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Investigation of the effect of *Polygonum cognatum* Meissn. ethanol extract Bax, Caspase-3, Bcl-2, NF-κB and NRF-2/HO-1 pathways in streptozotocin induced diabetic rats

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Abstract

In this study, it was investigated of the effect of *Polygonum cognatum* Meissn. ethanol extract Bax, Bcl-2, Caspase-3, NF-κB and NRF-2/HO-1 pathways in streptozotocin induced diabetic rats. A total of 24 healthy Spraque-Dawley male rats were randomly divided into four groups containing of 6 rats per group as Control; Diabetes mellitus (DM); *Polygonum cognatum* Meissn. ethanol extract (PCE) and DM+PCE. Experimental diabetes was induced by a single dose of 60 mg/kg/i.p Streptozotocin (STZ) injection for DM and DM+PCE groups. The animals showing diabetes (Blood glucose level >250 mg/dL) will be selected for diabetes groups in 7 th days. PCE was given at a dose of 10 mg/kg /day/p.o via gavage 20 days. All of rats were sacrificed on 20 th day, taken blood and dissected kidney tissues ender anesthesia. Bax, Bcl-2, Caspase-3, COX-2, HO-1, NF-κB and Nrf-2 expression levels were determined by western blotting in kidney tissue. Compared with the control and diabetes groups, Bax, Caspase-3, COX-2 and NF-κB expression levels increased ($p<0.001$); Bcl-2, HO-1 and Nrf-2 expression levels were decreased in diabetes group. PCE given with STZ decreased Bax, Caspase-3, COX-2 and NF-κB expression levels ($p<0.001$); Bcl-2, HO-1 and Nrf-2 expression levels were increased. PCE treats STZ-induced diabetic kidney injury by regulating apoptosis parameters such as Bax, Bcl-2, Caspase-3, and inflammation pathways such as, NF-κB, COX-2, Nrf-2 and HO-1 against STZ-induced diabetes. It was concluded that PCE can be used as a therapeutic agent after determining the molecular processes behind the therapeutic benefits of PCE against kidney damage in STZ-induced diabetes. kidney injury kidney damage. According to the biochemical findings, PCE 10 mg/kg treatment dose decreased in kidney Bax Bcl-2, Caspase-3 and NF-κB pathways, increased in kidney Nrf-2 and HO-1 protein expression levels when administered with STZ was presented as a pioneering study in the literature.

Keywords: Apoptosis; Diabetes mellitus; *Polygonum cognatum*; Nrf-2/HO-1

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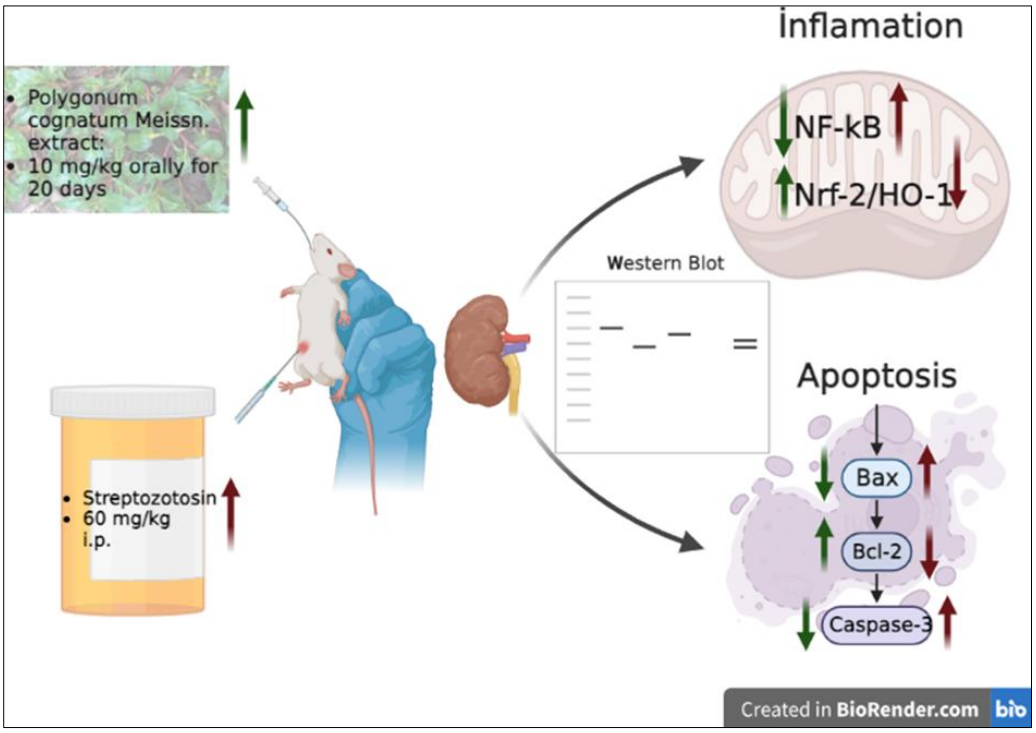
Graphical Abstract



1. Introduction

The prevalence of diabetes and other chronic diseases is increasing in many countries with the increase of the aging population. As a result, the complications are very common and very difficult to recover diabetes cause serious problems for public health. Diabetes mellitus (DM) is a metabolic disease that affects carbohydrate, fat and protein metabolisms, which occurs as a result of insufficiency in insulin secretion of the pancreas, impaired response of tissues to insulin or both. Pharmacological treatment of DM is based on the use of insulin and hypoglycemic (blood sugar lowering) drugs. However, due to the side effects of these treatment methods, herbal and alternative treatment methods are more popular. Traditional herbs are used to treat diabetes around the world. There are many scientific studies on the blood sugar lowering properties of many medicinal plants [1-2].

Polygonum cognatum Meissn. (P. cognatum), crude protein, vitamins C, E, carotenoids, Zn, Fe and Mn elements, rich in folic acid levels, a perennial wild edible edible belonging to the Polygonaceae family with antioxidant, antimicrobial, diuretic, antidiabetic, anthelmintic and antifungal properties [3].

Increased apoptosis plays an important role in the pathogenesis of renal damage. It maintains the production of antioxidant genes such as nuclear factor-erythroid 2-related factor 2 (Nrf-2), hemeoxygenase-1 (HO-1), which is the main regulator of the antioxidant defense mechanism, and stops oxidative stress-induced damage [2, 4, 5]. It has been reported that the Nrf-2/HO-1 pathway protects cells from damage by free radicals, apoptosis, and promotes cell survival against gentamicin-induced nephrotoxicity [6]. Nuclear factor kappa B (NF-κB) is upregulated in nephrotoxicity and diabetes [5, 7, 8].

In this study, *Polygonum cognatum* Meissn., which has antioxidant activity, in order to develop an alternative treatment method, considering the side effects of the treatment of diabetes mellitus with drugs. The aim of this study was to investigate the usability of ethanol extract in the prevention or amelioration of streptozotocin-induced diabetes.

2. Material and methods

2.1. Reagents

All chemicals were purchased from Sigma Aldrich (St. Louis, USA), Santa Cruz Biotechnology, (Inc., Texas, USA), PierceTM BCA Protein Assay Kit (Katalog No: 23225, ABD).

2.2. Materials

Polygonum cognatum Meissn. plants collected from Erzurum and dried under shade and protected it from sunlight. We roasted all of plant material and powdered in a blender coarsely before preperation of extract.

2.3. Preperation of *Polygonum cognatum* Meissn. ethanol extract

P. cognatum Meissn. plant was diagnosed by Prof. Dr. Şaban KORDALİ and herbarium was made and Atatürk University Faculty of Agriculture. It was kept in herbarium. The plants collected from Erzurum region were dried in the shade and ground in the mill, and the extracts were taken from the herbs (100 g each) ground in the Essential Oil Laboratory of the Faculty of Agriculture and put into 1000 mL balloons and 500 mL ethanol was added to the balloons separately. At the end of 48 hours, the plant materials and ethanol were filtered through a fine cheese cloth and the plant materials were separated from the suspension. Extracts were obtained by removing ethanol from the collected mixture with the help of an evaporator. The percentage yield of the obtained extract was determined and stored in a refrigerator at +4 °C to be used for the study. Since there is no literature on the use of *P. cognatum* in diabetes, it was decided to use *P. cognatum* ethanol extract as 10 mg/kg as a result of preliminary studies [2, 9].

2.4. Animals and experimental procedure

A total of 24 Sprague–Dawley 250–300 g male rats were used in the present study. The experiments were conducted according to the ethical norms approved by the Ethic Committee of Experimental Animal Teaching and Researcher Center (No: 10.11.2016 75296309-050.01.04-E.1600267147). Sprague–Dawley rats were purchased from the Medical and Experimental Application and Research Center (Erzurum, Turkey). Throughout the animal experiments were processed following the internationally accepted ethical guidelines for the care of laboratory animals. Prior to the experiments, rats were kept in standard laboratory conditions under natural light and dark cycle and were fed with standard food for one week, in order to adapt to the laboratory conditions. 18 h before from the experiments, they were fasted overnight, but allowed free access to water. Six animals were used for each group of study. Rats were divided into the four main groups such as Control (C), *P. cognatum* L. ethanol extract PCE) 10 mg/kg, diabetic (DM) and DM+ *PCE* (STZ-DM+NSE) groups.

2.5. Induction of diabetes

Diabetes was induced in rats by administration of a single (60 mg/kg b.w.) intraperitoneal injection of STZ (Sigma). STZ was dissolved in cold citrate buffer (0.1 M, pH 4.5). Seven days after the injection, the blood glucose levels were measured from tail vein. Each animal with a blood glucose level above 250 mg/dL was considered to be diabetic [2]. Blood glucose concentrations were determined using a Glucometer-elite commercial test (Bayer), based on the glucose oxidase method. In all experiments, rats were fasted for 18 h prior to STZ injection.

2.6. Taking kidney samples

On the 27 th day of the experimental application, kidney tissue samples were taken from all rats by decapitation under Xylazine (8 mg/kg) and Ketalar (60 mg/kg) anesthetics. Kidney samples were taken all of rats for western blotting analysis (-80 °C).

2.7. Western blotting analysis

Tissue homogenate was prepared. 600 µL of cold RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology, Inc., Texas, USA) was added and homogenized for 30 seconds with the Qiagen TissueLyser II homogenizer. Then, after centrifugation at 16.000 g for 20 minutes, the supernatants were taken. Protein concentrations of the supernatants we obtained were determined using PierceTM BCA Protein Assay Kit (Catalog No: 23225, USA). The BSA (bovine serum albumin) solution (2 mg/mL) supplied with the kit was used and the total protein amount of each tissue sample was calculated using the BSA-protein standard curve [10].

Electrophoresis of total proteins was done in 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to nitrocellulose membranes. After electrophoresis, the proteins in the gel were transferred to the polyvinylidene fluoride (PVDF) membrane. First, PVDF membranes were kept in methanol for 10 minutes. Stacks papers were placed in the prepared transfer buffer. Transfer process was done with Thermo blotting device and semi-dry system. Then, first stacks paper was placed on the Thermo blotting cassette and PVDF membrane was placed on top of it. The gel, whose execution process was completed, was placed on the PVDF membrane and covered with stacks paper again.

Proteins in the gel were allowed to pass to the PVDF membrane at 25 Volts for 10 minutes in a thermo blotting device. Proteins transferred to the PVDF membrane were blocked for 1.5 hours at room temperature in 5% BSA prepared with tris buffer (TBS-T) containing 0.1 % Tween 20 to prevent non-specific binding. After 1.5 hours, it was washed 3 times with 5 minutes intervals with tris buffer (TBS-T) containing 0.1 % Tween 20. Each time the previous dirty TBS-T was poured. Santa Cruz brand primary antibodies β-tubulin (sc-47778), Bax (sc-20067), Bcl-2 (sc-7382), caspase-3 (sc-56053) diluted 1/1000 with TBS-T to washed membranes, Nrf-2 (sc-365949), HO-1 (sc-390991), NF-κB (sc-8008) and COX-2 (sc-19999) were added and overnight it was incubated on a shaker at 4 °C. The next day, the primers were transferred to the falcon and placed at +4 °C for reuse. Membranes with primary antibody removed were washed 5 times with TBS-T for 5 minutes. Goat anti-mouse IgG-HRP (1:1000 dilution, sc-2005, Santa Cruz Biotechnology, Inc., Texas, USA) diluted 1:1000 with TBS-T as secondary antibody was incubated for 1.5 hours at room temperature. After 1.5 hours, it was taken to the falcon to be used again in secondary antibodies and placed at +4 °C. Membranes were washed again with TBS-T 5 times with 5 minutes intervals. Bands on the membrane were visualized with the enzyme-catalyzed chemiluminescence substrate Trident Femto Western HRP Substrate (Catalog No: GTX14698) brand ECL (5, 11). The ECL substrate solution was mixed in a 1:1 ratio in an epondorf tube by taking 200 µL of each of the dark and light bottles from the kit and poured onto the membrane. Since the working solution is light sensitive, this process was carried out in the dark. The bands were then imaged using the Biorad GelDoc XR gel imaging device (Model:0000076955, USA). Protein levels to be analyzed were calculated with Image Lab 6.1 software [12].

2.8. Statistical analysis

Statistical analysis was done by one-way analysis of variance (ANOVA) followed using SPSS software package, version 28.0.1.1. p values <0.05 were considered as significant. The results were presented as mean ± standard error (SEM) ($\bar{X}\pm S\bar{x}$) for 6 rats in each group.

3. Results

Western blotting results are shown in Figure 1-2 according to bands and all groups. When the expression levels of Bax and Caspase-3 proteins were examined, it was determined that the highest level was in the DM group. It was determined that there was a statistically significant decrease in the treatment groups treated with PCE compared to the DM group ($p<0.001$). Bcl-2 level was found to be the lowest in the DM group. It was determined that the PCE treatment groups significantly increased the Bcl-2 level compared to the DM group, which is the damage group ($p<0.001$). According to the results obtained, it was determined that PCE activates the Bcl-2 pathway and significantly reduces diabetes-related kidney damage (Fig. 1).

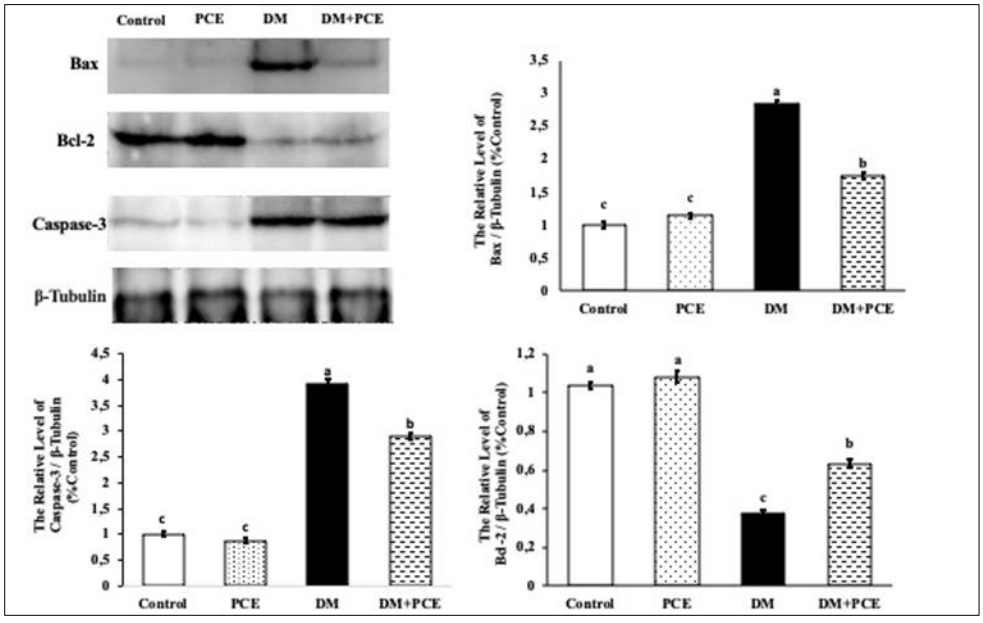


Figure 1 Expression levels of genes and proteins of Bax, Caspase-3 and Bcl-2 in the experimental groups. Immunoblots of Bax, Caspase-3 and Bcl-2; from kidney lysates. Densities of Bax, Caspase-3 and Bcl-2 protein bands in the experimental groups are presented. The bar graph shows the ($\bar{X}\pm S\bar{x}$) (one-way ANOVA and Tukey's test) ($p<0.001$). Abbreviations: PCE, plant extract rats; DM, diabetes rats; DM+PCE, plant extract-treated rats

When the expression levels of NF-κB protein were examined, it was determined that the highest value was in the DM group. It was statistically determined that PCE administered with DM significantly decreased the NF-κB protein expression level ($p<0.001$). When the Nrf-2 protein expression levels of the study groups were examined, it was observed that the highest value was in the PCE group and the lowest value was in the DM group. It was determined that the dose of PCE administered with DM significantly increased the Nrf-2 protein expression level ($p<0.001$). When the expression levels of HO-1 protein were examined, it was determined that there was a significant decrease in the DM group compared to the control group. It was determined that PCE applied together with DM significantly increased the HO-1 protein level ($p<0.001$) (Fig. 2).

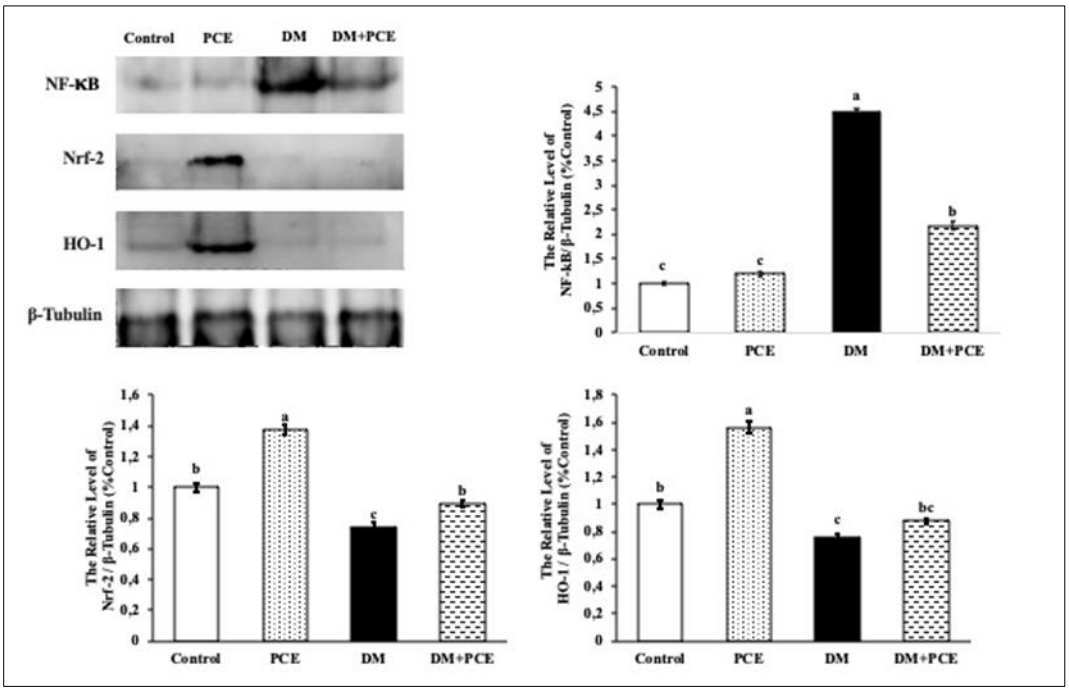


Figure 2 Expression levels of genes and proteins of NF-κB, Nrf-2 and HO-1 in the experimental groups. Immunoblots of NF-κB, Nrf-2 and HO-1; from kidney lysates. Densities of NF-κB, Nrf-2 and HO-1 protein bands in the experimental groups are presented. The bar graph shows the ($\bar{X}\pm S\bar{x}$) (one-way ANOVA and Tukey's test) ($p<0.001$). Abbreviations: C, control rats; PCE, plant extract rats; DM, diabetes rats; DM+PCE, plant extract-treated rats

4. Discussion

It is known that in the last 14 years, patients with diabetes prefer alternative treatment methods in their treatment and this rate is 17-73 %. It was determined that 67.9 % of the alternative treatment methods used herbal treatment. The fact that herbal applications are easy to apply, easy to access, economical and frequently used in the field of health makes people with diabetes prefer herbal applications more [2]. Diabetic nephropathy (DN) is a serious complication of diabetes and can lead to renal failure [13]. It is reported that many herbal hypoglycemic agents used in the treatment of DM today have serious side effects. Therefore, there is a need to develop new potential anti-diabetic drugs with few side effects [14]. Various studies have shown that PCE has hypoglycemic activity [15].

The apoptotic pathway plays an important role in nephrotoxicity pathogenicity. This pathway is controlled by the balance between the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax. If the Bax/Bcl-2 ratio increases, Caspase-3 activity increases and the apoptotic pathway becomes stronger [16].

The transcription factor Nrf-2 provides protection against excess ROS and oxidative damage by upregulating several antioxidant enzymes such as SOD and HO-1, which play an important role in cellular antioxidant defense. Therefore, Nrf-2 activation represents an effective strategy to reduce oxidative stress and prevent progression of DM [17, 18]. In line with the results obtained, down-regulation of Nrf-2 and HO-1 was detected in the kidney of diabetic rats, but no literature was found on this subject. It was revealed that the negative effects of STZ were prevented by increasing protein expression levels with PCE treatment. These findings can be shown to decrease ROS production and increase antioxidant defense on the basis of the renoprotective effect of PCE.

Nrf-2 is thought to protect cells from damage by free radicals, apoptosis and ensure cell survival and play a protective role in many diseases. However, HO-1 is considered to be one of the main influencers of Nrf-2-dependent cellular responses. HO-1 and its metabolic products have been reported to play an important role in maintaining cellular homeostasis and adaptive response to cellular stress and preventing the transformation of healthy cells into neoplastic cells [19].

NF-κB plays a crucial role in regulating immunological and inflammatory responses [20]. Overexpression of NF-κB has been observed in inflammatory demyelinating polyneuropathies, suggesting that NF-κB is important in regulating inflammatory demyelination [21]. In this study, STZ injection showed that it increased NF-κB immunoreactivity in the kidney. This activation of the NF-κB inflammatory axis was suppressed after PCE treatment. In a study, it showed anti-inflammatory effects on experimental diabetes by reducing NF-κB activation and cytokine release of PCE.

Recently, it has been reported that apaptosis parameters such as Bax, Bcl-2, Caspase-3, and inflammation pathways such as NF-κB, Nrf-2 and HO-1 are affected [22, 23]. The results showed the downregulation of Nrf-2 and HO-1 in the kidney of diabetic rats and revealed that this effect was prevented by PCE treatment by increasing protein expression levels. These findings can be shown to decrease ROS production and increase antioxidant defenses on the basis of the renoprotective effect of PCE [2].

5. Conclusion

Proapoptotic Bax, which is involved in apoptosis and inflammation in STZ-induced diabetes; It was determined that caspase-3 and NFκ-B protein expression levels increased, and the expression levels of antiapoptotic Bcl-2, HO-1 and Nrf-2 decreased. When PCE was applied to rats, Bax, caspase-3, and NF-κB protein expression levels decreased; Significantly increased expressions of Bcl-2, HO-1 and Nrf-2 were detected by western blotting. Although PCE is an antioxidant, the absence of any study on the effect of PCE on kidney Bax, Bcl-2, Caspase-3, NF-κB and Nrf-2/HO-1 signaling pathways in STZ-induced kidney damage was presented as a pioneering study in the literature.

Compliance with ethical standards

Acknowledgment

We appreciate the expert technical help of Prof. Dr. Saban Kordali. This study was produced based on the master's thesis of the second author (Eray ONAY), and the content was partially developed.

Disclosure of conflict of interest

Tuba Dogan, Eray Onay and Betul Apaydin Yildirim declare that they have no conflicts to declare.

Statement of ethical approval

The experiments were conducted according to the ethical norms approved by the Ethic Committee of Experimental Animal Teaching and Researcher Center (No: 19.10.2016 36643897-000-E.1600244918).

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