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Effect of Aqueous Extract from *Phaseolus vulgaris* Pods On Cytokine Profile Of Streptozotocin-Induced Diabetic Rats.

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ABSTRACT

This study was designed to evaluate the effect of long-term oral administration of aqueous *Phaseolus vulgaris* pods extract on pro- and anti-inflammatory cytokines profile in streptozotocin-induced diabetic rats. The results indicate that administration of the extract in dose of 200 mg/kg to diabetic rats resulted in significant lowering of blood glucose level along with a reduction of HbA1c content. It was revealed that hypoglycemic effect of studied extract was not related with the ability to stimulate of insulin secretion from pancreatic β-islets. Moreover, *Phaseolus vulgaris* extract administration acted beneficially on altered biochemical parameters of lipid metabolism caused by STZ-induced diabetes mellitus. Long-term oral administration of the extract in the group of diabetic rats caused significant increase of anti-inflammatory cytokines level (IL-4 and IL-10), which are known to be protective agents under DM1 conditions. Besides of that, administration of the extract to diabetic rats led to decreasing each of IgG level and pro-inflammatory cytokine IL-1β level, which are contributing to DM1 pathogenesis. Thus our data reveal anti-inflammatory properties of aqueous *Phaseolus vulgaris* pods extract that might have beneficial effect in treatment of diabetes.

Keywords: *Phaseolus vulgaris*, extract, streptozotocin-induced diabetes, cytokines.

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INTRODUCTION

Diabetes mellitus type 1 (DM1) is a chronic disease, which is characterized by progressive destruction of the insulin-producing beta cells in the pancreatic islets. This impairment causes disability to produce insulin, which results in elevated blood glucose level and its subsequent pathological effects [1]. More than 80% of patients have immune system mediated (type 1A) form of DM1 triggered by genetic and environmental factors and considered as autoimmune reaction. There is another form of phenotypic DM1, also known as the idiopathic DM1 (type 1B), which is reported in 10-20% of DM1 cases. This form of the disease is characterized by almost complete insulin deficiency and strong hereditary component but not associated with autoimmunity [2].

In the last years research has provided evidences that immune and inflammatory reactions have strong impact on DM1 pathogenesis. Abnormal immune response can cause islet inflammation or insulitis. Previously it was reported that insulin-producing beta cells destruction may lead to the development of a more aggressive T-cell profile e.g. change in the balance between Th1 and Th2 cells towards more pro-inflammatory milieu (Th1 dominant profile) [3]. T cells' cytokines (including pro-inflammatory cytokines INFγ, TNFα, IL-1, IL-2 and IL-12) mediate cell death via the Fas pathway [4] and participate in nitric oxide and oxygen-derived free radicals accumulation, which in turn triggers caspase-induced apoptosis of insulin-producing cells [5].

Disturbance of the cytokine profile in favour of pro-inflammatory cytokines overproduction leads to sustained destructive inflammatory processes in the insulin producing islets of pancreas. Misbalance between pro- and anti-inflammatory cytokines might be one of the most crucial factors of the diabetic complications progression [3-5]. This fact has been used for the development of the major approach for DM1 management. This approach is based on attenuation of the cytotoxic effects of inflammatory agents, such as β-cell autoreactive Th1 cells, pro-inflammatory cytokines and modulation of autoimmune response by increasing the amount of regulatory Th2/Th3 cells producing IL-4, IL-5, IL-10 and TGFβ1 [3, 6].

Since immune disorders influence the pathophysiology of DM1 it was suggested that herbal preparations rich in immune modulating components might be able to inhibit the development of diabetes and progression of DM1-related complications. Common kidney beans (*Phaseolus vulgaris*) are widely used in traditional medicine as source of natural products with potential antidiabetic effects. It was already shown that extracts from various parts of this plant can improve DM1 condition due to the hypoglycemic effect and by managing of lipid metabolism and antioxidant status [7-10]. We assumed that among natural product of *Phaseolus vulgaris* there might be a promising immune modulating agent for DM1 treatment. This study is an attempt to investigate possible effects of long-term administration of aqueous extract from *Phaseolus vulgaris* pods on pro- and anti-inflammatory cytokines profile (IFN-γ, IL-1β, IL-12, IL-4, IL-10) in streptozotocin-indused diabetic rats.

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MATERIALS AND METHODS

Preparation of plant extract

The aqueous extract was prepared by boiling 132 g of dried powdered pods of *Phaseolus vulgaris* in 1 liter of distilled water for 20 min [10]. After boiling, extract was left overnight to infuse. In order to remove plant debris obtained extract was filtered and centrifuged at 1000×g for 10 min. Supernatant was lyophilized using a freeze-dryer (The Telstar LyoQuest, Spain). Dry extract (8 g) was stored at -20˚C. Right before use, required doses were taken and resuspended in 2 ml of distilled water.

Experimental animals

Female white non-linear rats, each in the weight range of 100-120g, were obtained from the Animal house of Taras Shevchenko National University of Kyiv, Ukraine. All experimental protocols were approved by the Ethical Committee for Conduction of Animal Studies at the Educational and Scientific Center 'Institute of Biology' of Taras Shevchenko National University of Kyiv, Ukraine.

Experimental DM was induced by single intraperitoneal injection of 45 mg/kg b.w. streptozotocin (STZ; Sigma, USA) dissolved in 0.5 ml of 0.01 M citrate buffer, pH 4.5. Control group received 0.5 ml of buffer that was used for dissolving of STZ [11]. Two days after STZ injection DM induction was monitored by measuring blood glucose level by glucometer «Hlyukofot II» (Norma, Ukraine). Animals with glycemic values more than 15 mM were considered as diabetic and were chosen for the experiments.

Experimental design

The rats were weighed, tagged and randomly divided into four groups of six animals each as followed. "Control" and "Diabetes" (the untreated diabetic rats) were given by gavage de-ionized water (2 ml/day); "Control + Extract" and "Diabetes + Extract" were treated with *P. vulgaris* aqueous extract (200 mg/kg b.w. per day) dissolved in 2 ml of deionized water and applied orally. The experiment was conducted for 28 days [12]. During the experiment animals were kept under standard conditions (temperature, humidity, 12 hour dark-light cycle and were fed with standard commercial food and water available *ad libitum*).

Analytical methods

After 28 days, the animals were deprived from food overnight and killed by decapitation. Blood was collected and used for the estimation of blood glucose levels and glycosylated hemoglobin. Serum was separated by centrifugation at 2500×g for 25 min and stored at -20˚C until used for biochemical analysis.

Blood glucose level was evaluated using glucometer «Hlyukofot II» (Norma, Ukraine) and level of glycosylated hemoglobin was measured spectrophotometrically using commercial kit (ERBA-Lachema, Czech Republic). Other biochemical parameters of the blood

serum were estimated by Microlab 300 analyzer (Elitech, France) and commercial kits (Elitech Diagnostic, France) according to the standard protocols provided by manufacturers.

Serum levels of insulin, IgG and cytokines (IFN-γ, IL-1β, IL-12, IL-4, IL-10) were estimated by performing ELISA [13]. Serum samples were immobilized onto 96-well plate and incubated with corresponding specific primary antibodies (Santa Cruz, USA). After that secondary antibodies conjugated with horseradish peroxidase (Bio-Rad, USA) were added. To enable colorimetric detection, reaction with the substrate o-phenylenediamine/hydrogen peroxide (Sigma, USA) was performed and absorbance of each well was read at 492 nm. Values were expressed as optical density (OD) relative to total proteins as determined by the Bradford's method [14].

Statistical analysis

The data of biochemical estimations were reported as mean ± SEM for six animals in each group. Student's 't' test was used for statistical significance between groups. The difference between the parameters was considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSIONS

Levels of blood glucose, serum insulin and glycosylated hemoglobin (HbA_{1c}) of all experimental groups of animals after treatment with aqueous *Phaseulus vulgaris* pods extract for 28 days are represented in Figure 1. According to the obtained results, it seems that long-term administration of the extract had a positive influence on glucose metabolism. Blood glucose and HbA1c level in the group of diabetic rats was decreased. Blood glucose and HbA_{1c} contents in healthy rats treated with plant extract were not statistically different compared with group of healthy animals administered water (Fig. 1). Thus, it can be argued that the studied extract affects glucose metabolism only under conditions of high glucose concentration that occurs in diabetes.

Insulin level in both diabetic groups was decreased by 30-35% compared with control group. Our results showed that treatment of diabetic rats with the extract had no effect on the serum insulin level. But we think that our result can not contradict to recently published results concerning stimulated effect of *P. vulgaris* on insulin secretion [15]. Streptozotocin-induced model, used in this study, is based on the ability of streptozotocin to destroy pancreatic beta cells [16, 17]. That is why we can only assume that in diabetic rats treated with *Phaseolus vulgaris* pods extract beta-cells number or their insulin-producing function were recovered. Moreover, these data indicate that the mechanism of hypoglycemic action of the extract is not related with its ability to stimulate insulin secretion from pancreatic beta cells.

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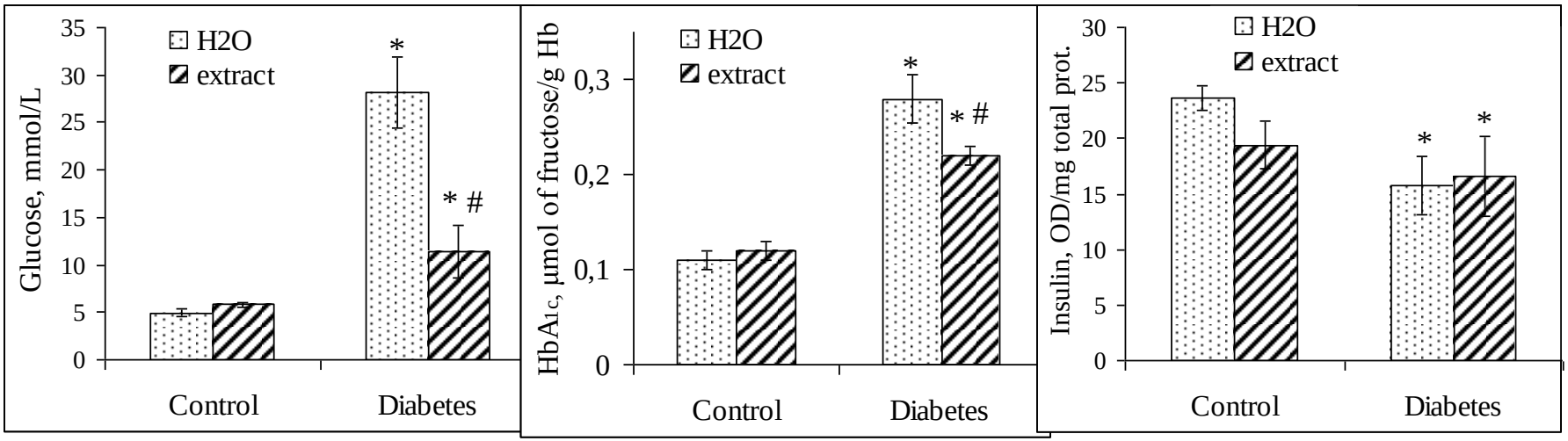

gure 1: Effect of *Phaseolus vulgaris* pods extract on blood glucose, glycosylated hemoglobin (HbA_{1c}) and insulin levels in control and diabetic groups of rats.

Given data are mean ± SEM for six animals in each group. *Significantly different from the control rats; #significantly different from the diabetes control rats. Values are statistically significant at p<0.05.

Chronic hyperglycemia, along with progressive inflammatory and immune reactions, causes not only a number of metabolic disorders but also a development of functional disorders in different tissues and organs [18, 19]. Biochemical serum markers can indicate either normal or pathological processes in the body. Hepatic function panel (alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (GGT)), renal function panel (creatinine, uric acid, urea) and lipid profile (total cholesterol, triglycerides, low- and high-density lipoproteins (LDL, HDL)) were investigated in the serum of all experimental groups of animals (Table 1).

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Table 1: Effect of *Phaseolus vulgaris* pods extract on various biochemical parameters of blood in control and diabetic groups of rats.

Group	Control	Control + extract	Diabetes	Diabetes + extract
ALT (U/L)	56.08 ± 8.23	59.30 ± 7.88	94.80 ± 6.00*	65.13 ± 6.09#
AST (U/L)	182.25 ± 15.16	200.45 ± 28.04	347.68 ± 9.62*	269.77 ± 22.20*#
Gama-GT (U/L)	4.28 ± 0.93	2.93 ± 0.48*	10.85 ± 0.65*	8.07 ± 0.38*#
Creatinine (µmol/L)	63.05 ± 3.70	64.45 ± 3.18	53.45 ± 1.07*	50.87 ± 3.96*
Uric acid (µmol/L)	111.23 ± 13.48	122.88 ± 13.73	155.40 ± 10.47*	97.53 ± 10.89#
Urea (mmol/L)	7.00 ± 0.74	6.38 ± 1.20	8.63 ± 1.51	15.33 ± 3.10*#
Total cholesterol (U/L)	1.77 ± 0.08	2.09 ± 0.27	1.18 ± 0.14*	1.42 ± 0.32
Triglycerides (U/L)	0.68 ± 0.11	0.63 ± 0.1	0.62 ± 0.17	0.82 ± 0.13
LDL (U/L)	0.21 ± 0.03	0.22 ± 0.01	0.17 ± 0.01	0.15 ± 0.02*
HDL (U/L)	0.68 ± 0.06	0.8 ± 0.14	0.27 ± 0.04*	0.39 ± 0.06*#

Given data are mean ± SEM for six animals in each group. *Significantly different from the control rats; #significantly different from the diabetes control rats. Values are statistically significant at $p<0.05$.

Untreated diabetic rats showed significant increase in serum liver enzymes (AST, ALT, GGT) and blood uric acid while creatinine, total cholesterol and HDL were significantly decreased in comparison to the healthy control. There was a significant restoration of these parameters to nearly normal in diabetic rats treated with *Phaseolus vulgaris* extract. That is why we concluded that the aqueous extract of the *Phaseolus vulgaris* pods beneficially influences functioning of various organs and systems under the conditions of STZ-induced DM.

DM1 is an organ-specific autoimmune disease characterized by the presence of different types of autoantibodies [20]. These antibodies and the corresponding antigens form immune complexes, which are circulating in blood stream. Presence of additional type of immunoglobulins, which bind these complexes, is one of the reasons of abnormal immune response under DM1 conditions [21]. It was shown that under the DM1 conditions IgG concentration in the serum is elevated. It suggests that IgG might play a role in pathogenesis of the vascular complications of DM1. Moreover, measurement of IgG level in serum can be used as a biomarker for DM1 diagnostics [22-24]. Due to these facts, it was study of great interest whether administration of *Phaseolus vulgaris* extract to diabetic rats would influence level of serum IgG.

Our results demonstrate that IgG level in diabetic control group of animals was 5 times higher compared to the control group of healthy rats (Fig. 2). Serum IgG level of diabetic rats treated with the extract was significantly lower in comparison with control group of untreated diabetic rats but still remained much higher than in control group (in group of control rats studied extract did not cause any effect).

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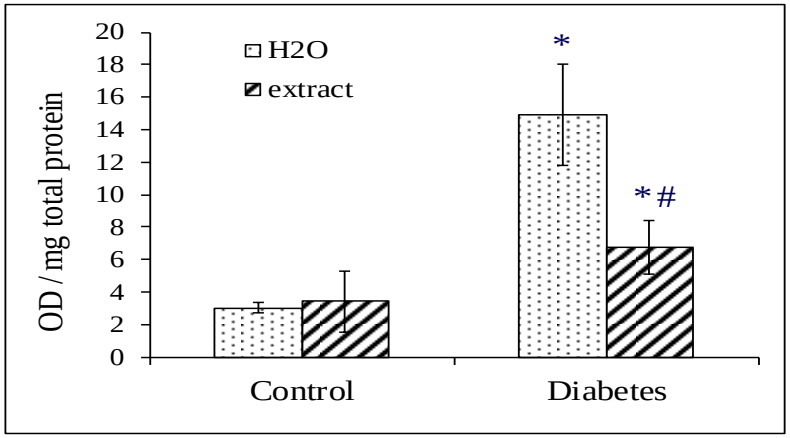

Figure 2: Effect of *Phaseolus vulgaris* pods extract on serum IgG levels in control and diabetic groups of rats.
Given data are mean ± SEM for six animals in each group. *Significantly different from the control rats; #significantly different from the diabetes control rats. Values are statistically significant at p<0.05.

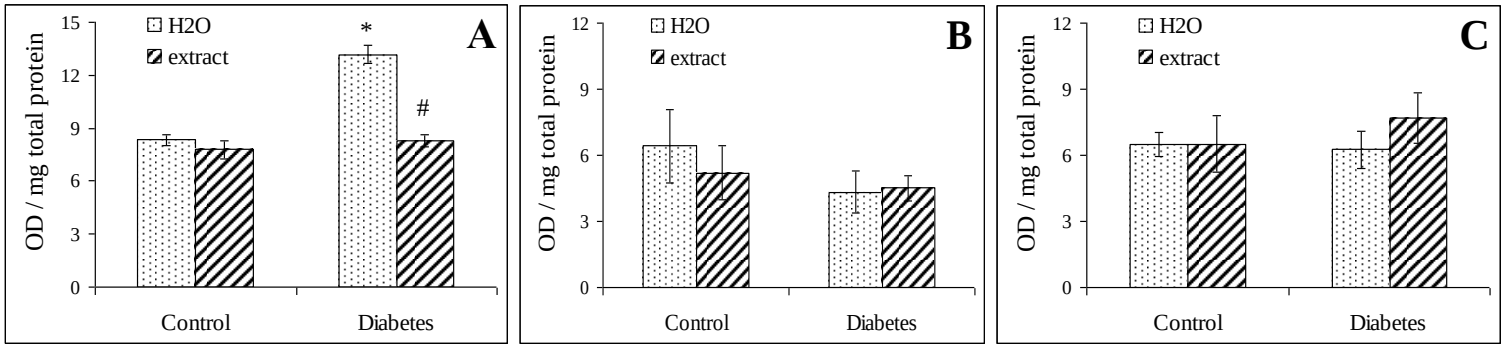

Figure 3: Effect of *Phaseolus vulgaris* pods extract on serum levels of pro-inflammatory cytokines IL-1β (A), IL-12 (B), IFN-γ (C) in control and diabetic groups of rats
Given data are mean ± SEM for six animals in each group. *Significantly different from the control rats; #significantly different from the diabetes control rats. Values are statistically significant at p<0.05

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The development of DM1 is associated with an imbalance between the Th1 and Th2 arms of the cellular immune system [2]. In DM1, Th1 (IFN-γ, IL-1, 2, 12) and Th2 (IL-4, IL-10) associated cytokines have been observed in insulitis lesions. It seemed that Th1 cytokines tend to promote and Th2 cytokines tend to regulate beta-cell destruction [25, 26]. The role of pro-inflammatory cytokines is associated with the initiation and progression of DM1. Pathological excess of pro-inflammatory cytokines impair islet function through the activation of MAPK pathway, intrinsic mitochondrial death pathway and subsequent oxidative stress. Anti-inflammatory cytokines, also contribute to the pathogenesis of DM1, decreasing the inflammatory process by down-regulation of pro-inflammatory cytokine production. On the other hand, elevated levels of inflammatory cytokines have been observed in type 1 diabetic patients and associated with the development of vascular complications [27-30]. Hence, an imbalance between pro- and anti-inflammatory cytokines may play a significant role in both autoimmunity and chronic inflammation, which probably leads to DM1 associated complications [27]. Based on these knowledge, we decided to examine whether administration of *Phaseolus vulgaris* extract to diabetic rats influences their level of pro- and anti-inflammatory cytokines.

Our results showed that serum level of IL-1β in untreated diabetic animal was elevated by 60% in comparison with control group (Fig. 3, A). In contrast, serum levels of IL-12 and IFN-γ appeared to be comparable with control group (Fig. 3, B and C). In the group of diabetic rats, treated with aqueous extract of *Phaseolus vulgaris* pods, serum IL-1β level was decreased by 40 % compared to untreated diabetic group. Administration of the extract to diabetic animals did not affect serum level of IL-12 and IFN-γ as well. In the group of control healthy rats administration of the extract did not cause any effect.

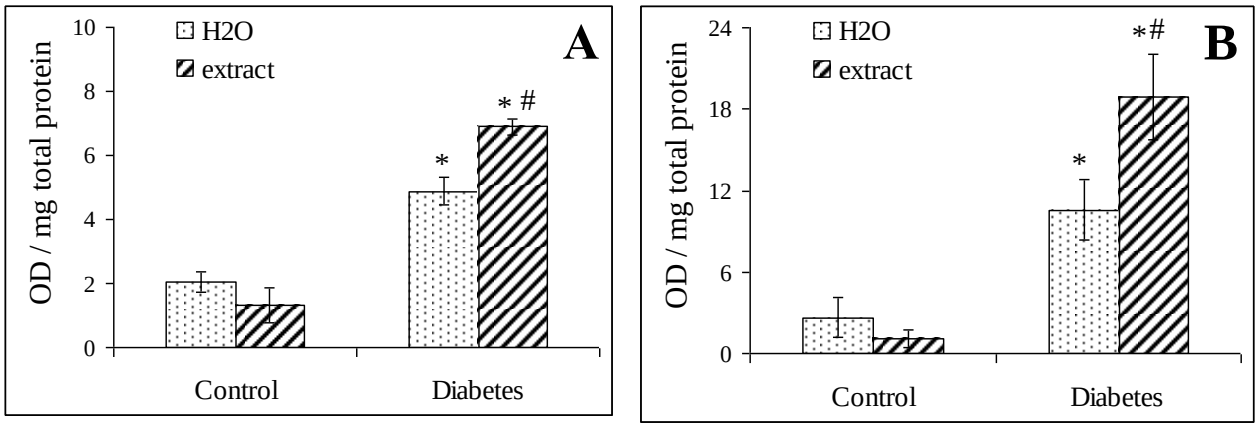

Figure 4: Effect of *Phaseolus vulgaris* pods extract on serum anti-inflammatory cytokines levels: IL-4 (A) and IL-10 (B) in the control and diabetic groups of rats

Given data are mean ± SEM for six animals in each group. *Significantly different from the control rats; #significantly different from the diabetes control rats. Values are statistically significant at p<0.05

It was also observed that IL-4 serum level in the group of untreated diabetic rats was elevated in 2.5 times compared to the control group. In diabetic rats treated with the extract serum level of IL-4 was increased by 40 % compared to the untreated diabetic control group

(Fig. 4, A). Serum level of IL-10 in the group of untreated diabetic rats was increased 4 times compared to the corresponding control group. Administration of the extract to the STZ-diabetic animals caused rising IL-10 by 80% compared with the group of untreated diabetic rats (Fig. 4, B). In the group of healthy rats administration of the extract did not cause any effect on levels of studied anti-inflammatory cytokines (Fig. 4, A and B).

CONCLUSIONS

This study was performed in order to investigate possible role of long-term oral administration of *Phaseolus vulgaris* pods extract on pro- and anti-inflammatory cytokines profile in streptozotocin-induced diabetic rats. We found that this extract had positive influence on the levels of blood glucose and glycosylated haemoglobin in diabetic rats. Importantly, it was shown that hypoglycemic effect of studied extract was not caused by the ability to stimulate release of insulin. Administration of *Phaseolus vulgaris* extract beneficially altered biochemical parameters of lipid metabolism dysfunction caused by STZ-induced diabetes mellitus. Long-term oral administration of this extract in the group of diabetic rats caused significant increase of the anti-inflammatory cytokines level, which are known to be protective agents under DM1 conditions. In contrast to this, administration of the extract to diabetic rats led to decreasing of IL-1β level (one of the pro-inflammatory cytokines contributing to DM1 pathogenesis). In the group of diabetic animals subjected to the extract administration decreasing of IgG level was observed. These results indicate that the effect of aqueous *Phaseolus vulgaris* pods extract on DM1 propagation could be attributed to the modulation of Th1/Th2 balance, which may ameliorate state of β-cell and inhibit T-cell infiltration into pancreatic islets.

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