dilated blood vessels could be seen (red arrow). (Scale bar = 100 µm).

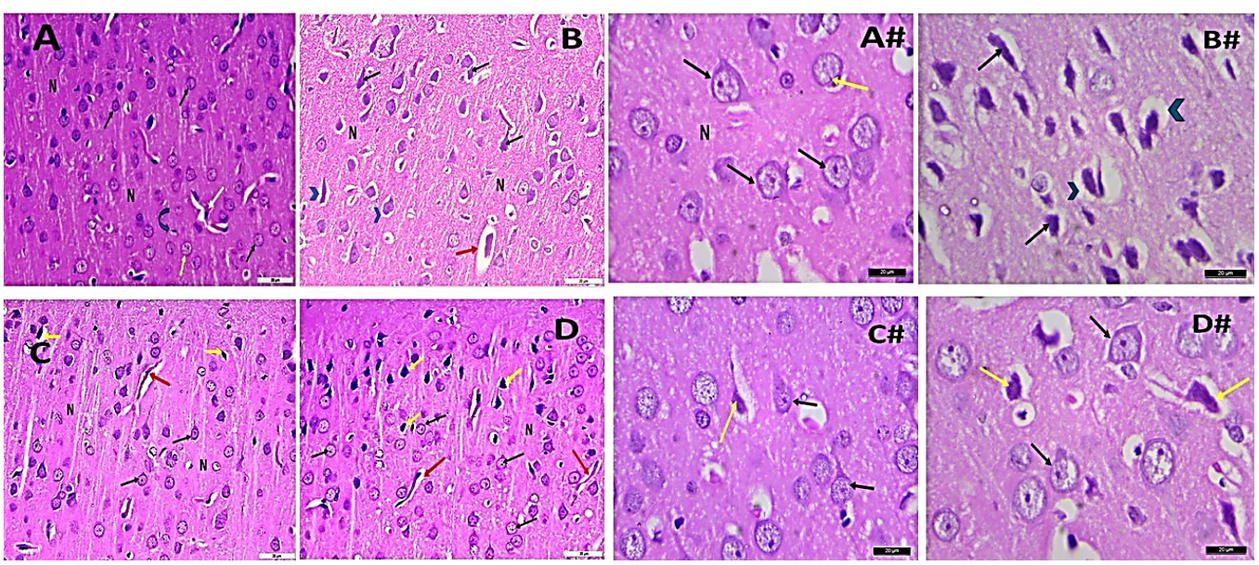


Fig. 4: H&E staining of brain sections of frontal cortex (A) A photomicrograph of higher magnification of a section of layer 3 (the external pyramidal layer) of frontal cortex from a rat of control group in which pyramidal cells appeared with large rounded vesicular nuclei (black arrows), granular cell (yellow arrow) and scattered neuroglia (curved arrow). normal blood vessels are also detected (white arrow) in the eosinophilic neuropil (N). (A#) A higher magnification of the previous photomicrograph showing the pyramidal cells appeared with large rounded vesicular nuclei (black arrows), granular cells with open face vesicular nucleus (yellow arrow) in the eosinophilic neuropil (N) that forms the background for the cells. (B) diabetic group in which pyramidal cells appeared with pyknotic nuclei which appear shrunken and deeply stained (black arrows) and some surrounded by halos and there is increase in the perinuclear space (blue arrow heads). dilated blood vessels are also detected (red arrow) in the neuropil (N). (B#) A higher magnification of the previous photomicrograph showing the pyramidal cells appeared pyknotic, shrunken with deeply stained nuclei (black arrows) and there is increase in the perinuclear space in some cells (blue arrow heads). (C) protected group in which pyramidal cells appeared with normal morphology (black arrows) and few distorted cells with deeply stained nuclei (yellow arrows). Dilated blood vessels are also detected (red arrow) in the neuropil (N). (C#) A higher magnification of the previous photomicrograph showing pyramidal cells appeared with large rounded vesicular nuclei (black arrows) and few cells with deeply stained nuclei (yellow arrow). (D) treated group in which some pyramidal cells appeared with normal morphology (black arrows) and some cells with darkly stained nuclei (yellow arrows). dilated blood vessels are also detected (red arrows) in the neuropil (N). (D#) A photomicrograph of higher magnification of the previous section in which some pyramidal cells appeared with large rounded vesicular nuclei (black arrows) and some cells with darkly stained nuclei (yellow arrows). (Scale bar = 20 µm& 10 µm).

group	Intensity color of toluidine blue$\bar{x}$+SD	F	P value
Control	91.79±1.36	94.95	P1 >0.05
Nigella Sativa	90.79±1.50		P2 <0.001
Diabetic	65.03±2.96		P3<0.001
Protected	84.46 ±1.42		P4 <0.001
Treated	80.81 ±4.7		P 5<0.05

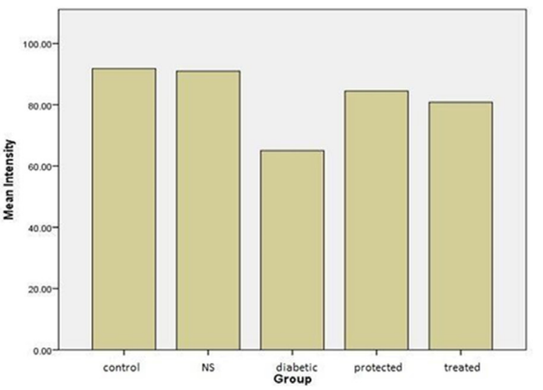


Fig. 5: Color intensity of toluidine blue of frontal cortex different experimental groups.

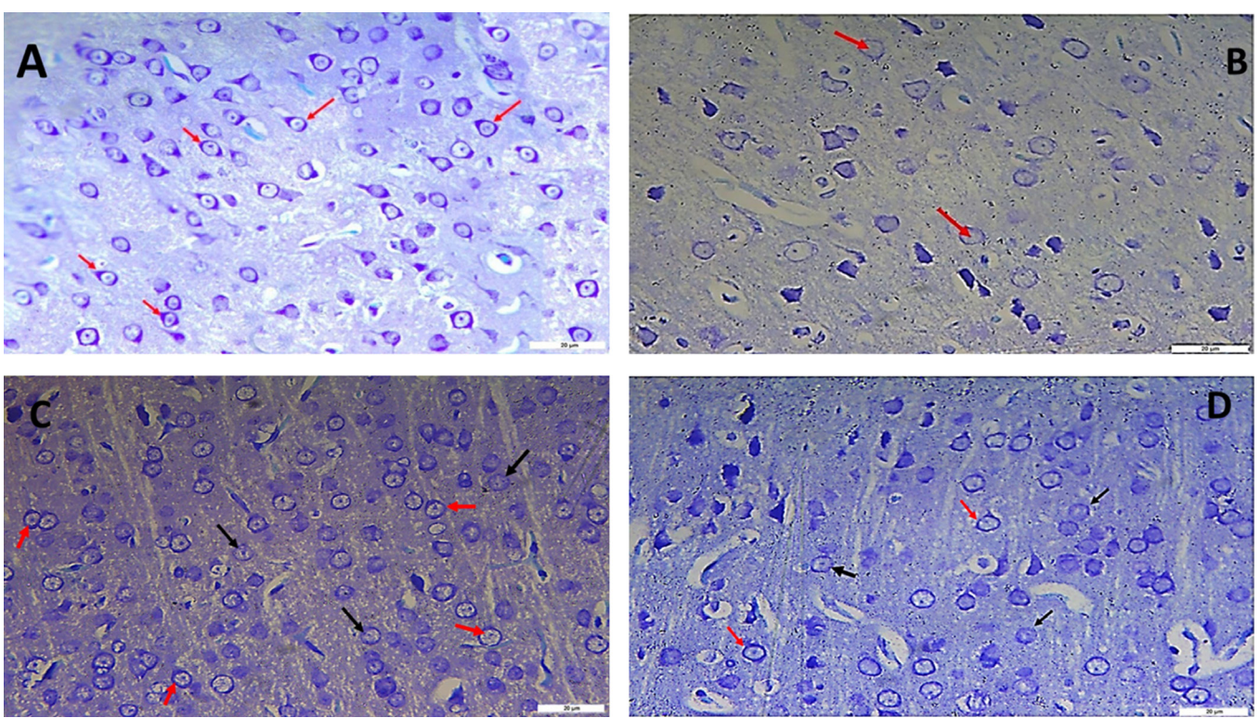
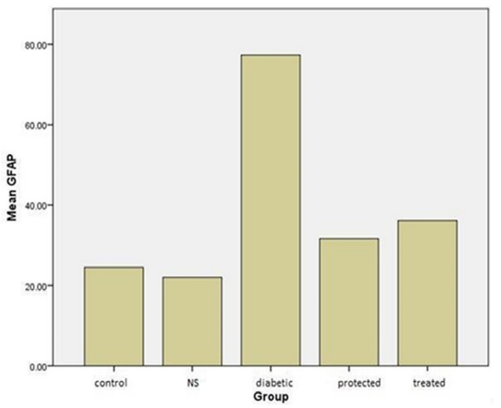


Fig. 6: A photomicrograph of a section of frontal cortex showing neurons of layer 3 (the external pyramidal layer) from a rat of (A) control group with dark blue staining of their cytoplasm and normal intensity of Nissl`s granules (red arrows). (B) diabetic group showing faint blue staining of the cytoplasm of most of the nerve cells with decrease in the intensity of Nissl`s granules compared with control group (red arrows). (C) protected group showing a maintenance of a good amount of Nissl's granules content in most neurons (red arrows) which appear dark blue in color but still few cells showing a decrease in their Nissl's granules content which appeared faint blue in color (black arrows) as compared with the diabetic group. (D) treated group showing a more or less dark blue staining of the cytoplasm of many of the pyramidal cells due to increase of Nissl's granules content of these cells (red arrows) while some cells showed decrease of their Nissl's granules content which appeared faint blue in color (black arrows). (Scale bar = 20 µm).



Group	number of GFAP positive cells.x̄+SD	F	P value
Control	24.5±1.87	241.46	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa	22±2.60		
Diabetic	77.33±5.24		
protected	31.66±2.16		
Treated	37.66±4.36		

Fig. 7: Number of GFAP positive cells in frontal cortex in different experimental groups.

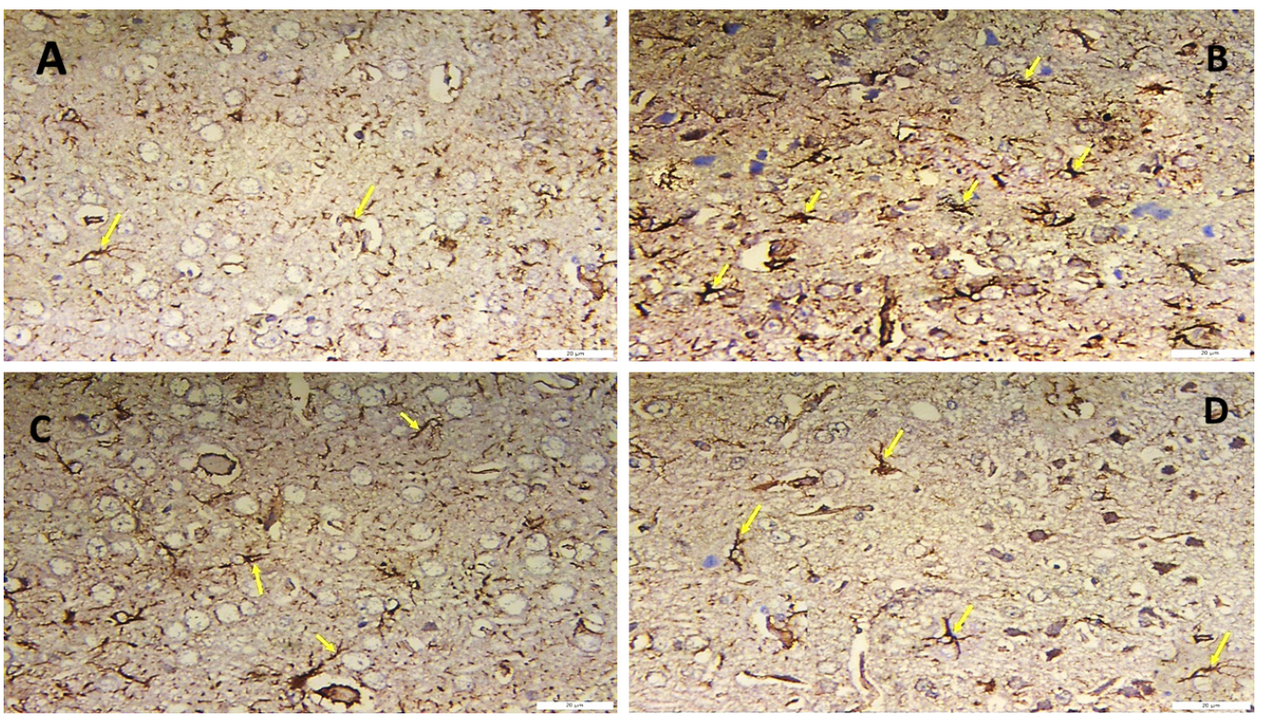
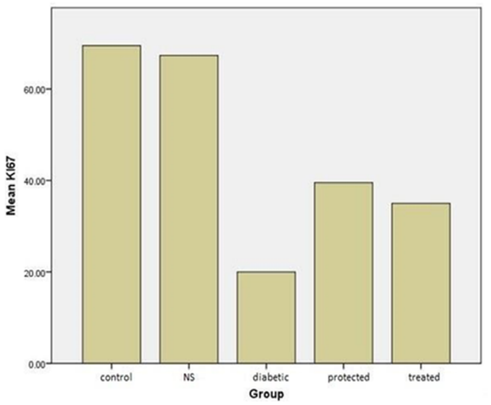


Fig. 8: A photomicrograph of a section of layer 3 (the external pyramidal layer) of frontal cortex from a rat of (A) control group showing a few small astrocytes (yellow arrows) with short processes and faint brown color with GFAP immunostaining. (B) diabetic group showing an apparent increase in the number of astrocytes. Some astrocytes (yellow arrows) are apparently increased in size with visible long processes and dense brown positive immunoreaction. (C) protected group showing an apparent decrease in the number of astrocytes which appeared small with short processes and faint brown GFAP immunoreaction as compared with the diabetic group (yellow arrows). (D) treated group showing decrease in the number of GFAP reactive astrocytes which are small with short branches and faint brown staining as compared with the diabetic group(arrows). (Scale bar = 20 µm).



group	number of Ki-67 positive cells $\bar{x}$+SD	F	P value
Control	69.5 ±4.88	235.22	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa	67.33±3.93		
Diabetic	20.0 ± 2.82		
protected	39.5± 1.87		
Treated	35 ± 2.82		

Fig. 9: Number of Ki-67 positive cells in frontal cortex in different experimental groups.

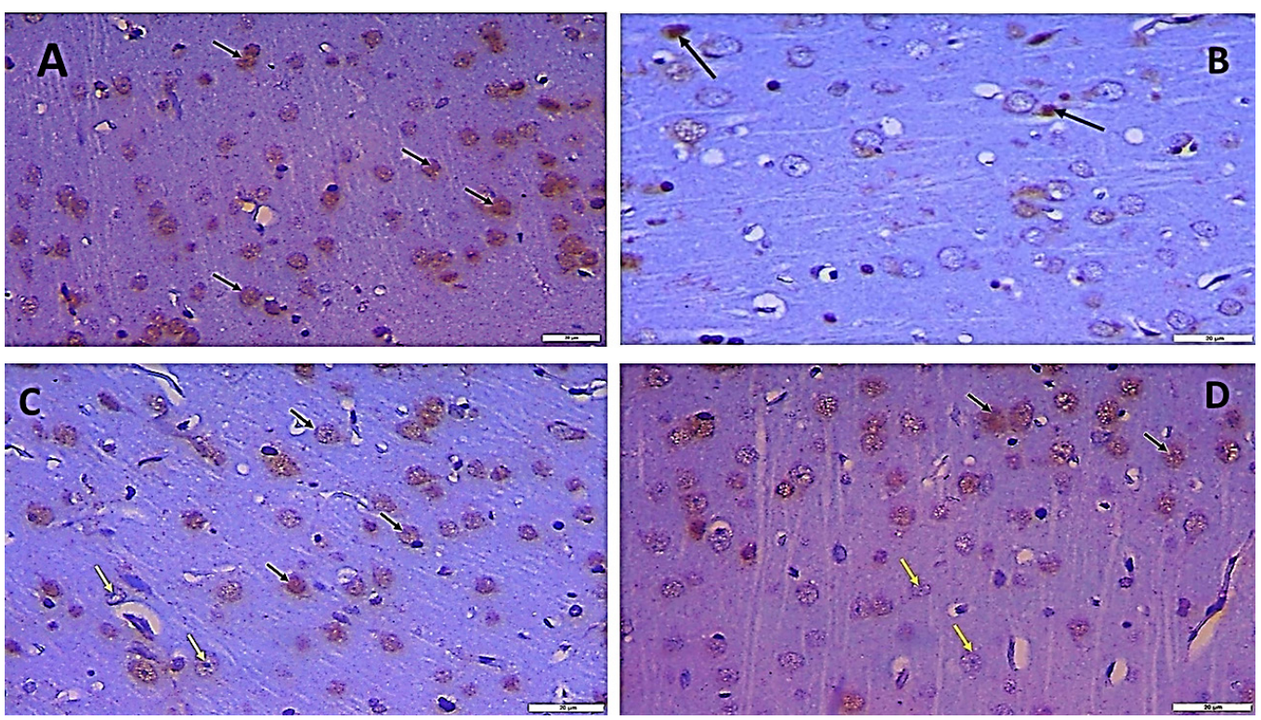
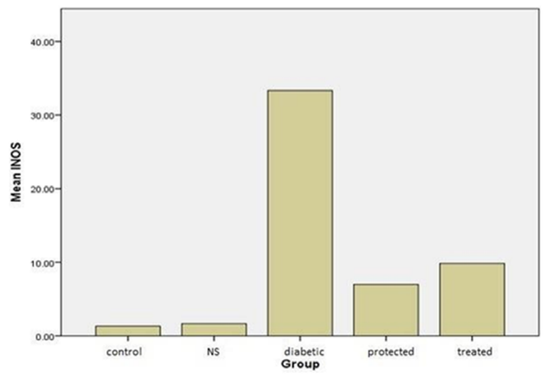


Fig. 10: A photomicrograph of a coronal section in layer 3 (the external pyramidal layer) of frontal cortex of (A) control rat showing Ki-67 positive cells (arrows). (B) diabetic rat showing a marked decrease in number of Ki-67 positive cells (arrows). (C) protected group showing a Ki-67 positive cells (black arrows) except for few cells which appeared negative (yellow arrows). (D) treated group showing some cells are Ki- 67 positive (black arrows) and others are negative (yellow arrows). (Scale bar = 20 µm).



Group	number of iNOS positive cells $\bar{x}$+SD	F	P value
Control	1.33±1.03	322.89	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa	1.66±.81		
Diabetic	33.3±3.07		
protected	7 ±1.67		
Treated	9.8 ±1. 47		

Fig. 11: Number of iNOS positive cells in frontal cortex in different experimental groups.

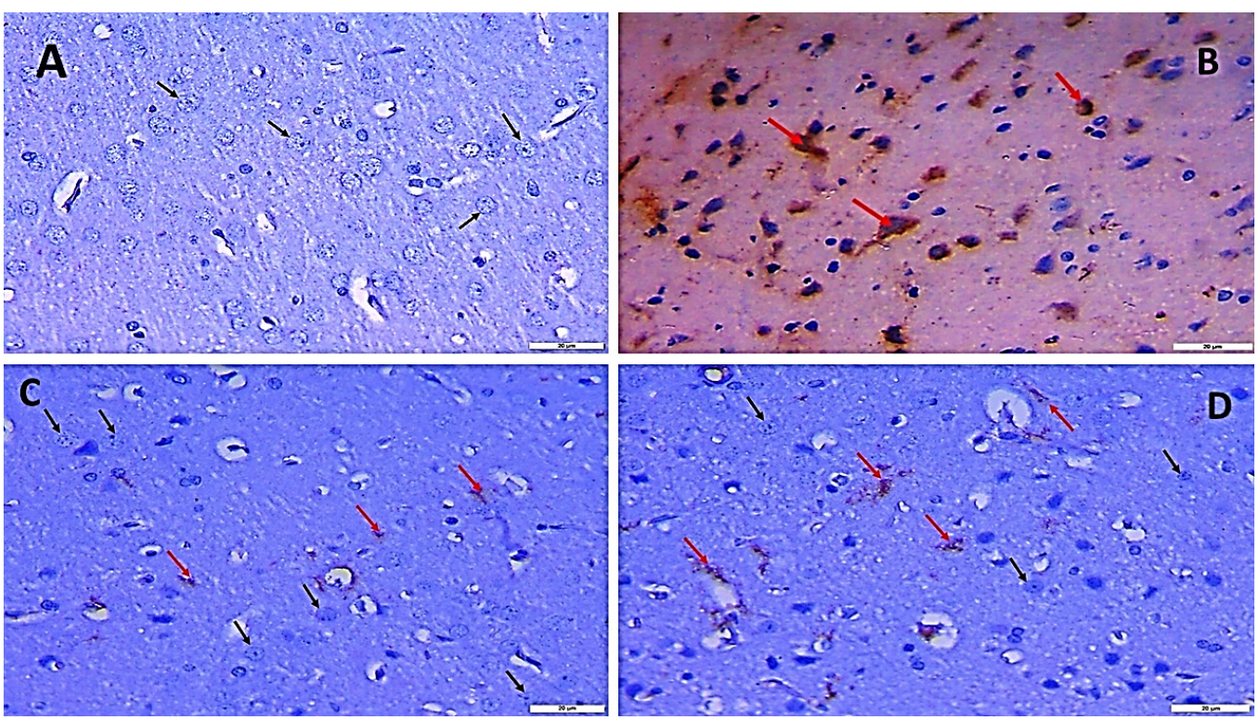
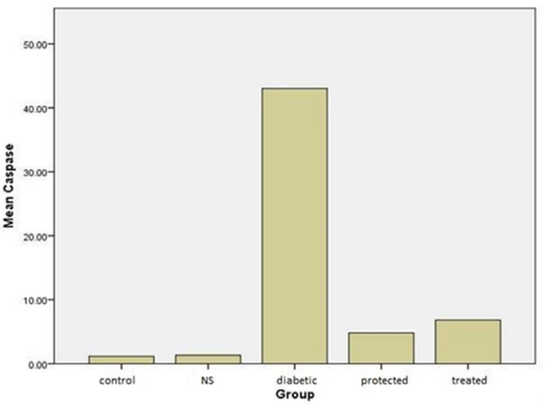


Fig. 12: A photomicrograph of a coronal section in layer 3 (the external pyramidal layer) of the frontal cortex of a rat from (A) control group showing a negative immunoreaction to iNOS (arrows). (B) diabetic rat showing a strong positive immunoreaction to iNOS in the form of brown color of the cytoplasm of the neuronal cells (strong iNOS immunoreactivity) (arrows). (C) protected group showing a negative immunoreaction of many neuronal cells (black arrows) to iNOS while few cells show a weak positive reaction (red arrows). (D) treated group showing showing negative immunoreaction of many neuronal cells (black arrows) to iNOS while some cells show a weak positive reaction (red arrows). (Scale bar = 20 µm).



Group	number of caspase-3 positive cells $\bar{x}$+SD	F	P value
Control	1.16 ±1.1	822.57	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa	1.33± .81		
Diabetic	43 ± 2.6		
protected	4.83± 1.32		
Treated	6.8± .98		

Fig. 13: Number of caspase 3 positive cells in frontal cortex in different experimental groups.

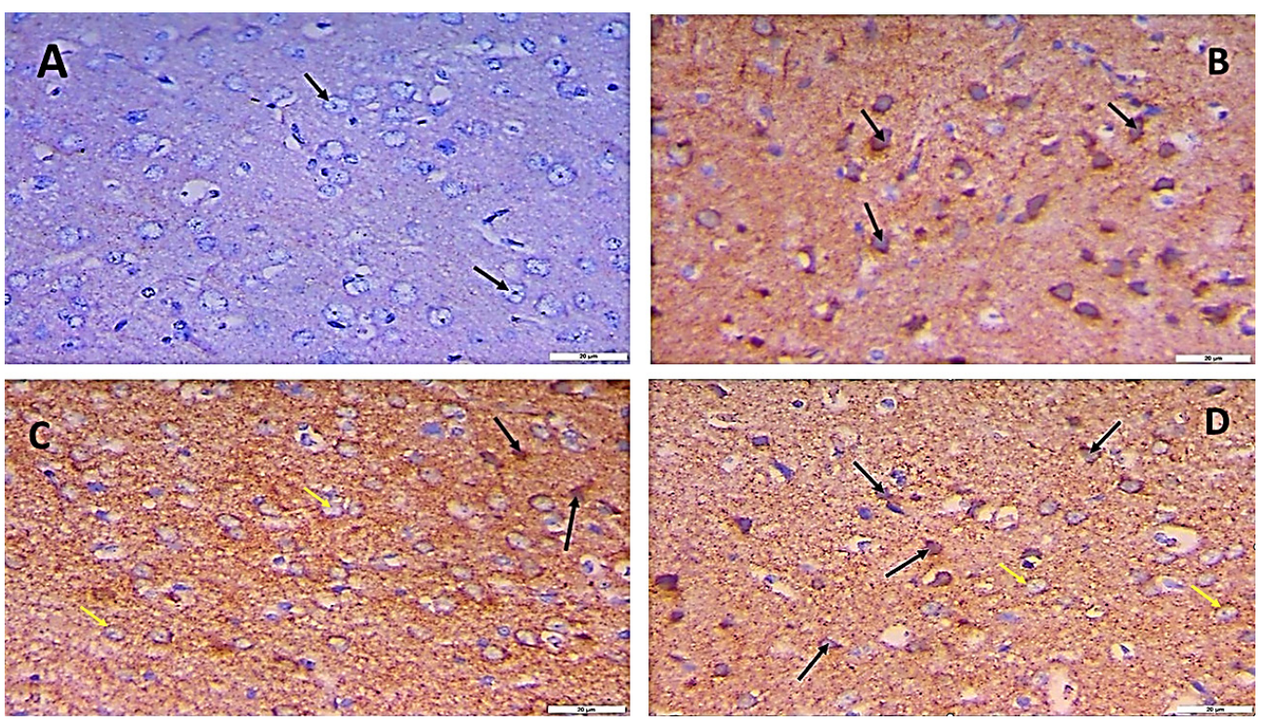


Fig. 14: A photomicrograph of a coronal section in the layer 3 (the external pyramidal layer) of frontal cortex of (A) control rat showing a negative reaction of caspase-3 immunoreaction (arrows). (B) diabetic rat showing many cells with positive reaction of caspase-3 immunoreaction (arrows). (C) protected group showing few number of cells with positive reaction of caspase-3 immunoreaction (black arrows) as compared to diabetic group. Others showed a negative reaction (yellow arrows). (D) treated group showing some cells with positive reaction of caspase-3 immunoreaction (black arrows) while others showed a weak positive reaction (yellow arrows). (Scale bar = 20 µm).

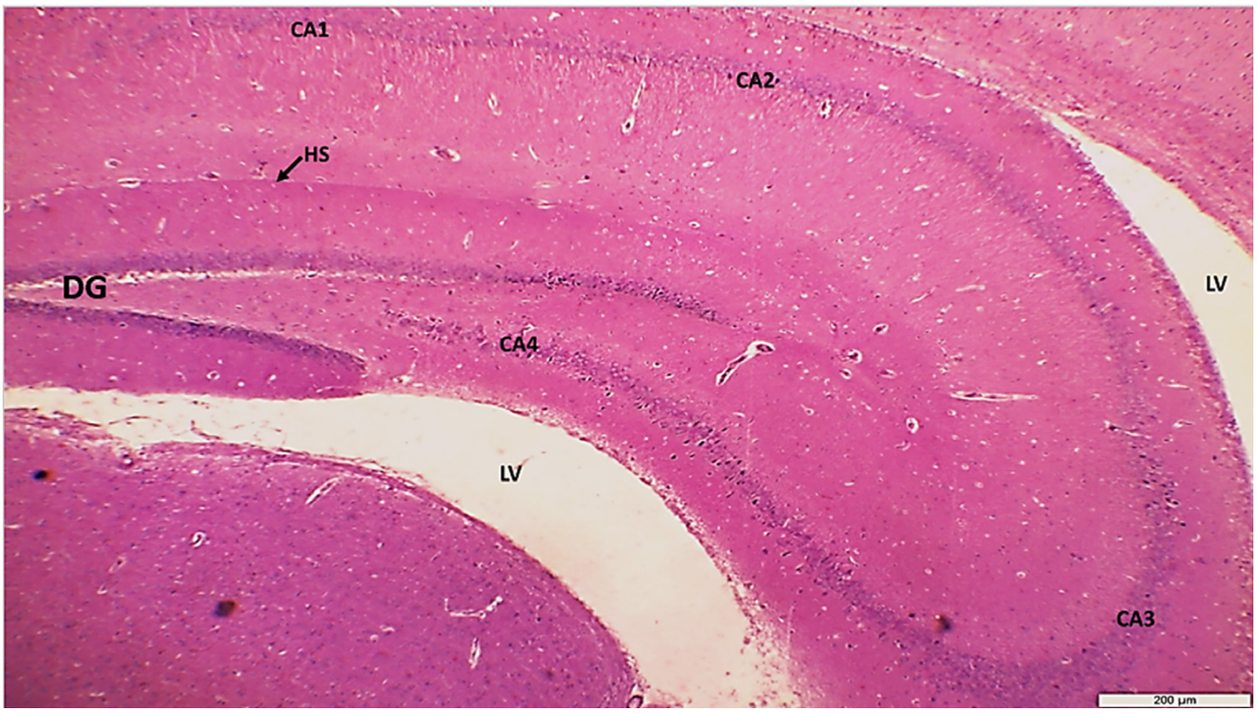


Fig. 15: A photomicrograph of a coronal section in the hippocampus of a control rat showing the major two parts of the hippocampal formation: the hippocampus proper and dentate gyrus (DG) with narrow hippocampal sulcus (HS) in between. The hippocampus proper is composed of four areas (CA1 to CA4). DG is seen surrounding CA-4 by its upper and lower limbs. Note the cavity of the lateral ventricle (LV). (Scale bar = 200 µm).

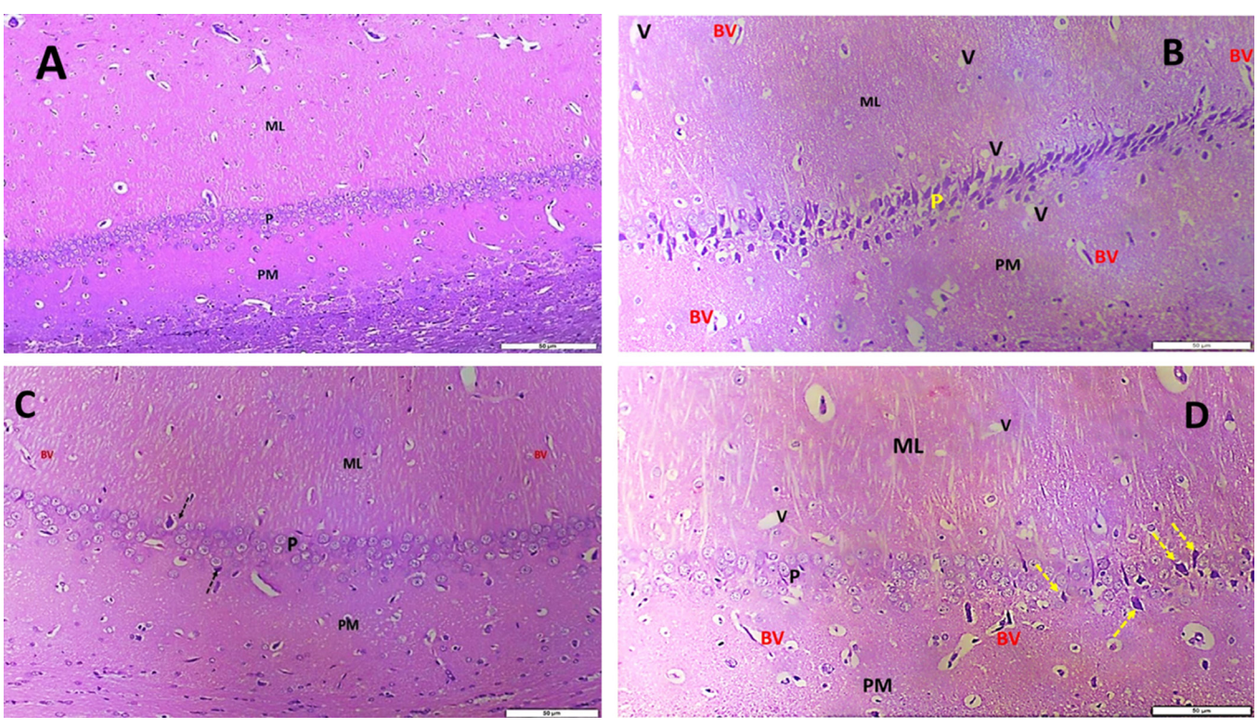


Fig. 16: A photomicrograph of a coronal section in the hippocampus proper at CA1 area of (A) a control rat showing its three layers arranged as outer molecular layer (ML), intermediate pyramidal cell layer (P) and inner polymorphic layer (PM). The pyramidal layer is formed of multiple regular rows of the pyramidal cells. (B) diabetic rat showing disturbed arrangement and decreased thickness of the pyramidal cell layer (P) with a marked shrinkage of the pyramidal cells. Dispersed vacuolations (V) and dilated blood vessels (BV) are seen in the neuropil of both molecular (ML) and polymorphic layers (PM). (C) protected group at CA1 area showing an apparent larger thickness and number of cells in pyramidal layer, few degenerated cells (dashed arrows) and dilated blood vessels (BV) are seen as compared to diabetic group. (D) treated group showing an increase in the thickness of pyramidal layer (P), some degenerated cells (dashed arrows), vacuolation (V) and dilated blood vessels (BV) are seen as compared to diabetic group. (Scale bar = 50 µm).

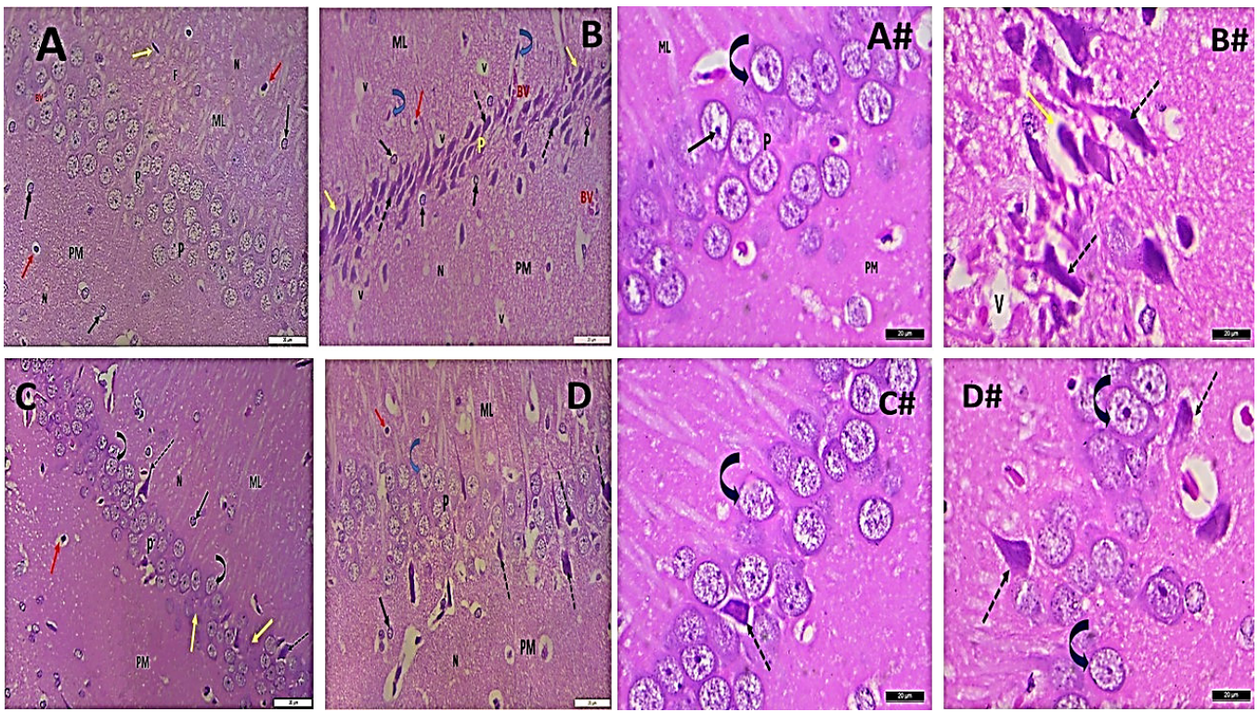
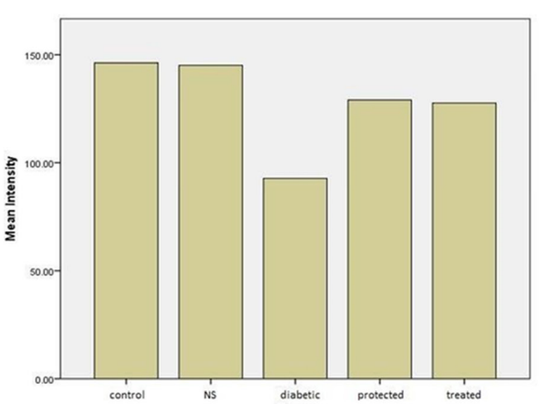


Fig. 17: A higher magnification of the previous section showing (A) control group with well-organized pyramidal cells compactly arranged in several rows inside the pyramidal layer (P). Each pyramidal cell appeared triangular with basophilic cytoplasm, large vesicular nucleus and prominent nucleolus. Different types of the neuroglia cells are scattered inside the neuropil (N) matrix; astrocytes (black arrows) with small vesicular nuclei, oligodendroglia (red arrows) with small dark nuclei and perinuclear halos and microglia (yellow arrow) with rod- shaped nuclei. nerve fibers (F) and normal blood vessels (BV) are also noticed in neuropil. (A#) A higher magnification of the previous section showing pyramidal cell appeared triangular with basophilic cytoplasm (curved arrow), large vesicular nucleus and prominent nucleolus (black arrow). (B) diabetic group showing pyramidal cells dark and hyperchromatic shrunken nuclei (dashed arrows) and perinuclear halos (yellow arrow). Vacuolations (V), dilated blood vessels (BV), oligodendroglia with dark nucleus (red arrow), microglia with rod-shaped nuclei (curved arrows) and apparent increase in number of astrocytes (black arrows) are observed in the neuropil of both molecular (ML) and the polymorphic (PM) layers. (B#) A higher magnification of the previous section showing pyramidal cells with dark and hyperchromatic shrunken nuclei (dashed arrows) and perinuclear halos (yellow arrow). Vacuolations (V) are observed in the neuropil. (C) protected group showing that that most cells in pyramidal cell layer (P) appear more or less normal with basophilic cytoplasm, large vesicular nucleus and prominent nucleolus (curved arrows) but few shrunken cells with hyperchromatic nuclei and perinuclear halos (dashed arrows) and few ghost like cells (yellow arrows) are also seen. Normal oligodendroglia (red arrow) and few astrocytes (black arrow) compared with the diabetic group are observed within the neuropil (N). (C#) A higher magnification of the previous section showing that most cells in pyramidal cell layer (P) appear more or less normal with basophilic cytoplasm, large vesicular nucleus and prominent nucleolus (curved arrow) but few shrunken cells with hyperchromatic nuclei and perinuclear halos (dashed arrow) are also seen. (D) treated group showing some pyramidal cells appeared more or less normal with basophilic cytoplasm, large vesicular nuclei and prominent nucleoli (curved arrow) but some cells appeared degenerated with hyperchromatic nuclei and perinuclear halos (dashed arrows). Normal oligodendroglia (red arrow) and astrocytes (black arrow) are observed within the neuropil (N). (D#) A higher magnification of the previous section showing many pyramidal cells appeared normal with basophilic cytoplasm, large vesicular nuclei and prominent nucleoli (curved arrows) but some cells appeared degenerated with hyperchromatic nuclei and perinuclear halos (dashed arrows). (Scale bar = 20 µm & 10 µm).



Group	Intensity color of toluidine blue*x*+SD	F	P value
Control	146.28±1.62	267.19	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa	145.11±1.68		
Diabetic	92.81±5.28		
protected	129.06 ±2.05		
Treated	127.65 ±3.83		

Fig. 18: Color intensity of toluidine blue of hippocampus different experimental groups.

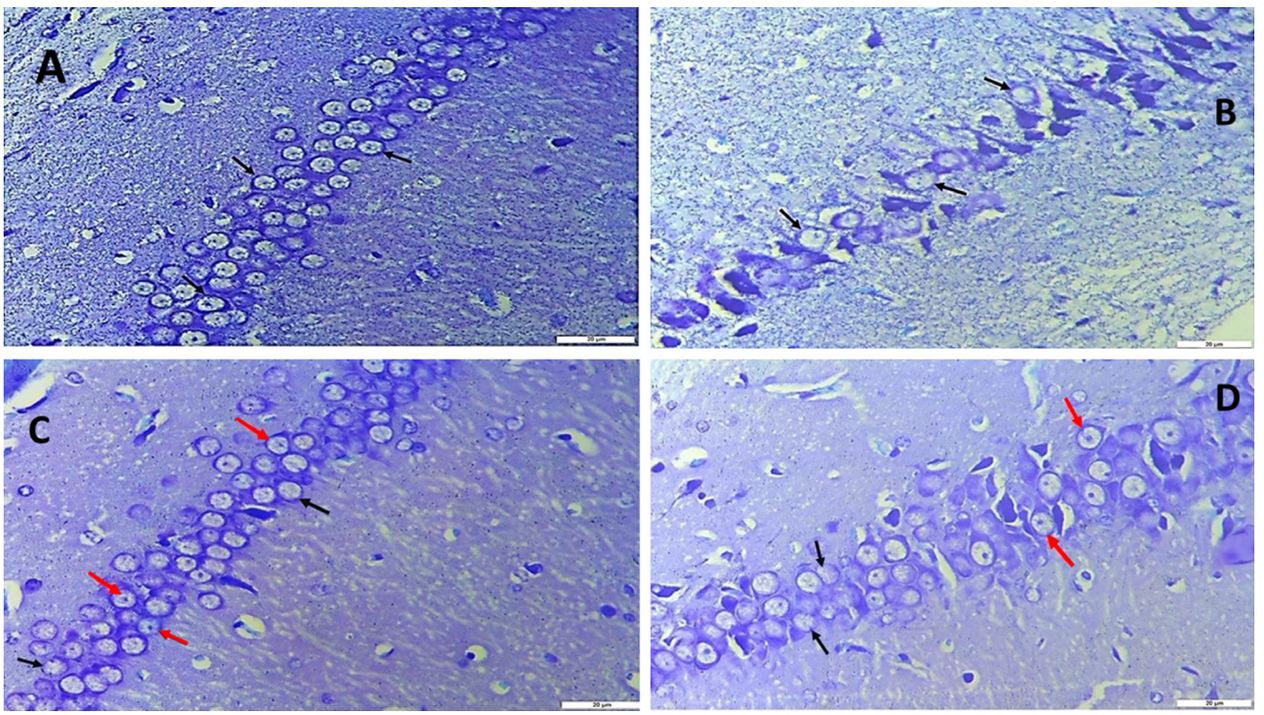
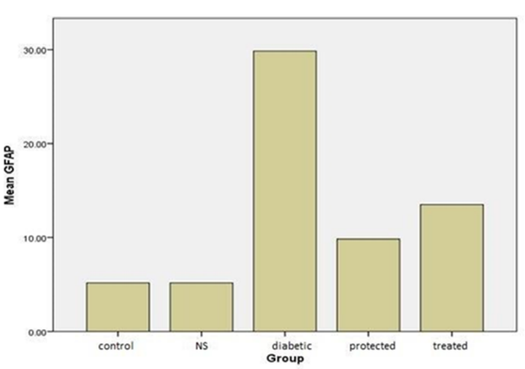


Fig. 19: A photomicrograph of a coronal section in the hippocampus proper at CA1 area of (A) a control rat showing the dense Nissl's granules content (arrows) inside the cytoplasm of the pyramidal cells which appear dark blue in colour. (B) diabetic rat showing apparent decrease of Nissl's granules content (arrows) in the cytoplasm of the pyramidal cells that appear faint blue in colour as compared with the control one. (C) protected group showing an apparent increase in Nissl's granules content in most of the pyramidal cells (red arrows) which appear dark blue in colour but still few cells showed decrease of their Nissl's granules content which appeared faint blue in colour (black arrows) as compared with the diabetic ones. (D) treated group showing an increase in Nissl's granules content in some pyramidal cells (red arrows) which appear dark blue in colour. some cells showed decrease of their Nissl's granules content which appeared faint blue in colour but more than the cells of protected group (black arrows). (Scale bar = 20 µm).



group	number of GFAP positive cells. $\bar{x}$+SD	F	P value
Control	5.17±.75	88.20	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa	5.16±1.47		
diabetic	29.83±4.07		
protected	9.83 ±3.54		
treated	13.5 ±1.87		

Fig. 20: Number of GFAP positive cells in CA1 of hippocampus in different experimental groups.

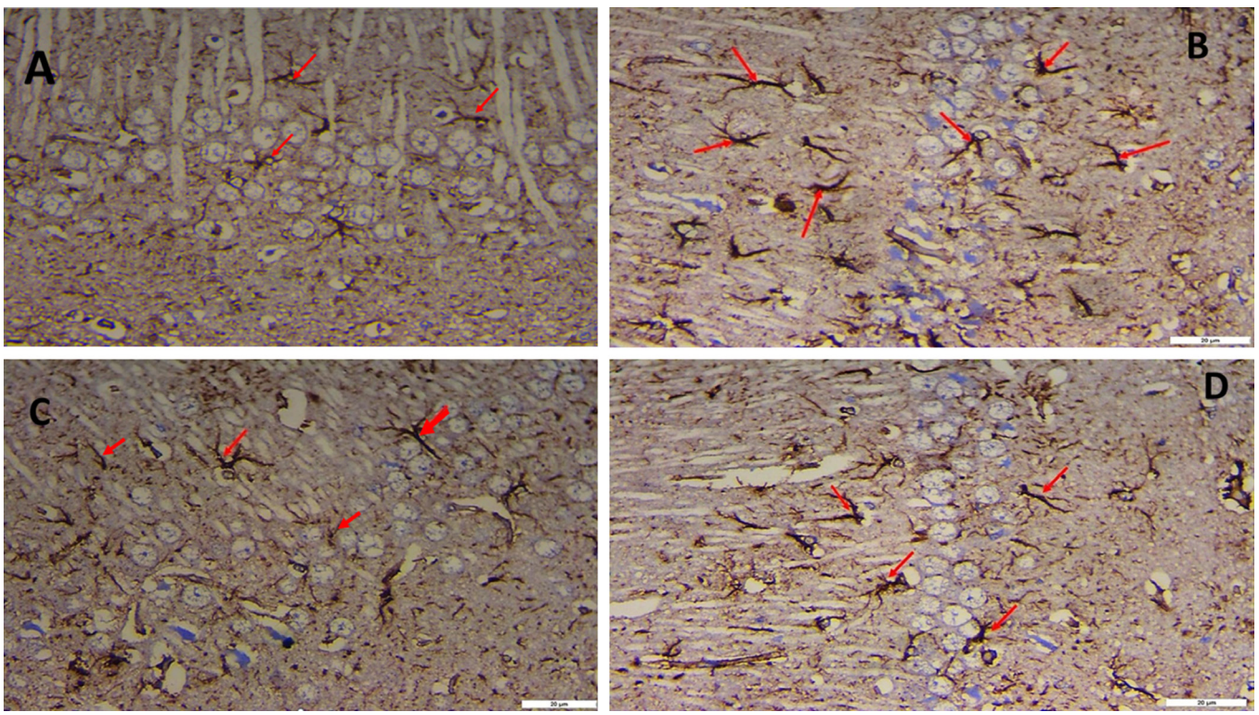
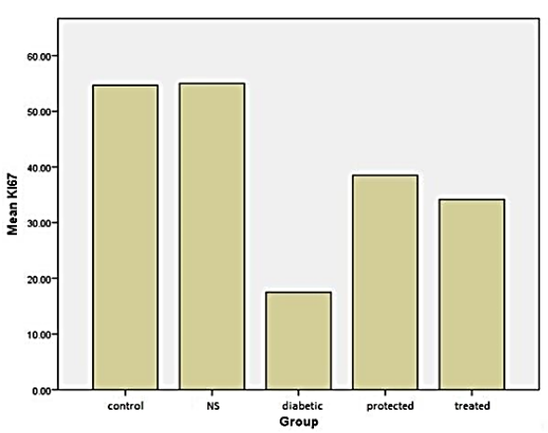


Fig. 21: A photomicrograph of a coronal section in the hippocampus proper at CA1 area of (A) a control rat showing few astrocytes which appear with small cell bodies, short cytoplasmic process and faint brown color withGFAP immune staining dispersed among the different layers of the hippocampus (arrows). (B) diabetic rat showing an apparent increase in the number of astrocytes as compared to control group. Some astrocytes are apparently increased in size with thick and increased branching of their processes and dense brown positive immunoreaction (arrows). (C) protected group showing decreased number of astrocytes which appeared small with short less branched processes and faint brown GFAP immunoreaction as compared with the diabetic group (arrows). (D) treated group showing apparent decrease in the number of GFAP reactive astrocytes which are small with short branches and faint brown staining as compared with the diabetic group (arrows) but still more than the protected group. (Scale bar = 20 µm).



group	number of Ki-67 positive cells $\bar{x}$+SD	F	P value
Control	54.66±3.88	128.31	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa	55±3.4		
Diabetic	17.5±3.4		
protected	38.5 ±2.88		
Treated	34.66 ±5.12		

Fig. 22: Number of Ki67 positive cells in CA1 of hippocampus in different experimental groups.

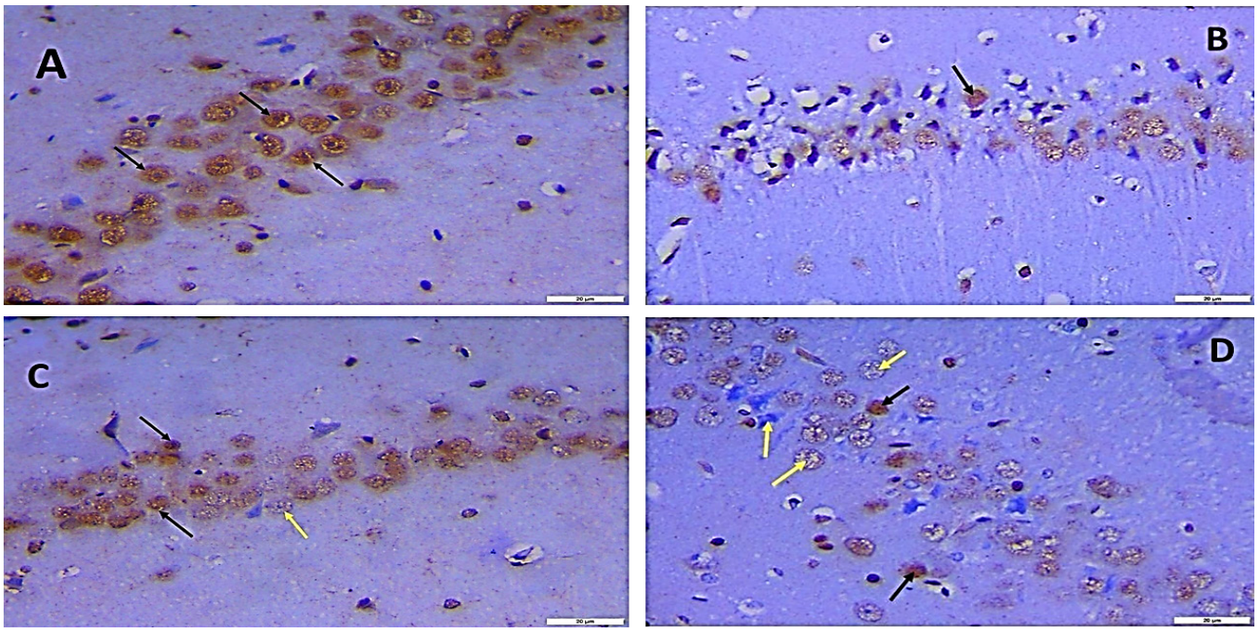
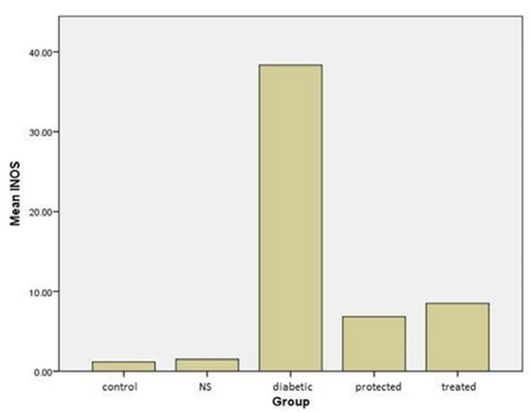
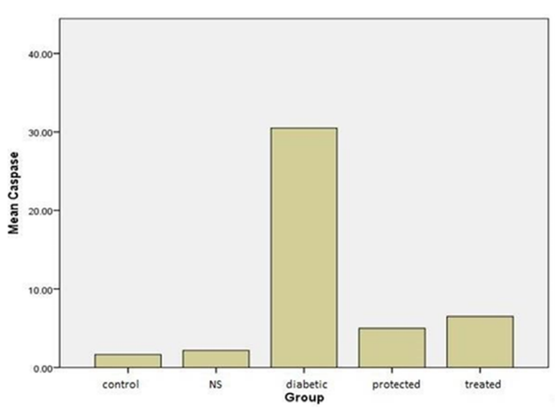


Fig. 23: A photomicrograph of a coronal section in the hippocampus proper at CA1 area of (A) a control rat showing Ki-67 positive cells (arrows). (B) diabetic rat showing a marked decrease in number of Ki-67 positive cells compared with control group (arrow). (C) protected group showing Ki-67 positive cells (black arrows) except for few cells which appeared Ki-67 negative (yellow arrow). (D) treated group showing g few cells are Ki-67 positive (black arrows) and the most are negative (yellow arrows). (Scale bar = 20 μm).



group	number of iNOS positive cells $\bar{x}$+SD	F	P value
Control	1.16±1.16	815.89	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa	1.5±.1.04		
Diabetic	38.5±2.16		
protected	6.83 ±.75		
Treated	8.5 ±1.04		

Fig. 24: Number of iNOs positive cells in CA1 of hippocampus in different experimental groups.



group	number of Caspase-3 positive cells$\bar{x}$+SD	F	P value
Control	1.66±.81	559.94	P1 >0.05 P2 <0.001 P3<0.001 P4 <0.001 P 5<0.05
Nigella Sativa	2.1±.75		
Diabetic	30.5±1.87		
protected	5 ±1.41		
Treated	6.5 ±1.04		

Fig. 25: Number of caspase3 positive cells in CA1 of hippocampus in different experimental groups.

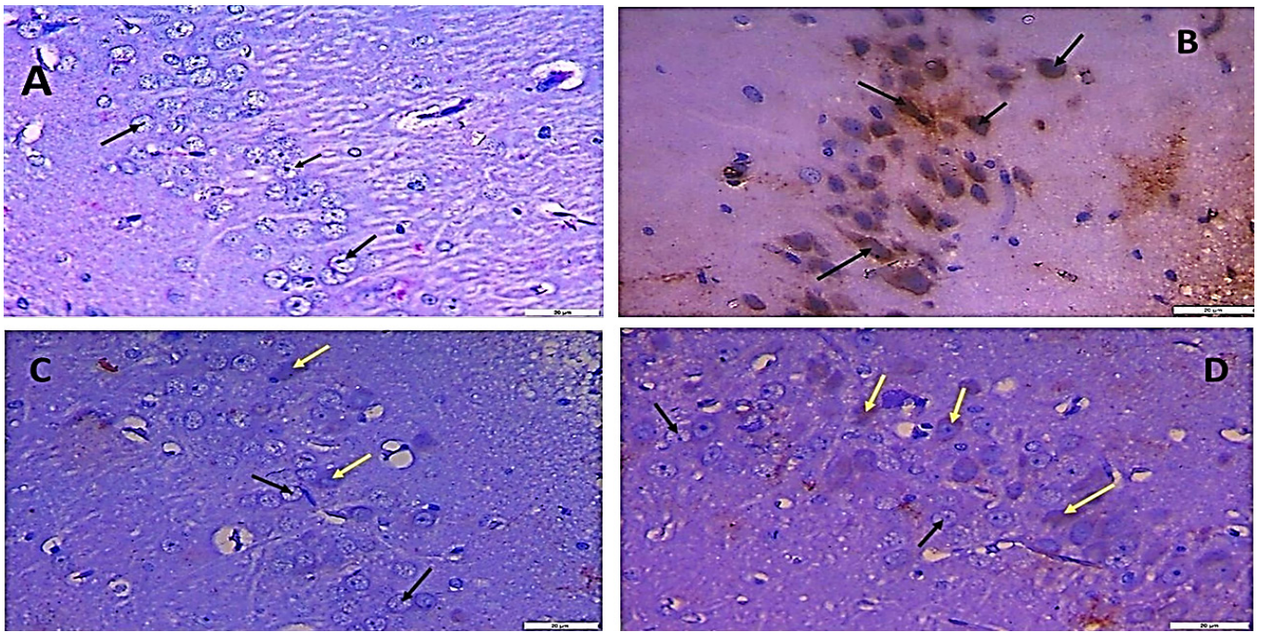


Fig. 26: A photomicrograph of a coronal section in the hippocampus proper at CA1 area of (A) a control rat showing negative immunoreaction to iNOS (arrows). (B) diabetic rat showing a strong positive immunoreaction to iNOS in the form of dark brown colour of the cytoplasm of the pyramidal cells (arrows). (C) protected group showing a negative immunoreaction to iNOS (black arrows) except for few neurons that show a weak positive immunoreaction (yellow arrows). (D) treated group showing a negative immunoreaction of pyramidal cells (black arrows) to iNOS while other cells showed an intermediate positive imunoreaction (yellow arrows). (Scale bar = 20 µm).

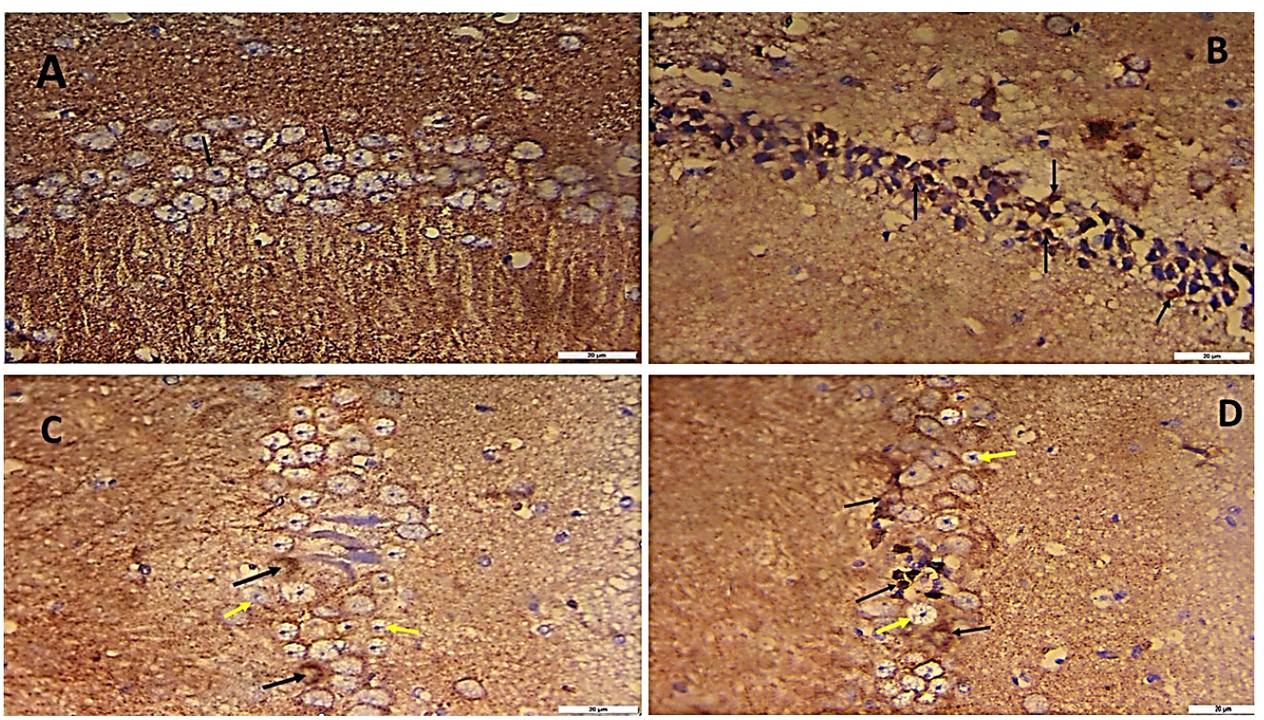


Fig. 27: A photomicrograph of a coronal section in the hippocampus proper at CA1 area of (A) a control rat showing negative Caspase-3 immunoreaction (arrows). (B) diabetic rat showing many cells with a strong Caspase-3 positive immunoreaction (arrows) with increase in brownish discoloration of the neurons. (C) protected group showing few number of cells with a weak Caspase-3 positive immunoreaction (black arrows) as compared to diabetic group. Other cells showed caspase-3 negative immunoreaction (yellow arrows). (D) treated group showing some cells with a strong Caspase-3 positive immunoreaction (black arrows). Others appeared with weak positive to negative reaction (yellow arrows). (Scale bar = 20 µm).

DISCUSSION

DM is a disorder of metabolism distinguished by persistently elevated levels of glucose in the blood stream and disturbances in the processes of metabolizing carbohydrates, proteins and lipids. The observed irregularities could potentially arise from resistance to insulin, insufficient production of insulin, or both. An estimated 537 million people between the ages of 20 and 79 were diagnosed as diabetics in 2021; by 2045, it is anticipated that this number will rise to 783 million[20].

Particularly in adipose and muscle tissues, glucose uptake is disrupted in DM, which causes hyperglycemia. Since neurons begin absorbing glucose in the absence of insulin, hyperglycemia in individuals with diabetes mellitus can result in a glucose uptake by neuronal cells that is up to four times greater. Increased glucose metabolism and concentration in the cytosol causes neuron damage[21].

The induction of apoptosis in the hippocampal CA1 region by diabetes has the potential to disrupt its homeostasis and function[22]. Additionally, pyramidal cells of external pyramidal layer (third layer) in the frontal cerebral cortex are degenerated by it[23]. Thus, the CA1 region and layer 3 of the frontal cortex of the brain were selected for this investigation.

Diabetes in experimental animal models display elevated levels of oxidative stress due to the chronic and persistent nature of hyperglycemia. As a result, the activity of the antioxidative defense system is diminished, resulting in the production of more free radicals[24]. Increased amounts of free radicals produced by the cell's auto-oxidation of increased glucose levels are linked to neuronal injury. Research has shown that the integration of spices and herbs into antioxidant therapy can effectively safeguard tissues against such damage[25].

Diabetes was established in rats by administering STZ at a dose of 50 mg/kg B.W. Serum glucose levels increased significantly in this group in contrast to the control group. The findings of the current study were just like to those detailed by Sobrevilla *et al*.[26] and Biswas *et al*.[27], which suggested that STZ was used to create animal models of diabetes mellitus because it could induce necrosis of pancreatic islet β-cells for at least sixteen weeks (72 hours) after administration. A statistically significant increase in both blood glucose and serum MDA concentrations was noted in the diabetic group in comparison to the control group in the current study. Blood glucose and MDA levels decreased significantly in the NS-protected and treated groups. The findings of Alimohammadi *et al*.[12], El Rabey *et al*.[28], Sangi and Jalaud[21], and Shrestha *et al*.[29] concerning the administration of NS extract to diabetic rats provide support for this.

A variety of mechanisms are involved in the antidiabetic activity of NS. By preserving the functional components of pancreatic beta-cells and preventing oxidative stress on beta-cells, NS exerts its anti-diabetic effects in addition to minimizing morphological changes. The dose-dependent reduction of intestinal glucose absorption is an additional possible explanation for the anti-diabetic effects of NS[29].

NS exhibits significant antioxidative properties, which establish it as a medicinal plant. The neuroprotective properties of NS and its principal component, thymoquinone, have been assessed in the context of epilepsy and neurotoxicity, among other conditions[30]. It has been demonstrated that thymoquinone inhibits lipid peroxidation in liposomes via non-enzymatic means and possesses antioxidant and cytoprotective properties. Moreover, NS oil inhibits lipid peroxidation in the rat hippocampal tissue injured by cerebral ischemia. According to a plethora of research, NS exhibits antioxidant and neuroprotective qualities against neurodegenerative diseases[31].

Histological analysis of H&E-stained sections of the frontal cortex and CA1 in diabetic rats revealed pyramidal cells that were deformed and possessed hyperchromatic nuclei with perinuclear halos. These characteristics are consistent with apoptotic changes, which are further supported by the upregulation of positive caspase-3 immunoreactivity. Furthermore, there were vacuolations and dilated blood vessels observed. A significant decline in both the number of pyramidal cells and Nissl's granules within the cytoplasm of the pyramidal cells was observed, suggesting a reduction in the survival of neuronal cells. Furthermore, an increase in the number of astrocytes was observed, as validated by GFAP immunostaining.

El-Kholy and El-Akabawy[23] documented comparable results, wherein they observed discernible neurodegenerative modifications in the dentate gyrus, frontal cortex, and cerebellum of rats induced with diabetes using STZ for durations of 4, 6, and 8 weeks. These changes suggest that the induction of oxidative stress by hyperglycemia is a primary factor contributing to neuronal damage within the diabetic brain.

In this experiment, sections of the frontal cortex and hippocampus of the group with diabetes revealed a considerable reduction in the color intensity of Nissl's granules. Akinola *et al*[32] and Elsaeed *et al*[33] reported comparable results, observing weak staining of pyramidal cells in the prefrontal cortex and hippocampus of diabetic rats, which indicates the absence or reduction of Nissl bodies in these cells.

The findings of our study indicate that diabetes significantly increases the number of GFAP-positive cells in every brain region that was examined. This finding is consistent with previous studies indicating that reactive gliosis results from oxidative stress in individuals with diabetes[5,23]. This phenomenon can be ascribed to reactive gliosis, which is caused by an elevation in ROS. This aligns with the results of Kaneko *et al*.[34] and Pekny and Nilsson[35], who reported that glial cells generate GFAP and S100B in response to the oxidative insult.

The current investigation observed a significant decline in the expression of Ki-67 immunoreactivity in diabetic group relative to control group. This finding is consistent with the research conducted by Jackson-Guilford *et al*.[36], El-Akabawy & El-Kholy[23] and others who have observed that the rate of cell proliferation in the frontal cortex and hippocampus of diabetic rats is lower than that of non-diabetic rats. While the precise mechanism underlying decreased neuronal production remains unknown, available evidence indicates that hyperglycemia and/or insulin deficiency in the brain could potentially be influential factors[37].

Hypothetically, oxidative stress is the primary etiology of neuropathy in individuals with diabetes. Oxidative stress is induced by the elevated intracellular glucose, leading to an overproduction of ROS and nitric oxide (NO)[38]. Morever, Nishikawa *et al*.[39] and Fukudome *et al*.,[40] added that, the excess generation of ROS and inducible NO synthase (iNOS) causes oxidative damage to cellular proteins, lipids, or DNA and subsequently inhibits their normal functions and disturbs homeostatics within the neuron, ultimately resulting in cell death via apoptosis. In addition, Gürpinar *et al*.,[41] reported that oxidative stress causes depolarization of the inner mitochondrial membrane with subsequent release of cytochrome c into the cytosol leading to induction of caspase mediated apoptosis.

The frontal cortex and hippocampus of diabetic rats exhibited elevated levels of iNOS, an indicator of oxidative stress, in our investigation. Consistent results were documented by[42,23]. In addition, Brahmachari,[43] reported that NO played an important role in the induction of GFAP expression in the astrocytes through the guanylate cyclase (GC)–cGMP–cGMP-activated protein kinase (PKG) signaling pathway. This goes in line with our results which showed significant upregulation of both iNOS and GFAP within the cortical and hippocampal specimens of the diabetic rats.

We observed a rise in the number of apoptotic cells in our investigation; this was validated by the upregulation of caspase-3 activities. Consistent with our study, a number of studies have demonstrated that cerebral cortex[44,45] and hippocampus[4,46,47,33] oxidative stress markers are elevated in diabetic rats, along with the apoptotic rate, suggesting that diabetes promotes oxidative stress-induced apoptosis in these brain areas.

The diabetic groups that were treated or protected with NS extract demonstrated histological and immunohistochemical recovery in the current study. The number of pyramidal cells and Nissl's granules within the cytoplasm of pyramidal cells increased significantly. These results are consistent with those of[21], who administered a hydroalcoholic extract of NS to diabetic rats induced with STZ. The extract prevented brain damage and repaired damaged neurons in the cerebral cortex, hippocampus, and cerebellum.

Additionally, a reduction in the number of astrocytes in the frontal cortex and hippocampus was observed in diabetic rats that were either protected or treated with NS extract, as opposed to the non-treated. Based on our findings, NS extract treatment ameliorated gliosis induced by diabetes. This finding aligns with prior research conducted on diabetic rats, wherein the protective impacts of antioxidants against reactive gliosis were ascribed to their ability to scavenge free radicals[48,49,50].

Positive Ki-67 immunoreactivity was dramatically increased in the protected and treated groups of the current study; this may counteract the diabetes-induced inhibition of neurogenesis. This aligns with the results of Imam *et al*.[51], who examined the effectiveness of NS oil (NSO) in protecting the hippocampus from Chlopyrifos (CPF) insults, an organophosphate known to cause brain damage and cognitive deficits. The study found that oxidative damage, suppression of AChE, and decreased potential adult neurogenesis in the hippocampus are some of the processes via which exposure to CPF impairs cognitive performance in rats. It is interesting that all neurocognitive indicators were improved and elevated by interventional treatment with NSO given soon after CPF insult. This improvement might be explained by the treated rats' hippocampal levels of stressor indicators being lower and their adult neurogenesis remaining intact. Rats administered with NSO showed significantly higher levels of Ki-67 expression than the control group; this shows that NSO has the ability to increase dentate gyrus cell proliferation, which aids in the integration of recently formed adult cells into the circuitry responsible for memory and learning. Several studies have found that NS extract may enhance cognitive performance and promote neurogenesis in animal models of neurodegenerative illnesses[52].

Furthermore, Asiaei *et al*.[31] documented that NS extract may possess the capability to avert hippocampal neural damage (specifically, the suppression of dark neuron production and apoptotic cell death) in rats neonatal and juvenile growth subsequent to inducing hypothyroidism with propylthiouracil (PTU). The current study observed that treatment with NS extract resulted in a substantial decline in the expression of iNOS, a substantial decline in the number of apoptotic cells, and a significant decline in the number of caspase-3-positive cells. This finding was in line with the findings published by Meral *et al*.[53]. Strong antioxidant properties are one of the most well-known therapeutic uses of Nigella sativa[30]. Antioxidant components of NS extract through previous proved strategies make NS specialized with neuroprotective effect on hippocampus and frontal cortex against diabetic neuropathy.

CONCLUSION

The current dose of STZ has a potent diabetogenic effect for creating animal model of diabetes resulting in elevation of the levels of blood glucose and serum MDA.

DM causes neurodegenerative effects on the cerebral frontal cortex and hippocampus due to oxidative stress; therefore, oxidative stress is recognized as a main mediating process in the pathogenesis of diabetic complications.

The current dose of NS extract proved to be an effective agent for protection and treatment of neurodegenerative changes in the diabetic rats in both cerebral frontal cortex and hippocampus, but it is more beneficial in protection than treatment.

CONFLICT OF INTERESTS

No conflicts of interest exist.

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الملخص العربى

التأثير الوقائى العصبى لمستخلص حبة البركة على القشرة المخية الجبهية وقرن آمون فى داء السكرى المستحث تجريبيا فى ذكر الجرذ الأبيض البالغ

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المقدمة: يعتبر الجهاز العصبي المركزي أحد الاجهزة العرضة للمضاعفات التي يسببها مرض السكرى. يعتبر الإجهاد التأكسدي عملية وساطة رئيسية في التسبب في مضاعفات مرض السكري. يعزى التأثير الوقائي لمستخلص حبة البركة إلى خصائصه القوية المضادة للأكسدة ، والتي ترتبط بقدرته على البحث عن أنواع الأكسجين التفاعلية المختلفة ، بما في ذلك أنيون الجذور الفائقة والجذور الحرة للهيدروكسيل ، لمنع اكسدة الدهون وتعزيز الإنزيمات المضادة للأكسدة.
الهدف: لدراسة وتقييم التأثير العصبي لمستخلص حبة البركة على القشرة الأمامية وقرن امون في داء السكري المستحث تجريبيا في ذكور الجرذان البيضاء البالغة. استند هذا إلى التحليلات البيوكيميائية والنسيجية والكيميائية المناعية والمورفومترية.
مواد وطرق البحث: تم تقسيم ستة وثلاثين من ذكور ذكور الجرذان البيضاء البالغين إلى خمس مجموعات: المجموعة الأولى شملت المجموعة الضابطة ، المجموعة الثانية (مجموعة حبة البركة) ، المجموعة الثالثة (مجموعة السكري)، المجموعة الرابعة (المحمية) ، المجموعة الخامسة (المعالجة). تم ذبح جميع الحيوانات بعد ٦ أسابيع. تم جمع عينات الدم للفحص الكيميائي الحيوي وتم إعداد أقسام القشرة الأمامية وقرن امون للدراسات النسيجية والكيميائية المناعية. تم إجراء التحليل المورفومتري والإحصائي.
النتائج: بالمقارنة مع المجموعة الضابطة ، أظهرت أقسام القشرة الأمامية وقرن امون ترتيبا مضطربا لطبقة الخلايا الهرمية ، ولوحظت العديد من الخلايا الهرمية المنكمشة وكان هناك أيضاً انخفاض في كثافة حبيبات نيسل. كما لوحظ زيادة في عدد وحجم الخلايا النجمية ذات التفاعل المناعي، وزيادة في عدد الخلايا الميتة التي تفاعلت مع صبغة كاسبيز ٣ وصبغةiNOS وانخفاض في التفاعل مع صبغة ال Ki٦٧ ، أيضا لوحظ ارتفاع مستوى المالون داى الدهاليد بالمقارنة مع المجموعات المحمية وايضا التى تم معالجتها بمستخلص حبة البركة والتى أظهرت تحسن جميع البارامترات في الدراسة.
الخلاصة: المكونات المضادة للأكسدة من مستخلص حبة البركة تجعل هذا المستخلص متخصص في التأثير الوقائي العصبي على القشرة الأمامية المخية وقرن امون .