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Inhibition of Streptozotocin-Induced Apoptosis in Beta Cells by *Peganum harmala* Seed Extracts in Adult Male Rats

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Abstract

Objectives: Many attempts have focused on controlling the progression of beta-cell destruction due to diabetes apoptosis. Therefore, any treatment with the least side effects inhibiting the progression of apoptosis and the destruction of the islets of Langerhans is valuable. This survey is the first one to compare the anti-apoptotic effect of the *Peganum harmala* seed extract with that of harmine in rats.

Materials and Methods: To this end, six equal groups (n=8) of rats (250-300 g) were selected in this experimental study. The diabetes condition was induced by the intraperitoneal administration of streptozotocin (STZ) (65 mg/kg). The methanolic extract of *P. harmala* seeds and harmine were gavaged to healthy and diabetic rats. The active substances of *P. harmala* were investigated by the high-performance liquid chromatography method. Flow cytometry was also employed to evaluate the apoptosis of beta cells. Hematoxylin and eosin and argyrophilic nucleolar organizer region staining were applied to examine pancreatic tissues.

Results: The obtained results from treatment with the seed extract and harmine were similar in the treatment groups. This treatment elevated plasma insulin levels while reducing plasma glucose levels and apoptosis ($P<0.05$). Finally, staining results showed that the seed extract could improve tissue damage more than harmine ($P<0.05$).

Conclusions: Our findings revealed that the seed extract of *P. harmala* is more effective in controlling diabetes and apoptosis due to diabetes compared to its active ingredient, namely, harmine. This feature seems to be due to its high percentage of antioxidants together with β-carboline compounds.

Keywords: Apoptosis, Harmine, Type 2 diabetes

Introduction

Apoptosis or cellular suicide is normally stimulated and activated by various signals including morphological changes in the cells (1,2). The decreased rate of apoptosis results in the survival and accumulation of abnormal cells that may lead to cancerous tumors (1). Apoptosis is responsible for several diabetes complications such as diabetic neuropathy (2) and diabetic nephropathy (3,4). Therefore, identifying factors influencing the apoptotic process can clarify the unknown aspects of disease progression and provide new therapies (5).

One of the factors inducing hyperglycemia in adult rats is using streptozotocin (STZ), the mechanism of action of which is through the selective destruction of pancreatic beta cells by oxidative stress. Accordingly, this process gives rise to the reduction of insulin in circulation, the elevation of the blood glucose level, and the presence of severe symptoms (2-4,6). Obesity and diabetes type 2 associated with insulin resistance have a link with decreased functional beta-cell mass (7, 8), affecting some cellular processes such as inflammation and apoptosis (9).

Peganum harmala belonging to the Zygophyllaceae family is famous for its active medical compounds, especially alkaloids, which are found in the seeds and roots and are used for the prevention of diabetes-associated complications. The main constituent of this plant is β-carbolines alkaloids viz harmine, harmaline, harmalol, and harman, as well as vasicin and vasicinone (10). Very few investigations have so far addressed the effect of the methanolic seed extract of *P. harmala* on the blood glucose level in rats (11). Moreover, studies on the insulin-secreting activity and anti-apoptotic effect of *P. harmala* seed extracts are scarce (5,12). In this regard, this survey attempted to first investigate the anti-apoptotic effects of the methanolic seed extract of *P. harmala* and its active ingredient (harmine) on the islets of Langerhans in rats.

Materials and Methods

Materials and Their Characterization

Harmine, as the main treatment agent, and STZ ($C_8H_{15}N_3O_7$) were purchased from Sigma-Aldrich (USA). All other chemicals were acquired from different valid brands such as Merck (Germany) and Sigma-Aldrich. *P. harmala* (recorded with number 107208 in the Central Herbarium of Iran) was collected from areas near Zahedan (Sistan and Baluchestan province, Iran) in spring.

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Key Messages

- The methanolic seed extract of *P. harmala* has an anti-apoptotic effect.
- The *P. harmala* seed extract is more effective in controlling diabetes compared to harmine.
- All the applied tests in the study were of high sensitivity.

Extraction Process

The plant sample was first crushed for the extraction of the methanolic seed extract of *P. harmala*. Subsequently, 100 g of the sample was added to a thimble, which was then placed in the middle of the soxhlet. Afterward, methanol (500 mL) was admixed with the as-collected samples and added to the soxhlet extractor at a rate of 20 siphons for 4 hours. The solvent was separated from the extract by rotary evaporation and stored at the temperature of 4˚C until subsequent use (13). Next, alkaloid was extracted from the seed extract by adding 5 mL of 5% sodium carbonate 1 g to the dry plant powder at room temperature for 24 hours. To the mixture, 10 mL of 85% ethanol was added and placed in a hot water bath at 60˚C for 15 minutes. The extraction process was repeated three times, and the solutions were mixed finally. After centrifugation at 3000 rpm for 10 minutes, the precipitates were separated from the alcoholic solution, which was then transferred to a beaker and evaporated at 50˚C. In the next step, the beaker was washed with 10 mL of each of 5% sulfuric acid and diethyl ether, and the resultant extract was separated from the lower acidic phase, the pH of which was adjusted at 10 with sodium. Next, chloroform solution was added, and the lower chloroform phase was collected, which was accomplished three times, and the chloroform was separated accordingly. Next, the total volume of the lower chloroform, the excess chloroform, and the lower chloroform were separated. The collected chloroform was evaporated at low temperature. Eventually, the beaker was rinsed with pure methanol (5 mL), and extracted alkaloids were prepared for analysis by the high-performance liquid chromatography (HPLC) to assess the quantitative analysis of the extracts. In this method, C_{18} column, as well as isopropyl alcohol, acetonitrile, water, and formic acid (100:100:300:3 ratio; as the mobile phase [1 mL/min]) were used, and chromatograms were produced in 330 nm (14). Anthocyanins and flavonoids were evaluated by the Shinoda test. Moreover, the ferric chloride and Borntrager tests were performed to verify the presence of tannins and check the presence of anthraquinones, respectively (15).

Animals and Experimental Design

Male Wistar rats (n = 48) with a body weight of 250-300 gr were maintained in 12-12 hours of lightness and darkness and had *ad libitum* access to water and standard food. The animals were randomly allocated to six equal groups including diabetic (D), control (C), harmine (H), diabetic + harmine (DH), seed extract (S), and diabetic + seed extract (DS).

Diabetics and Medication

After dilution in Milli-Q water (3 M), STZ (65 mg/kg) was administered (a single dose) intraperitoneally to the rats (16). Briefly, 16.25 mg of STZ was dissolved in distilled water and injected with a volume of 0.7 mL into the 250-g rats. To this end, all the rats fasted for about 12 hours. After 48-72 hours of fasting, rats with plasma glucose level of higher than 200 mg/dL and those with polydipsia and polyuria were selected for further study (17). On day 13 and after the STZ administration, all the rats underwent gavage. In addition, the blood glucose level of the rats was checked on days 10, 14, and 20. Each experimental group received 150 mg/kg of the seed extract and 6.5 mg/kg of harmine for 28 days in a gavage manner. Following the treatment period, the rats were anesthetized with ether and evaluated for apoptosis examinations, insulin and glucose assay, and histological examinations.

Preparation of Single-cell Solution

Trypsin (0.25 g) was diluted in 100 mL of the physiological serum according to the instruction recommended by the producer. DNAase was also prepared in accordance with the producer's protocol, and then DNAase solution (50 µL) was added to trypsin (5 mL). The pancreas tissue was rinsed with phosphate-buffered saline to eliminate the remaining blood, and then the tissue was crushed. Next, a mixture of 0.25% trypsin (100 µL) and 0.5% DNAase was added to the r amount of the crushed tissue in a microtube (18). Every 10 minutes, the content of the microtube was mixed by constant pipetting up and down for 30 minutes. This process was performed each time at 37˚C for 5 minutes. Thereafter, the bovine serum (5 µL) was added to the microtube until the inactivation of the enzyme, and the content of the microtube was centrifuged at 1500 rpm at 44˚C for 5 minutes. After discarding the supernatant, the remnants were placed in a new microtube and kept at -80 ˚C, then PBS was added as well. Finally, 10 minutes prior to flow cytometry analyses, 100, 10, and 5 µL of buffer, propidium iodide, and annexin were added, respectively, and apoptosis was examined accordingly.

Apoptotic Experiments

Primarily, the single cells were transferred to 1 mL of a binding buffer (1 mL). Subsequently, 100 µL of the binding buffer (containing 10^5 cells) and Annexin-FITC (5 µL) were added to cytometric tubes, respectively. The tubes were incubated in the dark at ambient temperature for 15 minutes, and then binding buffer (1 mL) was transferred to the tubes and centrifuged at 1500 rpm at 44˚C for 5 minutes. After discarding, the supernatant was added to the remaining 100 µL of binding buffer. Next, the propidium iodide solution (5 µL) was transferred to the tube, and the process was continued at ambient temperature for 15 minutes. Ultimately, the analysis of

the samples was accomplished by the flow cytometry apparatus (BD FACSCalibur; BD Bioscienes, San Jose, CA, USA) (16).

Insulin and Glucose Assay

The level of blood glucose was determined by a glucometer at the 10th, 14th, and 20th days, as well as an AutoAnalyzer (AutoAnalyzer Alpha Classic, Tajhizatsanjesh, Iran) at the end of blood sampling from the heart. The whole blood was placed in a centrifuge tube and incubated at ambient temperature for 30 minutes and then centrifuged at 5000 rpm for 10 minutes. At this stage, the serum was isolated, and insulin and glucose were measured by the mentioned AutoAanalyzer.

Histopathological Evaluations

To investigate histopathological evaluations, a standard protocol was applied as explained earlier (19). Briefly, a portion of the pancreas tissue stabled in a 10% formalin buffer solution was placed in paraffin, and sections (3-5 μm) were prepared accordingly. For H&E staining, the hydration stage, including staining with hematoxylin solution and eosin Y solution, was conducted, followed by the dehydration stage with ethanol and sample clearing by xylene. For argyrophilic nucleolar organizer region (AgNOR) staining, silver nitrate was used to stain the prepared samples on the slides. Finally, the prepared slides were observed using a microscope (Olympus BX51).

AgNOR Staining

To perform AgNOR staining, the solid sections (3 μm) of samples were constantly processed for each case from formalin-fixed, paraffin-embedded blocks, dewaxed in xylene, and dehydrated through alcohols to deionized water. Then, the sections were incubated in a new solution in darkness at ambient temperature at 37°C for 38 minutes. The solution was prepared by a mixture of two parts of gelatin 2% in 1% formic acid with one part of the 50% aqueous silver nitrate solution. After being placed in a gold chloride solution of 1% for 5 minutes, rinsing in deionized water, dehydrating in ascending alcohol concentrations, the sections were cleared in xylene and mounted in dibutyl phthalate in xylene. Under a light microscope, the AgNORs were observed as intranuclear dots with brown to black color (16).

Statistical Analysis

All data were analyzed by ANOVA and Tukey test and were estimated using the SPSS software, version 20. The values were represented as mean ± standard deviation, and $P<0.05$ was considered statistically significant.

Results

HPLC Study

HPLC was performed to measure β-carboline-type alkaloids and other compounds (i.e., harmaline, harmine, harmol, and harmalol) of *P. harmala* seeds. HPLC results are shown in Table 1.

Table 1. Characterized Metabolites by HPLC and DPPH Test

Extract Type	Seed (%)
Extraction	29.7
Alkaloid	
Harmalol	0.12
Harmol	0.02
Harmane	0.029
Harmaline	3.8
Harmine	2.93
Total flavonoid contents (mg QE/mg extract)	16.63
Total phenolic contents (mg GAE/mg extract) at 200 mg/mL	30.46
Anti-oxidant	
Scavenging (%)	68.9
IC_{50}	122.1

Note. HPLC: High-performance liquid chromatography; DPPH: 2,2-diphenyl-1-picrylhydrazyl; IC50: Half maximum inhibitory concentration.

Apoptosis Results and Insulin and Glucose Measurements

The effect of treatments on blood insulin and glucose levels was investigated in the examined rats. The results are represented in Figure 1. Treatments were applied via utilizing the methanol extract from seed (150 mg/kg) for 4 weeks. Figures 2 and 3 illustrate the effects of treatments on the apoptosis level in the experimental and diabetic groups, respectively. The comparison of groups C with D revealed the apoptosis induction by STZ in diabetic rats ($P\leq0.05$). Apoptosis has also been observed in experimental groups treated with seed extracts and harmine. In the S group, the seed extract resulted in the least amount of apoptosis while it had the highest rate of recovery in the DS group. It was noticeable that both harmine and the seed extract had similar effects on apoptosis and no significant difference was detected in this regard. Figure 2B depicts the effects of treatments on the insulin level in diabetic and experimental groups. The reduction in the insulin level was quite significant in group D relative to group C. The level of insulin, however, elevated significantly in both DS and S groups in comparison to other diabetic and experimental groups, respectively. Figure 2C displays the effects of treatments on the glucose level in diabetic and experimental groups. The level of glucose, the same as that of insulin, increased significantly in group D relative to the C group while it reduced only in DS and DH groups.

Results of Histopathological Evaluations

The prepared slides were examined via a microscope (Olympus, Japan), and then H&E and AgNOR staining techniques were applied to the samples. The results of staining are illustrated in Figure 4. As shown, Langerhans islands were normal and intact in the control group, and alpha and beta cells were distributed normally. However, Langerhans islands seemed to be smaller, and beta cells

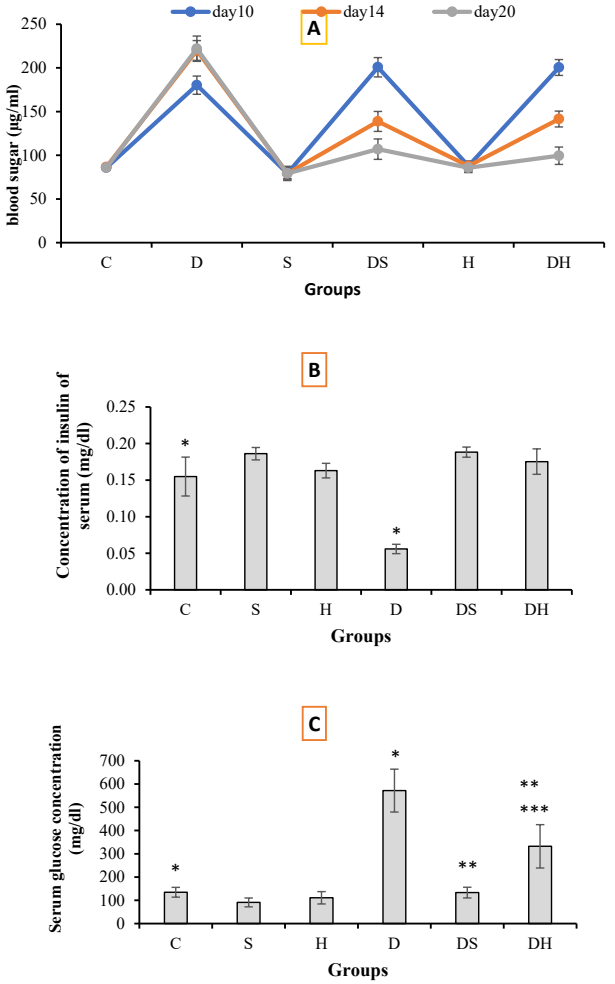

Figure 1. The Effect of Treatments on Blood Glucose on 10th, 14th, and 20th day (A), as well as Insulin (B) and Glucose (C) Levels. *Note*. SD: Standard deviation. All experiments were applied in triple and reported as the mean ± SD. **P*≤ 0.01, ***P*≤ 0.01, and ****P*≤ 0.05 compared with C, D, and DS groups, respectively; C: Control; S: Treatment with the seed extract; H: Treatment with harmine; D: Diabetic group; DS: Diabetic + seed extract; DH: Diabetic + harmine.

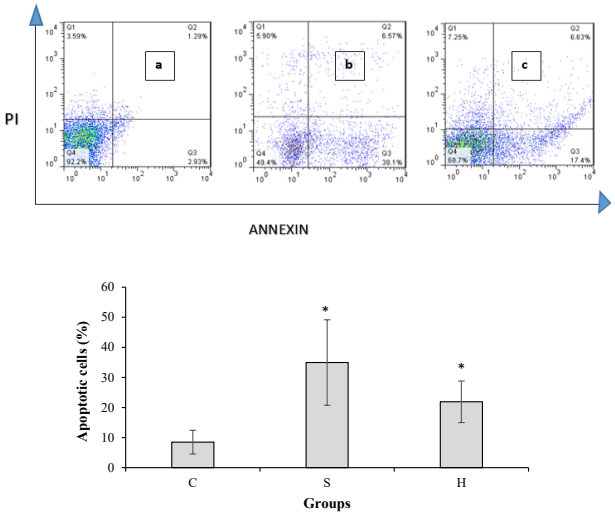

Figure 2. Effect of Treatments on Pancreas Cell Apoptosis for C (a), S (b), and H (c) Groups. *Note*. C: Control; S: Treatment with the seed extract; H: Treatment with harmine. **P*<0.05 compared with the C group.

were totally destructed in the diabetic control group. Based on the results, increased cell diameter, a large number of Langerhans islands, and higher beta-cell retrieval were observed in DS and DH groups after treatments. Interestingly, these improvements were more visible in the DS group in comparison with the DL group.

Discussion

Evidence revealed a relationship between diabetes type II and beta-cell mass reduction. It has also been approved that apoptosis plays a role in beta-cell disruption (20) such that apoptosis may frequently occur during diabetes complications (21). Inflammatory modulators such as interleukins (ILs) can target apoptosis pathways in beta cells and result in the loss of beta cells, consequently, causing complications in diabetes (22,23). It was previously reported that harmaline can interfere with cell cycle gene expression, and upregulation of genes is involved in the apoptosis process such as caspases 8 and 3 (7). Investigations also exhibited that phenolic compounds can alter the inflammatory activity of cytokines (e.g., IL-6, IL-1β, and tumor necrosis factor-α) and diabetes type II-related genes, along with elevating transpirational factor enzymes, namely, NF-κB and PPARγ (24). Plant secondary metabolites such as genistein and puerarin can reduce or even suppress apoptosis in a similar way (23). Therefore, unsurprisingly, naturally occurring compounds in *P. harmala*, especially the seed extract, are able to diminish induced apoptosis by diabetes. Imidazoline compounds can stimulate insulin secretion through activating imidazoline I_3 binding sites in beta cells situated in the pancreas (21-23,25). It has also been assumed that β-carbolines may have activity at imidazoline sites.

Based on our results, the methanolic seed extract of *P. harmala* enhanced plasma insulin levels in both diabetic and healthy rats while reducing the glucose level in diabetic rats. A former study found that the seed extract of *P. harmala* is unable to secret insulin. Therefore, its hypoglycemic activity can be ascribed to glucose usage or its absorption rather than the pancreas (25). Komeili et al (26) suggested that the anti-diabetic effects of *P. harmala* seeds may be either related to its antioxidant properties or enzymatic inhibition or the agonist/antagonistic effect on responsible receptors. They further proposed that the hypoglycemia effect of the hydroalcoholic seed extract of *P. harmala* may arise from the increased insulin secretion by the remaining pancreatic beta cells in diabetic rats. However, they reported no significant changes in the plasma glucose and HbA1C level of non-diabetic rats treated with the *P. harmala* extract (26). The results of another study uncovered that *P. harmala* extracts

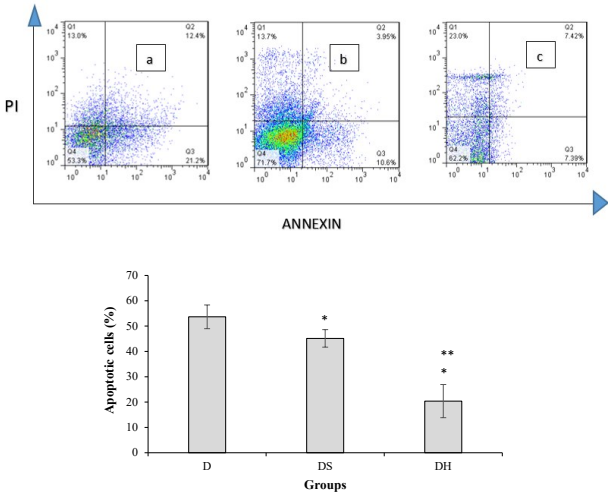

Figure 3. Effect of Treatments on Pancreas Cell Apoptosis for D (a), DS (b), and DH (c) Groups. *Note*. D: Diabetic group; DS: Diabetic + seed extract; DH: Diabetic + harmine. $^{*}P<0.05$ and $^{**}P<0.05$ compared with D and DS groups, respectively.

significantly reduce the blood glucose level in diabetic and non-diabetic rats in a dose-dependent manner (11). Based on the data in Figure 1, the seed extract increased the plasma level of insulin but reduced the blood glucose more effectively than harmine. Thus, the use of harmine, as the active ingredient of the *P. harmala* seed extract, can improve hyperglycemia in diabetic rats. However, due to its antioxidant and flavonoid compounds, the seed extract can more effectively influence insulin and glucose levels at least in cases with type 2 diabetes.

The results of the staining of the pancreatic tissue in the diabetic group demonstrated that 65 mg/kg of STZ can cause type 2 diabetes. An explanation for this observation could be the presence of a number of small and scattered islands in the pancreatic tissue. Another possible reason is the significant difference between the number of these islands in group D and that of group C.

The histopathological results of the current study in the control and diabetic groups are in agreement with those of previous research (27), implying that both normal and necrotic Langerhans islands are accompanied with reduced beta-cell mass. Our histological results indicated that the seed extract and harmine were both effective in preventing the process of apoptosis and biochemical factors. Although the treatment of DS and DH groups with both the seed extract and harmine showed tissue improvements, damage to tissues in the DH group was greater than that of the DS group. A possible reason could be the presence of flavonoid and antioxidant compounds in the seed extract that are effective when interfering with β-carbolines such as harmine. More survey is needed to determine the exact mechanism of these effects. In their study, Musman et al (28) exhibited that the methanolic extract of *S. oleana pericarp* improved beta-cell granulation while decreasing the blood glucose level

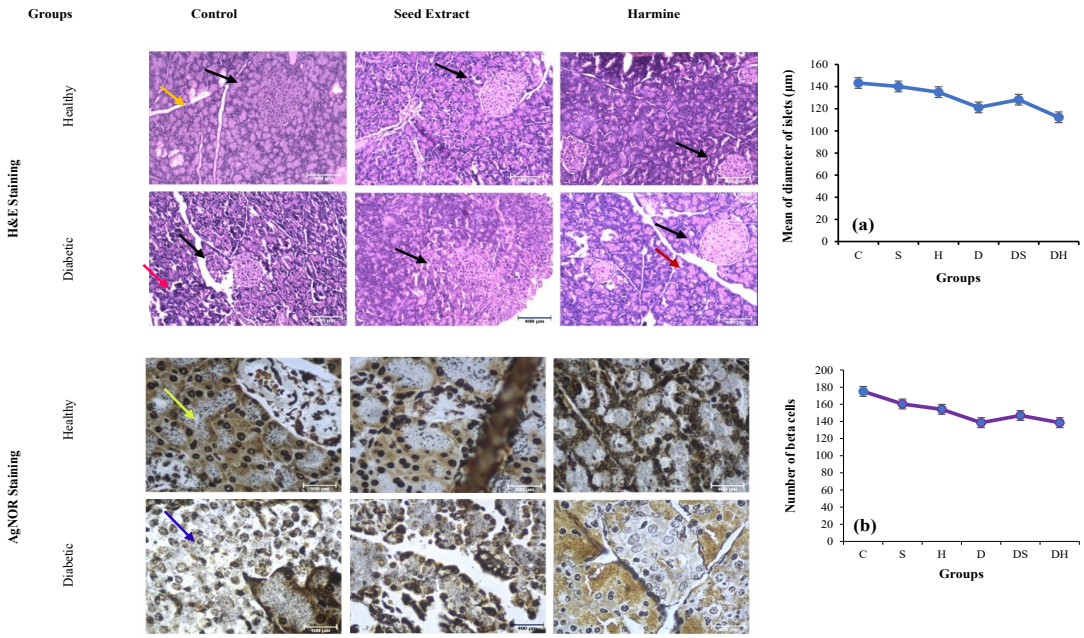

Figure 4. Micrograph of Pancreas Tissues Stained with H&E and AgNOR (Magnification, ×400): (a) Mean Diameter and (b) Number of Beta Cells in Pancreas on the Examined Rats ($P<0.05$). *Note*. H&E: Hematoxylin and eosin; AgNOR: Argyrophilic nucleolar organizer region. Black arrows represent the Langerhans islands in the treatment groups, and orange arrow indicates normal acinar arrangement with basal basophilia and apical acidophil. In addition, the pink arrow denotes fibrous tissue septa with the disruption of acinar arrangement appeared as entrapped acini with widened interlobular duct, as well as the degenerated entrapped islet of Langerhans. Further, red and blue arrows demonstrate widened interlobular duct passing through Langerhans islands and the decreased number of AgNOR-positive dots, respectively. Finally, the yellow arrow displays AgNOR-stained nuclei with favorable uniformity. C: Control; S: Treatment with the seed extract; H: Treatment with harmine; D: Diabetic group; DS: Diabetic + seed extract; DH: Diabetic + harmine.

owing to their flavonoids and phenolic compounds. This feature is mostly referred to the high amount of flavonoids in the seed extract that can act as strong antioxidants neutralizing oxidative stresses originated from diabetic complications (29,30). Therefore, the antioxidant effect of flavonoids and the β-carboline nature of harmine are together responsible for the effective impacts of the seed extract on damages due to STZ.

The present study has some limitations. In this study, only one single dose of the *P. harmala* seed extract and harmine was administered to rats. Another restriction was a failure to assess the gene pathways of apoptosis, which could have a significant effect on the results. Accordingly, it is recommended that future investigations determine the intracellular or extracellular pathways of apoptosis in the pancreatic tissue of diabetic rats by examining the expression of relevant genes.

Conclusions

The findings of our research work imply that the increased dose of the *P. harmala* extract can give rise to hypoglycemic activity reductions. Our findings also suggest that the selected doses of treatments in experimental groups have some side effects. In other words, the administration of the same dose of β-carbolines on the pancreas of healthy and diabetic rats has different effects.

Authors' Contribution

AS: Literature search, experimental studies, and statistical analysis; SO: Designing, defining intellectual content, and editing the manuscript; RA: Data analysis, manuscript preparation, and manuscript editing; KP, Designing, preparing the manuscript, and editing the manuscript.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

The protocol for animal care and use was approved by the Ethics Committee of the Islamic Azad University, Science and Research Branch, Tehran, Iran (IR.IAU.SRB.REC.1396.40).

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