Evaluation of Anti-diabetic Effects of *Eryngium Billardieri* on Streptozotocin-induced Diabetes in Male Mice

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Research

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Abstract

Background: One of the largest plant genera of the Umbelliferae family is *Eryngium* including 274 species, of which only 9 species are native to Iran and one of the most dominant species is *E. billardieri*. Numerous pharmacological effects of *E. billardieri* such as anti-inflammatory, antimicrobial, antioxidant have been reported in several studies.

Objective: The present study aimed to investigate the anti-diabetic effects of *E. billardieri* on streptozotocin-induced diabetes in male mice.

Methods: The extraction was performed by maceration with 70% ethanol solvent. Forty eight mice, weighing 32 g, were randomly divided into 8 groups (6 mice per group): healthy control, diabetic control (151 mg/kg STZ; IP), healthy extract control group (receiving the high dose of the extract orally), diabetic groups (receiving low dose and high dose of extract in the form of IP and Oral), the treatment group which received STZ (IP) and high dose of the extract group (Oral) for five days. To induce diabetes, a single dose of STZ (151 mg/kg) was injected intra-peritoneally. After diabetes, 100 mg/kg and 300 mg/kg of extract were injected into diabetic animals for 18 days as IP and Oral were given, and finally, serum samples were isolated and stored at -71 °C. Three days before surgery, OGTT test was performed.

Results: IP injection of 300 mg/kg of hydroalcoholic extract of *E. billardieri* reduced the weight of diabetic rats and significantly reduced glucose tolerance. Furthermore, gavage at a dose of 300 mg/kg of extract caused significantly the increase in serum insulin while the decrease in blood sugar, glucose tolerance and malondialdehyde.

Conclusion: *E. billardieri* extract, due to its components, probably increases serum insulin and decreases serum malondialdehyde by two mechanisms including protects pancreatic beta cells from further damage by streptozotocin and stimulation of insulin secretion from the remaining healthy pancreatic beta cells. In other words, part of the beneficial effects of the extract is associated with inhibition of oxidative stress and increased insulin secretion.

Introduction

Diabetes mellitus (DM) is a group of metabolic disorders that cause high blood sugar levels in a long time [1]. The cause of the disease depends on the amount of insulin production or insufficient production of insulin by pancreatic beta cells which are generally divided into three main types: type I diabetes, type II diabetes and gestational diabetes [2]. Common clinical manifestations of this disease include: weight loss, polyuria, polydipsia, polyphagia, headache, fatigue, slow wound healing, and if left untreated, can result in significant consequences such as neuropathy, retinopathy, and nephropathy [3, 4]. The global prevalence of adult diabetes was 6.4% in 2010, equivalent to 285 million people, and in 2012 was about 371 million people, estimated to reach 552 million by 2030 [5]. It has been estimated that the prevalence of diabetes in the Middle East will increase significantly and it is prognosticated that the annual growth rate of diabetes by 2030 in Iran will be second only to Pakistan [6]. Overall, the incidence of DM increased

from 11.3 million in 1990 to 22.9 million in 2017 [7]. Important risk factors for the disease include obesity, physical inactivity, family history, race, age, gender, high blood pressure and so on [8]. Exercise, weight control, proper diet, blood sugar lowering drugs and insulin injections are common treatments for this disease [9]. The study of natural compounds is very important in the treatment of various diseases, including diabetes. Due to the side effects of insulin and hypoglycemic drugs such as sulfonylureas, metformin, alpha-glucosidase inhibitors, troglitazone, etc., patients tend to use natural hypoglycemic products. One of the plant compounds with pharmacological properties is a plant of the Umbelliferae family called *Eryngium*. The distribution of this plant has been observed in the northern, northwestern, central and northeastern regions of Iran [10]. The anti-diabetic effects of some *Eryngium* species have been reported in previous studies [11, 12]. Considering the increasing prevalence of diabetes and the importance of medicinal plants in the treatment of diabetes and also taking into account that no study has been done to investigate the anti-diabetic effects of *E. billardieri*, this study aimed to evaluate the anti-diabetic effects of the extract of *E. billardieri* on diabetic mice.

Materials And Methods

Chemicals. Streptozotocin (Merck Germany), Ethanol 70% (Caledon Canada), Serum Normal Saline and Distilled Water, Sodium Citrate Dihydrate Buffer (Merck Germany), Citric Acid Monohydrate (Merck Germany), STAR Glucometer for Blood Glucose (Made in Taiwan), Insulin Kit (manufactured by Shibayagi Company), and Malone Devaldehyde Kit (Jiancheng Institute of Bioengineering Company, Nanjing, China).

Preparation of samples and plant powder. *E. billardieri* plant was collected in August 2016 from Urmia region, Iran. After identification, its herbarium specimen with number 1249 was kept in the herbarium of Tabriz University of Medical Sciences. At first, the aerial parts of the mentioned plant were dried naturally in the open air, and turned into a very fine powder. The powder obtained from this step was very light with a low density.

Extraction of the Plant Material. Extraction was performed by maceration method; first, pour 200 g of plant powder into a wide-mouthed Erlenmeyer flask and add 70% ethanol ratio. Then, the Erlenmeyer was placed in a quiet place away from sunlight and shook the Erlenmeyer flask once a day to mix its contents. We did this until the color of the solvent did not change. The supernatant is passed through a filter and the solvent of the extract is dried via an evaporator at low pressure and temperature. The dried extract was placed in a sealed container and stored at 4°C for further procedure.

Experimental Animals. This study was conducted in four stages including streptozotocin injection, injection and gavage of the extract, collection of blood and tissue samples, measurement of serum insulin and malondialdehyde. Forty eight male NMRI mice race purchased from Pasteur Institute of Iran with an average age of 12 weeks and a weight range of 32 g in standard clear polyethylene cages under standard conditions of 21°C, and humidity $\pm$ 12 hours light-dark, and in normal temperature of laboratory ($10 \pm 60\%$) were maintained. They had free access to sufficient water and food until the experiment. In general, mice were randomly divided into 8 groups (n = 6): Healthy control group, diabetic control group

(receiving STZ), healthy extract control group (gavage dose of 300 mg/kg extract), diabetic group (receiving 100 mg/kg extract from the third day as IP), diabetic group (receiving 300 mg/kg extract from the third day as IP), diabetic group (gavage 100 mg/kg extract from the third day), diabetic group (gavage 300 mg/kg extract from the third day), STZ receiving group and dose 300 mg/kg of extract (Oral) for 5 days.

Streptozotocin preparation. Since STZ is a powder and cannot be injected directly into the peritoneum, the powder was dissolved in a 0.1 M sodium citrate dihydrate buffer. Due to the sensitivity of STZ to light, all steps of STZ preparation were performed in the dark [13, 14].

Induction of diabetes in male mice by streptozotocin. First, the weight and blood sugar of each mouse was measured and recorded. Blood was taken from the tail vein of a mice to measure blood sugar; in this way, first the mice tail was disinfected with alcohol and then the tail picking method was used for blood sampling. The dose of streptozotocin (150 mg/kg) for each mice was calculated based on mouse weight [13, 14]. The injection site was disinfected with 70% ethanol and then streptozotocin was injected intraperitoneally. The mice were monitored for three days, during which time they had access to water and food. After three days, the rats' weight and blood sugar were measured again. Mice with blood glucose above 200 mg/dl were selected as diabetic groups and this procedure was repeated every three days.

Receiving the extract by diabetic mice as IP and Oral and their surgery. From the third day, diabetic mice (groups 4, 5, 6 and 7) received the extract for 18 days. After 18 days of receiving the extract, mice were anesthetized by intraperitoneal injection of ketamine (100–100 mg/kg) and xylazine (3–10 mg/kg) [15–17]. After deep anesthesia, cervical dislocation was performed to ensure the cessation of vital signs in mice. Blood samples were centrifuged at 4500 rpm and 4 ° C for 30 minutes and then isolated serum samples were stored at -70 ° C.

OGTT test. Three days before surgery, mice were given 2 g/kg glucose by gavage after 12 hours of fasting. At times, zero (before glucose consumption), 30, 60, 90, and 120 minutes after glucose consumption, blood samples were collected from the venous vein to measure blood glucose levels [18].

Measurement of serum insulin levels. Determination of insulin levels in serum samples was performed by ELISA method. For this purpose the company kit Shibayagi was used. 10 µl of serum samples were incubated in wells coated with biotin conjugated to insulin antimonoclonal for 2 hours at room temperature. After incubation, the wash buffer was added to the wells and incubated with streptavidin-conjugated HRP (Horseradish Peroxidase) for 30 minutes at room temperature. In the next step, the streptavidin-conjugated HRP residue reacted with the chromogenic reagent at room temperature for 30 minutes. The reaction was stopped by adding the solution. The absorbance was read with a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm [19].

Measuring the amount of malondialdehyde (MDA). The amount of malondialdehyde was measured using colorimetry method and thiobarbituric acid reagent (TBARS) according to the instructions of the

manufacturer (Jiancheng Institute of Bioengineering Company, Nanjing, China) and its absorption was determined at 500 nm [20].

Statistical analysis of data. Data were represented as Mean $\pm$ SEM. One-way ANOVA and post-hoc Tukey test were used as statistical methods. $P < 0.05$ is considered as a significant difference.

Results

Weight changes in healthy and diabetic groups. The weight of the healthy control group increased during the experiment. The weight of the healthy control group of the extract did not change much compared to the healthy control group. The diabetic control group lost weight until the ninth day, which is not significant compared to the weight of the healthy control group. In the diabetic group receiving 100 mg/kg of extract (IP) compared to the diabetic control group, weight loss was observed which was significant on the last day of the experiment (day 21) ($P < 0.05$). The weight of the diabetic group receiving 300 mg/kg of extract (IP) had a decreasing trend during the experiment, which was not significant compared to the weight of the diabetic control group. No weight loss was observed in the diabetic groups receiving 100 mg/kg and 300 mg/kg of the extract (Oral) compared to the weight of the diabetic control group (Fig. 1).

To better evaluate the results of weight changes during the experiment, a graph of weight changes compared to the first day is drawn. As illustrated in the chart (Fig. 2), streptozotocin caused significant weight loss in the diabetic control group compared to the healthy control group ($P < 0.05$ and $P < 0.01$).

Weight changes in simultaneous administration of STZ and 300 mg/kg dose (Oral). The weight of the samples in the simultaneous administration of STZ and the dose of 300 mg/kg of the extract did not change much compared to the weight of the diabetic control group (Fig. 3).

Change of blood sugar in healthy and diabetic groups. The blood sugar of the healthy control group did not show much change during the experiment and had a steady trend. Blood sugar in the healthy control group from extract did not change compared to the blood sugar in the healthy control group and had a steady trend as in the healthy control group. In the diabetic control group compared to the healthy control group, on the third day, an increase in blood sugar was observed and from the sixth day to the last day of the experiment (day 21), the increase in blood sugar was significant ($P < 0.001$). The diabetic groups receiving 100 mg/kg and 300 mg/kg extract (IP) did not have regular hypoglycemia compared to the diabetic control group. In the diabetic group receiving 100 mg/kg of extract (Oral), compared with the diabetic control group, no reduction in blood sugar was observed. From the ninth day to the last day of the experiment (day 21), the blood sugar of the diabetic group receiving 300 mg/kg of the extract (Oral) had a significant decrease compared to the diabetic control group ($P < 0.05$ for the ninth day, $P < 0.001$ for days 12–21) (Fig. 4).

To better evaluate the results obtained from changes in blood sugar during the test, a graph of changes in blood sugar compared to the first day is drawn (Fig. 5). Streptozotocin caused a significant increase in

blood sugar in the diabetic control group compared to the healthy control group. In addition, oral administration of 300 mg/kg of the extract significantly reduced the blood sugar of diabetic mice compared to the diabetic control group ($P < 0.05$, $P < 0.001$).

Change of blood sugar in concomitant administration of STZ and 300 mg/kg extract (Oral). As shown in Fig. 6, the blood glucose of the samples in the simultaneous administration of STZ and the dose of 300 mg/kg of extract (Oral), compared to the blood sugar of the diabetic control group, on the fifth day, was significantly reduced.

Change of blood sugar on the OGTT test. The healthy control group and the healthy control group of the extract had an increase in blood sugar at 30 minutes after glucose administration and in the following minutes, their blood sugar gradually decreased. In the diabetic control group compared to the healthy control group, after glucose administration, at all times, blood sugar was significantly high ($P < 0.001$). The diabetic groups receiving 100 mg/kg and 300 mg/kg of extract (IP), compared with the diabetic control group, after glucose administration, showed a decreasing trend in blood sugar, which decreased for the group receiving 100 mg/kg of the extract was significant at 120 min time. In the group receiving 300 mg/kg of the extract, it was significant in 120 – 60 minutes ($P < 0.001$). The diabetic group receiving 100 mg/kg extract (IP) had hyperglycemia at 30 minutes after glucose administration and no significant decrease in blood glucose was observed at subsequent times compared to the diabetic control group. The diabetic group receiving 300 mg/kg extract (Oral), compared with the diabetic control group, had a significant reduction in blood sugar at 90 and 120 minutes ($P < 0.001$) (Fig. 7).

In order to better evaluate the results obtained from changes of blood sugar in the OGTT test, a graph of changes in blood sugar compared to zero minutes (before glucose administration) has been drawn (Fig. 8). As can be seen, the diabetic group receiving 300 mg/kg extract (IP) had a significant reduction in blood sugar compared to the diabetic control group ($P < 0.05$ and $P < 0.001$).

Evaluation of insulin levels in the serum of mice. As shown in Fig. 9, the healthy control group of the extract did not change much in insulin levels compared to the healthy control group. In the diabetic control group, compared to the healthy control group, a significant decrease in insulin levels was observed ($P < 0.001$). The diabetic groups receiving 100 mg/kg and 300 mg/kg extract (IP) did not have a significant increase in insulin levels compared to the diabetic control group. No significant increase in insulin was observed in the diabetic group receiving 100 mg/kg extract (Oral) compared to the diabetic control group. Insulin levels in the diabetic group receiving 300 mg/kg of extract (Oral) had a significant increase compared to the diabetic control group ($P < 0.001$).

Evaluation of MDA in mice. As shown in Fig. 10, the amount of MDA in the healthy control group of the extract did not change significantly compared to the healthy control group. In the diabetic control group, compared to the healthy control group, a significant increase in MDA was observed ($P < 0.001$). The diabetic groups receiving 100 mg/kg and 300 mg/kg extract (IP) did not have a significant reduction in MDA compared to the diabetic control group. In the diabetic groups receiving the dose of 100 mg/kg and

the dose of 300 mg/kg of the extract (Oral), compared to the diabetic control group, a significant decrease in the amount of MDA was observed ($P < 0.01$ and $P < 0.001$, respectively).

Discussion

One of the chronic metabolic disorders and the leading causes of death in the world is diabetes mellitus (DM). Among the most common symptoms of this disease is high blood sugar levels, which can be caused by a defect in insulin secretion (type 1 diabetes) or insulin resistance (type 2 diabetes) [21]. Common treatments include exercise, weight control, proper diet, blood sugar-lowering drugs, and insulin injections. The reason why patients are more inclined to use natural products (herbs) to lower blood sugar is the side effects of blood sugar lowering drugs such as insulin, sulfonylureas, metformin, alpha-glucosidase inhibitors, troglitazone, etc. [22]. *Eryngium* species are cultivated as ornamental, herbal and medicinal products. However, the phytochemical and medicinal properties of most 251 species of this genus are not yet known. 23 species of the genus *Eryngium* have been identified, of which at least 127 compounds have been isolated and identified, most of which are compounds such as terpenoid and triterpenoid saponins, flavonoids, coumarins, polystylenes and steroids. According to *in vivo* and *in vitro* studies on extracts of some species of the genus *Eryngium*, they were found to have anti-cancer, anti-inflammatory, anti-snake and anti-scorpion, anti-bacterial, anti-fungal, anti-malarial anti-oxidants and anti-hyperglycemic effects [23].

Considering the increasing prevalence of diabetes and the importance of herbs in the treatment of diabetes and also considering that no study has been done to investigate the anti-diabetic effects of *E. billardieri*, this study was investigated to evaluate the effect of *E. billardieri* extract on glucose, insulin and malondialdehyde in diabetic mice. For this purpose, diabetes was caused by streptozotocin. Streptozotocin enters the pancreatic beta cells through the glucose transporter (GLUT2) and alkylates the cell DNA. DNA damage induces activation of Poly ADP-ribosylation which reduces NAD⁺ and cellular ATP; As a result, streptozotocin has toxic effect on the insulin-producing beta cells of the pancreas [24]. Based on the results of previous studies with STZ, this combination reduces the weight of mice [25]. In this study, it was observed that IP injection of 300 mg/kg of *E. billardieri* hydroalcoholic extract caused a decrease in the weight of diabetic mice, while gavage at 100 mg/kg and 300 mg/kg, as well as IP injection at a dose 100 mg/kg of extract did not cause significant change in weight of diabetic mice. In a study by Jagabir *et al.*, on the antidiabetic effects of *E. campestre* leaves has been shown that oral use of the aqueous extract of this plant reduced the blood glucose concentration of hyperglycemic rats [11]. In another similar study of *E. foetidum*, Rauter *et al.*, found that the plant's flavonoid compounds had anti-diabetic effects [26]. Afshari *et al.*, extracted the hydroalcoholic extract of *E. caucasisum* and found that the extract of this plant reduces blood glucose concentration in a dose-dependent manner (doses of 200 and 300 mg/kg) and has anti-diabetic properties [12].

The results of the present study also showed that the oral dose of 300 mg/kg of extract significantly reduced blood sugar in diabetic mice ($P < 0.001$) and increased insulin secretion ($P < 0.001$). To evaluate the effect of *E. billardieri* hydroalcoholic extract on glucose homeostasis in mice, OGTT test was

performed as an indicator to evaluate the function of Langerhans islands. OGTT results showed that diabetic control group had high glucose tolerance, while IP injection of 300 mg/kg and gavage of 300 mg/kg hydroalcoholic extract remarkably improved glucose tolerance in diabetic mice ($P < 0.001$).

To investigate the protective effect of *E. billardieri* extract on the development of diabetes by streptozotocin, concomitant administration of streptozotocin (IP) and 300 mg/kg extract (Oral) was performed in mice and developed on the third day of diabetes. However, on the fifth day, blood sugar dropped dramatically. It seems that the gavage at a dose of 300 mg/kg of the extract could not completely protect against damage to pancreatic beta cells by streptozotocin, so mice became diabetic on the third day. Significant reduction of blood sugar in diabetic mice on the fifth day can have two possible mechanisms including preventing further damage to pancreatic beta cells caused by streptozotocin and stimulating insulin secretion in the remaining healthy pancreatic beta cells. In addition, our experimental results showed that the use of 100 mg/kg and 300 mg/kg of extract orally significantly reduced MDA levels ($P < 0.01$ and $P < 0.001$, respectively) compared with diabetic control group, which in general shows the antioxidant effects of the extract which confirmed the anti-oxidant effects of *E. billardieri* in previous studies [23, 27]

Conclusion

Based on our findings, *E. billardieri* extract, increases serum insulin and decreases serum MDA. It is likely that *E. billardieri*, with two mechanisms protects the pancreatic beta cells from further damage by streptozotocin and stimulates insulin secretion by remaining healthy pancreatic beta cells which causes to increase serum insulin levels, lower blood sugar, and decrease serum MDA levels. Therefore, it seems that in oral administration of the extract, the substances in the extract are metabolized by chemical or enzymatic reactions in the gastrointestinal tract and become substances that have the ability to significantly inhibit oxidative stress and increase insulin secretion, which needs more studies. Moreover, according to the present study, it can be found that weight change in mice is often observed in diabetic groups and with increasing the dose of the extract, a decreasing trend was observed in the weight of mice.

Declarations

Ethics approval and consent to participate

The experimental procedures were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals and were approved by the Animal Ethical Committee of the Tabriz University of Medical Sciences (Permit Number: IR.TBZMED.VCR.RFR.1398.039). In addition, there is no involvement of human in this study.

Consent for publication

All other authors declare no conflict of interest.

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

Competing interests

We declare that we have no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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Authors' contributions

ET and KH performed the experiments; PA, HV, AG and VT analyzed of data; KH, VT, HV and PA prepared the manuscript; KH and VT wrote and edited the manuscript; PA designed the experiments; PA led and supervised the project.

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Figures

Figure 1

Weight changes in healthy and diabetic groups during the experiment. Data are reported as Mean ± SEM. One-way ANOVA and post hoc tukey statistical methods were used. * P < 0.05 compared with the diabetic control group (Dia: Diabetes, STZ: Streptozotocin, E.b: *Eryngium billardieri*, IP: Intraperitoneal)

Figure 2

Weight changes in healthy and diabetic groups compared to the first day. Data are reported as Mean $\pm$ SEM. One-way ANOVA and post hoc tukey statistical methods were used. * $P < 0.05$ and ** $P < 0.01$ compared with the diabetic control group (Dia: Diabetes, STZ: Streptozotocin, E.b: Eryngium billardieri, IP: Intraperitoneal).

Figure 3

Weight changes in the group receiving concomitant STZ and 300 mg/kg of extract (Oral). Data is reported as Mean $\pm$ SEM (STZ: Streptozotocin E.b: Eryngium billardieri)

Figure 4

Changes in blood sugar of healthy and diabetic groups during the experiment. Data are reported as Mean $\pm$ SEM. One-way ANOVA and post hoc tukey statistical methods were used. * P < 0.05 and *** P < 0.001 compared with the diabetic control group (Dia: Diabetes, STZ: Streptozotocin, E.b: Eryngium billardieri, IP: Intraperitoneal).

Figure 5

Changes in blood sugar of healthy and diabetic groups compared to the first day. Data are reported as Mean $\pm$ SEM. One-way ANOVA and post hoc tukey statistical methods were used. ** P < 0.01 and *** P < 0.001 compared with the diabetic control group (Dia: Diabetes, STZ: Streptozotocin, E.b: Eryngium billardieri, IP: Intraperitoneal).

Figure 6

Changes in blood glucose in the group receiving concomitant STZ and dose of 300 mg/kg of extract (Oral). Data are reported as Mean $\pm$ SEM. *** P < 0.001 compared with the diabetic control group (STZ: Streptozotocin, E.b: Eryngium billardieri).

Figure 7

Changes of blood glucose of different groups in the OGTT test. Data are reported as Mean $\pm$ SEM. One-way ANOVA and post hoc tukey statistical methods were used. *** P < 0.001 compared with the diabetic control group and **** P < 0.001 compared with the healthy control group (Dia: Diabetes, STZ: Streptozotocin, E.b: Eryngium billardieri, IP: Intraperitoneal).

Figure 8

Changes in blood glucose of different groups in the OGTT test compared to zero minutes (before glucose administration). Data are reported as Mean $\pm$ SEM. One-way ANOVA and post hoc tukey statistical methods were used. * $P < 0.05$ and *** $P < 0.001$ compared with the diabetic control group (Dia: Diabetes, STZ: Streptozotocin, E.b: Eryngium billardieri, IP: Intraperitoneal).

Figure 9

Serum insulin levels of healthy and diabetic groups during the experiment. Data are reported as Mean $\pm$ SEM. One-way ANOVA and post hoc tukey statistical methods were used. *** $P < 0.001$ compared with the diabetic control group and ◆◆◆ $P < 0.001$ compared with the healthy control group (Dia: Diabetes, STZ: Streptozotocin, E.b: Eryngium billardieri, IP: Intraperitoneal).

Figure 10

Serum MDA levels of healthy and diabetic groups during the experiment. Data are reported as Mean $\pm$ SEM. One-way ANOVA and post hoc tukey statistical methods were used. ** $P < 0.01$ and *** $P < 0.001$ compared with the diabetic control group and *** $P < 0.001$ compared with the healthy control group (Dia: Diabetes, STZ: Streptozotocin, E.b: Eryngium billardieri, IP: Intraperitoneal).