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Ameliorative effect of Myristica fragrans (nutmeg) extract on oxidative status and histology of pancreas in alloxan induced diabetic rats

Running head: Effect of nutmeg on pancreas histology

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Abstract

Background: Many traditional treatments have been recommended in the alternative system of medicine for the treatment of diabetes mellitus. The aim of this study was to assess oxidative stress and histological changes in the pancreas of alloxan-induced diabetic rats following Myristica fragrans seed (nutmeg) extract treatment.

Materials and methods: Forty-eight male Wistar rats weighing 200-250 g were randomly divided into six groups of eight rats each. Group I, non-diabetic rats; group II, diabetic rats;

groups III, IV and V, diabetic rats given orally nutmeg extract at levels of 50, 100 and 200 mg/kg, respectively; and group VI, diabetic rats given orally metformin (100 mg/kg). The experiment lasted for 28 days.

Results: Data showed that nutmeg extract (100 and 200 mg/kg) significantly decreased the blood glucose levels and increased the levels of serum insulin in diabetic rats. Administration of nutmeg extract to diabetic rats reduced oxidative stress and improved the antioxidant activities in pancreatic tissue. Histopathologic results of treated groups revealed marked improvement in the morphology of the pancreas compared with the control diabetic group. In addition, number of pancreatic islets and percent of β-cells increased significantly in these groups in comparison with diabetic untreated group.

Conclusions: These results suggest that nutmeg extract has potent antidiabetic and β-cell protection activities in alloxan induced diabetic rats, possibly via its antioxidant properties.

Key words: blood glucose, Myristica fragrans, regeneration, β-cells, oxidative stress

INTRODUCTION

Diabetes mellitus is an endocrine metabolic disease with many side effects. It is characterized by persistent hyperglycemia due to inadequate insulin production or insulin activity [1]. Oxidative stress plays an important role in the onset and progression of diabetes mellitus and its related complications. Hyperglycemia in diabetes mellitus increases oxidative stress, which could be a consequence of either increased free radical production or reduced antioxidant defenses [2]. Antioxidant enzymes, mainly superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and other antioxidants such as reduced glutathione (GSH) provide an important assistance in scavenging the free radicals and protect cells against oxidative damage [3].

Based on the role of oxidative stress in the development of diabetes and its complications, numerous studies were performed in order to characterize the cytoprotective or therapeutic benefit of antioxidants and to promote new class of antidiabetic drugs,

phytochemicals and antioxidant (vitamin) supplements [4]. There are new generation anti-diabetic drugs such as Linagliptin (dipeptidyl peptidase-4 inhibitor) and exendin-4 (glucagon-like peptide 1 analog) that reduce blood glucose levels with advantages of their neutral effect on body weight and low risk of the occurrence of hypoglycemia. Besides their potent effects on glycemic control, results of studies in animal models revealed their antioxidant properties [5, 6]. Zeng et al. [7] published evidence for a protective effect of exendin-4 on oxidative stress-induced retinal cell death in streptozotocin-diabetic rats. Linagliptin has been shown to have neuroprotective properties in stroke diabetic model [8], and to ameliorate inflammation and oxidative stress in animal models of type 2 diabetes [9]. However, there has been increasing interest regarding the role and use of natural antioxidants in the patient with diabetes. Recently herbal medicines are getting more importance in the treatment of diabetes as they are free from side effects and less expensive when compared to synthetic hypoglycemic agents [10]. The role of some medicinal plants in the treatment of diabetes have been attributed to their antioxidant activity [4].

Myristica fragrans belongs to the family Myristicaceae, is a dioecious or occasionally monoecious evergreen, aromatic tree [11]. Nutmeg is the actual seed of M. fragrans, while mace is the dried lacy reddish covering on the seed. Myristica fragrans is used in traditional medicine as a stomachic, carminative, aphrodisiac and anti-diarrheal agent [12, 13]. In the recent literature, M. fragrans has been investigated for its hepatoprotective [14], antitumor, and anti-inflammatory [15] activities. The cholesterol lowering, hypolipidemic and hypoglycemic effects of M. fragrans have also been reported [11, 16]. M. fragrans seed (nutmeg) has strong antioxidant properties and this correlates well with the total phenolic content [17]. Present study was conducted to investigate the effect of nutmeg extract on oxidative status and histopathology of pancreas in alloxan-induced type 1 diabetic rats.

MATERIALS AND METHODS

Experimental animals

Forty-eight healthy male Wistar rats (200–250 g; averaging 16 weeks old) were utilized in this study. The animals were acclimatized for 7 days prior to the commencement of the study. Before and during the experiment, the animals were maintained under

standard conditions, with a temperature of 22 ± 2˚C, a regular 12/12 hour light-dark cycle. The rats were allowed free access to standard pellet and tap water ad libitum. All procedures for animal care and use were performed as approved by Institutional Ethics Committee of Science and Research Branch, Islamic Azad University.

Induction of type 1 diabetes

To induce type 1 diabetes mellitus, after 12-hour overnight fasting, the rats received an intraperitoneal injection of freshly prepared alloxan monohydrate (Sigma Aldrich Co, Germany; 130 mg/kg b.w) in normal saline. After 72 h, animals with fasting blood glucose levels of >200 mg/dL were considered diabetic and selected for the subsequent experiments [11].

Preparation of extract

The seeds of M. fragrans were powdered and subjected to extraction with petroleum ether (60-80 ºC) using soxhlet apparatus. The extract was then concentrated to dryness under reduced pressure in rotary vacuum evaporator and later used in the experiment. The percentage yield of prepared extract was around 16% w/w.

Experimental design

Animals were randomly divided into six groups of eight rats each, as follows: Group I, non-diabetic rats received saline only; Group II, Diabetic rats received a single dose of alloxan (130 mg/kg, i.p.); Groups III, IV and V, diabetic rats treated with nutmeg extract at 50, 100 and 200 mg/kg doses respectively; and Group VI, diabetic rats treated with metformin at dose of 100 mg/kg b.w. (Merck Sante S.A.S., Lyon, France). The extract and metformin were administered through oral route using intragastric tube once daily for 21 days.

Sample preparation

At the end of the experiment, all the rats were fasted overnight and then sacrificed under anaesthesia. Blood samples were collected and serum was separated from the blood by centrifuging at 3000 rpm for 20 minutes. Pancreases were quickly removed and washed in ice-cold normal saline. Subsequently, a weight piece of each pancreas was minced and homogenized (10% w/v) in ice-cold 0.1 M Tris–HCl buffers; pH 7.4 (Merck, Darmstadt, Germany). The homogenate was centrifuged at 4000 g for 15 min at 4 °C and the resultant supernatant was used for biochemical measurements in tissue [18]. Another piece of each pancreas from each rat was kept immediately in formalin 10% for histopathological examination.

Determination of fasting blood glucose levels

Blood samples were collected from the tip of tail veins of the fasting animals on 1st and 21th days (the last day of experiment). Glucose concentration was determined in whole blood samples immediately after collection, using One Touch Glucometer (Accu Chek Active, Roche, Germany) based on the glucose oxidase method.

Biochemical assays

Serum insulin level was measured using a rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden). The levels of lipid peroxidation (LPO) in the pancreatic tissues were measured as malondialdehyde (MDA) according to the method of Ohkawa et al. [19]. superoxide dismutase activity was measured by the method described by Kakkar et al. [20]. The level of CAT was evaluated by the method given by Aebi [21]. Glutathione peroxidase was assayed by the method of Rotruck et al. [22]. Level of GSH assessed by the method of Ellman [23].

Histological examination

The pancreas tissues were fixed in 10% neutral formaline solution and embedded in paraffin. Sections of 5μm thickness were produced from the tissue blocks and stained with

Gomori chrome alum hematoxylin phloxine [24] for light microscopic examination of the pancreatic islets architecture.

Morphometric analysis

Tissue slides were examined using light microscope equipped with a high-resolution color digital camera. Eight sections were studied per group and morphometric parameters were counted. The number of islets per square millimeter was recorded in eight non-overlapping fields using ocular grid. Eight randomly selected islets per section (64 islets per group) were studied to count the number of β-cells in the islet using point counting method. The same islets in the same fields were used for counting both β-cells and the total number of cells. The Percent of β-cells was calculated by dividing the number of β-cells / the total number of cells in each islet x100 [25].

Statistical analysis

Results were analyzed using SPSS v18.0 software (SPSS Inc., Chicago, IL, USA), and expressed as means ± standard error of mean (SEM). The data were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple test was used for post hoc analyses. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Fasting blood glucose and serum insulin levels

The results presented in Table I revealed that initial fasting blood glucose levels after induction of diabetes were significantly ($p<0.05$) increased in all diabetic rats as compared to the non-diabetic rats. Oral administration of nutmeg extract at the doses of 100 and 200 mg/kg, as well as metformin, to diabetic rats caused significant ($p<0.05$) decrease in blood glucose levels at the end of the experiment, compared to the untreated diabetic group.

The levels of serum insulin in non-diabetic and experimental animals were determined and the values are depicted in Table I. Results indicated that serum insulin level of the diabetic group significantly decreased after 3 weeks of alloxan administration

compared with the non-diabetic group ($p < 0.05$). Oral administration of nutmeg extract (100 and 200 mg/kg) and metformin to diabetic rats increased the levels of serum insulin significantly ($p<0.05$) compared with the untreated diabetic rats.

Oxidative stress and antioxidant biomarkers

The results showed a significant elevation in tissue MDA in diabetic rats as compared with non-diabetic rats. Diabetic rats treated with nutmeg (100 and 200 mg/kg) showed significant decreases in pancreatic contents of MDA, when compared to the diabetic group. However, metformin produced significant antioxidant effect by restoring the increased MDA (Fig. 1). There was a significant positive correlation between blood glucose and pancreatic MDA levels in diabetic rats treated with the nutmeg extract (100 and 200 mg/kg) and metformin ($r = 0.41$, $P < 0.01$; $r = 0.66$, $p<0.001$ and $r = 0.61$, $p<0.001$, respectively).

Table II. illustrates the effect of nutmeg extract on the antioxidant biomarkers. Diabetic rats displayed significant decrease in GSH content and activities of antioxidant enzymes of pancreas; CAT, SOD and GPx with respect to the non-diabetic values. Administration of extracts (50, 100 and 200 mg/kg) showed a significant concentration dependent increase in the GSH status, as compared to diabetic rats. In comparison with diabetic group, nutmeg extract (50, 100 and 200 mg/kg) significantly increased CAT activity of diabetic rat's pancreas tissues. Extract at 200 mg/kg significantly restored the CAT activity towards normal in pancreatic tissue of diabetic rats. Pancreas SOD activity showed significant increase when rats treated with nutmeg extract (100 and 200 mg/kg) and metformin (100 mg/kg) as compared to diabetic control rats. Oral administration of 200 mg/kg of extract caused a significant ($p<0.01$) improvement in GPx level in pancreatic tissue as compared to that of untreated diabetic rats.

Histopathological study

The pancreatic islets of the non-diabetic group showed normal appearance of the islet cells (Fig. 2A). The Diabetic group showed degenerative changes, especially at the center of the islets of Langerhans with decrease in the density of bluish stained β-cells. The

remaining cells showed nuclear shrinkage and pycnosis. However, few functional β-cells were observed and α-cells were more prominent (Fig. 2B). Pancreatic sections of 50 mg/kg nutmeg extract treated rats appeared similar to those of the diabetic group (Fig. 2C). Treatment with the nutmeg extract at concentrations of 100 mg/kg (Fig. 2D) and 200 mg/kg (Fig. 2E), as well as metformin (Fig. 2F) showed restoration of pancreatic islet cells. There was an increase in the β-cell mass. The recovery of necrotic β-cells was especially more pronounced after treatment with 200 mg/kg of nutmeg extract than in the group treated with 100 mg/kg of the extract. Compared with the diabetic untreated group, apparently normal structure of pancreas was seen in diabetic rats treated with nutmeg extract (200 mg/Kg) and metformin.

Morphometric results

Morphometric results showed a significant reduction in the number of islets/mm2 in diabetic group compared to that of non-diabetic one. However, this parameter was significantly increased in 200 mg/kg nutmeg extract treated group (Table III). The number of β-cells decreased in diabetic rats, and treatment with nutmeg extract at doses of 100 mg/kg and 200 mg/kg significantly increased the number of β-cells in pancreatic islets (Table III). There was a significant decrease in the percent of β-cells after alloxan induced diabetes, while in extract (100 and 200 mg/kg) and metformin treatment groups an increase in the percent of β-cells was observed compared to the diabetic group (Fig. 3).

DISCUSSION

Alloxan is a well-known diabetogenic agent widely used to induce type 1 diabetes in experimental animals [26]. The diabetic effect of alloxan is mainly attributed to rapid uptake by the β-cells and the formation of free radicals. Thus, alloxan induced diabetes mellitus can be considered as a pathological biomodel for testing a substance with supposed antioxidant activities in vivo [27]. Several authors have used alloxan-induced diabetes model as a study tool to elucidate the pathophysiology of the disease and much more as a search engine for antidiabetic compounds with better therapeutic characteristics [28]. There

is a list of plants and their active compounds tested in diabetic animals induced by alloxan [10, 28].

In alloxan-induced diabetic rats, diabetes develops as a result of pancreatic β-cells destruction leading to degranulation and reduced insulin secretion. In this study, the diabetic rats were found to have higher fasting blood glucose levels and lower levels of serum insulin when compared to non-diabetic rats. Administration of nutmeg extract (100 mg/kg and 200 mg/kg) significantly reduced the raised blood glucose level and increased the serum insulin level in alloxan induced diabetic rats. The present results agreed with Somani and Singhai [11] who reported the hypoglycemic effects of nutmeg extract in diabetic rats. Decreased levels of glucose in extract treated groups could be due to improvement in the number of β-cells producing insulin in pancreatic islets. The pancreatic β-cells of these rats seemed to get repaired so that they were capable of producing insulin, as indicated by decreased blood glucose levels at the end of the treatment period.

Our findings indicated that treatment with nutmeg extract ameliorated the alloxan-induced islet injury in a dose dependent manner. Low dosage nutmeg treated group (50 mg/kg) failed to improve the morphological changes in the islets induced by alloxan while, treatment with nutmeg extract at doses of 100 and 200 mg/kg respectively inhibits alloxan-induced islet degeneration and necrosis and leads to the partial regeneration of the islet and β-cells of diabetic animals. This beneficial effect could be partially explained by considering the physiopathology of diabetes. Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications [18]. In the present study, elevated malondialdehyde (MDA, end product of lipid peroxidation), decreased GSH, and the inhibition of the antioxidant enzyme activities (GPx, SOD and CAT) are evidence of oxidative stress occurrence in the diabetic untreated rats. Both experimental and clinical studies suggested that there are dose links among hyperglycemia, oxidative stress and diabetic complications [2]. Therefore, drugs and phytochemicals that improve oxidative stress ameliorate or prevent islet lesions. Protective effect of some phytochemicals on pancreas has been found to be mediated through their antioxidant effects [4, 29]. Similarly, the protective effect of nutmeg on pancreatic Islets might be attributed to the antioxidant properties of this plant.

The present study revealed that nutmeg extract treatment of diabetic rats (100 and 200 mg/kg) ameliorated the lipid peroxidation, which was assessed by the decreased pancreas MDA level. Moreover, nutmeg caused increased levels of pancreas GSH, and antioxidant enzyme activity (CAT, GPx and SOD) of diabetic rats. These results confirm the antioxidant potency of nutmeg extract in the pancreas of diabetic rats. Several reports have been made on the antioxidant activity of Myristica fragrans, attributed to phenolic and polyphenolic compounds, belonging to the group of lignans [17, 30]. Argenteane, a dilignan, isolated from nutmeg is similar in power to vitamin E [31]. Reduction of lipid peroxidation in the pancreas of diabetic rats treated with nutmeg suggests the beneficial potential of this plant in the amelioration of ROS-induced pancreatic islet lesions.

Reduction of β-cell mass is critical in the pathogenesis of diabetes mellitus. In the present work, morphometric results showed a significant reduction in number of pancreatic islets and number of β-cells/islet in diabetic group compared with the non-diabetic one. These results are in agreement with those reported previously [29, 32]. However, nutmeg extract treatment was able to reduce islet cells loss and showed significant increase in the number of β-cells in a dose dependent manner. These findings suggest that the administration of nutmeg to diabetic rats causes beneficial effect in terms of regeneration of β-cells in damaged pancreas. It is believed that β-cell can regenerate through the replication of pre-existing β-cells or neogenesis from stem cells and progenitor cells inside or outside the islets [33]. The regenerative effect of nutmeg extract on β-cells might be due to its anti-oxidant potential. Recent insights indicated that oxidative stress and β-cell regeneration are highly interrelated biological process, not only demonstrated by the fact that they coexist under several physiological and pathological conditions but also reflected by the profound direct and indirect deleterious impacts of oxidative stress on β-cell regeneration [34]. Furthermore, several studies have shown the beneficial effects of antioxidants supplementation on β-cell regeneration [35, 36].

CONCLUSIONS

The present study reveals that Myristica fragrans seeds (nutmeg) extract has potent antidiabetic activity in alloxan-induced diabetic rats. The findings of our study demonstrate that nutmeg extract has a protective effect on pancreatic islets damage, possesses the ability to regenerate β cells and ameliorates oxidative stress in alloxan-induced diabetic rats in a dosage dependent manner, possibly via its antioxidant properties.

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Table 1. Effect of nutmeg extract on fasting blood glucose and serum insulin levels in alloxan induced diabetic rats

Groups	**Fasting blood glucose (mg/dl)**		**Serum insulin (µU/ml)**
	Initial (1st day)	**Final (21st day)**	
Non-diabetic	92.38±4.92#	93.35±1.1#	16.51±0.34#
Diabetic	410.58±5.28*	483.57±5.29*	8.54±0.24*
Diabetic + nutmeg extract (50 mg/kg)	402.67±8.72*	445.36±6.22*	9.29±0.41*
Diabetic + nutmeg extract (100 mg/kg)	405.38±7.42*	265.66±7.52*#	11.35±0.36*#
Diabetic + nutmeg extract (200	415.40±5.07*	214.83±5.28*#	13.24±0.43*#

mg/kg)			
Diabetic + metformin (100 mg/kg)	412.18±4.36*	180.35±3.17*#	15.43±0.52#

All values are expressed as mean ± SEM.; n=8

*Significant compared with non-diabetic group, $P < 0.05$, #Significant compared with diabetic group, $P < 0.05$.

Table II. Effect of nutmeg extract on antioxidant biomarkers in pancreas of alloxan induced diabetic rats

Groups	**Antioxidant biomarkers**			
	CAT (units/mg protein)	**SOD** (units/mg protein)	**GPx** (μg/min/mg protein)	**GSH** (mg/100 g tissue)
Non-diabetic	11.47±0.44#	15.91±1.06#	39.26±1.97#	27.51±2.16#
Diabetic	3.36±0.26*	5.08±0.46*	19.80±0.64*	10.58±0.73*
Diabetic + nutmeg extract (50 mg/kg)	6.48±0.27*	4.67±0.32*	21.15±0.45*	19.08±1.11*#
Diabetic + nutmeg extract (100 mg/kg)	8.26±0.41*	8.71±0.48*#	24.43±1.01*	17.90±0.99*#
Diabetic + nutmeg extract (200 mg/kg)	11.25±0.38#	9.20±0.70*#	27.51±1.32*#	25.22±1.01#
Diabetic + metformin (100 mg/kg)	10.80±0.36#	12.68±1.05*#	33.35±2.02*#	31.07±1.46#

All values are expressed as mean ± SEM.; n=8. *Significant compared with non-diabetic group, P < 0.05; #Significant compared with diabetic group, P < 0.05. GSH; reduced glutathione, GPx; glutathione peroxidase, CAT; catalase and SOD; superoxide dismutase.

Table III. The number of islets/mm^2 and β-cells/islet in the control and treatment groups

Groups	Islets/mm2	β cells/ islet
Non-diabetic	7.62±0.82$^{\#}$	89.87±4.75$^{\#}$
Diabetic	2.50±0.42*	22.00±0.70*
Diabetic + nutmeg extract (50 mg/kg)	2.12±0.39*	24.62±1.06*
Diabetic + nutmeg extract (100 mg/kg)	3.25±0.49*	39.12±1.63$^{* \#}$
Diabetic + nutmeg extract (200 mg/kg)	5.75±0.59$^{\#}$	55.62±2.69$^{* \#}$
Diabetic + metformin (100 mg/kg)	5.37±0.62$^{\#}$	60.87±3.46$^{* \#}$

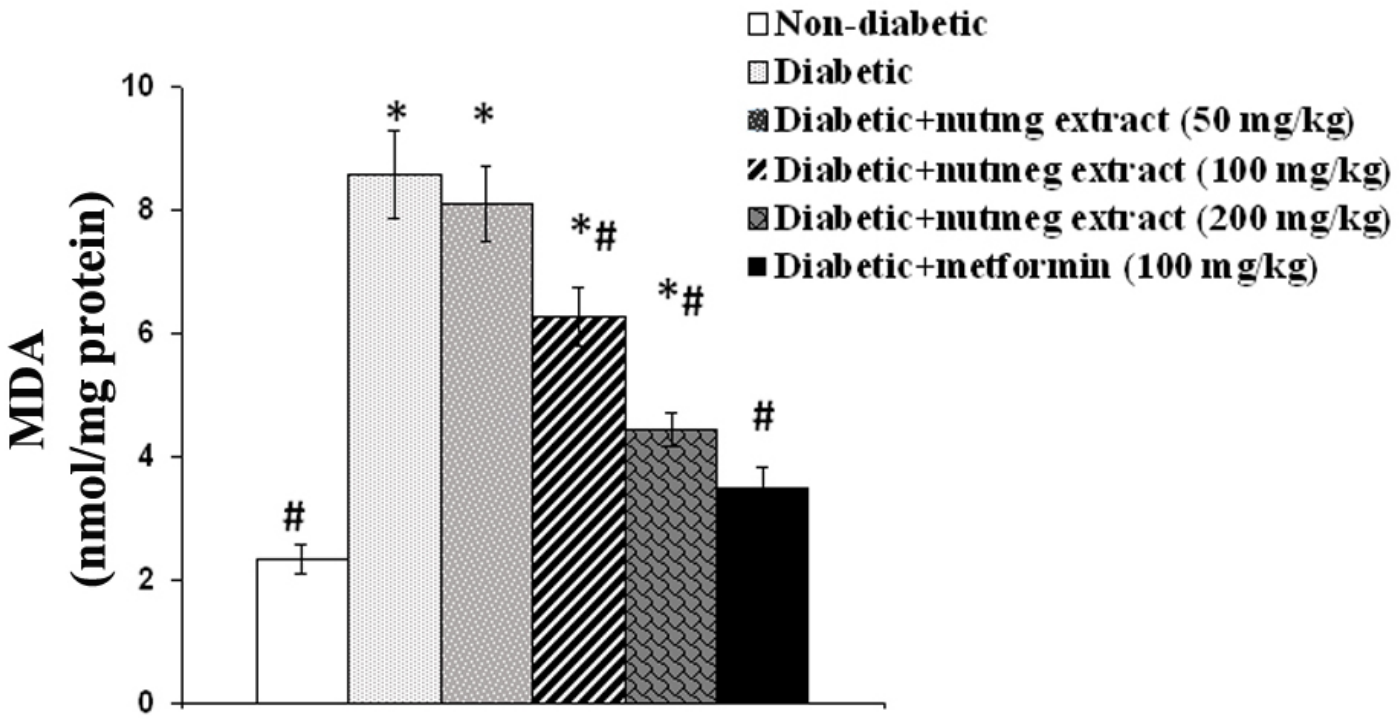
Values were represented as mean ± SEM. *Significant compared with non-diabetic group, P < 0.05, #Significant compared with diabetic group, P < 0.05.

FIGURE LEGENDS

Figure 1. Effects of nutmeg extract on MDA levels in the pancreas of experimental groups. *p < 0.05 as compared with the control group; # p < 0.05 as compared with the alloxan control group.

Figure 2. Photomicrographs of sections of the pancreas stained by Gomori Chrome Alum hematoxylin phloxine. **A**. Non-diabetic group. arrows: β-cells, arrowheads: α-cells; **B**. Diabetic group. arrow: β-cell necrosis, arrowheads: vacuolation; **C**. Diabetic treated (nutmeg extract 50 mg/kg) group. arrowheads: β-cells, arrows: α-cells, asterisk: vacuolation; **D** and **E**. Diabetic treated (nutmeg extract 100 and 200 mg/kg) groups. arrows: α-cells, arrowheads: β-cells, asterisk: vacuolation; **F**. Metformin treated group. arrows: β-cells, arrowheads: α-cells, asterisk: vacuolation. Gomori chrome alum Hx, scale bar 20 μm.

Figure 3. Percentage of β-cells per total number of islet cells (%) in the control and treatment groups. *$p < 0.05$ as compared with the control group; # $p < 0.05$ as compared with the alloxan control group.


Non-diabetic
Diabetic
Diabetic+nutmg extract (50 mg/kg)
Diabetic+nutmeg extract (100 mg/kg)
Diabetic+nutmeg extract (200 mg/kg)
Diabetic+metformin (100 mg/kg)
MDA
(nmol/mg protein)
10
8
6
4
2
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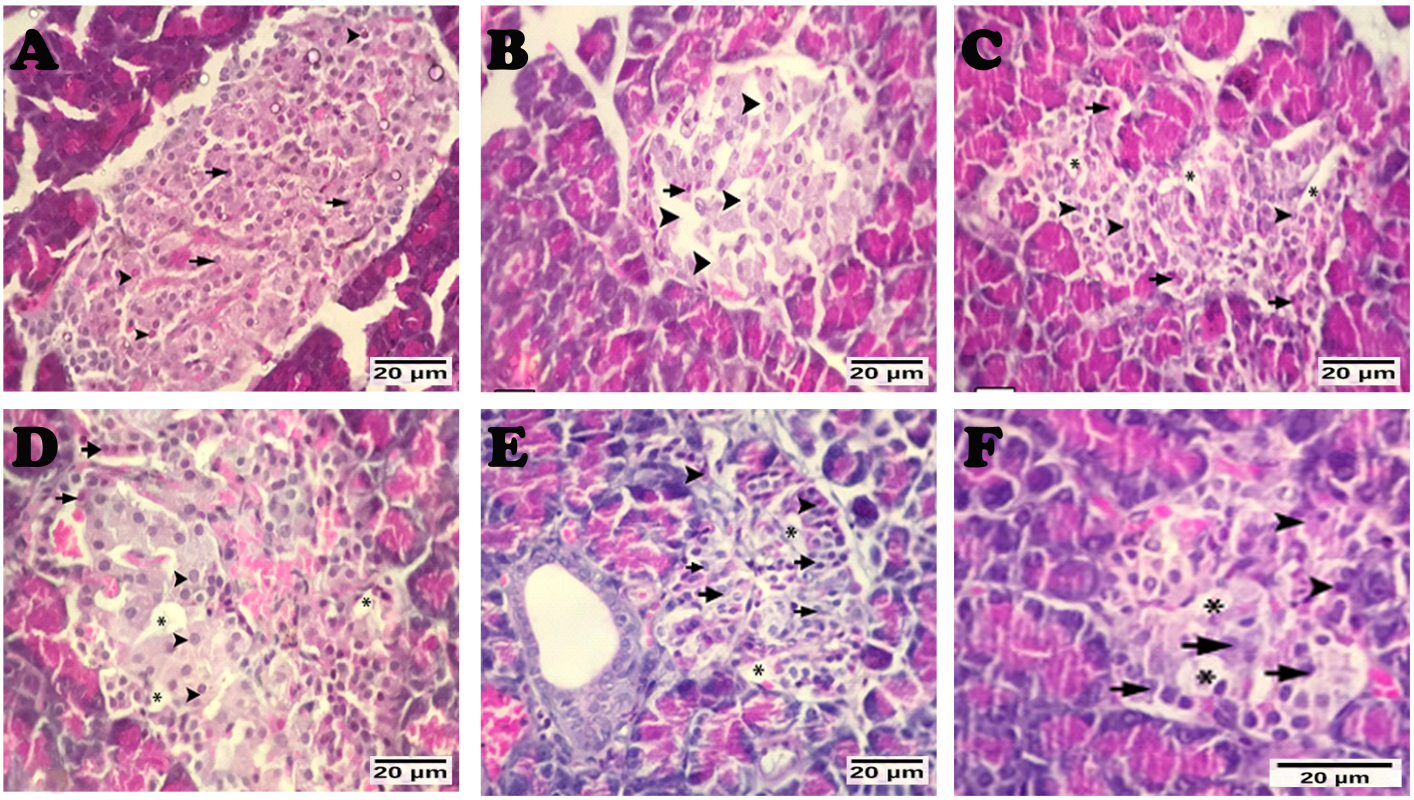
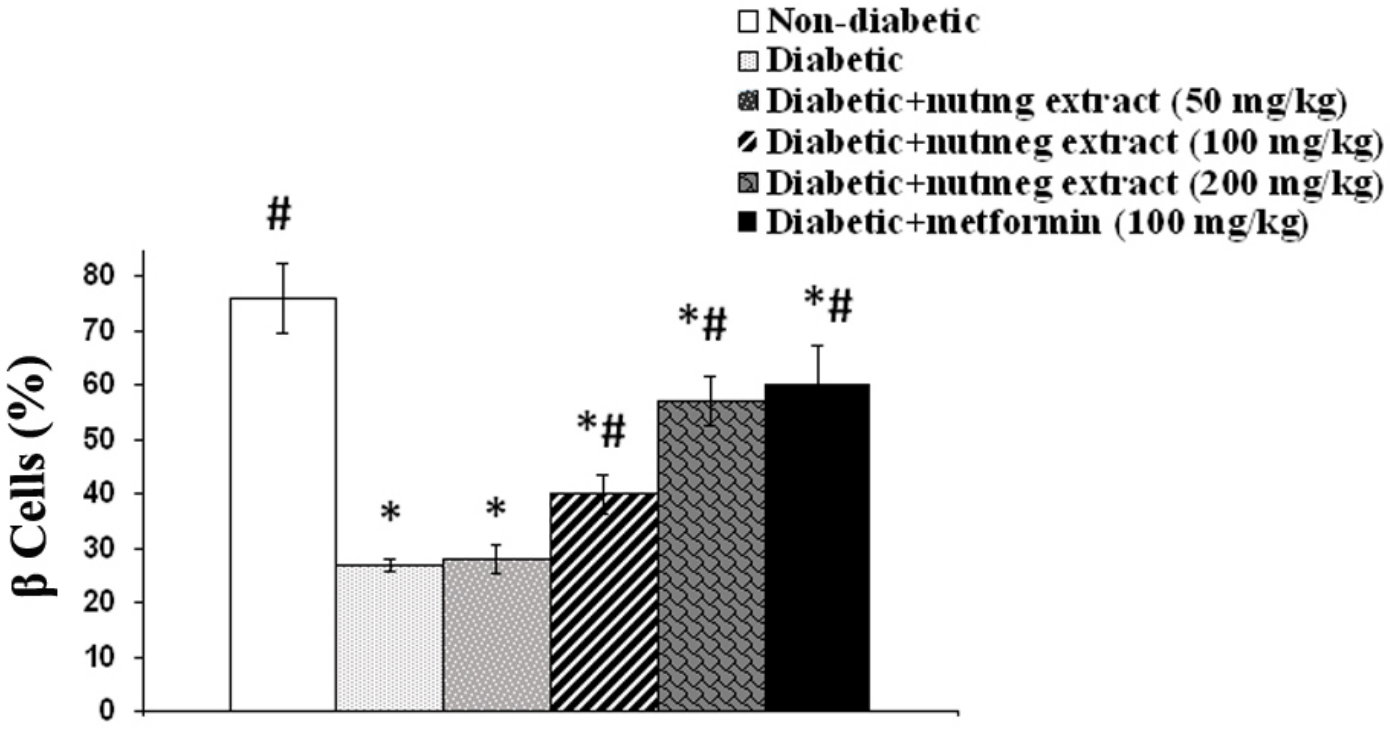

A
20 µm
B
20 µm
C
20 µm
D
20 µm
E
20 µm
F
20 µm


Non-diabetic
Diabetic
Diabetic+nutmg extract (50 mg/kg)
Diabetic+nutmeg extract (100 mg/kg)
Diabetic+nutmeg extract (200 mg/kg)
Diabetic+metformin (100 mg/kg)
β Cells (%)
0
10
20
30
40
50
60
70
80
#
*
*
*#
*#
*#