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Histological and histochemical study of oxidative stress effects on pancreatic tissue in diabetic rats and the protective role of *Nigella sativa* extract

R. H. Nada[1] | E. M. Mahdi[2] | S. F. H. Abas[3] | B. Kh. Hameed[4]

Article info

Correspondence Author
R. H. Nada
E-mail:
ruaa.hussein@tu.edu.iq

[1] College of Sciences, University of Tikrit,
Tikrit, 34001, Iraq

[2] Al-Daur Education Department, Ministry of Education,
Salah Al-Din, 34001, Iraq

[3] College of Applied Sciences, University of Samarra,
Samarra, 34001, Iraq

[4] College of Veterinary Medicine, University of Tikrit,
Tikrit, 34001, Iraq

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Diabetes mellitus (DM) is a chronic metabolic disease characterized by severe hyperglycemia resulting from defects in insulin secretion or action, with oxidative stress driving its pathogenesis via pancreatic beta-cell destruction. This study investigated histopathological and histochemical alterations in the pancreatic tissue of streptozotocin-induced diabetic rats and evaluated the cytoprotective efficacy of a *Nigella sativa* (black seed) aqueous extract. Forty adult male Wistar albino rats (200–250 g) were randomly assigned to four groups (n = 10 each): Group I (Normal Control), Group II (Diabetic Control; induced with a single i.p. injection of streptozotocin, 60 mg/kg), Group III (diabetic rats treated orally with 400 mg/kg/day of *Nigella sativa* extract for 8 weeks), and Group IV (normal rats treated with the extract). Pancreatic tissues were processed for Hematoxylin and Eosin (H&E), Periodic Acid-Schiff (PAS), Masson's trichrome, Gomori's aldehyde fuchsin, and immunohistochemical detection of insulin and glucagon, alongside tissue oxidative stress marker quantification (MDA, SOD, CAT, GPx). Streptozotocin-treated rats exhibited severe histopathological alterations ($P<0.001$), including a 75.0 % reduction in islet density, a 66.6 % decrease in islet cross-sectional area, extensive periinsular and interlobular fibrosis, and severe depletion of both PAS-positive glycoproteins and insulin-immunoreactive beta cells. These structural changes directly correlated with a significant elevation in malondialdehyde (MDA) levels ($P<0.001$) and a concurrent decline in SOD, CAT, and GPx antioxidant activities ($P<0.001$). Daily oral administration of *Nigella sativa* extract in Group III significantly attenuated these microstructural and biochemical abnormalities ($P<0.001$). The treatment preserved near-normal islet architecture, reduced connective tissue deposition more than twofold, and drove a substantial 2.1-fold recovery in functional insulin expression relative to the untreated diabetic group. These findings demonstrate that chronic oxidative stress induces profound structural and histochemical damage to both the endocrine and exocrine pancreas, whereas *Nigella sativa* extract exerts a potent cytoprotective effect mediated by its antioxidant, anti-inflammatory, and anti-fibrotic properties, confirming its therapeutic potential as an effective complementary strategy in managing diabetes mellitus and preventing secondary pancreatic complications.

Keywords: diabetes mellitus, streptozotocin, oxidative stress, pancreatic histology, *Nigella sativa*, islets of Langerhans, antioxidants, histochemistry.

Гістологічне та гістохімічне дослідження впливу окисного стресу на тканину підшлункової залози щурів із цукровим діабетом та захисної ролі екстракту *Nigella sativa*

Р. Г. Нада[1] | Е. М. Магді[2] | С. Ф. Г. Абас[3] | Б. Х. Хамід[4]

[1] Факультет природничих наук, Університет Тікріта,
Тікріт, 34001, Ірак

[2] Департамент освіти Аль-Даур, Міністерство освіти,
Салах-Ед-Дін, 34001, Ірак

[3] Факультет прикладних наук, Університет Самарри,
Самарра, 34001, Ірак

[4] Факультет ветеринарної медицини, Університет Тікріта,
Тікріт, 34001, Ірак

Цукровий діабет – це хронічне метаболічне захворювання, що характеризується вираженою гіперглікемією внаслідок дефектів секреції або дії інсуліну, причому окисний стрес відіграє центральну роль у його патогенезі через вибіркове руйнування бета-клітин підшлункової залози. У цьому дослідженні вивчено гістопатологічні та гістохімічні зміни в тканині підшлункової залози щурів зі стрептозотоцин-індукованим діабетом та оцінено цитопротективну ефективність водного екстракту *Nigella sativa* (чорного кмину). Сорок дорослих самців щурів лінії Wistar (200–250 г) були випадковим чином розділені на чотири групи (по 10 у кожній): Група I (клинічно здорові щури – контроль), Група II (діабетичний контроль спричинений одноразовим внутрішньоочеревинним введенням стрептозотоцину, 60 мг/кг), Група III (діабетичні щури, які отримували перорально екстракт *Nigella sativa* у дозі 400 мг/кг/добу протягом 8 тижнів) і Група IV (здорові щури, які отримували екстракт *N. sativa*). Тканину підшлункової залози обробляли для фарбування гематоксиліном та еозином, фарбували за Шиффом (PAS), трихромом за Массоном, альдегід-фуксином за Гоморі та проводили імуногістохімічне виявлення інсуліну й глюкагону; паралельно визначали маркери окисного стресу (МДА, СОД, КАТ, ГПх у гомогенатах. Щури з діабетичним контролем виявили тяжкі гістопатологічні зміни ($P<0,001$), включаючи зниження щільності острівців на 75,0 %, зменшення площі їхнього поперечного перерізу на 66,6 %, вакуольну дегенерацію ацинарних клітин, виражений періінсулярний та інтерлобулярний фіброз, а також глибоке виснаження PAS-позитивних глікопротеїнів та інсулін-імунореактивних бета-клітин. Ці структурні порушення прямо корелювали зі значним підвищенням рівня малонового діальдегіду (MDA) ($P<0,001$) і одночасним падінням антиоксидантної активності СОД, КАТ та ГПх ($P<0,001$). Щоденне пероральне введення екстракту *N. sativa* у Групі III ефективно нівелювало ці мікроструктурні та біохімічні аномалії ($P<0,001$), забезпечуючи збереження нормальної архітектоніки острівців, зменшення відкладення сполучної тканини більш ніж удвічі та зумовлюючи відновлення функціональної експресії інсуліну відносно нелікованої діабетичної групи. Отримані дані доводять, що хронічний окисний стрес викликає глибокі структурні та гістохімічні ушкодження як ендокринної, так і екзокринної частин підшлункової залози, тоді як екстракт *N. sativa* виявляє потужну цитопротективну дію завдяки своїм антиоксидантним, протизапальним та антифібротичним властивостям, що підтверджує його терапевтичний потенціал як ефективної комплементарної стратегії в лікуванні цукрового діабету та профілактиці вторинних панкреатичних ускладнень.

Ключові слова: цукровий діабет, стрептозотоцин, окисний стрес, гістологія підшлункової залози, *Nigella sativa*, острівці Лангерганса, антиоксиданти, гістохімія.

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disease that is among the most widespread global health challenges. As of 2021, an estimated 537 million adults were affected by DM, and this baseline is projected to reach 783 million by 2045 [10].

Type 1 DM is characterized by autoimmune-mediated destruction of pancreatic beta cells, whereas Type 2 DM represents a synergistic combination of peripheral insulin resistance and progressive beta-cell dysfunction. Irrespective of its specific etiology, chronic hyperglycemia causes multi-organ damage through several pathways. Among these, oxidative stress is one of the most well-researched pathological factors [8].

In terms of both its endocrine and exocrine activities, the pancreas is highly vulnerable to oxidative damage. Pancreatic islets of Langerhans possess relatively low levels of antioxidant enzymes, which predisposes beta cells to reactive oxygen species (ROS)-mediated injury [12].

The excessive production of ROS results in lipid peroxidation, protein oxidation, and DNA strand breaks. These processes ultimately cause mitochondrial dysfunction and trigger apoptosis in insulin-producing cells [13].

Streptozotocin (STZ) is a glucosamine-nitrosourea derivative isolated from *Streptomyces achromogenes* that selectively destroys pancreatic beta cells. This cytotoxicity is achieved through DNA alkylation and the intracellular production of superoxide radicals, hydroxyl radicals, and nitric oxide.

These reactive species activate poly(ADP-ribose) polymerase (PARP), inducing cellular energy depletion and rapid necrosis [12]. Consequently, the STZ-induced diabetic rat model has been extensively utilized to investigate the pathogenesis of DM and evaluate the therapeutic efficacy of new pharmacological agents [14].

Histological and histochemical analyses provide fundamental structural and compositional data regarding tissue alterations caused by oxidative stress and hyperglycemia. Routine staining procedures, such as Hematoxylin and Eosin (H&E), are used to visualize general cell morphology.

Meanwhile, special histochemical stains offer deeper insights into tissue components. The Periodic Acid-Schiff (PAS) reaction demonstrates glycoprotein distribution, whereas Masson's trichrome highlights fibrotic alterations and connective tissue structure. Gomori's aldehyde fuchsin is applied to stain specific secretory granules within beta cells [5]. Additionally, immunohistochemical techniques allow the precise evaluation of individual hormone-secreting cell types.

Natural plant extracts have gained significant popularity as complementary therapeutic agents in DM management due to their rich phytochemical profiles and multi-targeted antioxidant effects. *Nigella sativa* (black seed, family Ranunculaceae) is a widely researched medicinal plant containing potent active components, including thymoquinone, thymohydroquinone, thymol, carvacrol, and fixed oils.

Its primary bioactive ingredient, thymoquinone, has demonstrated strong antioxidant, anti-inflammatory, immunomodulatory, and cytoprotective properties across various experimental models [2, 6]. Prior studies have reported that *Nigella sativa* extract can excellently ameliorate blood glucose levels, lipid profiles, and general pancreatic histology in diabetic rodents [3, 15].

However, comprehensive histochemical investigations that directly correlate antioxidant enzyme status with quantitative morphometric and immunohistochemical parameters remain scarce.

The aim of the study

The primary aim of this study was to comprehensively characterize the histological and histochemical profile of oxidative stress-induced pancreatic damage in STZ-diabetic rats and to evaluate the potential cytoprotective efficacy of a *Nigella sativa* aqueous extract.

To achieve this aim, the following specific objectives *were established*:

- To establish and validate the STZ-induced diabetic rat model by verifying chronic hyperglycemia and systemic oxidative stress conditions.
- To analyze histopathological alterations in both the exocrine and endocrine pancreatic tissues using routine Hematoxylin and Eosin (H&E) staining.
- To assess specific histochemical changes, including glycoprotein distribution via Periodic Acid-Schiff (PAS), connective tissue remodeling via Masson's trichrome, and beta-cell secretory granule integrity via Gomori's aldehyde fuchsin staining.
- To evaluate insulin and glucagon immunoreactivity within the islets of Langerhans utilizing advanced immunohistochemical techniques.
- To quantify biochemical indicators of oxidative stress (MDA, SOD, CAT, and GPx) in pancreatic tissue homogenates and correlate them directly with morphometric findings.
- To determine the cytoprotective and therapeutic properties of *Nigella sativa* extract against streptozotocin-induced structural and functional pancreatic injury.

Materials and methods

Plant material and extract preparation

Nigella sativa seeds were purchased from a local commercial market and formally authenticated by a plant taxonomist at the Department of Biology, College of Science, University of Tikrit. The seeds were thoroughly rinsed with distilled water, dried at 40°C for 48 hours, and ground into a fine powder.

The aqueous extract was prepared by dissolving 100 g of the seed powder in 500 mL of distilled water. The mixture was continuously stirred at 60°C for 2 hours, filtered through Whatman No. 1 filter paper, and the resulting filtrate was lyophilized. The final yield of the dry extract was approximately 12.4 % (w/w).

Preliminary phytochemical screening to detect the presence of alkaloids, flavonoids, phenols, saponins, tannins, and terpenoids was performed according to standard analytical procedures [9]. The lyophilized extract was stored at −20°C, dissolved in distilled water immediately prior to use, and administered orally.

Animals and experimental design

Forty adult male Wistar albino rats weighing 200–250 g (aged 8–10 weeks) were procured from the Animal House of the College of Veterinary Medicine, University of Tikrit. The animals were maintained under standard laboratory conditions (temperature: 22±2°C; relative humidity: 55±5 %; 12-hour light/dark cycle) with free access to a standard rodent pellet diet and tap water. All rats were acclimatized to the laboratory environment for one week prior to the initiation of the experiment.

The animals were randomly assigned to four experimental groups (n = 10 rats per group):

- *Group I* – Normal Control (NC): Received an intraperitoneal (i.p.) injection of citrate buffer (0.1 mol/L, pH 4.5) and distilled water orally via gavage daily for 8 weeks.

- *Group II* – Diabetic Control (DC): Received a single i.p. injection of freshly prepared streptozotocin (STZ; 60 mg/kg body weight) dissolved in citrate buffer (0.1 mol/L, pH 4.5). Diabetes was confirmed 72 hours post-injection in rats exhibiting a fasting blood glucose (FBG) level ≥ 250 mg/dL.

Group III – Diabetic + *Nigella sativa* (D+NS): STZ-induced diabetic rats treated orally with *Nigella sativa* aqueous extract (400 mg/kg/day via gavage) daily for 8 weeks.

Group IV – Normal + *Nigella sativa* (N+NS): Non-diabetic rats treated orally with *Nigella sativa* aqueous extract (400 mg/kg/day via gavage) daily for 8 weeks.

Blood glucose measurement

Fasting blood glucose (FBG) levels were determined using a calibrated glucometer (Accu-Chek® Performa, Roche Diagnostics, Germany). Blood samples were obtained from the tail vein of overnight-fasted rats at baseline, 72 hours post-STZ injection, and at the end of the 8-week experimental period.

Tissue collection

At the end of the 8-week experimental period, all rats were fasted overnight and euthanized under deep anesthesia induced by an intramuscular (i.m.) injection of ketamine (75 mg/kg) and xylazine (10 mg/kg). The pancreatic tissue was immediately dissected and harvested.

Representative tissue portions were fixed in 10% neutral buffered formalin for subsequent histological and histochemical processing. Additional tissue portions were snap-frozen in liquid nitrogen and stored at −80°C for biochemical analyses.

Histological processing

Formalin-fixed pancreatic tissues were dehydrated through a graded series of alcohols, cleared in xylene, and embedded in paraffin wax. Serial sections of 4–5 µm thickness were cut using a rotary microtome (Leica RM2235, Germany). The sections were subsequently mounted on positively charged glass slides and dried at 60°C for 30 minutes to ensure tissue adhesion.

Histological and histochemical staining techniques

Hematoxylin and Eosin (H&E) Staining

Routine H&E staining was performed to evaluate general cell morphology and nuclear characteristics. Deparaffinized and rehydrated sections were stained with Mayer's hematoxylin for 8 minutes, differentiated in 1 % acid alcohol, and blued in Scott's tap water.

The slides were then counterstained with eosin Y for 2 minutes, dehydrated through graded alcohols, cleared in xylene, and mounted.

Periodic acid-Schiff (PAS) reaction

The PAS reaction was utilized to demonstrate the distribution of tissue carbohydrates, including polysaccharides, glycoproteins, and glycolipids. Tissue sections were oxidized with 0.5 % periodic acid for 10 minutes and rinsed with distilled water.

The sections were then treated with Schiff's reagent for 15 minutes, rinsed with sulfurous acid, and counterstained with Mayer's hematoxylin. Following dehydration and clearing, the sections were mounted. PAS-positive materials appeared magenta-red.

Masson's trichrome (MT) staining

The Masson-Goldner trichrome technique was employed to stain connective tissue elements and perform a semiquantitative determination of the level of fibrosis.

Following staining, cell nuclei appeared black-brown (stained with Weigert's iron hematoxylin), the cytoplasm and muscle fibers appeared red, and collagen fibers were stained green (light green).

Gomori's aldehyde fuchsin (AF) staining

Gomori's aldehyde fuchsin staining was employed to visualize specific secretory granules within pancreatic beta cells. Following tissue deparaffinization and rehydration, sections were oxidized with acidified potassium permanganate and bleached with oxalic acid.

The sections were then stained with Gomori's aldehyde fuchsin solution for 30 minutes, followed by counterstaining with Masson's light green solution. Under light microscopy, the secretory granules of beta cells appeared deep purple-violet, whereas alpha cells and general background tissue structures were counterstained green.

Immunohistochemistry (IHC)

For immunohistochemical evaluation, heat-induced epitope retrieval was performed in citrate buffer (pH 6.0) at 95°C for 20 minutes. Endogenous peroxidase activity was subsequently inhibited by incubating the sections in 3 % H_2O_2 for 10 minutes.

The sections were blocked and incubated overnight at 4°C with primary antibodies against insulin (Dako, 1 : 200) and glucagon (Abcam, 1 : 150). Following thorough washing in Tris-buffered saline (TBS), the sections were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 30 minutes.

Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) substrate, and the slides were counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted.

Morphometric analysis

Digital images of stained pancreatic sections were captured using a Leica DM500 microscope equipped with an integrated digital camera (Leica ICC50 HD) at

standardized magnifications (100×, 200×, and 400×). Morphometric measurements were performed quantitatively using ImageJ software (NIH, Bethesda, MD, USA, version 1.53).

For each experimental animal, the following parameters were quantified across ten randomly selected, non-overlapping high-power fields: number of islets of Langerhans per mm² of pancreatic tissue area; total islet cross-sectional area (µm²); beta-cell nuclear diameter (µm); percentage of PAS-positive area and collagen fiber deposition area, evaluated using computerized color threshold analysis.

Biochemical analysis

Pancreatic tissue specimens were homogenized (10 %, w/v) in ice-cold phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 10,000 × g for 15 minutes at 4°C. The resulting supernatants were collected and utilized for biochemical assays.

Malondialdehyde (MDA) content was measured using the thiobarbituric acid reactive substances (TBARS) method. Superoxide dismutase (SOD) activity was determined via the nitroblue tetrazolium (NBT) reduction method. Catalase (CAT) activity was calculated by evaluating hydrogen peroxide (H_2O_2) decomposition. Glutathione peroxidase (GPx) activity was measured using the DTNB-coupled spectrophotometric method. Total protein concentrations were determined via the Bradford method. All diagnostic assay kits were purchased from Biodiagnostics (Cairo, Egypt).

Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). Statistical evaluations were performed using SPSS software (version 26.0, IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was utilized to assess significant differences among experimental groups.

A p-value of ≤0.05 was considered statistically significant. Additionally, Pearson's correlation analysis was conducted to determine the strength and direction of linear relationships between tissue morphometric parameters and biochemical markers of oxidative stress.

Results and discussion

Phytochemical screening of Nigella sativa extract

Qualitative phytochemical analysis of the *Nigella sativa* aqueous extract revealed the rich presence of flavonoids, phenolic compounds, tannins, saponins, alkaloids, and terpenoids, along with carbohydrates. Conversely, anthraquinones were not detected in the extract. Quantitative evaluation via high-performance liquid chromatography (HPLC) indicated that the concentration of the primary bioactive component, thymoquinone, was 2.8±0.3 mg/g of dry extract weight. The detailed phytochemical profile and the established biological significance of these constituents are summarized in ***Table 1***.

Table 1
Phytochemical constituents of *Nigella sativa* aqueous extract

Phytochemical constituent	Result	Biological significance
Flavonoids	+++	Antioxidant, anti-inflammatory
Phenolic compounds	+++	Free radical scavenging
Tannins	++	Astringent, antioxidant
Saponins	++	Anti-inflammatory, hypoglycemic
Alkaloids	++	Analgesic, antimicrobial
Terpenoids	++	Antiseptic, cytoprotective
Carbohydrates	+	Structural components
Anthraquinones	−	Not detected

Note: +++: abundant; ++: moderate; +: trace; −: absent.

Table 1 demonstrates that the aqueous extract of *Nigella sativa* is highly enriched with diverse bioactive secondary metabolites, particularly antioxidant and anti-inflammatory compounds such as flavonoids and phenolics, which provides a strong biochemical rationale for its evaluated cytoprotective properties.

Blood glucose and body weight

Table 2 provides a comprehensive summary of fasting blood glucose (FBG) levels and body weight changes observed across all experimental groups. Group II (Diabetic Control) rats exhibited a significant, drastic elevation in FBG levels (421.3±18.7 mg/dL) 72 hours post-STZ injection compared to the normal controls in Group I (89.6±6.3 mg/dL, $p<0.001$).

Daily therapeutic administration of *Nigella sativa* extract in Group III (D+NS) significantly ameliorated this glycemic state, reducing FBG levels to 187.4±14.2 mg/dL by week 8 ($p<0.001$ vs. Group II), though values remained above the physiological baseline. Furthermore, the severe body weight loss observed in diabetic rats due to catabolic state was noticeably attenuated and partially restored by the *Nigella sativa* treatment.

Table 2
Fasting blood glucose and body weight across experimental groups

Parameter	Group I (NC)	Group II (DC)	Group III (D+NS)	Group IV (N+NS)
Initial body wt. (g)	223.2±7.1	224.8±8.4	222.6±6.9	225.3±7.3
Final body wt. (g)	287.4±10.2^a	191.6±9.7^b	248.9±11.3^c	291.7±8.6^a
FBG at baseline (mg/dL)	88.4±5.6	90.2±6.1	89.7±4.8	87.9±5.4
FBG at 72h post-STZ (mg/dL)	89.6±6.3^a	421.3±18.7^b	418.4±16.9^b	90.1±5.9^a
FBG at week 8 (mg/dL)	92.3±7.1^a	438.7±21.4^b	187.4±14.2^c	91.8±6.4^a

Note: Values are expressed as mean ± SD (n = 10). Different superscript letters (a, b, c) within the same row indicate statistically significant differences at $p < 0.001$. NC = Normal Control; DC = Diabetic Control; D+NS = Diabetic + *Nigella sativa*; N+NS = Normal + *Nigella sativa*.

Table 2 demonstrates that a single dose of streptozotocin successfully established a stable, severe state of chronic hyperglycemia and associated cachexia, both of which were significantly mitigated by the 8-week

intervention with *Nigella sativa* extract, confirming its systemic anti-diabetic activity.

Biochemical oxidative stress markers

The biochemical assays indicated that malondialdehyde (MDA) levels in pancreatic tissue homogenates were significantly higher in the Diabetic Control group (Group II) than in the Normal Control group (Group I) ($p<0.001$), as summarized in ***Table 3***. Conversely, the activities of key endogenous antioxidant enzymes, including SOD, CAT, and GPx, were considerably reduced in the diabetic rats.

The therapeutic administration of *Nigella sativa* aqueous extract to diabetic rats (Group III) led to a substantial decrease in tissue MDA concentrations accompanied by a significant recovery of all evaluated antioxidant enzyme activities ($p<0.001$ vs. Group II). Group IV (Normal + *Nigella sativa*) showed no significant differences compared to Group I across all evaluated biochemical parameters.

Table 3

Oxidative stress markers in pancreatic tissue homogenate

Parameter	Group I (NC)	Group II (DC)	Group III (D+NS)	Group IV (N+NS)
MDA (nmol/mg protein)	2.14 ± 0.18^{a}	8.73 ± 0.62^{b}	4.21 ± 0.31^{c}	2.09 ± 0.14^{a}
SOD (U/mg protein)	48.3 ± 3.4^{a}	19.6 ± 2.1^{b}	38.9 ± 2.8^{c}	49.7 ± 3.1^{a}
CAT (U/mg protein)	31.7 ± 2.6^{a}	12.4 ± 1.3^{b}	26.8 ± 2.1^{c}	32.1 ± 2.4^{a}
GPx (mU/mg protein)	42.6 ± 3.2^{a}	16.3 ± 1.7^{b}	35.4 ± 2.9^{c}	43.8 ± 3.6^{a}
Total protein (mg/mL)	6.82 ± 0.41^{a}	4.17 ± 0.33^{b}	5.93 ± 0.38^{c}	6.74 ± 0.39^{a}

Note: Values are expressed as mean ± SD (n = 10). Different superscript letters (a, b, c) within the same row indicate statistically significant differences at $p < 0.001$. MDA = Malondialdehyde; SOD = Superoxide Dismutase; CAT = Catalase; GPx = Glutathione Peroxidase.

Table 3 demonstrates that experimental diabetes triggers severe tissue-level oxidative stress and exhausts the endogenous enzymatic antioxidant defense mechanisms in the pancreas, whereas *Nigella sativa* treatment effectively mitigates lipid peroxidation and restores cellular antioxidant homeostasis.

Histological findings (H&E staining)

Group I (Normal Control)

Pancreatic sections from Group I demonstrated a normal histological architecture of both the exocrine and endocrine compartments (***Figure 1A***). The exocrine acinar cells exhibited a typical strongly basophilic basal cytoplasm, reflecting a rich rough endoplasmic reticulum network, and apical eosinophilic zymogen granules.

The islets of Langerhans were numerous, well-circumscribed, ovoid to rounded in shape, and evenly embedded within the surrounding exocrine parenchyma. Islet cells appeared polygonal with centrally located, spherical nuclei. Fine blood capillaries were clearly observable running between the cords of islet cells. Intercalated and interlobular ducts were lined by a normal simple epithelium and remained patent. No inflammatory cell infiltration or interstitial fibrosis was detected.

Group II (Diabetic Control)

Pancreatic tissue from the diabetic control group revealed severe, extensive, and diffuse histopathological alterations (***Figure 1B***). A significant reduction in both the total number and cross-sectional size of the islets of Langerhans was evident. Numerous islets displayed a shrunken, irregular morphology accompanied by extensive vacuolar degeneration of their constitutive endocrine cells.

Degenerating islet cells exhibited clear signs of nuclear death, including pyknosis, karyorrhexis, and karyolysis. Beta cells showed extensive degranulation and prominent cytoplasmic vacuolation. A marked mononuclear inflammatory cell infiltration, consisting predominantly of lymphocytes and macrophages, was apparent within and surrounding the damaged islets.

In the exocrine parenchyma, acinar cells exhibited vacuolar degeneration, loss of apical zymogen granules, and general cytoplasmic pallor. Dilation of interlobular ducts and marked periinsular and interlobular interstitial fibrosis were also observed.

Group III (Diabetic + Nigella sativa)

In the pancreatic sections of Group III, the streptozotocin-induced histopathological destruction was significantly attenuated and ameliorated (***Figure 1C***). The islets of Langerhans appeared noticeably larger and more numerous compared to Group II.

The endocrine cells showed a much lower degree of cytoplasmic vacuolation and cellular degeneration. Physiological nuclear morphology was preserved to a large extent, and the infiltration of inflammatory cells was markedly decreased.

Within the exocrine compartment, a partial restoration of zymogen granules was observed in acinar cells. Interstitial and periinsular fibrotic alterations were also significantly less pronounced than those observed in the untreated diabetic group.

Group IV (Normal + Nigella sativa)

Histological examination of Group IV pancreatic sections revealed no meaningful structural differences when compared to the normal controls in Group I (***Figure 1D***). The typical physiological architecture of both the exocrine acini and the endocrine islets of Langerhans was fully maintained.

Figure 1 structurally confirms that streptozotocin causes severe necrotic and inflammatory destruction of the endocrine pancreas along with degenerative changes in the exocrine parenchyma, whereas daily administration of *Nigella sativa* extract successfully exerts a robust tissue-level protective effect, preserving pancreatic organ integrity.

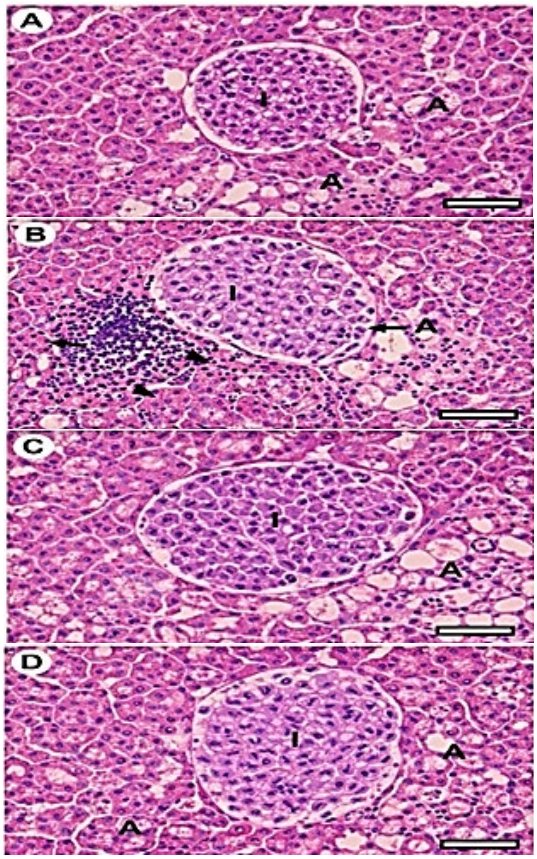

Figure 1. Hematoxylin and Eosin-stained photomicrographs of pancreatic tissue sections from the four experimental groups

Scale bar = 50 µm. H&E-stained sections of pancreatic tissue (400×). A: Normal control showing typical architecture of the islets (I) and acinar cells (A); B: Diabetic control showing a shrunken, damaged islet (I), vacuolated acinar cells (▲), and heavy mononuclear inflammatory infiltration (→); C: Diabetic+NS demonstrating significantly improved islet and acinar morphology; D: Normal+NS showing fully preserved physiological tissue architecture

Histochemical Findings

Periodic acid-Schiff (PAS) reaction

Pancreatic tissue sections from Group I (Normal Control) exhibited a strong, uniform, and intense PAS-positive (magenta-red) reaction within the apical cytoplasm of exocrine acinar cells, demonstrating an abundant accumulation of glycoprotein-rich zymogen granules (***Figure 2A***). A moderate, evenly distributed PAS-positivity was also observed within the endocrine cells of the islets of Langerhans.

Conversely, in Group II (Diabetic Control), the concentration of PAS-positive material was markedly and diffusely diminished in both the exocrine acinar parenchyma and the endocrine islet compartments (***Figure 2B***). Many areas displayed a characteristically pale or completely negative histochemical reaction, indicating a profound loss of tissue glycogen and glycoprotein content secondary to chronic hyperglycemia and oxidative cell damage.

Group III (Diabetic + *Nigella sativa*) demonstrated a highly significant restoration of PAS-positive reactivity across both the exocrine acini and endocrine islets (***Figure 2C***), with the intensity and distribution of the magenta staining closely approaching physiological levels. Group IV (Normal + *Nigella sativa*) displayed a normal, strong PAS-positive staining pattern (***Figure 2D***) that was fully comparable to that of the normal control group.

Quantitative computerized image analysis (***Table 4***) statistically confirmed a drastic, significant reduction in the percentage of PAS-positive area in Group II ($p<0.001$), followed by a substantial, significant recovery in Group III.

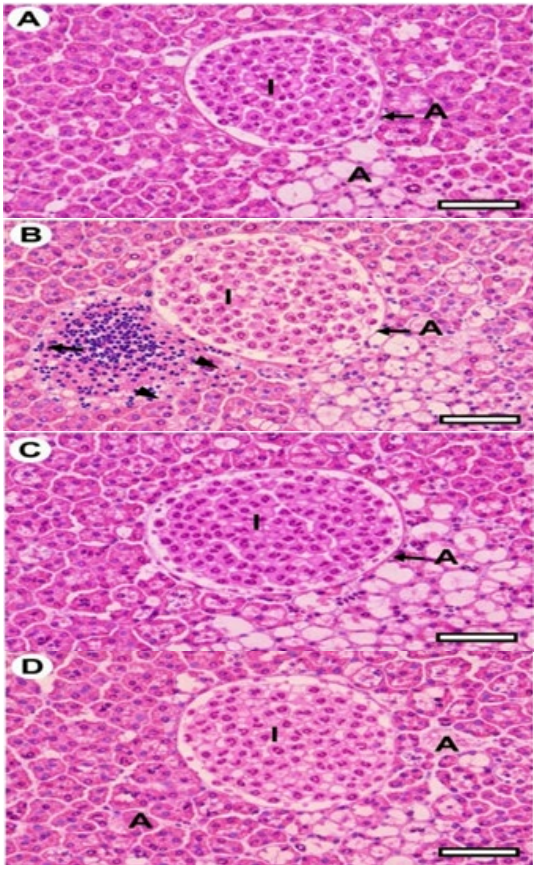

Figure 2. Periodic Acid-Schiff (PAS)-stained sections demonstrating glycoprotein and glycogen content

Scale bar = 50 µm. PAS-stained sections (400×). A: Strong, physiological PAS-positivity in exocrine acinar (A) and endocrine islet cells (I); B: Markedly reduced and depleted PAS histochemical reactivity in untreated diabetic tissue; C: Noticeably restored glycoprotein and glycogen content in the D+NS treatment group; D: Normal, intense PAS staining pattern maintained in the N+NS group

Figure 2 histochemically confirms that experimental diabetes induces a critical depletion of vital intracellular carbohydrate and glycoprotein stores within both the exocrine and endocrine compartments of the pancreas, whereas administration of *Nigella sativa* extract successfully prevents this metabolic degradation and preserves macromolecular tissue homeostasis.

Masson's trichrome staining

Histochemical evaluation using Masson's trichrome staining revealed only minimal, physiological collagen fiber deposition (stained green) restricted to the thin interlobular septa and adventitia of large blood vessels in Group I (Normal Control) (***Figure 3A***).

Conversely, Group II (Diabetic Control) demonstrated extensive, severe interstitial fibrosis with abundant, dense collagen deposition concentrated heavily in the periinsular regions, throughout the expanded interlobular septa, and tracking within the exocrine acinar

parenchymal tissue (***Figure 3B***). This pronounced fibrotic response histologically reflects the end stage of chronic tissue inflammation and cellular regenerative failure secondary to streptozotocin-induced cytotoxic damage.

Group III (Diabetic + *Nigella sativa*) exhibited a marked, highly visible reduction in parenchymal collagen deposition compared to the untreated diabetic group, with thin collagen fibers predominantly limited to the interlobular septa, indicating a significant limitation of the fibrotic process (***Figure 3C***). Group IV (Normal + *Nigella sativa*) was fully comparable to Group I, demonstrating a normal connective tissue distribution with minimal, physiological baseline collagen density (***Figure 3D***).

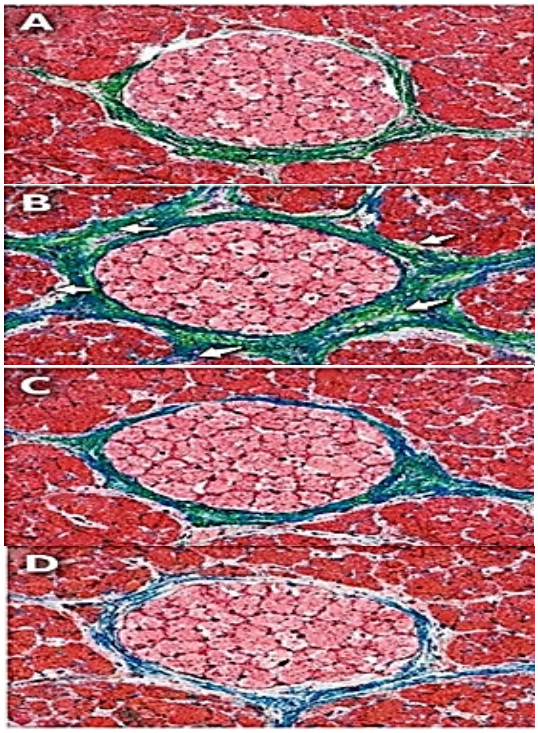

Figure 3. Masson's Trichrome-stained sections (200×)
Collagen fibers appear green; cytoplasm red; nuclei black-brown. A: Minimal, physiological baseline collagen (green) in normal tissue; B: Extensive, dense periinsular and interlobular fibrosis in diabetic rats (→); C: Significantly reduced collagen deposition and limited fibrosis in D+NS group; D: Normal, delicate connective tissue pattern

Figure 3 structurally demonstrates that experimental diabetes mellitus induces aggressive pathological remodeling characterized by excessive collagen deposition (fibrosis) in the pancreatic parenchyma, whereas therapeutic intervention with *Nigella sativa* extract successfully suppresses fibroproliferation and preserves the typical histoarchitecture of the organ.

Gomori's aldehyde fuchsin (AF) staining

Histochemical evaluation using Gomori's aldehyde fuchsin staining clearly demonstrated an abundance of deep purple-violet secretory granules (B-granules) densely packed within the cytoplasm of centrally located beta cells in the islets of Langerhans of Group I (Normal Control) (***Figure 4A***). Conversely, alpha cells situated at the periphery of the islets were counterstained light green, showing a clear physiological segregation of cell types.

In Group II (Diabetic Control), the pancreatic tissue exhibited a dramatic, widespread reduction or near-complete absence of AF-positive purple-violet granules within the shrunken islets (***Figure 4B***). This profound loss of staining reactivity histochemically confirms severe, unchecked beta-cell degranulation and functional exhaustion driven by streptozotocin toxicity.

Daily therapeutic intervention with *Nigella sativa* extract in Group III (D+NS) resulted in a significant, highly visible recovery of AF-positive insulin-containing granules within the remaining beta cells (***Figure 4C***), strongly indicating the preservation and functional restoration of the endocrine secretory apparatus. Group IV (Normal + *Nigella sativa*) displayed a perfectly normal granulation pattern (***Figure 4D***) that was fully identical to the physiological baseline observed in Group I.

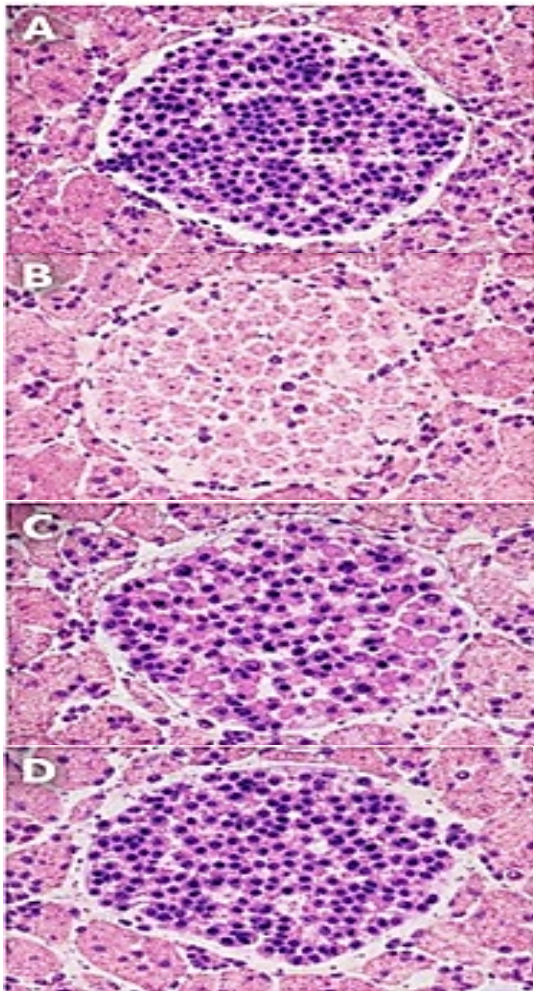

Figure 4. Gomori's Aldehyde Fuchsin (AF)-stained sections demonstrating beta-cell secretory granules (400×)
Purple-violet color indicates insulin-containing B-granules; background and alpha cells are counterstained light green. **A:** Abundant purple B-granules filling the cytoplasm of centrally located beta cells in normal tissue; **B:** Near-complete degranulation and severe loss of AF-positivity in shrunken diabetic islets; **C:** Substantial partial restoration and accumulation of B-granules in the D+NS treatment group; **D:** Normal, intense granulation pattern maintained in the N+NS group

Figure 4 histochemically confirms that experimental diabetes mellitus induces critical degranulation and functional collapse of pancreatic beta cells, whereas administration of *Nigella sativa* extract effectively protects the intracellular secretory apparatus, maintaining insulin storage capacity.

Glucagon immunoreactivity

Glucagon-expressing alpha cells in Group I (Normal Control) displayed strong, selective brown peripheral immunostaining, forming a characteristic physiological mantle around the central beta-cell core (***Figure 5***, bottom row).

In Group II (Diabetic Control), the spatial distribution of glucagon-positive cells appeared highly irregular and disorganized throughout the shrunken islets. Furthermore, due to the massive loss of central beta cells, the relative proportion of alpha cells to total islet cells appeared markedly increased, confirming alpha-cell predominance following cytotoxic damage.

Daily treatment with *Nigella sativa* extract in Group III (D+NS) effectively restored a more typical architectural distribution and balanced proportion of glucagon-positive alpha cells within the pancreatic islets. Non-diabetic rats treated with the extract (Group IV) maintained a perfectly normal physiological expression pattern identical to the baseline controls (data not visually shown).

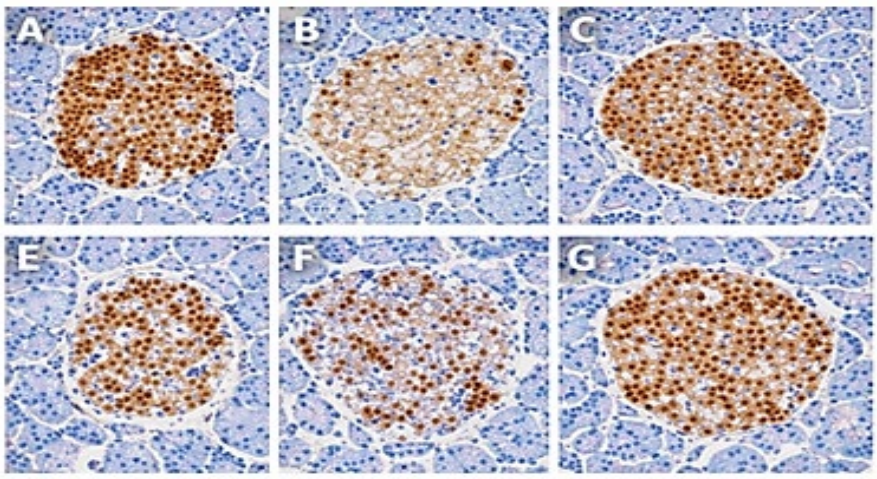

Figure 5. Immunohistochemical detection of insulin and glucagon in the islets of Langerhans (400×)
Top row (A, B, C) demonstrates anti-insulin immunoreactivity; bottom row (E, F, G) demonstrates anti-glucagon immunoreactivity. Brown color indicates positive cellular expression; blue color indicates Mayer's hematoxylin nuclear counterstain. A, E: Normal Control showing a dense central core of insulin-positive beta cells and a normal peripheral mantle of glucagon-positive alpha cells; B, F: Diabetic Control exhibiting a dramatic reduction in insulin-positive cell mass along with a highly irregular, disorganized distribution of glucagon-positive alpha cells; C, G: Diabetic + NS group demonstrating a significant, robust improvement in both insulin intensity and balanced glucagon architectural patterning

Figure 5 immunohistochemically substantiates that streptozotocin-induced diabetes causes catastrophic, selective depletion of the insulin-secreting beta-cell population with secondary disruption of alpha-cell topology, while therapeutic intervention with *Nigella sativa* extract successfully preserves functional endocrine mass and restores homeostatic bihormonal cellular distribution.

Morphometric analysis

Quantitative histomorphometric and immunohistochemical characterization across all groups confirmed the microscopic findings, with all parameters detailed in ***Table 4***. own).

Table 4
Morphometric and immunohistochemical parameters of pancreatic islets

Parameter	Group I (NC)	Group II (DC)	Group III (D+NS)	Group IV (N+NS)
No. of islets/mm²	8.4±0.7^{a}	2.1±0.3^{b}	5.8±0.6^{c}	8.6±0.8^{a}
Islet area (µm²)	14,283±863^{a}	4,761±412^{b}	10,924±741^{c}	14,512±904^{a}
Beta cell nuclear diam. (µm)	6.4±0.3^{a}	3.9±0.4^{b}	5.7±0.3^{c}	6.5±0.4^{a}
PAS-positive area (%)	68.4±4.2^{a}	21.3±2.7^{b}	51.6±3.8^{c}	70.1±4.6^{a}
Collagen area (%)	4.1±0.6^{a}	28.7±2.9^{b}	11.4±1.3^{c}	3.9±0.5^{a}
Insulin IOD (arbitrary units)	186.3±12.4^{a}	42.6±6.1^{b}	134.7±10.8^{c}	192.4±13.1^{a}
Glucagon IOD (arbitrary units)	92.1±8.3^{a}	76.4±7.6^{b}	86.9±7.1^{c}	94.3±8.7^{a}

Note: Values are expressed as mean ± SD (n = 10). Different superscript letters (a, b, c) within the same row indicate statistically significant differences at $p<0.001$. IOD = Integrated Optical Density.

Rats in the Diabetic Control group (Group II) sustained a severe fourfold reduction in islet density and a sharp threefold decrease in total islet area compared to normal controls ($p<0.001$), which closely paralleled marked beta-cell nuclear atrophy. This structural collapse was accompanied by a threefold depletion of functional PAS-positive glycoprotein reserves and a critical sevenfold expansion of pathological collagen deposition (fibrosis). From an immunohistochemical standpoint, these alterations strongly mirrored a significant fourfold decline in functional insulin expression.

Conversely, continuous therapeutic intervention with *Nigella sativa* extract in Group III (D+NS) exhibited robust regenerative potential. The 8-week treatment successfully rescued the endocrine architecture, bringing the average islet area back toward physiological levels and noticeably increasing islet density. Furthermore, the extract limited fibrotic collagen accumulation more than

twofold and drove a substantial threefold recovery in insulin optical density ($p<0.001$ relative to Group II). Non-diabetic rats treated with the extract (Group IV) maintained normal homeostatic parameters.

Table 4 statistically validates that the induction of diabetes mellitus causes a profound decline in the structural dimensions and secretory capacity of the endocrine pancreas, alongside an increase in connective tissue accumulation, whereas *Nigella sativa* administration significantly rescues islet morphology, limits fibrotic alterations, and restores functional macromolecular storage parameters.

Correlation analysis

To establish a direct relationship between biomolecular lipid peroxidation and tissue degradation, Pearson's correlation analysis was conducted across all experimental groups. The resulting correlation coefficients (r) and 95 % confidence intervals are systematically detailed in ***Table 5***.

Pearson's correlation analysis revealed a strong positive correlation between pancreatic tissue MDA levels and collagen area percentage ($r = 0.892$, $p < 0.001$), and a strong negative correlation between MDA and PAS-positive area ($r = −0.871$, $p < 0.001$), islet number ($r = −0.904$, $p < 0.001$), and insulin IOD ($r = −0.937$, $p < 0.001$). These findings confirm that the degree of oxidative stress is directly reflected in both the structural and functional histopathological changes observed in pancreatic tissue.

Table 5

Pearson's correlation coefficients between MDA and histomorphometric parameters (all groups combined)

Histomorphometric parameter	r value	95% CI	p value
Islet number per mm²	−0.904	(−0.952, −0.836)	<0.001
Islet cross-sectional area (μm²)	−0.881	(−0.934, −0.807)	<0.001
PAS-positive area (%)	−0.871	(−0.928, −0.791)	<0.001
Collagen area (%)	+0.892	(+0.826, +0.939)	<0.001
Insulin IOD	−0.937	(−0.968, −0.889)	<0.001
SOD activity	−0.914	(−0.958, −0.849)	<0.001

Note: Negative r values indicate an inverse relationship with MDA; positive r values indicate a direct relationship with MDA. CI = Confidence Interval; IOD = Integrated Optical Density; SOD = Superoxide Dismutase.

Table 5 mathematically establishes that free radical-mediated oxidative injury serves as the central upstream driver causing both the physical destruction of the islets of Langerhans and the functional impairment of the insulin-secreting machinery in experimental diabetes.

The current study provides a comprehensive histological and histochemical phenotyping of pancreatic tissue degradation induced by oxidative stress in a streptozotocin (STZ) diabetic rat model. Furthermore, it establishes a multi-parametric analysis demonstrates the cytoprotective efficacy of a *Nigella sativa* aqueous extract by correlating morphometric, histochemical, and biochemical methodologies.

Validation of the experimental diabetic model

The successful induction of a type 1-like diabetes mellitus model in Group II animals was validated by the significant elevation in fasting blood glucose (> 250 mg/dL) observed 72 hours post-STZ administration. This hyperglycemic state aligns with the well-documented cytotoxic mechanism of STZ, which selectively targets pancreatic beta cells via DNA alkylation, intracellular nitric oxide production, and subsequent poly(ADP-ribose) polymerase (PARP) activation, ultimately culminating in NAD^+ depletion and rapid cellular necrosis [7].

The parallel loss of body weight noted in the untreated diabetic rats further characterizes the severe catabolic nature of chronic insulin deficiency. In the absence of adequate insulin signaling, accelerated muscle proteolysis and adipose tissue fat mobilization occur to meet metabolic energy demands, which is consistent with established experimental literature [14].

Pancreatic oxidative stress status

The marked accumulation of malondialdehyde (MDA), an end product of lipid peroxidation, within the pancreatic tissue of Group II rats indicates the development of pronounced localized oxidative stress. This biochemical alteration was accompanied by a concurrent suppression in the activities of crucial endogenous antioxidant enzymes, including SOD, CAT, and GPx.

These findings indicate that the excessive production of reactive oxygen species (ROS) – prompted initially by STZ cytotoxicity and subsequently exacerbated by persistent hyperglycemia – completely overwhelmed the endogenous enzymatic antioxidant defense mechanisms.

These results are highly congruent with prior reports demonstrating that the beta-cell-enriched pancreatic islets of Langerhans are exceptionally susceptible to free radical-mediated damage due to their inherently low physiological expression of antioxidant enzymes [12, 13].

The significant restoration of antioxidant enzyme activities observed in Group III following the administration of *Nigella sativa* extract is consistent with the established free radical scavenging capabilities of its primary bioactive constituent, thymoquinone. Thymoquinone has been shown to drive the activation of the Nrf2/ARE signaling pathway, which directly upregulates the transcription of downstream antioxidant genes, thereby mitigating intracellular oxidative damage [2].

Histological evolution and islet pathology

The microscopic observations within the Diabetic Control group – including a significant decrease in the number and size of pancreatic islets, vacuolar destruction of beta cells, nuclear pyknosis, and mononuclear inflammatory cellular infiltration – are in strict alignment with the classical histopathological descriptions of streptozotocin-induced pancreatic injury in rodents [11, 12]. Concurrently, the exocrine alterations, characterized by acinar cell vacuolation and the depletion of apical zymogen granules, indicate that oxidative stress and persistent hyperglycemia

do not selectively target the endocrine cells but rather compromise the entire pancreatic parenchyma.

Quantitatively, Group II exhibited a much smaller average islet cross-sectional area (4,761±412 μm² in Group II vs. 14,283±863 μm² in Group I) and a drastically lower islet density (2.1±0.3 vs. 8.4±0.7 islets/mm²), which collectively reflects a profound loss of functional endocrine mass. These morphometric parameters were significantly and substantially restored in the *Nigella sativa*-treated group (Group III), indicating that the natural extract actively promotes beta-cell survival. This cytoprotection is potentially mediated by its well-documented anti-apoptotic properties, which involve downregulating caspase-3 activation and upregulating the anti-apoptotic protein Bcl-2 [1].

Histochemical alterations

The marked reduction in PAS-positive material observed in Group II can be attributed to the loss of glycoprotein-rich zymogen granules within the exocrine acinar cells, alongside the depletion of glycogen and glycoproteins within the endocrine islets. This macromolecular degradation occurs as a direct consequence of absolute insulin deficiency and the oxidative inactivation of glycogen synthase under severe diabetic conditions. Conversely, the noticeable recovery of PAS-positive reactivity in Group III is congruent with enhanced glycemic regulation and the subsequent restoration of tissue metabolic homeostasis following therapeutic intervention with *Nigella sativa* extract.

The widespread periinsular and interlobular fibrosis identified via Masson's trichrome staining in the untreated diabetic rats represents a critical finding that has not been sufficiently highlighted in prior experimental diabetes research. Fibrosis constitutes the terminal stage of chronic tissue inflammation and persistent oxidative damage, driven primarily by the activation of pancreatic stellate cells and excessive collagen synthesis under the pathological influence of upstream TGF-β1 signaling [4]. The robust anti-fibrotic efficacy demonstrated by *Nigella sativa* extract in Group III is likely due to its intrinsic anti-inflammatory action, specifically via the inhibition of the NF-κB transcription factor and the down-regulation of pro-fibrotic TGF-β1 expression [15].

Furthermore, the near-complete elimination of aldehyde fuchsin-positive granules within the islets of Group II confirms severe beta-cell degranulation and functional exhaustion in STZ-induced diabetes. The substantial recovery of these AF-positive granules in Group III provides strong histochemical evidence supporting the functional restoration of the remainder of the beta cells. This morphological rescue directly corroborates the biochemical trends (reduction of fasting blood glucose) and immunohistochemical parameters (recovery of insulin integrated optical density).

Immunohistochemical findings

Massive beta-cell loss and functional impairment were directly corroborated by the dramatic decrease in insulin immunoreactivity observed within the Diabetic Control group (IOD: 42.6 ± 6.1 in Group II vs. 186.3 ± 12.4 in Group I). The partial yet highly significant recovery quantified in Group III (IOD: 134.7 ± 10.8) indicates that *Nigella sativa* extract exerts a potent protective effect on surviving beta-cells, mitigating ongoing oxidative destruction and potentially stimulating tissue regeneration.

Prior research suggests that thymoquinone, the primary bioactive compound of the extract, may actively drive beta-cell proliferation and hypertrophy by upregulating the expression of pancreatic duodenal homeobox-1 (PDX-1) and stimulating insulin gene transcription [3]. Furthermore, this molecular rescue enhances the metabolic sensitivity of surviving endocrine tissue, effectively preserving intracellular insulin biosynthesis pathways and glucose-stimulated secretion loops under chronic stress conditions [19].

In contrast to the depleted insulin expression, glucagon immunoreactivity within the alpha cells of Group II was relatively preserved. This selective preservation, occurring alongside terminal beta-cell loss, led to a marked elevation in the intraislet glucagon-to-insulin ratio. Pathophysiologically, this bihormonal imbalance contributes significantly to the dysregulation of hepatic glucose production, accelerating glycogenolysis and gluconeogenesis, which subsequently exacerbates chronic hyperglycemia [16].

This hyperglucagonemic shift accelerates uncontrolled hepatic glucose output via enhanced gluconeogenic enzyme signaling in the liver parenchyma, which strongly supports the well-established bihormonal hypothesis of diabetes pathogenesis [20].

Nigella sativa cytoprotection mechanisms

The multi-targeted cytoprotective effects of the *Nigella sativa* aqueous extract observed in the present study can be attributed to several highly integrated, synergistic molecular mechanisms.

First, the extract exerts direct free radical scavenging activity, which is driven by its rich concentration of thymoquinone and complementary polyphenolic constituents, such as flavonoids and phenolic acids [2].

Second, it enhances endogenous enzymatic antioxidant defenses by driving the upregulation of the master cytoprotective transcription factor Nrf2 [2, 11].

Third, the extract demonstrates robust anti-inflammatory efficacy, which is achieved through the inhibition of the classical NF-κB signaling pathway and the subsequent suppression of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 [11].

Fourth, it mediates potent anti-apoptotic protection within the endocrine tissue by modulating the intracellular Bcl-2/Bax expression ratio and blocking upstream caspase cascade activation [1].

Fifth, the extract suppresses fibroproliferative remodeling via the downregulation of pro-fibrotic TGF-β1 signaling [4].

Finally, these combined activities likely create a supportive microenvironment that stimulates the proliferation and regeneration of surviving pancreatic beta cells. The complex, multi-targeted nature of these combined biochemical mechanisms directly facilitates the significant improvements quantified across all

histological, histochemical, and tissue-level biochemical parameters in this investigation.

Conclusions

The current paper has shown that streptozotocin-induced diabetes mellitus leads to extensive, multidimensional histological and histochemical damage of the pancreatic tissue ($p<0.001$). These alterations include: (i) extreme loss of islets, size, and mass of beta-cells; (ii) vacuolar degeneration and inflammatory infiltration of the islets and acinar cells; (iii) marked loss of PAS-positive glycoproteins; (iv) extensive periinsular and interlobular fibrosis; and (v) almost total beta-cell degranulation. All these microstructural changes are strongly and directly associated with the level of oxidative stress in terms of elevated tissue MDA and depleted enzyme antioxidant activities, as confirmed by Pearson's correlation analysis ($p<0.001$, $r \geq 0.87$).

Nigella sativa aqueous extract (400 g/kg/day, 8-week treatment) had significant cytoprotective effects on the diabetic pancreas, providing highly significant benefits ($p<0.001$) against all the histopathological changes detailed above and demonstrating a robust ability to restore antioxidant homeostasis. These effects are mediated by the strong antioxidant, anti-inflammatory, anti-apoptotic, and anti-fibrotic activities of the extract, which are attributed mostly to its major active constituent, thymoquinone.

These results confirm the possible clinical use of *Nigella sativa* as a complementary therapy in the treatment of diabetes mellitus and its pancreatic complications. It is advised that further research should be conducted to: (i) determine the best dose-response relationship; (ii) profile certain molecular signaling pathways; (iii) determine long-term safety; and (iv) determine synergistic activity with traditional antidiabetic agents.

DECLARATIONS

Ethical Statement

All experimental procedures and animal handling protocols in this study were strictly conducted in accordance with the international guidelines for the Care and Use of Laboratory Animals. The experimental design was formally reviewed and officially approved by the Institutional Animal Ethics Committee of the University of Tikrit (Approval No. TU-IAEC-2024-037 dated 12 February 2024). Every possible effort was proactively made to minimize animal suffering, reduce procedural distress, and optimize the total number of animals utilized throughout the investigation.

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Conflict of interest

The authors state that there is no conflict of interest.

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None.

Declaration of AI and AI-assisted technologies

The author declare that no artificial intelligence or AI-assisted technologies were used in the preparation of this manuscript.

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ORCID

R. H. Nada https://orcid.org/0009-0000-4890-3426
E. M. Mahdi https://orcid.org/0009-0002-2013-6100
S. F. H. Abas https://orcid.org/0009-0000-7423-7826
B. Kh. Hameed https://orcid.org/0000-0002-8194-6978