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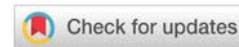
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Research Article

Mitigation with plant ethanol extracts of STZ-induced histopathological injuries in the tissues of laboratory rats

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Summary

This work studied the use of several plant extracts as probable medications in the treatment of diabetes. In laboratory rats, the diabetes model was developed by injection of Streptozotocin (STZ) in a dose of 40 mg/kg of body weight. The animals, in which the blood glucose level increased 4-5 times, were considered as diabetic. The diabetic animals were treated by ethanol extracts of plants traditionally used in folk medicine and/or as food in Armenian cuisine. During four weeks, the STZ-induced diabetic rats were administered orally three times in a week with the ethanol extracts from sorrel leaves (SL, *Rumex Confertus*), pellicles of walnut kernel (WP, *Juglans regia*), grape leaves (GL, *Vitis vinifera*) and rose petals (RP, *Rosa damascena*). At the end of the experiment, certain decrease of blood glucose level was registered in these groups. An oxidative stress marker, malondialdehyde, which increased in the blood of the STZ-induced diabetic animals by 38%, decreased in the SL and WP groups to the level of the intact animals. The histopathological examination of the pancreas, kidney and liver sections from the diabetic animals, fed with the extracts from SL, RP and WP, revealed positive changes compared with the STZ-control animals. The obtained results manifest the benefit of using the studied herbal extracts in the STZ-induced diabetes model.

Introduction

Diabetes Mellitus (DM) is known to be a metabolic disorder resulting from variable interactions of hereditary and environmental factors. It is characterized by abnormalities in the insulin metabolism with subsequent distortions in carbohydrates, proteins and lipid metabolism. Chronic hyperglycemia activates different cellular pathways associated with tissue damage and development of diabetic complications [1], which are often considered as results of pathogenic effects of reactive oxygen species (ROS) [2].

Medicinal herbs contain diverse bioactive compounds [3-5]. Many of drug substances are derived or developed from plant compounds [6]. For instance, a first line drug for type 2 diabetes metformin is developed on the basis of biguanide from

French lilac [7]. The efficacy of many plants as useful agents in the management of hyperglycemia and related complications is evidenced [8,9].

It is known that DM affects the pancreatic exocrine and endocrinesystems. The most common cells in islets of Langerhans in endocrine system are β -cells, which produce insulin, the major hormone in regulation of carbohydrate, fat and protein metabolism. The β -cell mass in islets of Langerhans decreases during the development of DM [10,11].

One of the most widespread complications related to DM is diabetic nephropathy. Disturbances in antioxidant defense systems and ROS lead to accumulation of renal disorders, which is usually associated with kidney damage [12,13]. DM can cause chronic kidney diseases, damage both the nodular apparatus and the proximal and distal tubules. Prevention and management of



diabetic nephropathy are parts of the comprehensive care of DM patients.

DM can also have serious effects on the liver. The odds of having serious liver problems at DM are almost twice as high as having cardiovascular disease. Several investigations have shown the injurious effects of hyperglycemia on liver in different *in vitro* and *in vivo* diabetic models and the amelioration of these effects by natural agents [14]. The agents, which protect the diabetic liver or decrease the severity of its injury, include flavonoids, catechins, various polyphenolic compounds, curcumin derivatives, etc [15]. Hence, it is prospective to continue studying natural compounds, plant compounds in particular, for developing new antidiabetic drugs.

Earlier, inhibition of activities of DM-relating enzymes Dipeptidyl peptidase IV and Adenosine deaminase by aqueous extracts from Armenian Highland plants, was demonstrated [16]. The *in vitro* and *in vivo* effects of ethanol extracts from several plants and their constituents on the activities of these enzymes were described [17]. The transmission and scanning electron microscopy study proved the ability of the ethanol extract from rose petals and of its phenole glycoside fraction to hinder the aggregation of the pancreas peptide hormone, amylin, which is one of the causes of developing DM [18]. The *in vitro* protection of islet β -cells in the presence of cytotoxic aggregated amylin and decrease of its aggregation with the ethanol extracts and fractions from several plants, were demonstrated [19–21]. These results prove the usefulness of herbal preparations in prevention and treatment of DM.

The present work describes the beneficial influence of several plant extracts on the STZ-induced histopathological disturbances in the pancreas, kidneys and liver of rats. The work studied the plants, which are traditionally used in folk medicine and/or as food in Armenian cuisine, but are not studied widely.

Materials and methods

Plants

The leaves of grape (GL, *Vitis vinifera*) and sorrel (SL, *Rumex Confertus*), the rose petals (RP, *Rosa damascena*) and the walnut kernel pellicles (WP, *Juglans regia*) were collected in the Armenian Highland and dried in shade. A voucher specimen has been deposited in the herbarium of the Botanical Department of Yerevan State University (Dr. Narine Zaqaryan). The ethanol extract preparation and characterization by chemical analyses, thin layer chromatography and UV-Vis absorbance were described earlier [22].

Animals

The male white rats of 150–200 g body weight were taken from vivarium of the Institute of Biochemistry. The rats were housed in one room, on an identical diet, in separate cages, 3–5 animals in each, under normal day and night cycles, at temperature of $23 \pm 4^\circ\text{C}$. They were given free access to standard food and tap water.

Ethical approval

Care and treatment of animals, and the experiments were approved by the Ethics Committee on Animal Use at the Institute of Biochemistry (Protocol number: 8, 25.05.2016), based on the main provisions of the “Appendix A of the European Convention for the protection of Vertebrate animals used for experimental and other scientific purposes (ETS N 123)”.

Creation of diabetes model in laboratory rats

The diabetes model was developed by intraperitoneal injection of streptozotocin (STZ, AppliChem GmbH, Germany, dissolved in citrate buffer, pH 4.5, in a dose of 40 mg/kg of animal) to 150–200 g starving rats. The animals were considered as STZ-induced diabetics, if blood glucose index (measured by glucometer) in 3 days raised from 4.8 ± 0.2 (87 mg/L) to 19.6 ± 1.7 (356 mg/L) – $2.6.5 \pm 1.9$ (481 mg/L).

Histological investigations

During four weeks, the STZ-induced diabetic rats were given ethanol extracts of the chosen plants three times a week. At the end of the experiment the animals were subjected to instant euthanasia, samples of the pancreas, kidney and liver were fixed in 10% buffered formalin and embedded in paraffin; 3–5 μm sections were prepared, stained with H&E [23] and examined/photographed with Zeiss Jeneval light microscope.

The average surface area of islets of Langerhans was determined using the Axio Vision LE 4.8.2.SP3 software. Digital data were expressed as mean \pm standard deviation (s.d.).

Measurement of malondialdehyde

The oxidative stress marker, malondialdehyde (MDA) assay mixture in 1.1 ml contained: 100 μl of blood plasma, 750 μl of 2% H_2PO_4 , 250 μl of 0.8% thiobarbituric acid. It was incubated in boiling water bath for 45 min, and the absorbance at 535 nm was registered after cooling [24].

Evaluation of protein carbonylation

The protein carbonylation assay mixture in 400 μl contained: 20 μl of blood plasma, 280 μl H_2O , 100 μl of 10 mM 2,4-dinitrophenyl hydrazine in 2 M HCl. After shaking for 15 min, 100 μl of 6 M NaOH was added, the shaking was continued for additional 15 min to form 2,4-dinitrophenyl hydrazone derivatives, and samples were centrifuged at 6000 g for 5 min. Then the absorbance at 450 nm was registered [25].

Data were analyzed using the statistical software InStat, version 3 for Windows (GraphPad Software, Inc., San Diego, CA, USA). Specific differences were examined using Student's two-tailed t-test. The data showing $P < 0.05$ were considered as statistically authentic. Results were expressed as mean \pm s.e.m.

Results

Treatment of diabetic rats with plant extracts

Usually, in each of 5 independent *in vivo* experiments, a group of 3–5 of 30 laboratory rats was kept as intact control

group. In the other animals, after a 12 hour fast, STZ was injected intraperitoneally in a dose of 40 mg/kg of body weight. The rats were given 5% glucose for the next 24 hours to avoid hypoglycemia. After three days, the development of diabetes was confirmed by measuring glucose in blood samples obtained by cardiopuncture with a heparinized syringe. The STZ-induced diabetic animals, which blood glucose level increased 4-5 times as compared with the intact animals, were divided to groups of 3-5 rats in each. One of these groups was kept as STZ-control. During four weeks, the animals in the rest groups were fed orally three times a week with water suspension of dried ethanol extract from walnut kernel pellicles (WP), Rose petals (RP), leaves of grape (GL) and Sorrel (SL), in a dose of 400 mg/kg of body weight. This dose was chosen as the most suitable after preliminary testing of doses between 150-500 mg/kg.

At the end of four weeks, in the rats with STZ-induced diabetes fed with ethanol extracts from RP, GL, WP and SL, the average values of blood glucose, summarized by all independent experiments (number of animals 8-15) was lower than the value in the STZ-control group by: 7.5%, 18.9%, 27.5%, and 29.8%, respectively.

The mortality of diabetic animals during the experimental period decreased in the order: STZ-control > WP > SL > RP > GL (4.4%, 30%, 17%, 7.7% and 0% of total number of animals, respectively). The numbers of recovered animals in percentage to the total number of diabetics in these groups were: 5%, 35%, 28%, 15% and 25%, respectively.

Lipid peroxidation and protein carbonylation

Lipid peroxidation is implicated in many diseases, including diabetes. Malondialdehyde (MDA) is recognized as a marker of lipid peroxidation. In our experiments, the level of MDA in the STZ-control group was higher as compared with the intact control group by 38% \pm 1.2. At the end of the experiment, in the groups, fed with extracts from WP and SL, the MDA differed from the STZ-control significantly ($p < 0.05$), approaching to the level in the intact animal group, 110% \pm 8.1 and 94% \pm 6.6, respectively.

The level of protein carbonylation is one of the indexes of protein damage caused by oxidative stress [26]. In our experiments, contrary to the lipid peroxidation, the difference in the protein carbonylation was not registered in any of experimental groups. In the groups of the STZ-control and those receiving ethanol extracts from SL and WP for four weeks, the level of protein carbonylation was around 90% of the level in the intact control. Presumably, the expression of this damage demands more longitude following the diabetic conditions.

Histopathology of tissues in diabetic rats

Histopathological characteristics of pancreas in the experimental animals: Normally, the pancreas consists of the exocrine and endocrine parts, of which the former includes pancreatic acinus and excretory ducts, while the latter includes round or oval pale stained islets of Langerhans, interspersed between exocrine acinus. In the islets, the endocrine cells create irregular, branched anastomosing chords, separated by blood

capillaries. Interlobular connective tissue septa are clearly defined (Figure 1a).

It is known that STZ is cytotoxic for insulin-producing cells of the islets of Langerhans, for β -cells in particular. In the pancreas of the non-treated, STZ-control animals (Figure 1b), the islets of Langerhans undergo destructive and dystrophic changes. The insulocytes are necrotic, nuclear pyknosis and karyolysis are observed, the cytoplasm is eosinophilic. Some insulocytes and exocrine cells are vacuolated. The interlobular connective tissue septum is almost absent. The acini and lumen of excretory ducts in the exocrine part are narrowed.

In the pancreas of the STZ-diabetic animals fed with SL, vacuolization, pyknosis, and karyolysis of insulocytes are seen (Figure 1c). However, these pathological destructive changes are less prominent compared to those in the STZ-control group.

A relatively different histological picture is observed in the pancreas of the STZ-diabetic animals fed with the ethanol extract from RP (Figure 1d). The histostructure of parenchymal elements and islets of Langerhans are restored. The destructive and dystrophic changes are moderate. The interlobular connective tissue septum is well detected. The lumen of pancreatic acini and excretory ducts is normal. Yet, in the tissue of this group, cells with vacuolated cytoplasm, dark and pyknotic nuclei are seen.

An identical morphological picture is seen in the pancreas of the STZ-diabetic animals fed with the ethanol extract from WP (Figure 1e). The destructive changes are less prominent as compared to the STZ-control group. In the exocrine part of the gland, the histological structure of the pancreatic acini and

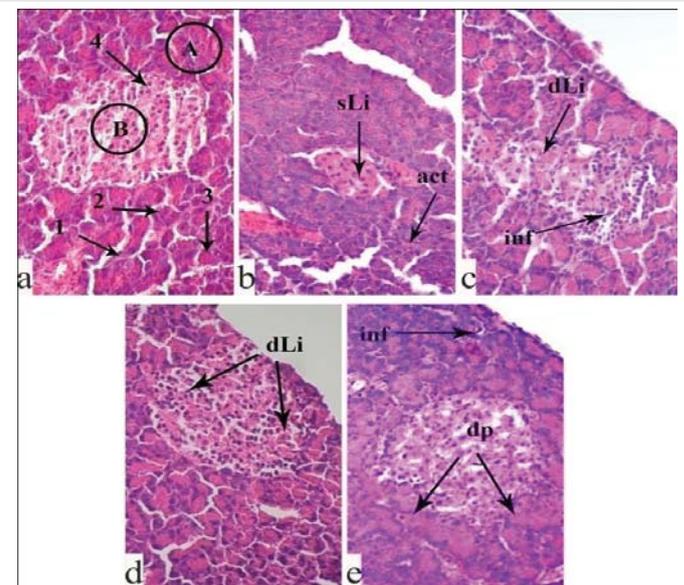


Figure 1: Photomicrographs of pancreas of animal groups: a) intact-control; b) STZ-control; c) STZ-induced diabetic, fed with SL; d) STZ-induced diabetic, fed with RP; e) STZ-induced diabetic, fed with WP. A - exocrine part, B - endocrine part; 1 - connective tissue septa, 2 - pancreatic acini, 3 - excretory ducts, 4 - islets of Langerhans, act - absence of connective tissue septa, dLi - destruction of islets of Langerhans, dp - dystrophic processes, inf - infiltration, sLi - shrinkage of islets of Langerhans; Stained with H&E \times 250:

excretory ducts are mostly preserved. In the endocrine part, the dark pyknotic nuclei are fewer in number, however, insulocytes with vacuolated cytoplasm are registered.

In the pancreas of the non-treated, STZ-control animals, the morphometric analysis showed significant decrease of the area of islets of Langerhans due to the shrinkage of the size and number of the islets (Figure 2).

Histopathological characteristics of kidney in the experimental animals: Our histological examination showed normal microscopic structure of the cortex and medulla in the kidney of the intact group. There are no degenerative and inflammatory processes in the proximal and distal tubules (Figure 3a).

In the renal tissue of the STZ-control rats, nephropathy structural changes, characteristic for diabetes, are seen (Figure 3b). In the microscopic fields, many necrotic glomeruli with Bowman's capsule expansion, as well as thickening in the basement membrane of some glomeruli are observed. Degeneration processes of the proximal and distal tubules with prominent inflammatory infiltration are registered. The end of the distal tubules is markedly expanded. The collecting duct system has an irregular arrangement.

The histological study showed that the abovementioned pathological changes were less in the kidney of the SL fed animals (Figure 3c) compared with the STZ-control group. The renal corpuscles are mostly preserved; the proximal and distal tubules are without visible pathological changes, although in some places, there are inflammatory foci and destructive processes in the parenchyma.

The analysis of the histological picture of the kidney of the RP fed STZ-diabetic animals (Figure 3d) showed relatively less severity of pathological changes compared with the STZ-control group. The histostructure of renal corpuscles and capillary glomeruli are mostly preserved. The destructive and dystrophic processes in the proximal and distal tubules are moderate. The collecting ducts are well-shaped. However, along with this, the epithelial cells are vacuolated in some places. Mesangial expansion of some glomeruli, as well as thickening of the basal membranes are seen.

In the kidney of the STZ-diabetic rats fed with the ethanol extract from WP (Figure 3e), the observed nephropathic changes

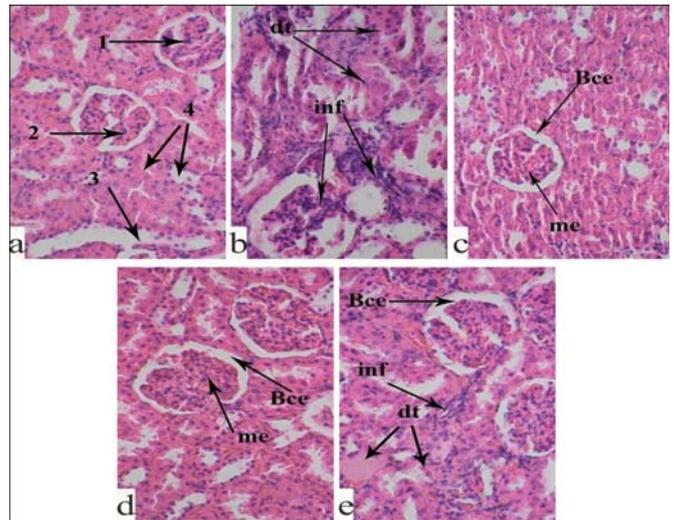


Figure 3: Photomicrographs of kidney of animal groups: a) intact-control; b) STZ-control; c) STZ-diabetic, fed with SL; d) STZ-diabetic, fed with RP; e) STZ-diabetic, fed with WP.

1 - renal corpuscle,
2 - capillary glomeruli,
3 - collecting ducts,
4 - distal and proximal tubules, inf - infiltration, Bce - Bowman's capsule expansion, dt - destruction of distal and proximal tubules, me - mesangial expansion;
Stained with H&E \times 250.

are moderate. The renal capsules are preserved, but there are foci of destructive processes in the capillary glomeruli. Mesangial expansion of the glomeruli, as well as thickening of the basal membrane are observed. Lymphohistiocytic infiltrations are found in all the histological sections.

Histopathological characteristics of liver in the experimental animals: Along with the increase of the risk of kidney disease, nerve damage, blood vessel damage, infections, blindness and heart disease, diabetes can also have profound effects on the liver. Various pathological changes in the liver can occur, in particular, fatty degeneration, leading to the steatosis and probability of developing liver cirrhosis.

In intact rats, we observe a normal histological structure of the liver tissue (Figure 4a); around the central vein, hepatocytes are arranged in cords. There are no pathological abnormalities in the portal tracts and periportal zones. No inflammatory processes are identified; the sinusoidal capillaries are normal.

In the histological structure of the liver tissue of the STZ-induced diabetic rats (Figure 4b), fatty degeneration, destructive processes of parenchymal elements, *pyknosis* and *karyolysis* of hepatocytes are revealed. In the portal triad, a phenomenon of hyperplasia and perivascular infiltration and, in some places, tissue fibrosis are seen.

In the liver tissue of the STZ-diabetic animals, fed with the extract from SL (Figure 4c), the destructive and dystrophic changes are less than in the STZ control animals. The cords of hepatocytes are mostly preserved; there are much fewer foci of fatty changes and *pyknosis* of hepatocyte. At the same

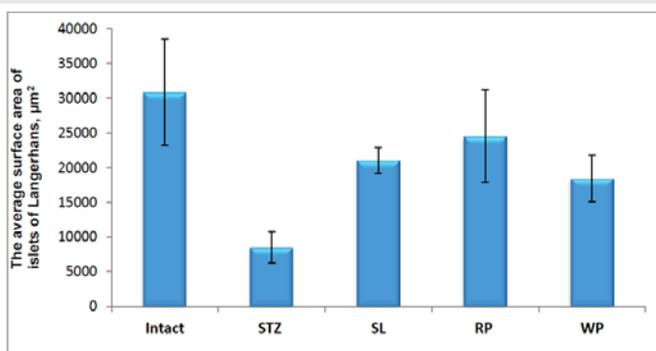


Figure 2: The average surface area of the islets of Langerhans (\pm sd).

time, in the parenchyma, the sinusoidal capillaries are dilated; hyperplasia of the bile ducts and perivascular infiltration in the portal triads are seen.

A similar morphological picture is recorded in the liver of the RP fed STZ-diabetic animals (Figure 4d). Moreover, along with the above pathological changes, the parenchyma of the liver lobules is destroyed; in the intercellular spaces of hepatocytes, bile appears.

In WP fed animals (Figure 4e), dilation of sinusoidal capillaries was observed. In the portal triads, hyperplasia, lymphohistocytic infiltrations and early fibrosis are registered.

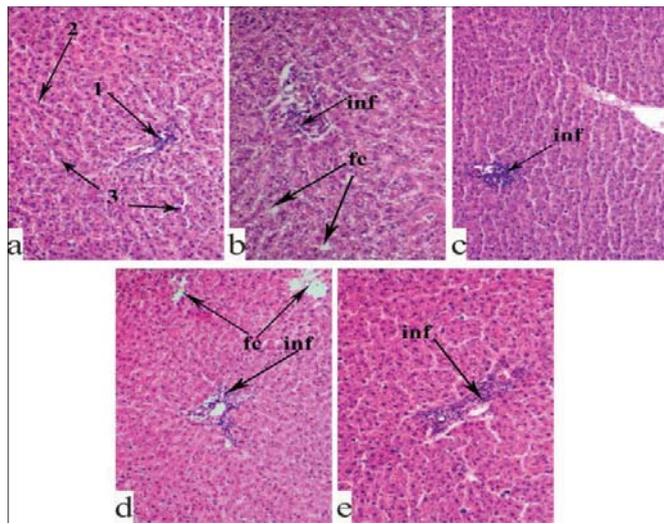


Figure 4: Photomicrographs of liver of animal groups:

- a) intact-control;
 b) STZ-control;
 c) STZ-diabetic, fed with SL;
 d) STZ-diabetic, fed with RP;
 e) STZ-diabetic, fed with WP.

1 – portal tract,
 2 - hepatic cords,
 3 - sinusoids, inf – foci of infiltration, fc – fatty changes; Stained with H&E $\times 125$.

Discussion

In the present work we performed the *in vivo* study on STZ-induced diabetes model of laboratory rats. Feeding the diabetic animals with extracts from SL, WP, GL and RP decreased the blood glucose level only by 7%–30% of the STZ-controls, remaining essentially higher than the level in the intact controls. It is noteworthy that a higher rate of glucose improvement was registered in the animals of the WP and SL groups. Worth noting, that at the end of the experiment, higher percentages of animals (35% and 28% of the number in each group, respectively) recovered in the same groups. Moreover, feeding of the diabetic animals with ethanol extracts from SL and WP during one month decreased the MDA level down to the level in the intact animals.

Anyhow, in the pancreas, kidney and liver of the STZ-diabetic animals fed with ethanol extracts from SL, RP and WP, the histopathological study revealed positive changes compared with the STZ-control animals.

- In the SL fed animals: in the pancreas, the degenerative phenomena are less prominent, the number and size of insulin islets increases; in kidney, fewer nephrotic changes and necrotized glomeruli are seen; in liver, there is no fatty degeneration, the cords are preserved, although processes of pyknosis and cytolysis of hepatocytes are observed.
- In the WP fed animals: in the pancreas, the size and area of the islets of Langerhans are larger; in kidney, the pyknosis, degenerative and nephritic changes are less prominent.
- In the RP fed animals: in the pancreas, fewer destructive and dystrophic changes in both the exocrine and endocrine parts are seen, the average surface area of islets of Langerhans is larger compared to the other experimental groups (except the intact); in the kidney, the morphological changes are moderate, but necrosis of glomeruli and destruction of tubules are not so prominent; in liver, karyopyknosis and karyolysis of hepatocytes are moderate.

While carrying out our previous and the present investigations, special attention was given to sorrel leaves (*Rumex Confertus*), historically used in Eastern, particularly, in Armenian cuisine. Previously, relatively low antioxidant activity of the ethanol extract from sorrel leaves has been registered [22]. Nevertheless, this extract was surprisingly very effective in protecting pancreas β -cells from toxic action of aggregated amylin (IC_{50} as low as $0.03 \pm 0.002 \mu\text{g/ml}$) [20] and had a significant toxic effect on cancer cells (particularly, IC_{50} in inhibition of growth of Ehrlich ascites carcinoma cells was as low as $0.1 \pm 0.04 \text{ ng/ml}$, the lowest among the studied plant extracts) [22]. Interestingly, the ethanol extract from SL can also serve as a source for a low-cost production of the known anticancer agent emodin, with additional anti-diabetic and anti-amyloidogenic features [27].

Actually, in the present work, the ethanol extract from SL proved to be most effective in improving the blood glucose level and the histopathological distortions in the pancreas, kidney and liver of the STZ-induced diabetic rats. This observation is contrary to our expectations, since the efficacy of the SL in inhibiting both the ADA and the DPPIV enzymes is rather low ($IC_{50}=0.68 \pm 0.06 \text{ mg/ml}$ and $>100 \text{ mg/ml}$, respectively) [17]. It has to be noted, that many reserchers consider inhibition of these enzymes, especially the DPPIV enzyme, as highly beneficial in the treatment of hyperglycemia [28].

Based on the described results, which manifest the benefits of using the studied herbal extracts in the STZ-induced model of diabetes, we recommend a well-designed clinical trial to confirm the positive effects of these extracts (particularly of SL) on diabetic patients.

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