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Comparative studies of antidiabetic activity of bilberry leaf extract in Wistar rats with STZ-induced diabetes and Zucker diabetic fatty rats

Sidorova, Y.S., *Shipelin, V.A., Mazo, V.K., Zorin, S.N., Petrov, N.A. and Kochetkova, A.A.

Federal State Budgetary Scientific Institution "Federal Research Centre of Nutrition, Biotechnology and Food Safety", Russia, 109240, Moscow, Ustyinsky proezd, 2/14

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Abstract

The use of biologically active substances (BAS) in the composition of the antidiabetic specialized food requires the in vivo pre-testing using experimental models of diabetes in laboratory animals. The aim of this work was the comparative research of antidiabetic activity of *Vaccinium myrtillus* L. bilberry leaf extract (VLE) in Wistar rats with diabetes induced by STZ injection and a high-carbohydrate diet and in Zucker Diabetic Fatty (ZDF) male rats (Zucker Rat Crl: ZUC-Leprfa rats). The prospects of using the extract for the dietary management of diabetes are discussed. In the1st experiment male Wistar rats with streptozotocin (STZ) and high-fructose diet-induced diabetes were fed with a 2% solution of bilberry leaf extract ad libitum for the 50 days. In the 2nd experiment, ZDF rats were treated once daily with 2 g/kg-day of bilberry leaf extract orally via a gastric tube for the following 28 days. During daily inspection were observed the general conditions of all animals in appearance, quality of coat, and behavior. At different intervals, blood glucose and body weight were recorded. Blood samples were collected to determine glycated hemoglobin. Oral glucose tolerance test and insulin tolerance test before and after treatment were performed. Food and water consumption was measured. Results of the glycated hemoglobin and blood glucose level analysis showed the ameliorative effect of VLE on carbohydrate metabolism in diabetic rats. A significant decrease of the fluid intake and increase of the weight gain in animals are the beneficial effects of VLE treatment that speaks in favor to the normalization of metabolic processes in animals. After 2 weeks of VLE consumption, the body weight of ZDF rats was significantly decreased and the growth's slowdown was not due to the different food intake. VLE to a certain extent also reduced hyperglycemia and improved insulin sensitivity in diabetic animals.

Introduction

The efficiency of type 2 diabetes diet therapy can be enhanced with the use of plant minor BAS with hypoglycemic and hypocholesterolemic effects. The potential of flavonoids – high-active natural antioxidants – for type 2 diabetes prevention and treatment are connected to their efficiency and safety in the realization of one of the main phytotherapy principles – they affect as on a damaged organ as on connected organism systems (Pandey and Rizvi, 2009). Flavonoids` effects are mostly determined by the interaction with cell membranes, changing its structural organization and the phase state of membrane lipids. The wide range of flavonoids (anthocyanins, flavonols, and other polyphenols) was found in bilberry leaf extracts. For over a century in vivo and clinical studies investigated the antidiabetic effects of bilberry. It should be noted that the bilberry leaves have richer content of flavonoids than the berries, which are traditionally used in such studies (Ferlemi and Lamari, 2016). Presently the prospects of using extracts for the dietary management of diabetes are discussed.

The use of BAS in the composition of the antidiabetic specialized food requires the in vivo pre-testing using experimental models of diabetes in laboratory animals. The model choice that reproduces the clinical, biochemical and morphological disorders of type 2 diabetes, is essential for the correct interpretation of results obtained in the evaluation of the effectiveness of tested minor BAS. The extrapolation of results, acquired from the rodent diabetes model using genetic lines of animals, for the human organism is more reasonable than the use of so-called medicamental models. ZDF fa/fa rats is a traditional test-system in type 2 diabetes studies. ZDF rats are hyperinsulinemic, hyperlipidemic, and hypertensive as well and show impaired glucose tolerance. Type 2 diabetes develops in male rats as a

*Corresponding author.
Email: *v.shipelin@yandex.ru*

result of feeding a high-energy diet on the background of a homozygous (fa/fa) mutation of the receptor of the hormone leptin (Al-Awar *et al.*, 2016).

Nevertheless, use of chemical models is also actual to optimize the search of BAS with anti-diabetic action, because of the high cost of the genetic lines of laboratory animals and the complexity of the model playback, as well as particular conditions of care and a high degree of inbreeding. STZ-induced chemical models (modeling mixed type or type 2 diabetes) are the most widely used in modern experimental diabetology. Among them, the feeding with high-fructose diet in combination with STZ-injection is an easier and quicker way for the development of type 2 diabetes in rodents (Wilson and Islam, 2012). Combined action of STZ injection and high-fructose diet allows inducing the development of diabetes complications in male Wistar rats in relatively short period.

For complex preclinical evaluation first, it was of interest to examine inhibitory effect of bilberry leaf extract on hyperglycemia and insulin resistance progression in rodents with genetically induced diabetes and then to characterize possible beneficial effects on glucose level in chemically (STZ) induced hyperglycemia. The aim of this work was the comparative research of bilberry leaf extract hypoglycemic effect in Wistar rats with diabetes induced by STZ injection and a high-carbohydrate diet, and in diabetic ZDF male rats.

Materials and Methods

Chemicals and reagents

All the chemicals used in the experiments were of analytic grade. Commercially available individual substances: rutin, hyperoside, isoquercitin, avikulyarin, vitexin, isovitexin, luteolin-7-glucoside, kaempferol-3-glucoside, myricetin, quercetin, kaempferol, luteolin, apigenin, chlorogenic acid, caffeic acid, ferulic acid, rosmarinic acid, p-coumaric acid, produced by Sigma-Aldrich, Extrasynthese, PhytoLab, and ChromaDex were used. STZ (purity >98%) was purchased from Sigma-Aldrich Chemical Co. (United States). Glycated hemoglobin (HbA1C) assay kit was obtained from ELTA (Russia). One Touch Ultra glucometer strips and fructose were purchased from a local pharmacy.

Preparation of extracts

VLE in dry brown powder form (manufactured by Harms, Russia) was dissolved in distilled water (10%); the solution was subjected to microfiltration through a 0.45 micron filter (Millitan, Russia). The filtrate was further diluted with distilled water five times, and the obtained solution was used in the diet of animals instead of water ad libitum (Gavrilov, 2010).

Extract characterization

Research on the content and composition of flavonoids and hydroxycinnamic acids in extracts was performed by the HPLC-MS method developed for the analysis of these substances in *Leonurus quinquelobatus* grass (Zhogova *et al.*, 2014). The content of proanthocyanidins (condensed tannins) was determined by the Bates-Smith method (Tutelyan and Eller, 2010). Caffeine content was analyzed by HPLC with diode array spectrophotometric detector and time-of-flight mass selective detector.

Animals

Experiment №1. Forty adult male Wistar rats were purchased from the Stolbovaya nursery of laboratory animals of the Scientific Center for Biomedical Technology of the Federal Medical and Biological Agency (Russia).

Experiment №2. Twelve male ZDF rats were purchased from Charles River Laboratories (Italy). The animal manufacturer has provided the latest data on animals, confirming their SPF-status (animals free from specific pathogens).

The studies were carried out in strict accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 2011). The experimental designs were approved by the Animal Ethics Committee of the Federal Research Centre of Nutrition and Biotechnology (Protocol No. 04-00041-16 from 04/02/2016) in accordance with the order of the Ministry of Health, and Social Development of the Russian Federation dated 08/23/2010, N 708, "On Approval of the Rules of Laboratory Practice".

Design of the experiment №1

Animals were kept two rats per cage with a controlled 12-hour light/dark cycle. Room temperature and relative humidity were maintained at 23 °C ± 2 °C and 60% ± 5%, respectively. Rats were provided with a standardized pellet diet consisting of protein (19.0%), fat (5.0%), fiber (4.0%), calcium (1.8%), phosphorus (1.1%), vitamin A (5000 ME/kg), vitamin D (500 ME/kg), vitamin E (30 ME/kg), cinders (9%), and water (13.5%), energy 310 kcal/kg (Laboratorkorm, Russia) ad libitum.

After one week of acclimation, animals with a mean body weight (bw) of 170 ± 3 g were randomly divided into two groups as follows: normal control with 10 animals in the group (NC) and high fructose–

induced (HFD) rats with 30 animals in the group. The NC group was supplied with normal drinking water ad libitum, whereas HFD rats were given 10% fructose solution ad libitum for the initial 4 weeks only and then given normal drinking water for the remaining period of the experiment. Body weight was recorded weekly; food and water consumption were recorded daily. STZ was dissolved in physiological saline. On the 31st day of the experiment, each HFD animal in the diabetic group (body weight 325 ± 4 g) were injected with STZ (50 mg/kg bw); the animals in the NC group (body weight 326 ± 8 g) were injected with physiological saline only (Ragbetli and Ceylan, 2010).

One week after the STZ injection, the blood glucose level was measured by using a portable glucometer (OneTouch Select Simple) in blood collected from the tail vein. Animals with blood glucose level >11 mmol/l were considered diabetic (Kumar *et al.*, 2014). The diabetic animals were divided into two groups according to their weights and blood glucose levels to make the average weights and blood glucose levels similar among the groups: hyperglycemic eight rats without any treatments (Diabetic Control Group (D1)) and eight rats in Diabetic Experimental Group 2 (D2).

Healthy rats of the NC group and the D1 group rats were treated with a standard pellet diet and water ad libitum during the remaining period of the experiment. Animals of group D2 were treated with a 2% solution of bilberry leaf extract ad libitum for the following 50 days. Animals were provided with food ad libitum during the whole experiment.

On the 22nd day of feeding (on the 60th day of experiment), blood from the tail vein was again taken from animals to check glucose levels of all experimental groups. Animals were fed with experimental diets for 50 days; the total duration of the experiment was 88 days.

At the end of the experimental period, overnight fasted animals were anesthetized through a slight diethyl ether exposure. After decapitation, fresh blood samples were collected from rats into EDTA-contained (0.2 ml 0,1M Na_2 EDTA) test tubes for further analysis of glycated hemoglobin.

Design of the experiment №2

The second experiment was conducted in Laboratory of biological testing "Branch of the Institute of Bioorganic Chemistry" Russian Academy of Sciences (Russia, Pushchino) which is accredited by AAALAC International. For animal welfare standards adopted standards defined by the Directive 2010/63/EU on the protection of animals used for scientific purposes. Among the acceptable boundaries of climate parameters in animal rooms accepted boundaries defined guidance of the "Guide for Care and Use of Laboratory Animals" (National Research Council, 2011). The animals were caged individually in enclosed ventilated cabinets controlled for temperature (22 °C), relative humidity (50%), and light conditions (12 hour light/dark cycles). After one week of acclimatization, rats aged 10 weeks were randomly divided into two treatment groups (n = 6 each group): control K1 and experimental E1 groups. The initial body weight did not differ between control and experimental groups (335.7 ± 32.2 g; 336.5 ± 19.0 g, respectively). Fasting glucose level was also similar for both groups and amounted 4.8 ± 0.8 mmol/l for the control group and 4.6 ± 0.5 mmol/l for the experimental group.

Rats were treated with high fat diet consisting of crude protein (16.4%), fat (6.4%, of them pork lard 3.7%), fiber (4.2-4.4%), carbohydrates (54-57%), crude ash (5.5%) and water (10-11%), energy 360-363 kcal/kg ad libitum. Animals of an experimental group were treated once daily with 2 g/kg of bilberry leaf extract orally via a gastric tube for the following 28 days.

Body weight and water intake were determined once a week. Twice a week was determined food intake. On the 10th, 19th and 29th day of the study, the content of glycated hemoglobin was determined.

Blood glucose levels were determined weekly using blood samples from the tail vein by a glucose analyzer (One Touch Select, Life Scan-Johnson and Johnson, USA).

Overnight fasting rats were subjected to oral glucose tolerance test (GTT) and insulin tolerance test (ITT) before and after treatment. In GTT the rats were given intragastrically 40% glucose (w/v) solution at a dose of 5 g/kg bw. Blood glucose levels were taken at 0 (before oral glucose), 15, 45, 120 and 240 min after glucose administration. ITT with oral glucose load was carried out on the 28th day of treatment. The rats were given intragastrically 40% glucose solution at a dose of 5 g/kg bw and then insulin was injected intramuscularly at a dose of 0.1 mg/kg bw. Blood glucose levels were taken at 0 (before oral glucose), 15, 45, 120 and 240 min.

Glycated hemoglobin determination

The method is based on the principle of affinity separation of glycated and unglycated hemoglobin fractions of blood hemolysate (Little *et al*., 1983). The affinity sorbent with bonded 4-aminomethylbenzeneboronic acid provides specific glycated hemoglobin binding and its separation from

unglycated fraction at the 1st stage. At the 2nd stage, boronic acid turns into free ionic form with releasing and following full elution of glycated hemoglobin fraction. The determination of optical dense for both fractions (wavelength – 414 nm (405-420 interval) allows valuing the relative concentration of glycated hemoglobin in the analyzed sample.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 20 software (Statistical Package for Social Sciences, USA) by means of descriptive statistics tools (M: arithmetic mean; SD: standard deviation; m: standard error of the mean; n: number of observations), ANOVA test (to evaluate the likeness of variable dispersion in groups), Mann–Whitney U-test, and Fisher`s ratio test. The critical level of significance of the statistical null hypothesis (p) was taken to be 0.05.

Results and Discussion

Bilberry leaf extract characterization

The total content of flavonoids in the extract was 14.01 ± 0.34 mg/g. The total content of hydroxycinnamic acid derivatives in the extract was 11.68 ± 0.22 mg/g. The number of proanthocyanidins in the extract was 15.5 ± 1.5 mg/g. In investigated bilberry extract, the amount of caffeine was 1.9±0.16 mg/g. The total content of polyphenols was 43.6 ± 1.1 mg/g.

Experiment 1

Food and fluid intake and average body weight change

Figure 1 shows the mean food intake (g per rat per day) before and after STZ injection over the whole experiment and fluid intake (ml per animal per day) over 50 days of treatment. Before STZ injection food intake was not significantly different between the NC group and HFD animals. After STZ injection food intake was significantly higher in the diabetic groups D1 and D2 compared with NC group. Significantly higher fluid intake was observed in the D1 and D2 groups compared to the NC group and also significant difference was found between the D1 group, treated with drinking water, and the D2 group, treated with the bilberry leaf extract 2% solution. Fluid intake in the D2 group was more than 2 times lower than in the D1 group.

Figure 2 shows the average body weight differences of the animals of all groups from the 38th to the 88th day of the experiment (over 50 days of treatment).

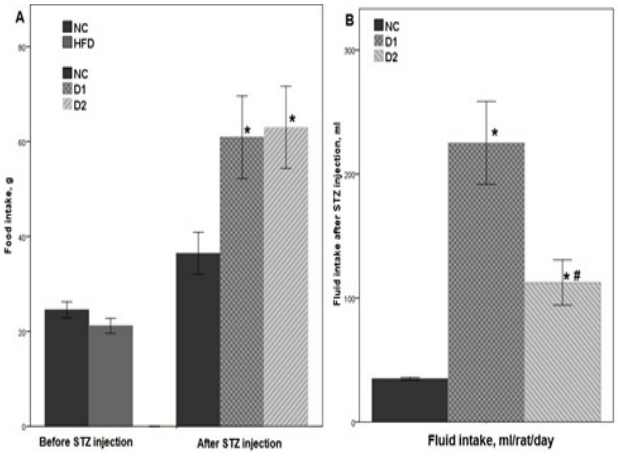

Figure 1. (A) Mean food intake (g) per animal per day; (B) Fluid intake (ml) per animal per day.
Values are mean ± SEM of 8-10 rats per group.
*P<0.05 vs. NC rats
#P<0.05 vs. D1 diabetic rats

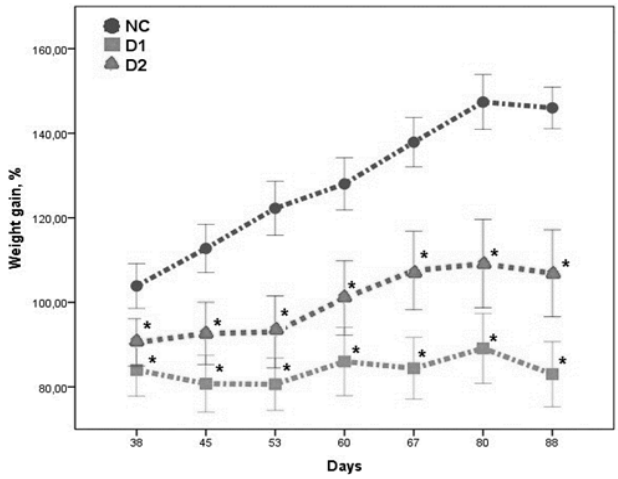

Figure 2. The average body weight gain differences of the animals of all groups from the 38th to the 88th day of the experiment.
Values are mean ± SEM of 8-10 rats per group.
*P<0.05 vs. NC rats

Ranging from the 38th day of the experiment, as soon as STZ was injected, a significant loss of body weight was observed in animals of the diabetic groups D1 and D2. Although it should be noted that there was a significant increase in the average bw of animals in the D2 group, which consumed a diet rich in the polyphenols of bilberry leaves.

Polydipsia that manifested in the increased fluid intake in diabetic animals accompanied by abundant polyuria and weight loss are hallmarks of STZ-induced diabetes (Akbarzadeh *et al*., 2007). A significant decrease of the fluid intake and increase of the weight gain in animals D2 group are the beneficial effects of VLE treatment that speaks in favor to the normalization of metabolic processes in animals. The obtained results are consistent with the previous data (Wilson and Islam, 2012) and showed

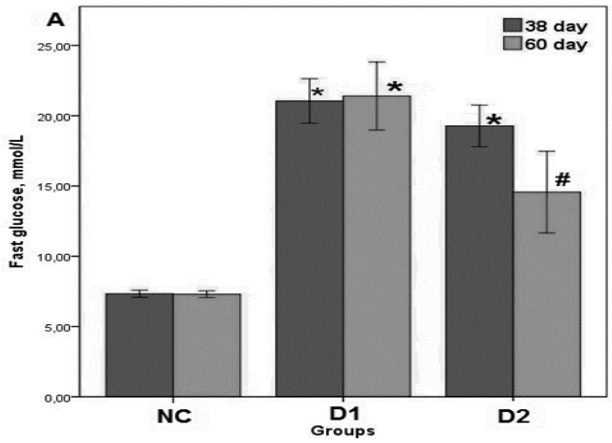

Figure 3. The glucose level in the blood of animals after 7 days and 29 days of administration of 50 mg/kg of STZ.
Values are mean ± SEM of 8-10 rats per group.
*P<0.05 vs. NC rats
#P<0.05 vs. D1 diabetic rats

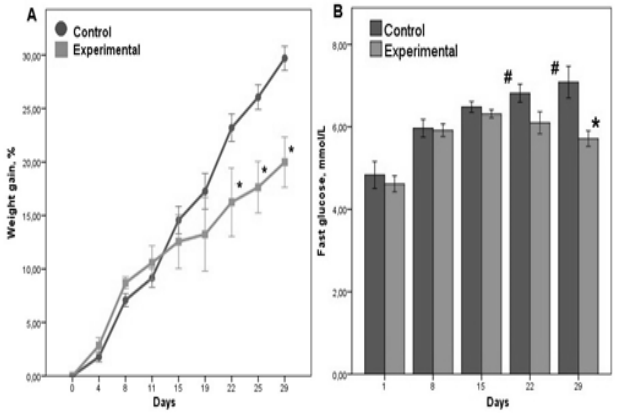

Figure 4. Effects of 4-week treatment with bilberry leaf extract (A) The average body weight gain (B) Dynamics of fast glucose level during the experiment.
Values are mean ± SEM of 6 rats per group.
*P<0.05 vs. Control rats
# P<0.05 vs. 1st day

that STZ injection in a dose 50 mg/kg bw to Wistar rats, treated with a 10% fructose solution during 4 weeks, resulted in a significant decrease in bw gain.

Development of STZ-induced diabetes in rats

During the first 30 days of the experiment, the general condition of all animals in appearance, quality of coat, behavior, and growth rate during daily inspection did not differ. The gain of body weight in control group №1 (NC) and group of rats treated with a solution of 10% fructose (HFD) was consistent with the growth rate characteristic of the species and age of the animals (Cossio-Bolanos *et al.*, 2013). The growth did not differ significantly between groups and was, respectively, 89.2 ± 4.5% and 88.9 ± 4.6%.

On the 31st day of the experiment (day of administration of STZ, 50 mg/kg), the average body weight of the animals in the control group was 326 ± 8 g and was not significantly different from the mean body weight of animals treated with fructose (326 ± 5 g).

The results of determining the fast glucose average levels on 38th and 60th days of the experiment shown in Figure 3. On the 38th day of the experiment, the glucose level of more than 11.0 mmol/l in the blood was determined in 16 of 30 rats that, during the preceding 7 days were given STZ intraperitoneally. The mean value of blood glucose levels for these diabetic animals was 20.5 ± 3.7 mmol/L and for NC animals – 7.5 ± 2.3 mmol/L. The average blood glucose level in the D1 group was 21.1 ± 7.4 mmol/L and after 22 days of feeding (60th day of the experiment) remained unchanged – 21.4 ± 7.1 mmol/L. The average blood glucose level in the group D2, which consumed bilberry extract, after 22 days of feeding decreased to 14.6 ± 4.9 mmol/L, moreover, the level of hyperglycemia decreased to normal values in four animals out of eight. The differences that determined between group D1 and group D2 are significant.

After measurement of glucose on the 60th day of the experiment, the experimental diets were continued for next 28 days. After 88 days, the animals were taken out of the experiment.

Results of the glycated hemoglobin analysis show the ameliorative effect of bilberry leaf extract on carbohydrate metabolism in diabetic rats. The average level of glycated hemoglobin level in blood serum was significantly higher compared to normal control group. However, half of the animals (4 of 8) treated with bilberry leaf extract had normal glycated hemoglobin levels at a value of 6%, as in the NC group. Whereas only in one animal out of 8 rats in the D1 group the normal level of glycated hemoglobin was determined. The testing of glycated hemoglobin can avoid the problem of day-to-day variability of glucose values (World Health Organization, 2011).

Experiment 2

Body weight and food intake

As shown in Figure 4 (A) a body weight gain increased gradually for both groups of animals during the experiment. Starting from the 15th day of the experiment the average weight gain of rats which consumed bilberry extract began to decrease compared to the control group. Moreover, on the 22nd day of the experiment, the mean body weight gain of this group was significantly lower than that of the control group, and this difference was stable until the end of the experiment (P<0.05). The growth's slowdown in the experimental Zucker rats (E2

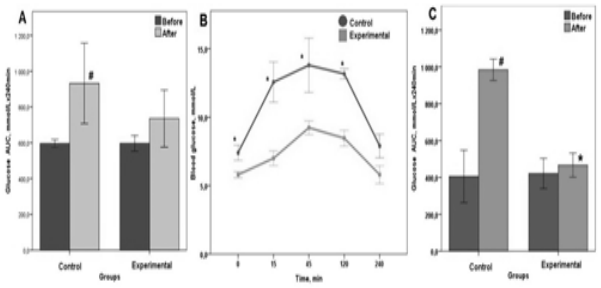

Figure 5. Effects of 4-week bilberry leaf extract treatment of ZDF rats in GTT and ITT. (A) AUC of glucose determined by GTT before and after intervention. (B) Glucose levels determined by an ITT in control and experimental rats after intervention. (C) AUC of glucose determined by ITT before and after intervention.
Values are mean ± SEM of 6 rats per group.
*P<0.05 vs. Control rats
# P<0.05 vs. 1st day

group) was not due to the different food intake, as the average cumulative value of this factor was 72.6 ± 8.0 g per day for K1 and 68.4 ± 6.8 g per day for E2 groups (differences not significant). The ZDF rat is a popular obese, type 2 diabetes model (King, 2012) and this fact can clearly be seen as a favorable factor in the effect of VLE extract.

Glucose metabolism

The dynamics of blood glucose level is presented on Figure 4 (B) for both groups. Starting from the 22nd day of experiment the average blood glucose level of control animals was significantly higher about to the 1st day of the experiment. The blood glucose level of experimental animals, treated with bilberry leaf extract, didn`t change during the experiment. Fasting blood glucose was significantly lower in the bilberry group compared with control group at the end of the experiment.

The data, presented on Figure 5 (A, B, C) characterizes the GTT and ITT (with glucose load) results at the beginning and the end of the experiment. The GTT and ITT are classic tests in diabetes studies to evaluate the glucose metabolism in the organism and insulin sensitivity (Ghezzi *et al.*, 2012).

The results of GTT and ITT at the beginning of the experiment didn`t show any significant difference between the groups, which meant their randomized distribution in groups. The GTT and ITT revealed a significant decrease in the glucose tolerant capacity of control rats (Figure 5) after 4 weeks of treatment. The results of GTT and ITT after intervention showed a significant increase in average area under the curve (AUC) value for the control group. Average AUC value for animals, treated with bilberry leaf extract, remained almost the same, as at the first test before intervention (Figure 5 (A), (C)). The blood glucose levels in control rats at baseline, 15, 45, 120 min were all significantly higher than those in the experimental group (P< 0.05) (Figure 5 (B)).

The above results suggest decreased insulin effects and an increased susceptibility of control rats to diabetes mellitus. While the consumption of bilberry leaf extract contributed to the improvement of the reaction of insulin sensible tissues to exogenous administration of glucose and insulin.

The blood glycated hemoglobin was determined in all groups on the 10th, 19th and 29th day of the experiment. There were no significant changes as between groups, as in dynamics of this parameter ($p \geq 0.05$, data not shown). The absence of changes may be explained, that the parameter shows the average blood glucose concentration for the last 1-2 months (Albright *et al.*, 1994).

Conclusion

In male Wistar rats with hyperglycemia-induced with STZ injection and high fructose diet treatment with bilberry leaf extract during 7 weeks lowered glucose and blood glycated hemoglobin levels and had a positive effect on body weight gain. In male Zucker fatty rats, treatment with bilberry leaf extract during 4 weeks inhibited hyperglycemia development, glucose intolerance, insulin tolerance and also inhibited body weight increase in obese rats. The results of both experiments showed the positive effects of the use of VLE extract for the dietary management of diabetes. In studies of both the medical model of diabetes and the genetic, have been confirmed the relevance of including VLE to specialized food products for patients with type 2 diabetes.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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