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Role of pumpkin seed extract in improving the apoptotic pathways of hepatocytes in STZ-induced diabetic rats

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ABSTRACT

In diabetic rats given an experimental treatment, the impact of alcoholic pumpkin seed extract (Cucurbita pepo L.) on hepatocellular mortality and inflammation was studied. One intraperitoneal injection of streptozotocin (STZ; 40 mg/kg) caused diabetes in male rats, followed by a dose of pumpkin seed extract (200 mg/kg), for three months, given every other day. TNF-α levels in diabetic rats treated with pumpkin seed extract improved to normal. Pumpkin seed extract also prevented arise in p53, Bax, caspases-3, 8, and 9, lessened hepatic damage of DNA, and slowed the rise in TNF-α levels. It also regulated apoptosis by increasing the production of Bcl-2 protein. Based on current research, pumpkin seeds may shield the liver from necrosis, apoptosis, inflammation, and DNA damage by enhancing the redox state, inhibiting p53 and caspases, and raising Bcl-2 protein. As a result of enhancing apoptotic signals, pumpkin seeds have a great deal of therapeutic promise for managing diabetes and its adverse effects on the liver.

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Introduction

Diabetes mellitus is a metabolic disorder characterized by high blood sugar (Hyperglycemia) and altered energy metabolism caused by a relative or complete deficiency in insulin secretion, a lack of insulin action, or both. It is a chronic disease that occurs when the pancreas is incompetent to produce enough insulin or the body cannot use the produced insulin effectively. Insulin is a hormone that controls the amount of glucose in the blood. Persistent high blood sugar in diabetes causes various biochemical and structural changes in the body's cells, tissues, and organs. These changes explain the widespread complications of diabetes that can occur in many body organs, including the liver, kidneys, eyes, nerves, blood vessels, etc [1]. Medicinal plants have been a suitable alternative to these materials. This trend was reinforced by the World Health Organization (WHO) Expert Committee report on diabetes, which recommended conducting more studies and research on conventional methods used for treating diabetes [2]. Thus, the interest of many researchers in this aspect has focused on identifying the most important plants that reduce blood sugar levels, which are used in particular by those working in the field of folk medicine. Other researchers investigate the Histotoxicity of several of these commonly used plants.

The study aim

In the present study, we addressed one of the plants familiar to those working in folk medicine, the pumpkin plant (Cucurbita), by using the alcoholic extract of its seeds to study its therapeutic effectiveness in reducing blood sugar in male rats induced with diabetes and improving the apoptosis (programmed cell death) pathways of the liver and some its biochemical and histological aspects.

Material and methods

Preparation of extracts

The alcoholic extract of Cucurbita pepo was prepared using a Soxhlet extractor device, taking 25 g of powder and adding 500mL of absolute ethanol alcohol. The solvent was absorbed for 10min, with a 20:1 ratio between the substance and solvent. The device was operated at boiling temperature for 2.5–3h. The extracted solution was dried in an oven at 40–50°C and collected in a sterilized vial for use [3].

Chemicals

Sigma Chemical (St. Louis, MO) supplied the streptozotocin (STZ). The highest grade was used for all other compounds.

Initiation of diabetes

Rats were given diabetes by injecting intraperitoneally (i.p.) 40 mg/kg body weight of newly synthesized streptozotocin in 0.01 M citrate buffer at pH 4.5. [4]. The level of blood glucose was tested 48 hours following the STZ injection using an Elegance glucose monitor set (CT-X10, Convergent Technologies GmbH & Co. KG, Marburg, Germany). For the purposes of the studies, the rats were classified as diabetic if their blood glucose level was more than 250 mg/dl.

Experimental Animals

This study was conducted in the animal house of the Department of Biology/ College of Education for Pure Sciences / University of Anbar. The study comprised 40 adult male albino rats of the Sprague Dawley, aged 3 to 4 months and weighing 300 to 320 g. The experiment needed three months to complete. The animals were categorized according to similar weights into four groups containing ten rats each. The first group, Control, was orally dosed with 5 mL/kg distilled water daily; the second group, Pumpkin, was orally dosed with the alcoholic extract of pumpkin seeds at a concentration of 200 mg/kg [5] every other day for three months at a dose of 2 ml and the third group, STZ, was injected intraperitoneally (IP) with STZ at a concentration of 40 mg/kg in a single dose (0.1ml) and left without treatment. It was given water and food normally and was considered a positive control group, Group 4th, The rats were injected intraperitoneally with (40 mg/kg) of streptozotocin, and were orally dosed with alcoholic extract of pumpkin seeds at a concentration of 200 mg/kg every other day for three months after confirming that the rats had diabetes.

Collection of samples:

Rats were given 0.1 ml/100 g intraperitoneally of ketamine/xylazine to induce anesthesia at the end of the experiment.

After administering anesthesia, a cardiac puncture was used to extract blood from the heart. The animals' livers were then removed during dissection. Serum was collected after blood coagulation, by centrifugation at 3000 rpm for 10 minutes, and it was maintained for biochemical assays at − 20 °C.

Inflammation investigations

Kits from R&D Systems (Minneapolis, MN, USA) were used to measure the amount of TNF-α in blood serum.

Flow cytometry study

Analysis of liver sample apoptosis for flow cytometry. The cells were separated into aliquots, suspended in BSA-containing PBS, and kept for analysis at 4 °C. Utilizing Becton Dickinson's Cell Quest Pro software for data gathering and processing, the flow cytometry studies were carried out on a FACSCaliburTM cytometer (BD Biosciences, San Jose, CA). [4].

P53, CD95, Bcl-2, and Bax

The cell suspensions were made in a PBS/BSA buffer, and for 30 minutes, they were treated with anti-Bcl-2 [100/D5] and anti-Bax [6A7] antibodies (ab692), respectively, for the purpose of Bcl-2 and Bax flow cytometry analysis. For P53 analysis via flow cytometry, a rat anti-P53 "aa20-25" FITC, Clone: DO-1, was employed. To assess CD95, the anti-Fas (CD95/Apo-1) monoclonal antibody was used. Flow cytometry was used to evaluate the cells after they were incubated for 30 minutes at room temperature, centrifugation after PBA/BSA washing for 5 minutes at 400 × g, and then again suspended in PBS/BSA containing 0.5% paraformaldehyde.

Caspases 3–8, 9 regarding cases -3, -8, and -9: The following antibodies were used: monoclonal antibody against caspase-9 (E23) in rats (ab32539), anti-caspase-8 (E6) antibody (Abcam), and FITC rabbit anti-active caspase-3 (CPP32; Yama; Apopain, BD Bioscience).

Analytical statistics

The data were expressed as means and standard error of mean (±SEM) using GraphPad Prism version 6.01. For statistical differences between two groups, the student's t test was employed; for multiple comparisons, one-way analysis of variance was followed by Tukey's test. P-values less than 0.05 were regarded as statistically significant.

Results

Proinflammatory cytokines

Using enzyme immunoassay, influence of pumpkin seed extract on TNF-α production in the serum of several rats groups was assessed. TNF-α levels were considerably greater in animals that received STZ than in control values (Fig. 1). When compared to diabetic rats, the administration of pumpkin seed extract improved the rise in serum inflammatory proteins and demonstrated only minor alterations.

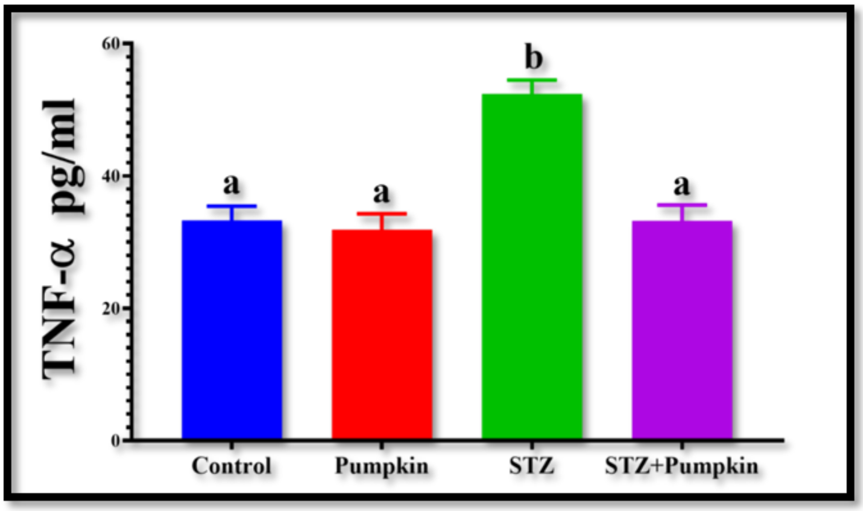

Fig. 1: The impact of pumpkin seed extract (Pumpkin) and streptozotocin (STZ) on the level of serum TNF-α in rats across several groups, expressed as pg/ml. Values represent mean ± standard error. The different letters indicate the presence of significant differences at a significant level (P<0.05) between the groups. Similar letters indicate that there are no significant differences at a significant level (P<0.05) between the groups (n = 10).

Apoptosis and apoptotic proteins: a flow cytometric analysis

P53 and CD95

In the liver of rats treated with STZ, there was also a notable rise in the expressions of P53 and CD95 (Fig. 2a, b). Compared to the diabetic rats, the treatment of pumpkin seed extract following diabetic induction greatly reduced the rise in CD95 and P53 in the liver. Diabetes's effects and pumpkin seed extract on certain apoptotic proteins have been assessed.

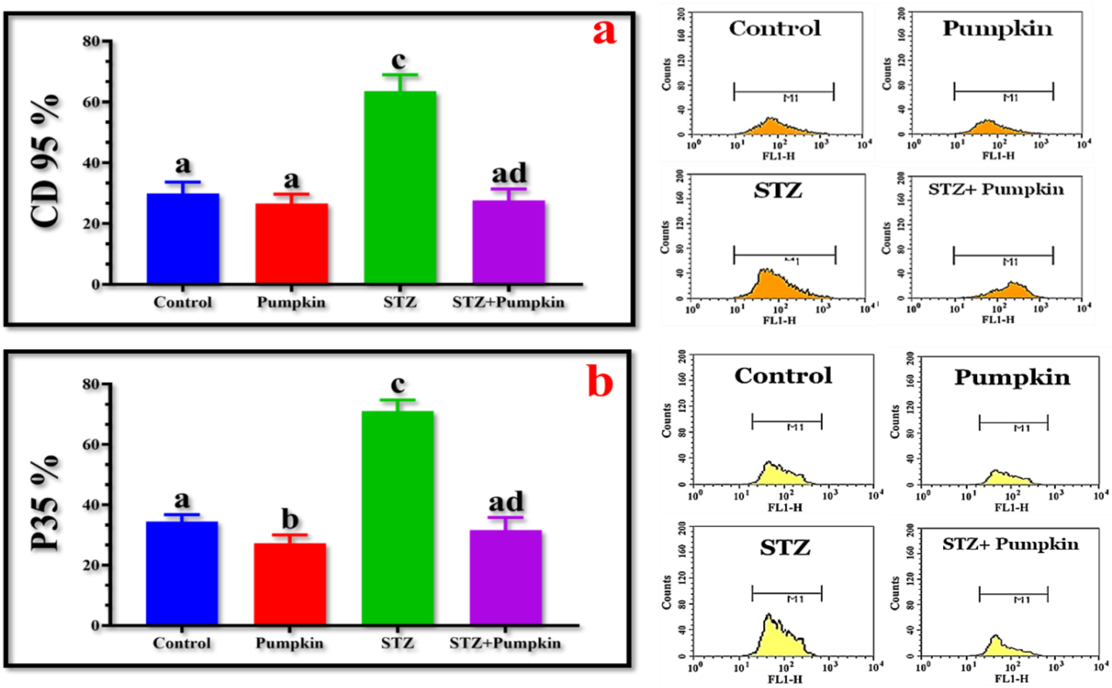

Fig. 2: Impact of pumpkin seed extract (Pumpkin) and streptozotocin (STZ) on the proportion of P53 (b) and CD95 (a) in rats across various groups. Each histogram has flow cytometry findings on the side that display samples of the data produced by FACS.. Values represent mean ± standard error. The different letters indicate the presence of significant differences at a significant level ($P<0.05$) between the groups. Similar letters indicate that there are no significant differences at a significant level ($P<0.05$) between the groups ($n = 10$).

Bax and Bcl-2

The examination of the flow cytometry data showed that in the liver of STZ-induced diabetic rats, the apoptotic protein Bax was increased and the antiapoptotic protein Bcl-2 was considerably decreased (Fig. 3a, b). The application of pumpkin seed extract (Pumpkin) significantly restored the STZ-induced alterations in these proteins' expression

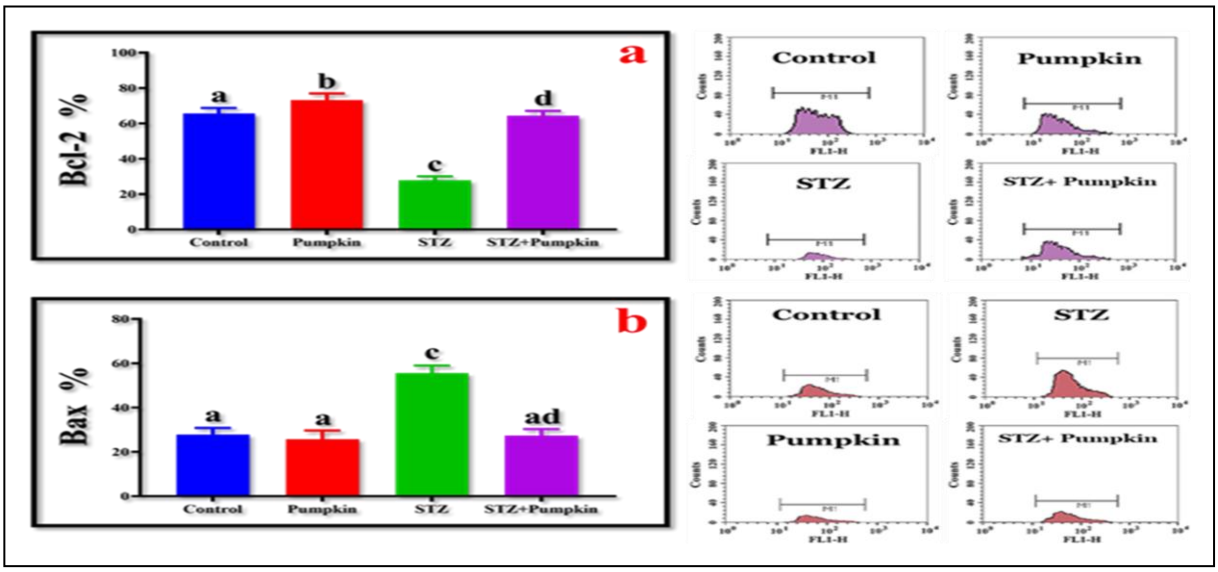

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Fig. 3: Effects of pumpkin seed extract (Pumpkin) and streptozotocin (STZ) on the proportion of Bcl-2 (a) and Bax (b) in rat livers for various groups. Each histogram has flow cytometry findings on the side that display samples of the data produced by FACS. Values represent mean ± standard error. The different letters indicate the presence of significant differences at a significant level (P<0.05) between the groups. Similar letters indicate that there are no significant differences at a significant level (P<0.05) between the groups (n = 10).

Caspases-3, 8 and 9

The livers of the STZ-induced diabetic rats showed a significant up-regulation of caspase-3, 8 and 9, as shown in Fig. 4. The treatment of pumpkin seed extract greatly restored these apoptotic proteins to normal (Figs. 4 a, b, c).

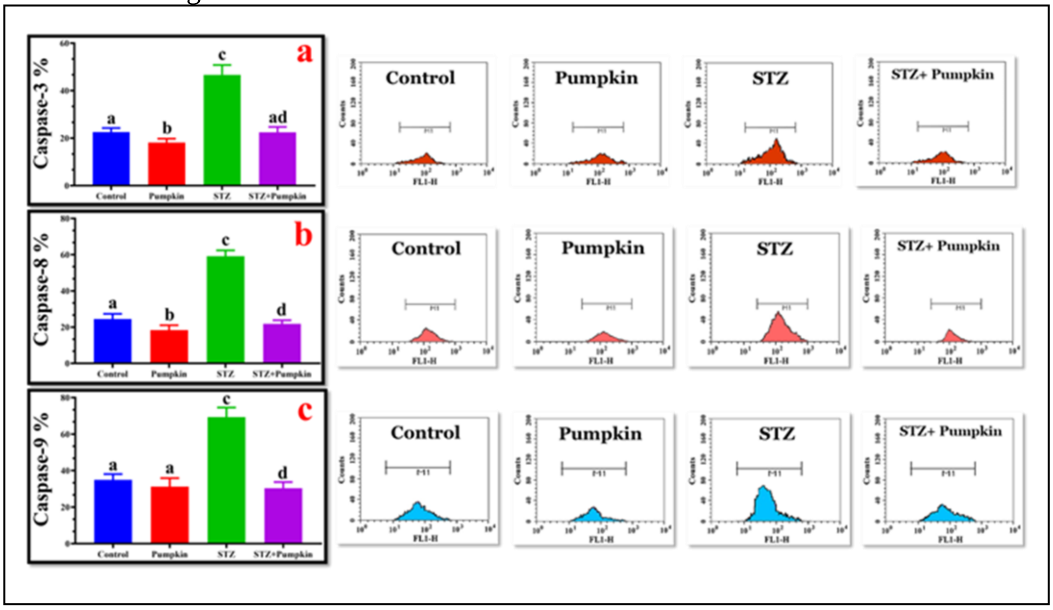

Fig. 4: Impact of pumpkin seed extract (Pumpkin) and streptozotocin (STZ) on the expression of caspases 3 (a), 8 (b), and 9 (c) in rat livers for various groups. Each histogram has flow cytometry data on the side that illustrates samples of the data produced by FACS. Values represent mean ± standard error. The different letters indicate the presence of significant differences at a significant level (P<0.05) between the groups. Similar letters indicate that there are no significant differences at a significant level (P<0.05) between the groups (n = 10).

Discussion

Effect of alcoholic extract of pumpkin seeds Cucurbita pepo L. on TNF-α level

Tumor necrosis factor alpha (TNF-α) is an active inflammatory cytokine. Its activity is increased in acute inflammation caused by proinflammatory cytokines, and it is also a key mediator of various physiological processes, cell proliferation, apoptosis, insulin resistance, and endothelial cell functions. Many types of cells in the body contain TNF-α. It is generated in the liver mainly by Kupffer cells [5].

The results showed a significant increase in the level of tumor necrosis factor TNF-α in the animals of the third group treated with streptozotocin, and this is consistent with many studies, proving that the increase in TNF-α is correlated with the occurrence of diabetes and insulin resistance, either directly or indirectly [6, 7]. The study results align with research demonstrating that diabetic animals exhibited elevated levels of TNF-α, which was correlated with an inflammatory response resulting in beta cell death [6]. High concentrations of inflammatory cells may lead to apoptosis of beta cells, which may result in decreased numbers of insulin-producing cells and decreased insulin levels. Elevated levels of MDA and TNF-α in diabetes have also been shown to be due to increased oxidative stress or decreased antioxidant defense mechanisms.

In the current study, rats treated with streptozotocin, and pumpkin seed extract had lower serum levels of TNF-α than the third group of rats with diabetes. This is due to the anti-inflammatory and anti-tumor activity of some compounds of pumpkin seed extract, including carnosic acid, carnosol, and caffeic acid, which have an inhibitory effect on cancer cell migration and an enhancing effect on programmed cell death in cancer cells [8].

In this study, Because of its antioxidant qualities, we postulated that pumpkin seed extract may alter cell function during inflammation. The results showed that pumpkin seed extract reduced the concentration of TNF-α, which may be due to the active substances or biologically active substances in pumpkin seeds, especially phytosterols, polyphenols, and carotenoids, which have anti-inflammatory properties, in particular polyphenols, which get interest to researchers as potential therapeutic agents for various diseases due to their antioxidant activity [9].

Effect of alcoholic extract of pumpkin seeds Cucurbita pepo L. on apoptotic pathways of hepatocytes of diabetically induced male rats

To comprehend the process by which pumpkin seed extract inhibits apoptosis in the kidney and liver of diabetes patients, we studied the intrinsic and extrinsic apoptotic pathways induced by STZ injection in experimental rats. The doses administered to the rats decreased STZ-induced apoptosis in the liver and kidney tissues, according to flow cytometry analysis, by upregulating proteins of pro-apoptotic like Bax, CD95, caspases 3, 8, 9, and p53 in addition to anti-apoptotic proteins like Bcl2. These findings show that all mitochondrial death receptors and apoptosis pathways were significantly altered in the liver and kidney of diabetic rats given an alcoholic pumpkin seed extract treatment. These findings so demonstrate that pumpkin seeds can influence apoptotic pathways in diabetics.

Pumpkin seeds are rich in antioxidants and substances and elements biologically active in protecting the liver by stimulating the effectiveness of antioxidants and raising the level of the anti-apoptotic protein Bcl2, as the apoptosis of liver cells was evident through DNA damage, which was confirmed by the study results through the increase in the level of the pro-apoptotic protein P53, as studies have indicated that the increasing this protein is a sign of cell DNA damage [10, 11]. On the other hand, in the current study, treatment with pumpkin seed extract protected liver tissue from DNA damage caused by STZ injections. This result was evident from the decrease in the P53 protein percentage, indicating that pumpkin seeds prevented DNA strand breakage, which can be attributed to the antioxidant capacity of pumpkin seeds by eliminating ROS in diabetic patients.

It is worth noting that the present results reveal for the first time, to our knowledge, that pumpkin seeds have cellular biological activities through their anti-apoptotic and DNA-protective effects, as the dosing pumpkin seed extract to rats with induced diabetes effectively downregulated or reduced pro- and anti-apoptotic proteins through their antioxidant activity. Pro-apoptotic proteins can induce apoptosis via a ROS-dependent pathway [12], Thus the rise in the ratio of anti-apoptotic and pro-apoptotic proteins is directly correlated with the cell's levels of ROS and GSH. [13].

The results of our current study confirmed that pumpkin seeds reduced apoptosis in the liver tissues of diabetic rats induced by intraperitoneal injection of STZ by preventing DNA damage and suppressing caspases and pro-inflammatory cytokines, which may be related to their effects on blood sugar. The increase in the percentage of GSH observed in the current study results is evidence of the scavenging of H_2O_2 and its disassembly from the cell's internal environment, so it represents protection against oxidizing agents. In addition, glutathione acts to remove a number of toxic molecules in the cells, maintain oxidation Redox inside the cells, and thus reduce apoptosis [14]. Overexpression of Bcl2 acts as a pro-antioxidant that helps protect cells from damage caused by oxidant agents through accurate regulation of the antioxidant defense mechanisms, making cells more resistant to additional hazards [15].

The deficient antioxidants in the serum of STZ-induced diabetic rats increase lipid peroxidation of liver and kidney cell membranes, which increases the cell membrane permeability. Our study confirmed this by observing an increase in MDA and H_2O_2. Also, an increase in ROS increases the Bax expression and decreases the Bcl2 expression, noting that the increase in the expression of Bax changes the mitochondria permeability by forming some pores or small openings in its outer membrane, leading to the release of the apoptosis-inducing factor, Cytochrome-C from the mitochondria to the cell cytoplasm, thus activating caspase and then activating the apoptosis pathway [16].

The increase in Bcl2 expression, obtained in our study as a result of pumpkin alcoholic extract dosing, works to bind to Bax, and restoring the permeability of mitochondrial membranes to normality and preventing the release of Cytochrome-C, thus protecting cells from apoptosis. The apoptosis-inducing protein Bax opens channels in the mitochondrial cell membrane, allowing Cytochrome-C to exit, while the apoptosis-inhibiting protein Bcl2 works to close these channels [17]. Our results indicate that membrane permeability is a reversible stage by increasing the level of antioxidants and decreasing the level of oxidative stress caused by free radicals, leading to the removal of inhibitory factors of apoptotic cell death pathways and thus protecting liver cells from death.

Antioxidants are nutrients—such as vitamins or carotenoids—that play a role in protecting cells from everyday damage. Because of the way cells process oxygen, they produce molecules called free radicals, which are unstable molecules that damage the cells' DNA as they restore stability.

Because it contains biologically active components that act as external antioxidants, pumpkin seed extract increases insulin secretion and improves cell receptors for insulin, thus consuming glucose by cells naturally. In addition, it improves the cells' bioactivities, which will result in a weak accumulation of toxic waste, in particular free radicals, and make the risks weaker, which will help cells continue living.

In conclusion, based on the current study results, we can affirm that the alcoholic extract of pumpkin seeds reduced the physiological changes caused by STZ injection in the experimental rat group and might be a promising and safe alternative to blood sugar-lowering drugs.

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دور مستخلص بذور اليقطين في تحسين مسارات موت الخلايا الكبدية المبرمج في الجرذان المصابة بمرض السكري المستحث بالستربتوزوتوسين

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الملخص

في الجرذان المصابة بداء السكري التجريبي المستحث بالستربتوزوتوسين ، تمت دراسة تأثير مستخلص بذور اليقطين الكحولي (Cucurbita pepo L.) على معدل موت الخلايا المبرمج والالتهابات في الخلايا الكبدية. تسبب حقنة واحدة داخل الصفاق من STZ؛ (40 مجم/كجم) في الإصابة بمرض السكري لدى الفئران الذكور، تليها جرعة من مستخلص بذور اليقطين (200 مجم/كجم)، لمدة ثلاثة أشهر، كل يومين. تحسنت مستويات TNF-α في الفئران المصابة بمرض السكري التي عولجت بمستخلص بذور اليقطين إلى المعدل الطبيعي. كما منع مستخلص بذور اليقطين ظهور p53 وBax وCaspases-3,8,9، وخفف من تلف الحمض النووي في الكبد، وأبطأ ارتفاع مستويات TNF-α ، كما نظم موت الخلايا المبرمج عن طريق زيادة إنتاج بروتين Bcl-2 ، استنادًا إلى الدراسة الحالية، قد تحمي بذور اليقطين الكبد من النخر، وموت الخلايا المبرمج، والالتهاب، وتلف الحمض النووي من خلال تعزيز حالة الأكسدة والاختزال، وتثبيط بروتين p53 و Caspases ، ورفع مستوى بروتين Bcl-2، ونتيجة لتعزيز إشارات موت الخلايا المبرمج، تتمتع بذور اليقطين بقدر كبير من الوعود العلاجية للسيطرة على مرض السكري وآثاره الضارة على الكبد.

الكلمات المفتاحية: مرض السكري، اليقطين، موت الخلايا المبرمج، الإجهاد التأكسدي، STZ