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Aqueous anise extract alleviated the pancreatic changes in streptozotocin-induced diabetic rat model via modulation of hyperglycemia, oxidative stress, apoptosis and autophagy: A biochemical, histological and immunohistochemical study

Running head: Effect of Anise on Diabetes Mellitus

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Abstract

Background: The present study aimed to investigate, for the first time to the best of our knowledge, the effect of aqueous anise extract on the pancreatic damage in the streptozotocin (STZ)-induced diabetic rat model with referral to some of its underlying mechanisms.

Materials and methods: Forty adult male albino rats were divided equally into four groups; control, anise extract treated (500 mg/kg orally once daily), diabetic control group (STZ 50 mg/kg once intraperitoneally) and diabetic group treated with anise extract. At the end of experiment (7weeks); body weight, blood glucose and serum amylase levels were assessed. Pancreatic tissues were subjected to biochemical, histological (light and electron microscopic), and immunohistochemical studies.

Results: The diabetic group exhibited significant decrease in body weight and increase in blood glucose and serum amylase levels. Marked degenerative changes affecting both β -cells and acinar cells of the pancreas in the form of a significant decrease in islet's perimeter,

vacuolated cytoplasm, pyknotic nuclei, depletion of zymogen granules, dilated congested blood vessels and degenerated organells were reported. Hyperglycemia induced oxidative stress with subsequent upregulation of caspase-3 and beclin-1 immunoreaction were suggested to be implicated in DM pathogenesis. Anise extract ameliorated the all examined parameters via its hypoglycemic and antioxidant properties with subsequent downregulation of apoptosis and autophagy.

Conclusions: Anise extract can be a promising agent in the control of DM for further clinical trials.

Key words: Anise, diabetes, pancreas, autophagy, apoptosis, oxidative stress, histopathology

INTRODUCTION

Diabetes mellitus (DM) is increasingly becoming a global health problem affecting even young adults. Its prevalence all around the world is expected to increase at an alarming rate annually [72,21]. About 415 million adult people suffer from DM which is expected to rise to 642 million people in 2040; and every six, a person dies due to DM [48].

Diabetes mellitus is considered as a group of metabolic disorders characterized by hyperglycemia with disturbances of carbohydrate, protein and fat metabolism resulting either from defective insulin secretion (type 1), insulin action (type 2) or both [23]. Interestingly, it is now identified that both major types of DM affect β -cell mass and insulin secretion [46]. Thus, one of the important goals in the treatment of DM is the preservation of functional pancreatic β -cell that is mandatory to sustain euglycemia [19].

Moreover, symptoms of exocrine pancreatic insufficiency have been observed in diabetic patients [37]. It is unclear whether the exocrine alteration in DM is related to the same factors that affect the β -cells causing their destruction or it is secondary to the loss of β -cell function [12].

Based on several experimental and clinical studies, it is now well accepted that DM is associated with increased production of free radicals and impaired antioxidant defenses [15]. Recently, autophagy is known to play a pivotal role in a variety of degenerative conditions including Alzheimer's disease, DM and ageing [32].

Autophagy is a complicated and highly regulated process that plays an important role in maintaining the intracellular homeostasis and surviving via degrading and recycling intracellular proteins and damaged organelles. So, it is known as a "clear out" process [54]. The autophagic pathway can be stimulated by various factors such as oxidative stress, hypoxia, infection and physical exercise [10].

A lot of antihyperglycemic drugs are available in the market nowadays but unfortunately with significant side effects [56]. Accordingly, there's increasing demands for antioxidant herbal products with anti-diabetic activity, less side effects and relatively low costs.

Pimpinella anisum L. (Anise) is an annual herb with white flowers and small green to yellowish-brown seeds. It grows in Turkey, Iran, India, Egypt and many other warm regions of the world [52]. The principal constituents of anise are volatile oil, coumarins, fatty acids, flavonoid glycosides e.g. quercetin-3-glucuronide and rutin, proteins and carbohydrates. Anise powder and aqueous extract are used as carminatives, antiseptics, diuretics, digestives and as a remedy for insomnia and constipation [40]. Anise has also been reported to have antihemolytic, anti-inflammatory [24,30], anti-cancer [34], as well as anti-ulcer [5] and anti-osteoporosis characteristics [28]. Moreover, supplementation of diabetic patients with anise was previously found to have antioxidant, antihyperglycemic and hypolipidemic effects but this was based only on biochemical studies [53,64].

In this work, we aimed to evaluate, for the first time to the best of our knowledge, the effect of aqueous anise extract on the structure of pancreas in streptozotocin- induced diabetic rats with referral to the possible underlying mechanisms through biochemical, histological and immunohistochemical studies. We hypothesized that the antioxidant and

antihyperglycemic of anise extract would be reflected as amelioration of the histopathological changes in pancreas.

MATERIALS AND METHODS

Chemicals

Streptozotocin (STZ) powder was obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). The powder was stored at -20°C . The amount needed was freshly dissolved in distilled water immediately before use [26].

Aqueous anise extract

The dry and ripe seeds of *pimpinella anisum* L. plant (Anise) were purchased from a local market, Menoufia, Egypt. These seeds were identified, and the extract was prepared by an expert taxonomist, Faculty of science, Menoufia University, Egypt. To obtain the aqueous extract, the seeds of *P. anisum* L. were ground; 100 g of the powder were immersed in 1 L of distilled water, boiled for 15 minutes and filtered through Whatman paper No.1 [9]. The filtrate was then evaporated to dryness under reduced pressure in a rotary evaporator [57].

Animals

This study was carried out on forty adult male albino rats with an average weight of 180–200 g. The animals were housed at Theodor Bilharz Research Institute Animal House, Cairo, Egypt, at room temperature $25\pm 2^{\circ}\text{C}$ with a natural lighting cycle (12 h dark/light cycle). They had free access to diet and tap water. Strict care and hygiene were provided to keep them in normal and healthy conditions. All experimental procedures were conducted with the approval of the Research Ethics Committee, Faculty of Medicine, Menoufia University, Egypt. This was in strict accordance with the requirement of National Research Council 2011

“Guide for the Care and Use of Laboratory Animals: Eighth Edition. Washington, DC: The National Academies Press.

Experimental design

After an adaptation period of one week, the rats were randomly divided into four equal groups (ten rats each):

- **Group I: control group:** received no special treatment.
- **Group II (Anise extract treated group):** received anise extract, dissolved in 2 ml distilled water, once daily, orally by a gastric tube, at a dose of 500 mg/kg [9] for seven weeks.
- **Group III (Diabetic control group):** included STZ-induced diabetic rats.
- **Group IV: (Diabetes + Anise extract group):** included STZ-induced diabetic rats which received anise extract at the same dose, route of administration and duration as group II.

Induction of diabetes

In groups III and IV, diabetes was induced by administration of a single intraperitoneal injection of streptozotocin (50 mg/kg) to overnight fasted animals [60]. After 72h of STZ injection, blood sample was withdrawn by retro-orbital puncture under light ether anesthesia and the blood glucose level was measured. Rats with blood glucose level more than 250 mg/dl were considered diabetic [55] and chosen for this study.

At the end of the experimental period (7 weeks), the rats were weighed then anesthetized by inhalation of pentobarbital overdose (200mg/kg). Blood samples were obtained from the retro-orbital venous plexus from all groups for biochemical study. A longitudinal incision of the anterior abdominal wall was made, and the pancreas was dissected out.

Evaluation methods

Biochemical study:

The collected blood was divided into two portions. One portion was used to estimate fasting blood glucose level using blood glucose test strips (Accu-Chek; Diagnostic Solutions Inc., Irvine, California, USA) and One Touch Basic Blood Glucose Meter (LifeScan Inc., Milpitas, California, USA). The other portion was left to clot then centrifuged at 3000 rpm for 15 min. The serum was separated and used for estimation of α -amylase activity by commercially available kits.

Pancreatic specimens were homogenized. Homogenates were centrifuged at $10000 \times g$ for 15 minutes at 4°C . The supernatant was used for the measurement of tissue superoxide dismutase (SOD) and catalase according to the methods of Kono [38] and Aebi [2] respectively. Meanwhile, the concentration of malondialdehyde (MDA) was measured as an index of lipid peroxidation [49].

Histological studies

Light microscopic examination. The pancreas was removed immediately from each animal and then washed with physiological saline (0.9% NaCl) for removal of the blood that might hinder the fixation process. Pancreatic specimens were fixed in 10% neutral formalin for 24 h, dehydrated in ascending grades of alcohol, cleared and embedded in paraffin. Sections of 5 μm thick were cut by microtome and subjected to hematoxylin and eosin (H&E) staining for routine histological examination.

Ultrastructural examination. Small pancreatic specimens, 1 mm each, taken from all experimental groups were prepared for transmission electron microscopic (TEM) study. The specimens were fixed in 3% glutaraldehyde in sodium phosphate buffer, pH 7.3, for 3 hours at 4°C , routinely osmicated in 1% osmium tetroxide and then processed. Semithin sections were stained with toluidine blue stain and were examined under light microscope as a preliminary step. Ultrathin sections were stained with lead citrate and uranyl acetate and were examined under transmission electron microscope.

Immunohistochemical study

Paraffin sections (5 µm thick) on poly-L-lysine coated slides were deparaffinized in xylene for 1–2 min and rehydrated in descending grades of ethanol (100%, 95%, and 70% ethanol) two changes 5 min each, then brought to distilled water for another 5 min. Endogenous peroxidase was blocked by inserting the sections in 3% hydrogen peroxide (H₂O₂). The microwave antigen retrieval procedure was performed. The sections were incubated with primary anti-insulin antibody (guinea pig polyclonal, Abcam, dilution 1:100), anti-caspase 3 antibody (rabbit polyclonal, Lab Vision, USA, dilution 1:500) and anti-beclin1 antibody (rabbit polyclonal, Abcam, dilution 1:200). After that, biotinylated goat-polyvalent secondary antibody was applied. The sections were then incubated in preformed streptavidin peroxidase and finally the prepared DAB substrate chromogen (3,3'-diaminobenzidine tetrahydrochloride) was applied and the slides were counterstained with hematoxylin to be examined under light microscope.

Quantitative assessment

Using Image J software, version K 1.45, the following parameters were measured:

- 1) The islet's perimeter (µm).
- 2) The area % of insulin, caspase-3 and beclin 1 immunoreaction.

For each parameter, five non-overlapping fields (40 x) for every specimen (from five different rats/each group) were randomly taken using a Leica DML B2/11888111 microscope equipped with a Leica DFC450 camera.

Statistical analysis

The data were collected, tabulated and analyzed by SPSS (statistical package for social science) version 23.0 on IBM compatible computer (SPSS Inc., Chicago, IL, USA). Results were expressed as mean ± SD. The significance differences between groups were evaluated using one way-ANOVA followed by post hoc Bonferroni test. A p value of < 0.05 was considered statistically significant.

RESULTS

There was no a significant difference in the all examined parameters between the control and anise extract treated groups.

Body weight results

At the end of the experiment, there was a significant decrease ($P<0.001$) in the body weight in the diabetic group (130.64 ± 4.29 vs 248.15 ± 5.22) compared to the control group. On the other hand, the diabetic group treated with anise extract showed a significant increase ($P<0.001$) in its weight (205.06 ± 2.91 vs 130.64 ± 4.29) compared to the diabetic control group (Fig. 1a).

Biochemical results

There was a significant increase ($P<0.001$) in the blood glucose level in the diabetic group (396.78 ± 6.15 vs 96.77 ± 1.34) compared to the control group. This level was significantly decreased ($P<0.001$) in the diabetic group treated with anise extract (195.46 ± 5.09 vs 396.78 ± 6.15) when compared to the diabetic control group (Fig. 1a).

Regarding the serum amylase level, a significant increase ($P<0.001$) in its level was noted in the diabetic group (244.36 ± 4.69 vs 99.08 ± 2.80) compared to the control group, while the diabetic group treated with anise extract exhibited a significant decrease ($P<0.001$) in the serum amylase level (125.81 ± 3.60 vs 244.36 ± 4.69) compared to the diabetic control group (Fig. 1a).

The assessment of MDA, SOD and catalase levels in the pancreatic homogenates revealed a significant increase ($P<0.001$) in MDA level (4.88 ± 0.59 vs 1.57 ± 0.35) and a significant decrease ($P<0.001$) in SOD and catalase levels in the diabetic group (11.73 ± 0.90 vs 23.89 ± 0.87 - 7.10 ± 0.69 vs 13.20 ± 0.83 respectively) compared to the control group. Indeed,

the diabetic group treated with anise extract displayed a significant decrease ($P<0.001$) in the MDA level (2.59 ± 0.27 vs 4.88 ± 0.59) and a significant increase ($P<0.001$) in SOD and catalase levels (18.32 ± 0.84 vs 11.73 ± 0.90 , 10.60 ± 0.57 vs 7.10 ± 0.69 respectively) when compared to the diabetic control group (Fig. 1b).

Histological results

Light microscopic study. Hematoxylin and Eosin (H&E) stained pancreatic sections from the control group showed normal histological architecture. The endocrine part showed pale stained islets of Langerhans scattered between the acini, most of its cells are centrally located β -cells with rounded nuclei (Fig. 2a). The diabetic group exhibited marked degenerative changes with apparent decrease in the islet's size. Loss of many cells of islets of Langerhans was a prominent feature. Marked cytoplasmic vacuolations were observed (Fig. 2b). On contrast, the diabetic group treated with anise extract showed apparent increase in islet's size with marvelous amelioration of most of degenerative changes except for presence of mildly vacuolated β -cells (Fig. 2c). Statistically, there was a significant decrease ($P<0.001$) of the islet's perimeter in the diabetic group (148.24 ± 2.80 vs 210.51 ± 2.37) compared to the control group. The diabetic group treated with anise extract showed a significant increase ($P<0.001$) in islet's perimeter (196.42 ± 2.85 vs 148.24 ± 2.80) compared to the diabetic control group (Fig. 2d)

The exocrine portion of the control group displayed highly packed acini within the pancreatic lobules that were separated by narrow interlobular septa. Each acinar cell had a rounded basal nucleus and apical acidophilic zymogen granules (Fig. 3a). However, the diabetic group revealed marked separation between the pancreatic lobules, congested dilated blood vessels, extravasation and intense inflammatory infiltration. Most of acinar cells showed pyknotic nuclei with cytoplasmic vacuolation and decreased zymogen granules. (Fig. 3b-e). On contrary, the diabetic group treated with anise extract showed restoration of the normal architecture except for the presence of mild separation of pancreatic acinar lobules and a few cells with cytoplasmic vacuolation (Fig. 3f).

Transmission electron microscopic (TEM) study. Transmission electron microscopic examination of the β -cells of rats' pancreas of the different studied groups was done. The control group revealed normal architecture of the islets of Langerhans formed mainly of β -cells; each with an euchromatic nucleus, well developed rough endoplasmic reticulum (rER) and mitochondria in addition to many electron dense secretory granules surrounded by lucent halos (Fig 4a). On the other hand, the diabetic group exhibited distortion of the architecture of β -cells. Some nuclei showed chromatolysis and others were pyknotic. Intense cytoplasmic rarefaction and marked vacuolations as well as degenerated mitochondria, cystic dilatation of the rER and dilated Golgi were obviously noted (Fig 4b-c). Diabetic group treated with anise extract displayed more or less normal architecture of the β -cells. However, slightly condensed chromatin, few vacuoles, dilated Golgi and few slightly degenerated mitochondria were encountered (Fig 4d)

Transmission electron microscopic study of the acini of rats' pancreas was examined. The control group showed acinar cells each with euchromatic nucleus, well developed organelles as mitochondria and rER. Numerous electron dense secretory granules (zymogen granules) at the apical part were noted (Fig 5a). The diabetic group revealed marked acinar changes with widening of the intercellular spaces. Nuclear changes in the form of peripheral chromatin condensation, irregularity of the nuclear membrane with perinuclear space were observed. Some cells exhibited shrunken and pyknotic nuclei. In addition, swollen degenerated mitochondria with disintegrated cisternae, dilated rER, decreased electron density and depletion of zymogen granules and large cytoplasmic vacuolations were noted. Vacuoles contained cellular debris mostly autophagic vacuoles were also seen (Fig 5b-e). On contrast, the diabetic group treated with anise extract exhibited restoration of the normal pancreatic architecture except for some scattered few small vacuolations and mildly degenerated mitochondria and slightly condensed chromatin (Fig 5f).

Immunohistochemical results

Immunohistochemically, there was a significant decrease ($P<0.001$) in insulin immunoreaction in the diabetic group (5.10 ± 0.57 vs 23.28 ± 1.23) compared to the control one. Moreover, diabetic group treated with anise extract showed a significant increase ($P<0.001$) in insulin immunoreaction (17.69 ± 0.81 vs 5.10 ± 0.57) compared to the diabetic control group (Fig. 6a-c, j).

Regarding the caspase-3, a significant increase ($P<0.001$) in its immunoreaction was noted in the diabetic group (52.96 ± 2.32 vs 1.07 ± 0.11) compared to the control group. On the other hand, there was a significant decrease ($P<0.001$) in its immunoreaction in the diabetic group treated with anise extract (22.34 ± 1.27 vs 52.96 ± 2.32) compared to the diabetic control group (Fig. 6d-f, j).

Moreover, there was a significant increase ($P<0.001$) in beclin-1 immunoreaction in the diabetic group (46.85 ± 1.30 vs 30.31 ± 0.86) compared to the control group. The diabetic group treated with anise extract exhibited a significant decrease ($P<0.001$) in its immunoreaction (31.55 ± 1.05 vs 46.85 ± 1.30) compared to the diabetic control group (Fig. 6g-I, j).

DISCUSSION

Currently, diabetes mellitus (DM) is one of the major health concerns globally [6]. Unfortunately, uncontrolled hyperglycemic condition for a long period can cause end-organ damage with high rate of morbidity and even mortality [42].

Although several drugs, either insulin or oral hypoglycemic drugs, are used in attempt to control DM, perfect control is rarely achieved. Moreover, these drugs have many side effects as hypoglycemia at higher doses, hepatic affection, neurological disturbance and digestive disorders. So, searching for new medications with safe and effective properties for DM control is highly important. Recently, the use of medical plants as alternative remedies is encouraged for the treatment of several disorders including DM [16,63].

Therefore, the present study aimed, for the first time to the best of our knowledge, to investigate the role of aqueous anise extract in ameliorating the biochemical and histological alterations in STZ diabetic rat model with referral to some of its underlying mechanisms.

Streptozotocin is a well-known diabetogenic agent with longer half-life and sustained hyperglycemia for longer duration than other diabetogenic agents [69]. A single high dose STZ produced a rapid ablation of β -cells and hence used for induction of type 1 DM [67] as confirmed, in this study, by the significant increase in blood glucose level and significant downregulation of insulin immunoreaction as well as by the degenerative changes of β -cells observed in the diabetic control group. It specifically destroys β -cells via alkylation and thus fragmentation of deoxyribonucleic acid (DNA) [14]. This model presents a chronic pathological pattern like human as clarified by Tourreal et al. [70]; Srinivasan et al. [68]. So, the commonly used chemical induced models in the field of diabetes researches for assessing the mechanism of DM, searching for potential therapies for this disease, and evaluating treatment options are the STZ induced models [75,33].

Increased glucose concentration leads to free radical production and depletion of antioxidant defenses producing a state of oxidative stress that in turn produces more β -cells destruction [44]. The dose of aqueous aniseed extract used in the study, 500mg/kg, was proved previously to have antioxidant properties [9]. According to the toxicity classification, anise is particularly non-toxic at this dose because its LD₅₀ value is more than 5 g/kg [29]. Furthermore, aqueous anise extract is preferred than ethanolic one as it had much more antioxidant capacity as reported by Gulcin et al. [24].

In the present study, anise extract exerted a significant hypoglycemic effect compared to the diabetic control group. This is mostly attributed to its ability to increase the insulin secretion as confirmed by the significant upregulation of insulin immunoreaction. previous researches postulated that some of phenolic compounds in anise extract interfere with the glucose absorption [51], facilitate peripheral tissue utilization of glucose via an insulin dependent glucose transporter [17] and restore insulin sensitivity [31]. This agreed with Shobha and Andallu [64] who investigated the hypoglycemic effect of anise extract in vitro. They

attributed its hypoglycemic effect to its antioxidant property, as established in our study by the significant decrease in MDA and increase of catalase and SOD by anise extract supplementation.

The diabetic group treated with anise extract in the current study showed a significant increase in body weight compared to the diabetic control group. This may be attributed to its hypoglycemic properties preventing hyperglycemia induced protein loss. Moreover, Bekara et al. [9] referred the role of anise in body weight gain to its bioactive compounds that have stimulant effect on the digestive system such as anethole, eugenol, anisaldehyde, estragol and methylchavicol. Furthermore, anise is rich in nutritive compounds that have a positive effect on body weight gain like: proteins 18%, fatty oil 8-23%, essential oil 2-7%, sugars 3-5% and crude fiber 12-15% [8,9].

In this research, β -cells and exocrine acini of pancreas were affected in the diabetic group. The islets of Langerhans in the diabetic group, in this study, showed a significant decrease in their perimeter in addition to marked distortion in the β -cell architecture in the form of presence of pyknotic nuclei, mostly apoptotic as proved by the significant upregulation of caspase-3, chromatolysis, cytoplasmic rarefaction and vacuolation in addition to degenerated mitochondria, dilated Golgi and cystic dilatation of the rER. Moreover, the exocrine portion demonstrated marked separation of the pancreatic lobules, dilated congested blood vessel, darkly stained pyknotic acinar nuclei and vacuolations. Wide intercellular spaces, swollen degenerated mitochondria, dilated rER, decreased electron density and depletion of zymogen granules were also noted. These findings were in consistence with previous studies that examined the histological pancreatic changes in diabetic models [1,4,22,47,66].

Although the antioxidant and hypoglycemic properties of anise extract was studied previously at the biochemical level [64], the present study correlate the biochemical results with histological and ultrastructure changes in diabetic rat model and investigate the role of anise extract on DM induced apoptosis and autophagy in the pancreatic tissue.

The diabetic group treated with anise extract presented with a significant improvement of the structure of β -cells, with a significant increase of insulin immunoreaction, and the pancreatic acini, with a significant decrease of amylase as compared to the diabetic control group.

This beneficial effect of anise extract could be explained by considering the pathophysiology of DM. Oxidative stress plays a crucial role in the pathogenesis of development and progression of DM [59]. This was confirmed in this research by the significant decrease of antioxidant enzymes (SOD and catalase) and increase in the level of MDA, a marker for lipid peroxidation, and this was in line with Sifuentes-Franco et al. [65] who postulated that the increased production of oxidative stress as a result of the persistent hyperglycemic state is capable of producing oxidative damage to the macromolecules (lipids, carbohydrates, proteins, and nucleic acids).

Previous studies referred the histological changes observed in the diabetic pancreas to the reactive oxygen species (ROS) generation. Bogolepov [11] considered the vacuolation as one of the structural indications of permeability disorders of the membranes that could be caused by ROS mediated formation of lipid peroxides as mentioned by Halliwell and Chirico [27]. In-turn, the disturbed plasma membrane integrity, and subsequent edema could disrupt the intercellular junctions causing wide intercellular spaces [13].

As a result of increasing lipid peroxidation, lipid-containing membranes as Golgi and rER membranes were dilated as stated by El-Kordy and Alshahrani [20]. Moreover, Schönthal [61] stated that rER dilatation represented a well-documented ultrastructural response to ER stress that led to accumulation of unfolded, misfolded, insoluble or damaged proteins that might damage cellular functions and pose a threat to the cell survival. In addition, some studies referred the mitochondrial degeneration to the increased glucose concentration that overwhelms the mitochondrial electron transport chain by increasing the oxidative phosphorylation and generation of ROS [39].

From all the above, the beneficial effect of anise extract can be attributed to its hypoglycemic and antioxidant properties as nearly all the histological alterations in the diabetic control group was linked primarily to the oxidative stress accompanied hyperglycemic state.

Moreover, anise extract exerted a marked improvement in the exocrine portion of pancreas mostly due to its ability to secrete insulin and hence preservation of acinar cells with significant decrease in serum amylase level compared to the diabetic control group. This was in line with Williams et al. [73] who referred the exocrine affection to loss of insulinotropic effects on acinar cells and considered this factor as a primary cause for exocrine affection in case of DM and with Akpan et al. [3] who referred increased amylase level in the diabetics to its leakage from the broken acini.

In addition to the role of ROS in the pathogenesis of DM [59], recent studies suggested a pivotal role of autophagy in DM [10]. Moreover, the connection between autophagy and apoptosis or other forms of cell death is a burgeoning area of research [25]. In accordance with this, a significant upregulation of beclin1, an autophagy regulator marker, and caspase3, an apoptotic marker, was observed immunohistochemically in the pancreas of the diabetic rats in the present study indicating the role of autophagy and apoptosis in DM. This may be attributed to the oxidative stress associated with the hyperglycemic state.

This was in line with previous studies that revealed upregulation of caspase-3 [43,45] in DM and they referred the activation of apoptotic pathway to the state of oxidative stress. Indeed, upregulation of beclin 1 in the diabetic group was in line with previous studies who demonstrated an elevated level of beclin1 within the pancreatic tissues of diabetic rat using enzyme-linked immunosorbent assay [50] and in the diabetic retinopathy [76]. This may be an adaptive process to protect organisms during periods of enhanced cellular distress as clarified by Kang et al. [35]. Okasha et al. [50] demonstrated that DM leads to imbalances in the antioxidant capacity within the cell resulting in oxidative/nitrosative stress and suggested an intimate relationship between autophagy and ROS production as major pathogenic mechanisms of DM.

In this research, the diabetic group treated with anise extract showed a significant downregulation of beclin1 and caspase3 compared to the diabetic control group. This was attributed to its antioxidant properties. Although the molecular connections between autophagy and apoptosis are complex and still poorly understood [25], the previous

researchers [62,18,71] clarified that excessive autophagy leads to apoptotic cell death and that ROS induce both autophagy and apoptosis simultaneously.

Hence the anise extract exerted its beneficial role via its hypoglycemic and antioxidant effects. This was consistent with Kucukkurt et al. [41] who clarified that supplementation of anise affects positively the antioxidant defense. The antioxidant activity of anise extract was studied previously [7,58]. They attributed its antioxidant properties to its high content of phenolic compounds. Kesharwani et al. [36]; Wintola and Afolayan [74] clarified that phenolic compounds are hydrogen donors so, they can scavenge free radicals and reduce oxidative damage.

From the results of this work, it could be concluded that aqueous anise extract alleviated the pancreatic damage induced in STZ diabetic rat model via modulation of insulin secretion, oxidative stress, autophagy and apoptosis. Further studies are needed to throw more light on the effect of anise extract on different diabetic complications and to investigate the effect of different doses of anise extract on DM.

Work was conducted in: Theodor Bilharz Research Institute Animal House, Cairo, Egypt; Faculty of Medicine, Menoufia University, Menoufia, Egypt; Faculty of Agriculture, Mansoura University, Mansoura, Egypt.

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Figure 1. a): A histogram showing a significant decrease in the body weight and increase in the blood glucose level and serum amylase in the diabetic group compared to the control group (*P<0.001). These values are significantly improved in the diabetic group treated with anise extract compared to the diabetic control group (o P<0.001). b): A histogram revealing a significant increase in the pancreatic MDA level and decrease of SOD and catalase compared to the control group (*P<0.001) that was significantly alleviated in diabetic group treated with anise extract (o P<0.001 compared to the diabetic control group).

Figure 2. (a-c): Representative photomicrographs of H&E stained sections of rat pancreas in the different experimental groups. a): Islet's of Langerhans of the control group showing centrally placed normal β -cells (B). b): STZ induced diabetic group showing apparent reduction in the islet size. Most of its β -cells are lost and marked vacuolations (V) are seen. c): Diabetic group treated with anise extract revealing regaining of nearly normal β -cells

except for mild vacuolation (V) (H&E, scale bar=20, 40x). d): A histogram showing a significant decrease in the islet's perimeter in the diabetic group compared to the control group (*P<0.001) as well as a significant increase in its perimeter in the diabetic group treated with anise extract compared to the diabetic untreated group (o P<0.001).

Figure 3. Representative micrographs of H&E stained sections showing exocrine portion of the pancreas of the different experimental groups. a): control group showing pancreatic lobules separated by interlobular septa (asterisk). Each lobule consists of acini (A) with rounded nuclei (n) and supranuclear zone contains acidophilic zymogen granules (Z). b-e): STZ induced diabetic group showing many degenerative changes in the form of marked separation between the pancreatic lobules (asterisk), darkly stained pyknotic acinar nuclei (arrow), cytoplasmic vacuolations (V), decrease in zymogen granules (bent arrow), dilated congested blood vessel (BV), extravasation (Ex) and accumulation of inflammatory infiltrates (double arrows). f): diabetic group treated with anise extract showing restoration of the nearly normal lobular architecture except for mild separation between the pancreatic lobules and a few cytoplasmic vacuolation (V). (H&E, scale bar=20µm, 40x)

Figure 4. Electron photographs of part of rats' islets of Langerhans of the different experimental groups. a): control group showing normal architecture of the β-cell with euchromatic nucleus (N), well developed mitochondria (M), rough endoplasmic reticulum (arrow) and electron dense secretory granules surrounded by lucent halos (thin arrow). b-c): STZ induced diabetic group showing distortion of the architecture with marked degenerative changes of β-cells in the form of nuclear chromatolysis (N), electron dense pyknotic nuclei (P), cytoplasmic rarefaction and marked vacuolation (V), degenerated mitochondria (M), cystic dilatation of the rough endoplasmic reticulum (arrow) and dilated Golgi (G). d): diabetic group treated with anise extract revealing a more or less normal beta cell except for slight chromatin condensation (N), few small vacuoles (V), dilated Golgi apparatus (G) and few slightly degenerated mitochondria (M). (TEM X 17500, Scale bar= 1µm)

Figure 5. Electron micrographs of rat exocrine pancreas of the different experimental groups. a): control group showing acinar cells with euchromatic nuclei (N), well developed mitochondria (M), rough endoplasmic reticulum (R) and numerous electron dense secretory granules (arrow) at the apical part. b-e): STZ induced diabetic group revealing marked acinar changes represented by marked widening of the intercellular spaces (arrow head), nuclear changes in the form of peripheral chromatin condensation (N) with irregularity of the nuclear membrane and perinuclear space (double arrows). Some nuclei are shrunken (S) and pyknotic (P). Swollen degenerated mitochondria (M), dilated rough endoplasmic reticulum (R), decreased electron density and depletion of zymogen granules (thin arrow) and large cytoplasmic vacuolation (V) compressing the nuclear membrane are seen. Vacuoles with cellular debris mostly autophagic vacuoles (thick arrows) are also noted. f): diabetic group treated with anise extract showing restoration of the normal pancreatic architecture except for slight chromatin condensation (N), few small vacuoles (V) and mild mitochondrial lysis (M). (TEM x 12000, Scale bar= 2 μ m)

Figure 6. (a-i) Representative micrographs of immuno-stained pancreatic sections from the different studied groups showing decrease of insulin and increase of caspase 3 and beclin 1 positive immunoreaction in the diabetic group compared to the control group. Diabetic group treated with anise extract reveals increase of insulin and decrease of caspase 3 and beclin 1 immunoreaction compared to the diabetic untreated group. Inserts indicates the positive immunoreaction. (scale bar=20 μ m, 40x). j): A histogram showing a significant decrease of insulin and increase of caspase 3 and beclin 1 area % immunoreaction in the diabetic group compared to the control group (* P<0.001). The diabetic group treated with anise extract shows a significant increase of insulin and decrease of caspase 3 and beclin 1 area % immunoreaction compared to the diabetic group (o P<0.001).

Fig 1

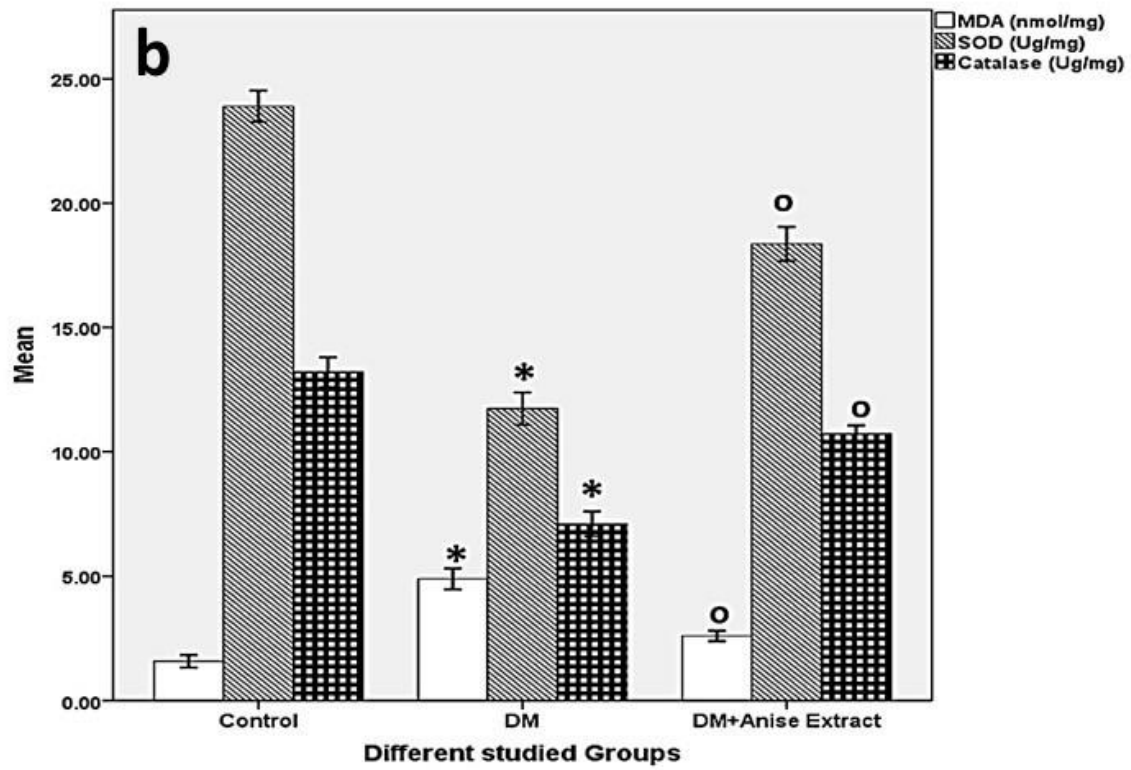
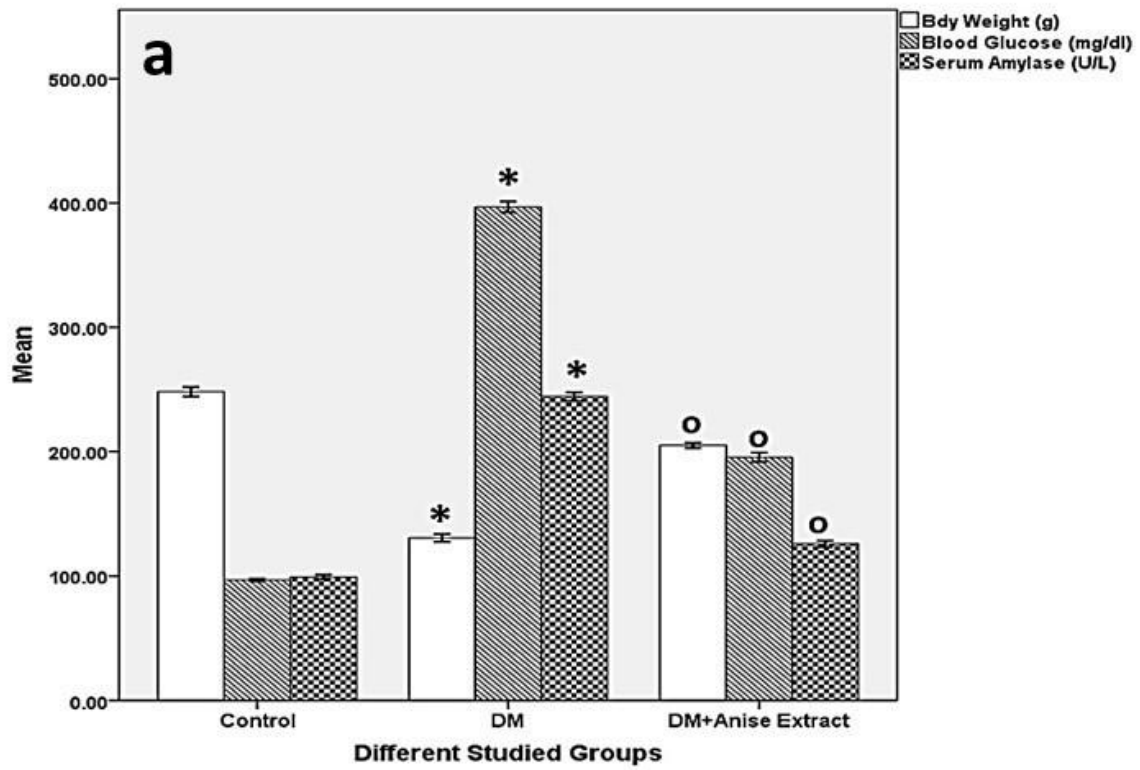


Fig 2

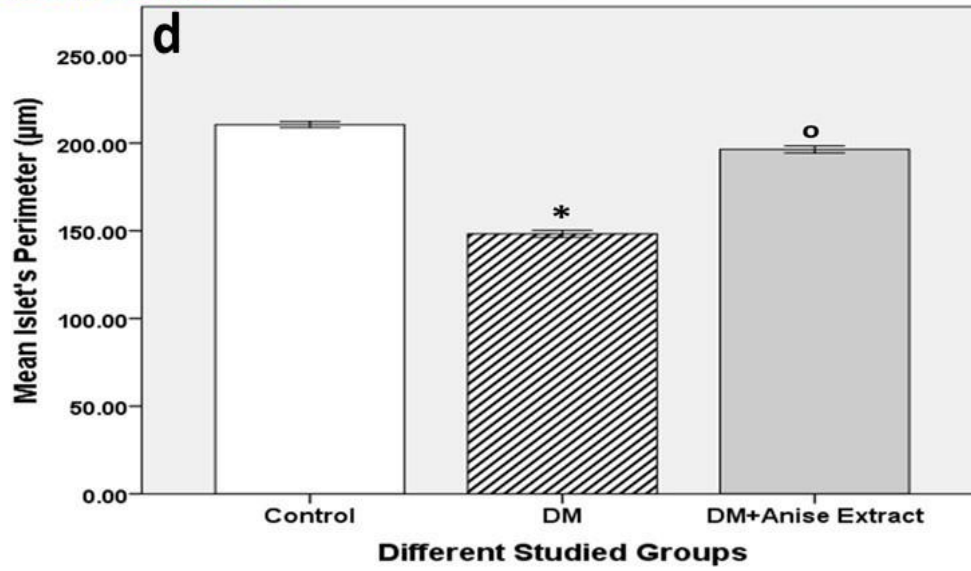
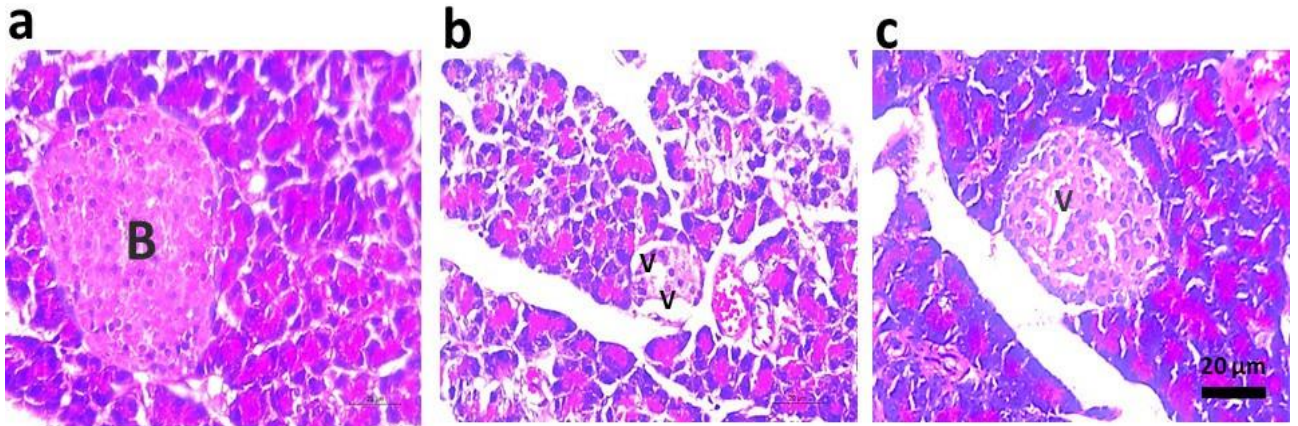


Fig 3

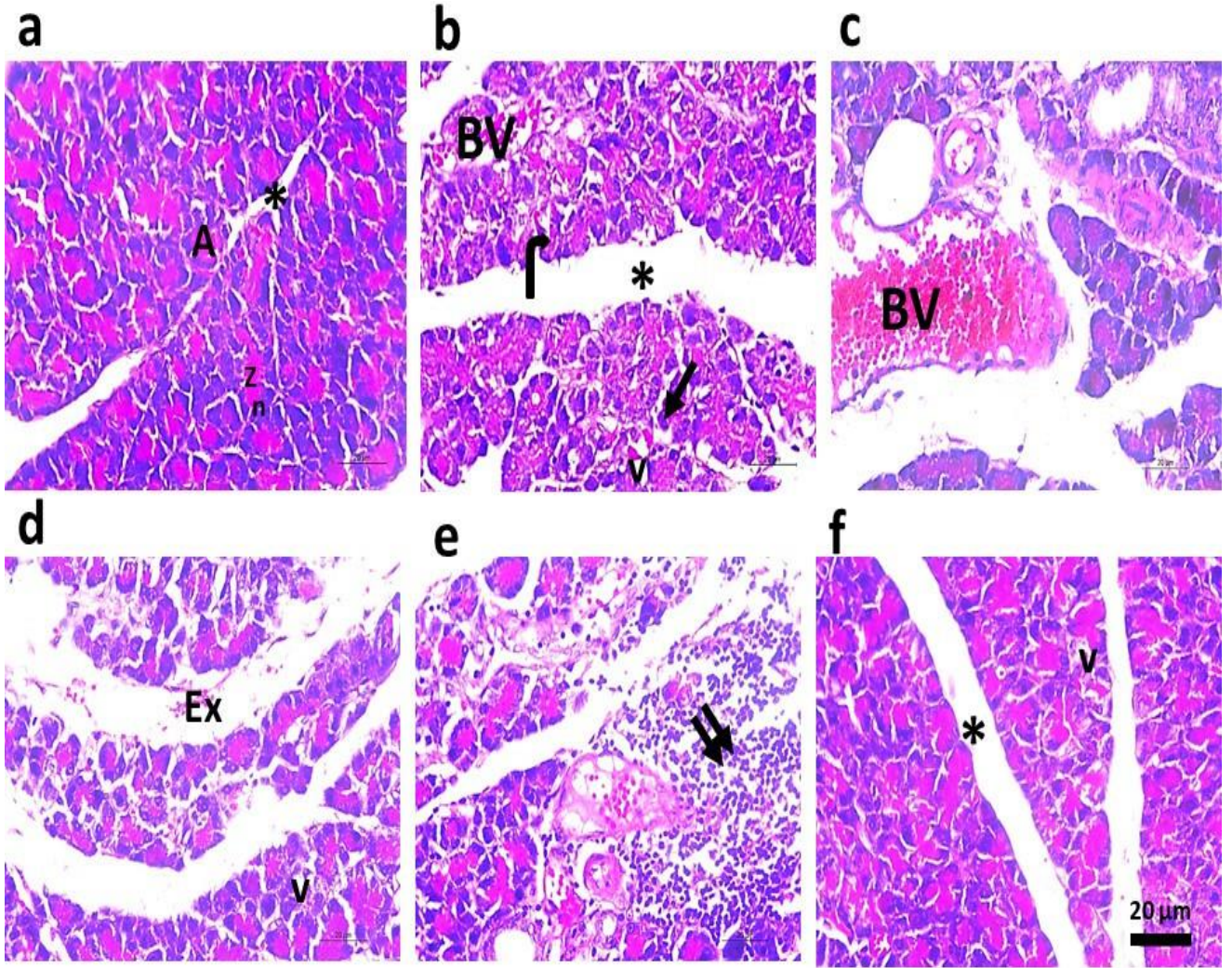


Fig 4

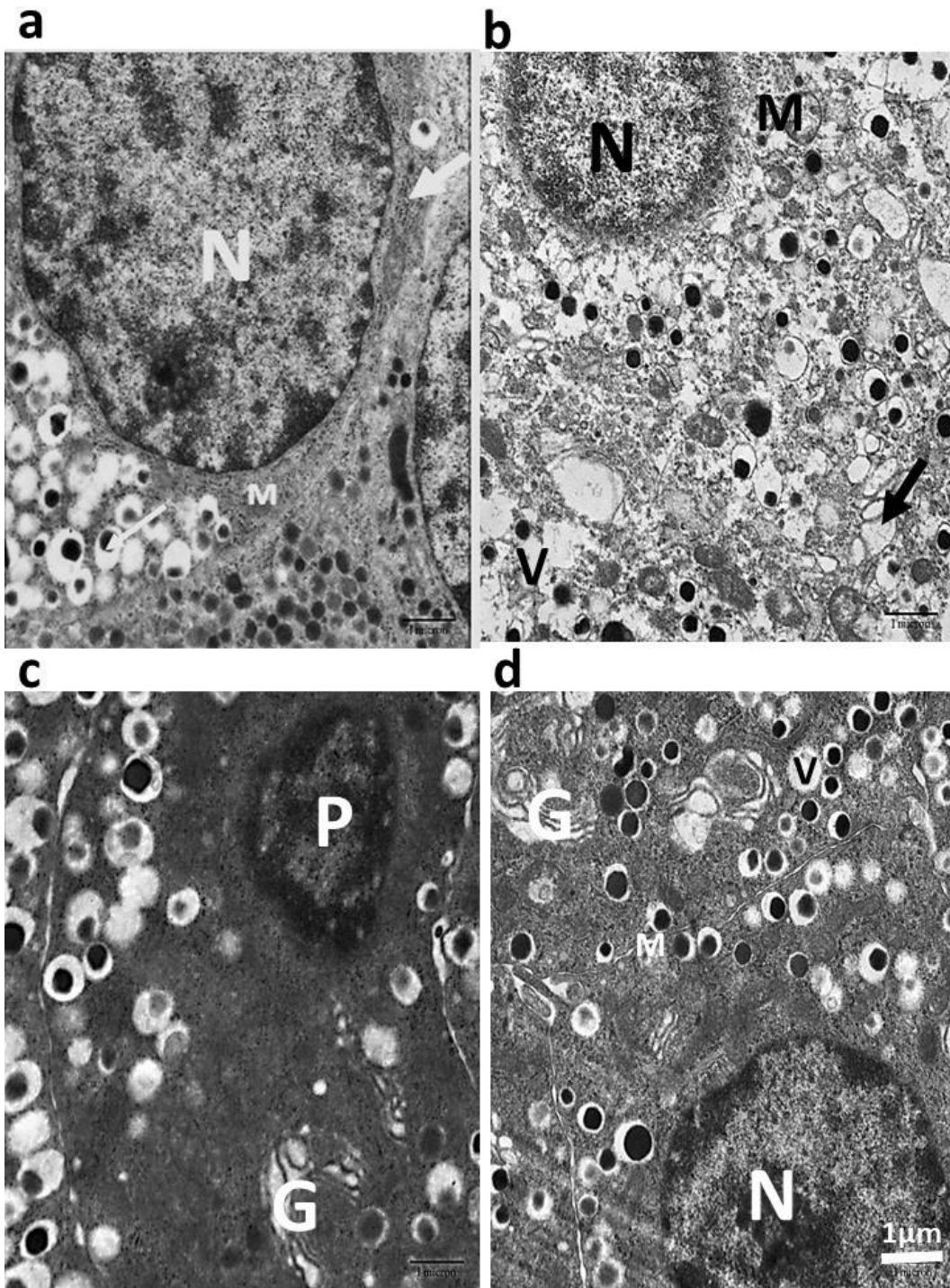


Fig 5

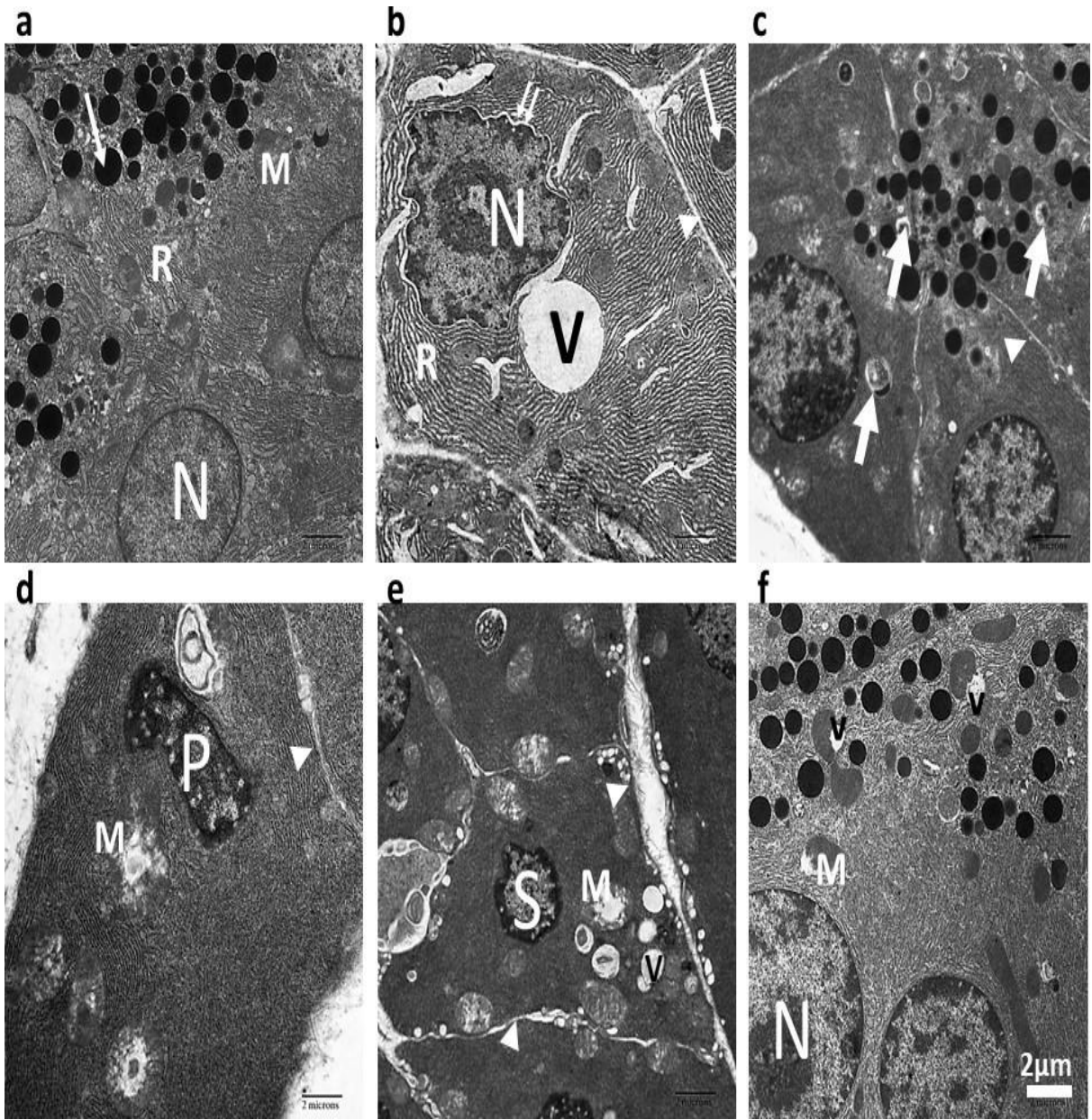


Fig 6

