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Influence of *Ocimum sanctum* (L.) Extract on the Activity of Gliclazide in Alloxan-induced Diabetes in Rats

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Abstract: *Ocimum sanctum L. is widely used as traditional remedy to manage hyperglycemia. This study was aimed to evaluate the effect of aqueous-methanolic extract of O. sanctum leaves (OSE) on the anti-diabetic activity of gliclazide in alloxan-induced diabetes in Wistar rats. Diabetes was induced by intraperitoneally injecting alloxan (120 mg/kg b.w.) in rats. Treatments including OSE (100 mg/kg b.w.), gliclazide (100 mg/kg b.w.), and in combination were given daily to diabetic rats till the 21st day of study. Body weight and fasting blood glucose levels were determined at regular intervals, while blood and organ samples were taken at the end of the study for biochemical and histopathological studies. Results showed that treatments exhibited anti-hyperglycemic activity through significantly ($p < 0.05$) restoring body weight, fasting blood glucose level, and serum levels of glucose, insulin and HbA1c. The anti-lipidemic activity was noticed as total cholesterol (TC), triglyceride (TG), high-/low density-lipoproteins (HDL-C, LDL-C) levels were restored in treated diabetic rats. Ameliorative effects of treatments were observed as significant ($p < 0.05$) reduction in serum levels of liver function biomarkers (alanine aminotransferase; ALT, aspartate aminotransferase; AST, alkaline phosphatase; ALP and bilirubin; BIL) and restoration of oxidative stress biomarkers (catalase; CAT, superoxide dismutase; SOD and malondialdehyde; MDA) in liver tissue. Histopathological findings supported these results as an increase in pancreatic islets size and protective effects on liver tissue was observed in diabetic rats treated with gliclazide and OSE alone and their combination. Conclusively, the combination of OSE and gliclazide produced a synergistic anti-diabetic effect as compared to that of alone treatment.*

Keywords*: Ocimum sanctum, gliclazide, antioxidants, alloxan, oxidative stress, diabetes*

1.Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder which can be characterized by persistent high blood glucose level of an individual due to absolute or relatively low level of insulin or secretion of non-functional insulin. İt is estimated that the total number of diabetic patients will increase to 366 million in 2030 and about 95 % of them will suffer from type 2 DM [1]. Type 2 diabetic patients show the persistently high level of blood glucose, the inability of pancreatic β-cells to secrete insulin or insulin resistance and impaired glucose consumption. Progressive pathological condition exacerbates ROS production, diminishes antioxidants enzymatic activities, dyslipidemia and oxidative stress-mediated cellular damage, consequently causes secondary complications such as cardiovascular morbidity, neuropathy, nephropathy and retinopathy [2,3]. Experimental studies suggested that hyperglycemia induces excessive ROS generation through glucose oxidation [4]. The impaired free radical scavenging capacity and excessive production of ROS results in pancreatic β-cells damage [5]. Oxidative stress alters signaling pathways that lead to advanced glycation end (AGEs) product formation, pro-inflammatory cytokines release and cell necrosis [6]. Hence, hindrance in oxidative stress generation highlights the importance of antioxidants in diabetes treatment [7].

Gliclazide is a member of second-generation sulfonylureas (Figure 1) and is primarily used as an oral hypoglycemic drug in NIDDM (non-insulin-dependent diabetes mellitus). İt produces an anti-diabetic effect through selectively inhibiting pancreatic K^+ ATP channels [8].

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Synthetic drugs, being used in diabetes management, are associated with severe side effects include abdominal discomfort, weight gain, renal and hepatic disorders [9].

Medicinal plants rich in phenolics and flavonoids have gained attention because of their numerous therapeutic properties [10]. The phytoconstituents present in plants exert free radical scavenging activities by strengthening antioxidant defense mechanism and regulating inflammatory pathways for hyperglycemia management [11].

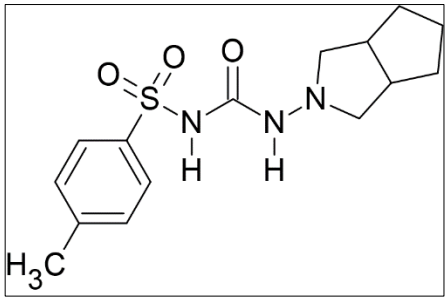

Figure. 1 Chemical structure of gliclazide

Ocimum sanctum L., also known as Holy basil, is a part of the Lamiaceae family and has been used as a folk remedy for various ailments such as fever, pain, hypertension, cancer, diabetes, renal and liver diseases [12]. Many phytoconstituents include flavonoids (luteolin, orientin, vicenin-2, isothymusin, cirsimaritin), phenolics (rosmarinic acid), phenylpropanoids (eugenol, furaldehyde), fatty acid derivatives, coumarin, cerebroside, terpenoids (ursolic acid, oleic acid), steroids (stigmasterol), fixed and essential oils are present in *O. sanctum* and are believed to be responsible for its antioxidant, anti-cancer, anti-stress, anti-inflammatory and anti-diabetic activities [13].

In Asian countries such as China, Pakistan, and India, it is common practice to use synthetic and herbal products to manage diabetes. It could result in drug-herbal interaction, thereby hinders or enhances drug activity. A literature review revealed that few studies were conducted to observe drug-herbal interactions as some herbal products can interact with oral hypoglycemic agents [14]. Therefore, the present study was designed to evaluate the efficacy and safety of an aqueous-methanolic extract of *O. sanctum* leaves and gliclazide combination in alloxan-induced diabetes in rats.

2. Materials and methods

2.1. Chemicals

Gliclazide (Nidonil®, Merck, Pvt., Ltd.) and Alloxan (Sigma-Aldrich, USA) were purchased. Serum insulin (Calbiotect®, CA, USA), glucose, glycated hemoglobin (HbA1c), hepatic function parameters and lipid profile were assessed by commercially available kits of QCA® (Spain). All other chemicals of analytical grade were used for biochemical and histopathological studies.

2.2. Plant collection and extraction

O. sanctum leaves were collected and specimen (herbarium number: 245-1-18) was submitted to the Department of Botany, University of Agriculture, Faisalabad. Plant material was shade dried for three weeks, then pulverized and sieved. The aqueous-methanolic extract was prepared by macerating 250 g of powdered material in 3 L (methanol: water, 3:1 v/v) for one week with vigorous shaking at regular intervals. The extract was filtered and concentrated by using a rotary evaporator (Heizbad Hei-VAP, Heidolph, Germany). Then, the percentage yield was calculated and stored at 4^{o}C until further used.

2.3. Animals

This study was conducted after approval of the Ethical Standards of Animal Care and Institutional Biosafety Committee of the University of Agriculture, Faisalabad (D. No. 3267/ORIC). All animals were cared for according to devised guidelines of the National Institute of Health (NIH publication No.

85-23, revised 1996).

Forty-two male Wistar rats of body weight 170-200 g were selected and kept at the animal facility of Institute of Microbiology, University of Agriculture, Faisalabad-Pakistan under standard conditions of temperature (25 ± 1°C) and 12 h light/dark cycle. All animals were provided with fresh water and food. After acclimatization for one week, animals were randomly allotted to seven groups (n = 6). Alloxan monohydrate (120 mg/kg b.w.), dissolved in normal saline, was injected intraperitoneally to induce diabetes. After confirming the diabetes induction, treatments were started as; (NC) healthy rats received normal saline (3 mL/kg b.w.) and 100 mg/kg b.w. of OSE given to healthy rats (N+OSE). Alloxan induced diabetic rats were given 3 mL/kg b.w. of normal saline (DC), 10 mg/kg b.w. of gliclazide (D+G), 100 mg/kg b.w. of OSE (D+OSE), and a combination of the same dose of gliclazide and OSE (D+G+OSE), respectively.

2.4. Biochemical analysis

A commercially available glucometer (OnCall® *Plus II*) was used to assess the fasting blood glucose level. Blood samples were collected at the end of study and serum was separated by centrifuging at 1010x g for 15 min and stored at -20°C until further analysis. Biochemical parameters including serum glucose, insulin, liver function biomarkers (AST, ALT, ALP and BIL) and lipid profile (TC, TG, HDL-C and LDL-C) were analyzed by using an automated serum analyzer (Bio-Ray 310 diagnostic).

Liver glycogen was determined according to a previously described method [15]. Oxidative stress markers of liver tissue were assessed by preparing 10% (w/v) of tissue homogenate in a buffer (1.15% KCl, 50mM Tris-HCl, *p*H 7.4) and centrifuging at 9000x g for 5 min. Supernatants were collected and used to determine CAT, SOD, and MDA levels by using a microplate spectrophotometer (Multiskan GO™ with SkanIt software 4.1) [16-18].

2.5. Histopathological examination

Pancreas and liver tissues were collected, washed and stored in NBS (10%, v/v) for fixation [19]. Tissues were embedded in paraffin, sectioned (5µm) and H&E staining was performed. Histo-pathological changes in tissues were observed under a light microscope (Model IM-910 IRMECO Gmbh & Co., Germany) attached to a camera (TOUPCAM, ToupTek Photonics Co., China).

2.6. Statistical analysis

The obtained data were subjected to one-way and two-way ANOVA and post-hoc Tukey's multiple comparison test to find significance ($p < 0.05$) between different groups by using GraphPad Prism® (Version 6) software.

3. Results and discussions

3.1. Effect of treatments on body growth and fasting blood glucose level

Body weight of normal and diabetic rats treated with gliclazide, OSE, and their combination is shown in Figure (2A). A regular gain in body weight was demonstrated in normal control and normal rats received OSE for 21 days. While diabetic rats demonstrated a significant ($p < 0.05$) drop in body weight till day 21st as compared to the normal control group. Diabetic received gliclazide and OSE exhibit significant ($p < 0.05$) increase in body weight while their combination demonstrated better effect on weight gain as compared to alone treatment. The body weight loss may be due to the inability of diabetic rats to effectively utilize carbohydrates as an energy source [20].

The fasting blood glucose levels in experimental rats were monitored weekly from diabetes induction to the 21st day of study (Figure 2B). The results showed that a significant ($p < 0.05$) rise in glucose level after diabetes induction. A considerable fall in glucose level was observed in diabetic rats treated with gliclazide and OSE, while their combination produced a more prominent effect in hyperglycemic rats.

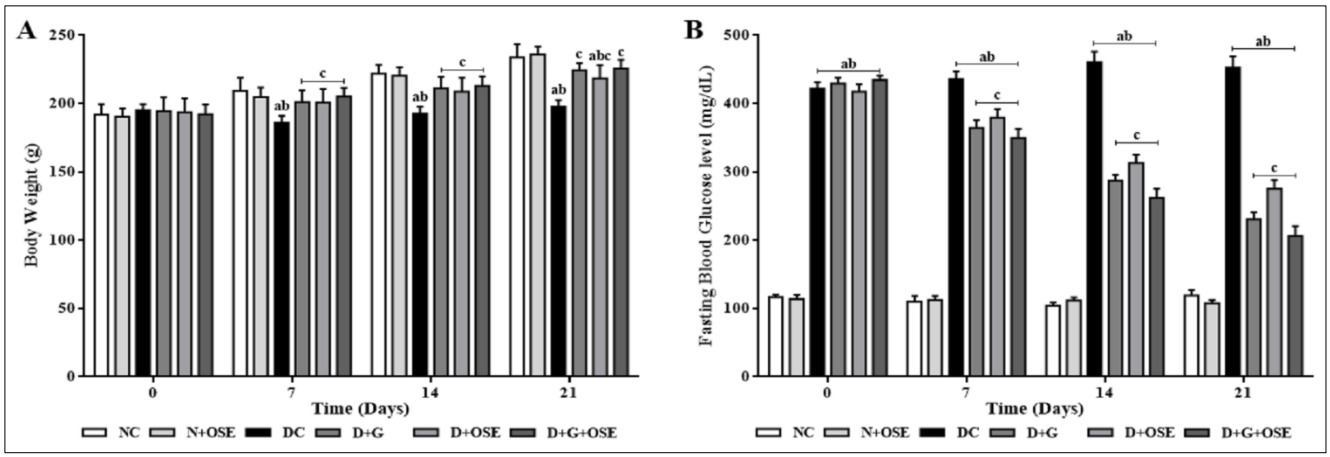

Figure 2. Effect of OSE, gliclazide and their combination on **(A)** body weight (g) and **(B)** fasting blood glucose level (mg/dL) in diabetic rats. Results statistically analyzed by two-way ANOVA, following post-hoc Tukey's test and presented as mean ± SD (n = 6). Where, statistical significant difference of treatments from NC ($^{a}p < 0.05$), N+OSE ($^{b}p < 0.05$) and DC ($^{c}p < 0.05$), respectively

3.2. Effect of treatments on hyperglycemic parameters

The findings of the present study showed significantly ($p < 0.05$) increased serum glucose and HbA1c level while decreased insulin level in serum of diabetic rats as compared to normal control. Diabetic rats treated with gliclazide, OSE and in combination markedly ($p < 0.05$) lowered serum levels of glucose and HbA1c and raised serum insulin level. It might be possible due to increased secretion of insulin from β cells and increased cellular uptake of glucose [21].

Results exhibited that liver glycogen level was significantly ($p < 0.05$) decreased in diabetic control as compared to healthy rats (Table 1). Reduction in glycogen synthetase and lower insulin levels are associated with lower glycogen in the liver [22]. Administration of gliclazide and OSE and their combination significantly ($p < 0.05$) raised the level of liver glycogen resulting in improved glucose metabolism.

Table 1. Effect of OSE, gliclazide and their combination on serum glucose, insulin, HbA1c, and liver glycogen

Treatments	Parameters			
	Serum Glucose (mg/dL)	Serum Insulin (U/L)	HbA1c (%)	Liver Glycogen (mg/g)
NC	113.36 ± 5.39	18.44 ± 0.95	5.61 ± 0.60	42.87 ± 3.19
N+OSE	108.32 ± 5.53	17.65 ± 1.05	5.87 ± 0.38	43.77 ± 2.86
DC	435.00 ± 13.49^{ab}	6.76 ± 0.58^{ab}	11.88 ± 0.85^{ab}	15.48 ± 0.89^{ab}
D+G	222.17 ± 10.38^{abc}	15.67 ± 0.56^{abc}	6.84 ± 0.66^{ac}	30.19 ± 1.07^{abc}
D+OSE	304.33 ± 9.37^{abc}	13.39 ± 0.42^{abc}	7.14 ± 0.58^{abc}	28.59 ± 1.12^{abc}
D+G+OSE	197.53 ± 12.81^{abc}	16.32 ± 0.69^{abc}	6.75 ± 0.69^{ac}	34.94 ± 1.35^{abc}

Results statistically analyzed by one-way ANOVA following post-hoc Tukey's test and presented as mean ± SD (n = 6). Where, statistical significant difference of treatments from NC ($^{a}p < 0.05$), N+OSE ($^{b}p < 0.05$) and DC ($^{c}p < 0.05$), respectively.

3.3. Effect of treatments on lipid profile

Hyperlipidemia is one of the common complications in diabetes. In this study, a significant ($p < 0.05$) change in lipid parameters was noticed. Diabetic rats treated with gliclazide, OSE and their combination showed a significant ($p < 0.05$) reduction in TC, TG and LDL-C levels and increased level of HDL-C. HDL-C is considered as good cholesterol as it acts as a scavenger and transport LDL-C away from arteries. Previous studies revealed that elevation in HDL-C is associated with lowering the CVD development risk [23]. The lipid-lowering potential of OSE might be linked to the

presence of phytocompounds, such as flavonoids [24].

Table 2. Effect of OSE, gliclazide and their combination on lipid profile

Treatments	Parameters			
	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
NC	119.50 ± 4.76	67.83 ± 9.64	43.27 ± 8.03	67.12 ± 11.18
N+OSE	116.79 ± 4.20	65.18 ± 9.14	46.02 ± 8.01	63.62 ± 10.09
DC	207.17 ± 17.19^{ab}	155.50 ± 10.04^{ab}	19.82 ± 3.84^{ab}	213.92 ± 9.39^{ab}
D+G	162.16 ± 6.24^{abc}	90.63 ± 4.59^{abc}	30.64 ± 5.28^{abc}	82.31 ± 7.63^{bc}
D+OSE	175.67 ± 8.09^{abc}	107.33 ± 5.61^{abc}	27.33 ± 4.41^{ab}	101.85 ± 8.95^{abc}
D+G+OSE	143.34 ± 5.57^{abc}	82.51 ± 7.74^{abc}	35.17 ± 2.86^{bc}	80.11 ± 11.46^{c}

Results statistically analyzed by one-way ANOVA following post-hoc Tukey's test and presented as mean ± SD (n = 6). Where, statistical significant difference of treatments from NC ($^{a}p < 0.05$), N+OSE ($^{b}p < 0.05$) and DC ($^{c}p < 0.05$), respectively.

3.4. Effect of treatments on liver function biomarkers

In diabetes, as a metabolic disorder, hepatocellular damage occurs that can be evidenced by elevated levels of hepatic function enzymes in the systemic circulation. Determination of these enzymes level in serum gives valuable information for liver injury. Alloxan induced diabetes causes an upsurge of ALT, AST and ALP activities in diabetic rats in contrast to healthy rats. The elevation of these enzymes in experimental diabetic rats might be due to the release of these hepatic enzymes into circulation. Diabetic rats treated with gliclazide, OSE alone and their combination significantly ($p < 0.05$) reduced the serum levels of ALT, ALT and ALP as similar findings were observed in previous studies [25,26]. A significant ($p < 0.05$) increase in serum bilirubin level was found in diabetic control in comparison to normal rats, which might be due to decreased capacity of liver uptake, increase formation or conjugation of bilirubin. However, the administration of gliclazide, OSE and their combination significantly ($p < 0.05$) reduced the bilirubin level [27].

Table 3. Effect of OSE, gliclazide and their combination on hepatic function biomarkers in diabetic rats

Treatments	Parameters			
	ALT (U/L)	AST (U/L)	ALP (U/L)	BIL (mg/dL)
NC	37.50 ± 3.45	44.08 ± 3.16	118.10 ± 7.16	0.46 ± 0.04
N+OSE	36.53 ± 2.91	42.82 ± 3.19	114.79 ± 6.65	0.43 ± 0.04
DC	91.50 ± 4.50^{ab}	145.17 ± 7.55^{ab}	202.68 ± 8.64^{ab}	1.87 ± 0.07^{ab}
D+G	44.67 ± 3.01^{c}	57.33 ± 2.58^{abc}	145.43 ± 7.05^{abc}	0.59 ± 0.04^{abc}
D+OSE	58.83 ± 8.59^{abc}	76.83 ± 4.96^{abc}	162.17 ± 8.23^{abc}	0.83 ± 0.13^{abc}
D+G+OSE	42.50 ± 3.68^{c}	53.56 ± 4.80^{abc}	127.86 ± 7.53^{c}	0.53 ± 0.04^{c}

Results statistically analyzed by one-way ANOVA following post-hoc Tukey's test and presented as mean ± SD (n = 6). Where, statistical significant difference of treatments from NC ($^{a}p < 0.05$), N+OSE ($^{b}p < 0.05$) and DC ($^{c}p < 0.05$), respectively.

3.5. Oxidative stress biomarkers in liver tissue

Persistent hyperglycemia in diabetic patients and experimental animals destabilizes the anti-oxidant defense system and accelerates the generation of *de novo* free radicals, consequently promotes oxidative stress-mediated cellular damage [28]. Antioxidants of either natural or synthetic sources can partially or completely alleviate this damage [29]. In the present study, elevated MDA level was found in diabetic control in contrast to the normal group, which is indicating an increased generation of free radicals. Diabetic rats treated with gliclazide and OSE alone and in combination

caused a reduction in MDA level (Table 4). This reduction on MDA level may elevate glutathione peroxidase (GPx) activity in treated diabetic rats and hence diminished LPO reactions [30].

The CAT and SOD are considered primary antioxidant enzymes as they directly eliminate ROS. CAT, being a hemoprotein, reduces H_2O_2 and is known to be involved in H_2O_2 detoxification. Among different important defense enzymes, SOD regulates proper cellular function by dismutation of superoxide radicals mediated ROS production and exaggeration of oxidative stress. Oxidation and non-enzymatic glycosylation inhibits CAT and SOD enzymes in DM [31,32]. In the current study, reduced CAT and SOD activities were observed in diabetic control as previously reported as alloxan-mediated ROS production could cause inactivation of these enzymes [29,32]. Administration of gliclazide and OSE alone and their combination significantly ($p < 0.05$) restored the CAT and SOD activities, which might be due to a reduction in oxidative stress as observed by reduced LPO (Table 4). Previous studies showed the presence of antioxidant compounds that could be responsible for the antioxidant activity of OSE and its synergistic property in combination with gliclazide in diabetic rats.

Table 4. Effect of OSE, gliclazide and their combination on oxidative stress biomarkers in liver tissue

Treatments	Parameter		
	MDA (mg/dL)	CAT (mg/dL)	SOD (mg/dL)
NC	2.42 ± 0.44	54.42 ± 5.71	6.80 ± 0.68
N+OSE	2.31 ± 0.42	56.28 ± 5.68	7.07 ± 0.55
DC	5.58 ± 0.54^{ab}	26.83 ± 3.76^{ab}	3.55 ± 0.43^{ab}
D+G	3.47 ± 0.78^{abc}	43.79 ± 5.09^{abc}	5.87 ± 0.24^{abc}
D+OSE	4.21 ± 0.43^{abc}	36.81 ± 4.92^{abc}	4.93 ± 0.53^{abc}
D+G+OSE	3.30 ± 0.69^{c}	47.06 ± 4.34^{bc}	6.20 ± 0.26^{bc}

Results statistically analyzed by one-way ANOVA following post-hoc Tukey's test and presented as mean ± SD (n = 6). Where, statistical significant difference of treatments from NC ($^{a}p < 0.05$), N+OSE ($^{b}p < 0.05$) and DC ($^{c}p < 0.05$), respectively.

3.6. Histopathological findings of pancreas and liver tissues

The histopathological sections of pancreatic tissue of experimental diabetic rats are shown in Figure 3. Histology of the pancreas of healthy control depicted normal features of pancreatic islets with abundant cytoplasm and active nuclei (Figure 3A). The diabetic control group revealed the presence of inflammatory cells, degenerated cells with atrophied and congested pancreatic islets (Figure 3B), as evidenced in a previous study [33]. Treatment of diabetic rats with gliclazide reduced cellular injury, restored regenerating cells and larger pancreatic islets in contrast to diabetic control (Figure 3C). Administration of OSE to diabetic rats (Figure 3D) showed comparatively less regenerating cells and pancreatic islets as compared to gliclazide treated rats. The combination of OSE and gliclazide markedly improved pancreatic islets and regenerating cells and reduction in tissue necrosis in comparison to diabetic control and gliclazide alone treated diabetic animals (Figure 3E) and these findings are in agreement to histopathological results observed in the previous study [34].

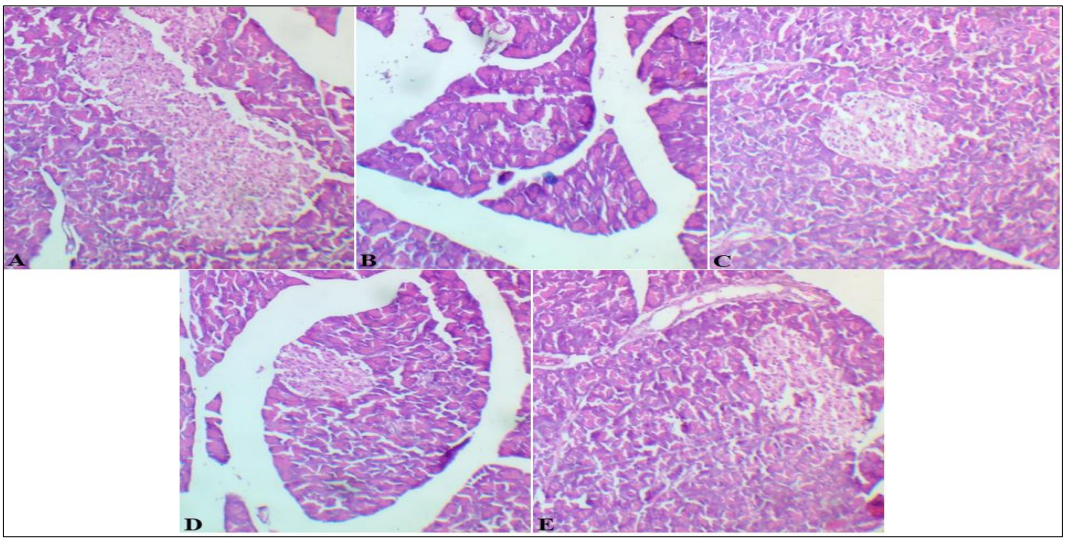

Figure 3. Histological changes in the pancreas (H&E, x100). Normal group (**A**), diabetic control (**B**), diabetic rats treated with gliclazide (**C**), *O. sanctum* extract (**D**), and with the combination of gliclazide and *O. sanctum* extract (**E**), respectively

The liver histopathological changes in experimental diabetic rats are shown in Figure 4. Normal rats showed normal tissue architecture with hepatic lobules and normally dilated hepatic sinusoids (Figure 4A). Diabetic control (Figure 4B) showed infiltration of mononuclear cells, vacuolization, congested nuclei, necrotic foci and lesions of parenchymatous tissue. Treatment of diabetic rats with gliclazide revealed the lesser histopathological injury, while diabetic rats treated with OSE showed some condensed nuclei, vacuolization to a lesser extent (Figure 4C and 4D). Administration of OSE and gliclazide combination to diabetic rats exhibited prominent protective effects in liver tissue, as shown in Figure 4E.

Histopathological findings of the current study showed similar outcomes as observed in previous studies where a marked improvement in pancreatic islets and insulin secretion was observed. The ameliorative activity of treatments on oxidative stress-mediated liver damage in diabetic animals was noticed [34,35].

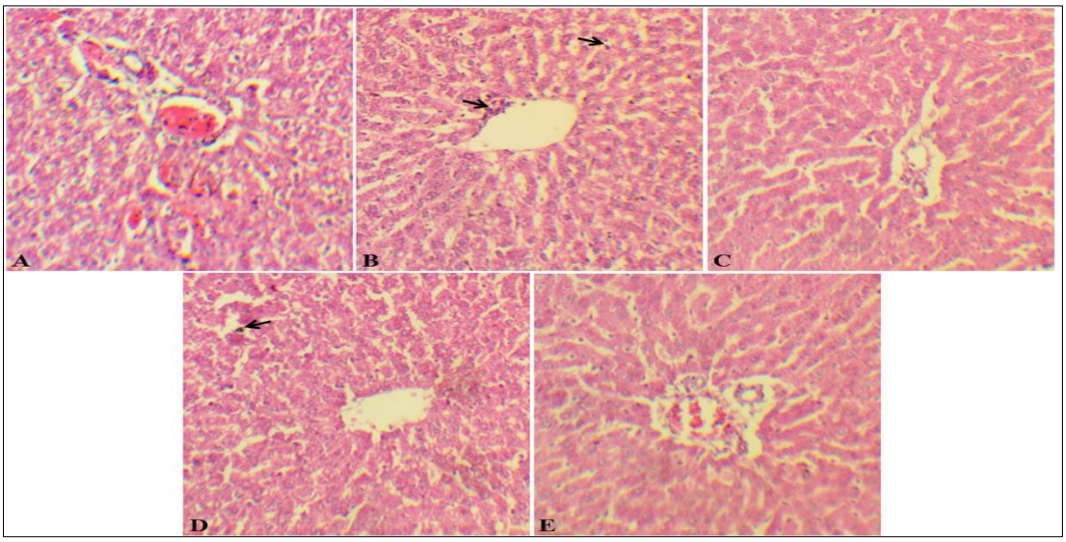

Figure 4. Histological changes in the liver (H&E, x100). Normal group (**A**), diabetic control (**B**), diabetic rats treated with gliclazide (**C**), *O. sanctum* extract (**D**), and with the combination of gliclazide and *O. sanctum* extract (**E**), respectively

4. Conclusions

We evidenced that *O. sanctum* extract improved the anti-diabetic activity of gliclazide and can be used safely to achieve the prolonged and sustained hypoglycemic effect. However, further animal and human studies are required to establish a long-term safety profile.

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APPENDICES

Appendix 1: Effect of treatments on body weight (g) of alloxan-induced diabetes in rats

Treatments	Body weight (g)			
	Day 0	Day 7	Day 14	Day 21
NC	191.83 ± 7.71	209.33 ± 9.48	221.62 ± 6.65	233.79 ± 10.17
N+OSE	189.43 ± 6.07	204.51 ± 7.11	220.13 ± 6.27	236.21 ± 5.89
DC	195.88 ± 4.56	186.47 ± 4.98^{ab}	192.54 ± 5.24^{ab}	197.68 ± 4.96^{ab}
D+G	194.34 ± 10.15	201.01 ± 8.78^{c}	211.159 ± 8.32^{c}	224.16 ± 5.28^{c}
D+OSE	193.56 ± 10.41	200.83 ± 9.81^{c}	208.79 ± 9.88^{c}	218.58 ± 9.74^{abc}
D+G+OSE	192.38 ± 7.40	205.26 ± 6.46^{c}	213.11 ± 6.78^{c}	225.53 ± 6.41^{c}

Results statistically analyzed by two-way ANOVA following post-hoc Tukey's test and presented as mean ± SD (n = 6). Where, statistical significant difference of treatments from NC ($^{a}p < 0.05$), N+OSE ($^{b}p < 0.05$) and DC ($^{c}p < 0.05$), respectively.

Appendix 2: Effect of treatments on fasting blood glucose level (mg/dL) of alloxan-induced diabetes in rats

Treatments	Fasting blood glucose level (mg/dL)			
	Day 0	Day 7	Day 14	Day 21
NC	116.23 ± 3.76	110.13 ± 8.18	104.21 ± 4.53	118.84 ± 8.23
N+OSE	114.09 ± 5.55	112.52 ± 5.96	111.58 ± 4.29	107.88 ± 4.49
DC	422.86 ± 9.51^{ab}	436.48 ± 11.43^{ab}	460.53 ± 15.37^{ab}	452.24 ± 17.11^{ab}
D+G	429.45 ± 8.12^{ab}	364.76 ± 11.22^{abc}	286.56 ± 8.86^{abc}	230.49 ± 10.69^{abc}
D+OSE	417.30 ± 11.04^{ab}	379.61 ± 11.93^{abc}	312.36 ± 12.44^{abc}	274.64 ± 12.29^{abc}
D+G+OSE	434.28 ± 5.92^{ab}	349.88 ± 13.04^{abc}	262.10 ± 13.32^{abc}	206.68 ± 14.01^{abc}

Results statistically analyzed by two-way ANOVA following post-hoc Tukey's test and presented as mean ± SD (n = 6). Where, statistical significant difference of treatments from NC ($^{a}p < 0.05$), N+OSE ($^{b}p < 0.05$) and DC ($^{c}p < 0.05$), respectively

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