Protective Effects of *Bryonia multiflora* Extract on Pancreatic Beta Cells, Liver and Kidney of Streptozotocin-Induced Diabetic Rats: Histopathological and Immunohistochemical Investigations

Ahmet Uyar[1*], Turan Yaman[1], Omer Faruk Kele [1], Elif Ebru Alkan[2], Ismail Celik[2], Zabit Yener[1]

[1]Department of Pathology, Yuzuncu Yil University, Faculty of Veterinary Medicine, Van, TURKEY.
[2]Department of Molecular Biology and Genetics, Yuzuncu Yil University, Faculty of Bioscience, Van, TURKEY.

ABSTRACT

Objective: In this study, the ameliorative potential and antioxidant capacity of treated with different doses of *Bryonia multiflora* extract (BE) was investigated using histopathological and immunohistochemical changes in pancreatic beta cells, liver and kidney tissues of streptozotocin (STZ)-induced diabetic rats. **Material and Methods:** A total of forty-two healthy adult Wistar albino male rats were divided randomly into six groups as Control (C); Diabetes mellitus (DM); DM + Akarboz 20 mg/kg; DM + 100 mg/kg BE extract (BE1); DM + 200 mg/kg BE extract (BE2); DM + 400 mg/kg BE extract (BE3). Experimental diabetes was established by a single-dose [45 mg/kg, intra-peritoneal (i.p)] STZ injection. Essential dosages of BE extracts and Akarboz were applied with gastric gavage for 21 day. Blood glucose levels were recorded throughout the all experiment period. **Results:** Histopathological studies showed that hepatorenal and pancreatic protection by depending on the dose level of BE extracts was further supported by the almost normal histology in DM + BE extract-treated group as compared to the degenerative changes such as disorder of architectural structure, inflammatory cell infiltration, hydropic degeneration and necrosis in pancreas, liver and kidney tissues of STZ-treated rats. Immunohistochemical investigation revealed that STZ-induced degenerative changes in beta-cells in the pancreas of the diabetic rats has reduced insulin immunoreactivity. On the other hand, insulin immunoreactivity in the β cells of pancreas of BE –treated diabetic rats has significantly increased. Additionally, Glutathione peroxidase 1 (GPx1) immunoreactivity was lower in the tissues of diabetic rats (DM group) compared to the other groups. **Conclusion:** In conclusion, BE extract has a protective effect on tissue damage probably due to its antioxidant activity and possess the ability to regenerate β-cell in STZ-induced diabetic rats.

Key words: Diabetes mellitus Type I, *Bryonia multiflora* extract, Histopathology, Immunohistochemistry, Rat.

INTRODUCTION

Diabetes mellitus is a chronic metabolic diseases and is characterized by hyperglycemia, which is formed as a result of insulin secretion, activation or defect in both.[1,2] Chronic hyperglycemia in the course of diabetes contributes to the oxidative stress, causing the redox balance of the body to change with excessive production of reactive oxygen species.[3] Oxidative stress leads to an increase in protein, lipid, carbohydrate and DNA oxidation. Accordingly, it causes tissue and organ damage.[4] It is emphasized that this condi-

tion causes micro- and macrovascular complications of diabetes.[5]

Oxidative stress is observed to be increased in many organs, particularly in the liver, due to diabetes.[6] Since the liver has a central and critical role in the regulation of carbohydrate metabolism,[7] various structural and functional disorders affecting glycogen and lipid metabolism are formed in the liver as a result of diabetes.[8] Similarly, the oxidative stress which is caused by hyperglycemia is effective in the pathogenesis of diabetic kidney complications.[9] In addition, damage observed in β cells of pancreas, which is known to be one of the most sensitive structures to oxidative stress, is thought to be caused due to toxic effects of hyperglycemia.[10] Glycation-mediated free radical production leads to beta cell apoptosis and decreases the gene transcription of insulin[11].

Glutathione peroxidase (GPx), which is one of the natural antioxidant defense systems against free radicals, prevents lipid peroxidation by catalyzing the reduction of hydrogen peroxide (H_2O_2) or organic hydroperoxides to water or alcohols by using reduced glutathione,[12] and it protects cell membranes against oxidative damage.[13] GPx1 is expressed in most tissues, and it is an intracellular selenoprotein with high expression levels in erythrocytes, liver, and kidneys.[14] In patients with diabetes, oxidative stress is important in the pathogenesis of chronic complications of diabetes due to the reduction of antioxidant parameters such as vitamins C and E, glutathione, superoxide dismutase (SOD), catalase (CAT) and GPx.[15] In several studies, polyphenolic compounds are displayed to be potential antioxidants in the treatment and prevention of oxidative stress-related diabetic complications in rats induced by using streptozotocin (STZ).[16,17]

Byronia is a perennial herb belonging to the *Cucurbitaceae* family and contain four *Byronia* species, B. *multiflora* **Boiss. & Heldr.,** B. cretica, B. *alba* ve B. *aspera* Stev.ex Ledeb, are known in Europa and Asia.[18] Among the people in Turkey are known as Ulungur.[19] From the *Byronia* ose, steroidal saponin, triterpenic saponin, essential oil, cucurbitacin I and B have been isolated.[20] According to literature reviews cucurbitacins and triterpenic acids are present in B types as triterpenic substance while sterols are present as steroidal substance. Cucurbitacins also have cytotoxic, ovulatory, cardiovascular, gastric antitumoral, anti inflammatory, purgative and antihelminthic activity.[21] *Bryonia multiflora* including saponin have displayed various biological effects especially as hypocholesterolemic, antiprotozoal, antioxidant, anti-inflamma-

tory, antimicrobial and antihypertensive.[22] On de other hand fresh roots of BM is used for the treatment of rheumatism and itchiness.[23]

In this study, we aimed to present the protective effects of *Bryonia multiflora* extract on the liver, kidney and pancreas against the experimental diabetic complications in rats histopathologically and immunohistochemically.

MATERIAL AND METHODS

Plant material and preparation of lyophilized extract

Plant material and preparation of lyophilized extract plant samples were collected from Turkey (Hizan/ Bitlis). The identity of the plant materials was confirmed by Süleyman Mesut PINAR, PhD, at the Biological Sciences Department, Science Faculty, Yuzuncu Yil University, Turkey, and a voucher specimen stored at the university's herbarium (Herbarium code: 165060). The plant materials were promptly washed with distilled water and were left at room temperature in the dark until dry. Subsequently, the samples were ground and stored at 20 C until analyzed. The lyophilized extract was prepared as described previously with some modifications.[24] Briefly, the ground plant materials were mixed with a five-fold volume of purified water (g/ml), shaken for 2 h at room temperature (22 ^{0}C) and centrifuged for 20 min with the supernatant collected. The solvent of supernatants were evaporated under reduced pressure at 40 ^{0}C. The derived fraction was dissolved in purified water and freeze-dried under a vacuum at 51 ^{0}C to obtain a fine lyophilized powder.

Animals

Forty-two Wistar albino male rats 4-6 months of age, weighing 200–300 g, were provided by the animal house of the Medical School of Yuzuncu Yil University. Animals were housed at 20 ± 2 °C in daily light/dark cycle. All animals were kept in stainless cages and fed with standart pellet diet. Water and feed were offered ad libitum. The animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. The ethic regulations have been followed in accordance with The National and Institutional guidelines for the protection of animal welfare during experiments. This study was approved by The Ethic Committee of the Yuzuncu Yil University (Protocol number: 02.01.2014-01).

Acute toxicity test

The method of Ibeha and Ezeaja (2011) were employed in this study. Twelve rats were randomly grouped into three groups; rats were dosed with 250, 500 or 1000 mg/kg of the extract orally via gastric gavage. The animals were given food and water ad libitum. No signs of toxicity and mortality were observed over a period of 72 h.

Induction of diabetes

The animals were fasted for 12 h prior to the induction of diabetes. Streptozotocin prepared in citrate buffer (0.1 M, pH 4.5) was injected intraperitoneally (i.p.) at a only one dose of 45 mg/kg. Seventy-two hours after, blood glucose levels of STZ-treated fasted rats greater than 200 mg/dl were considered as diabetic.

Experimental design

The rats were randomly divided into six groups each containing seven (n¼7) rats.

Control group (C): rats received citrate buffer (pH 4.5) (1 ml/kg, i.p.).

Diabetes mellitus group (DM): Rats only received STZ in a one dose.

Diabetes mellitus + Acarbose (DM + AC) (20 mg/kg b.w.) group: Acarbose (20 mg/kg, per day) was treated to diabetic rats via orally during 21 d.

Diabetes mellitus + *Bryonia multiflora* Extract (100 mg/kg b.w.) (DM+ BE1) group: *Bryonia multiflora* lyophilized extract (100 mg/kg, per day) was treated to diabetic rats via orally during 21 d.

Diabetes mellitus + *Bryonia multiflora* Extract (200 mg/kg b.w.) (DM+ BE2) group: *Bryonia multiflora* lyophilized extract (200 mg/kg, per day) was treated to diabetic rats via orally during 21 d.

Diabetes mellitus + *Bryonia multiflora* Extract (400 mg/kg b.w.) (DM+ BE3) group: *Bryonia multiflora* lyophilized extract (400 mg/kg, per day) was treated to diabetic rats via orally during 21 d.

DM, DM + AC, DM+ BE1, DM+ BE2 and DM+ BE3 rats received STZ in a only one dose (45 mg/kg, i.p.). Seventy-two hours later, the glucose levels in blood samples taken from the tail vein were determined by Accu-Chek Go (Roche) brand glucometer equipment and through its strips. The rats with blood glucose 200 mg/dl and above were regarded as diabetic.[25]

Histopathological examination

At the end of experiments, the rats were anesthetized by injection of ketamine (50 mg/kg) i.p. Tissue samples were taken from the liver, kidney and pancreas and fixated in 10% buffered formalin solution solution. After routine tissue follow-up, the samples were embedded into paraffin blocks; 4 µm-sections were taken using microtome (Leica RM 2135); stained with hematoxylin-eosin and examined under light microscope (Nikon 80i-DS-RI2).

Immunohistochemistry (IHC) staining

Immunohistochemistry was performed to investigate GPx-1 and insulin expressions. The streptavidin-peroxidase method (ABC) was used to stain the sections. Commercial antibodies were visualized on 5-µm-thick paraffin sections using an indirect streptavidin/biotin immunoperoxidase kit (Histostain Plus Bulk Kit, Zymed, South San Francisco, CA, USA). All steps were carried out following the procedure described by Sağsöz and Saruhan.[26] Tissue sections were incubated with the GPx-1 (abcam-ab22604) (1:400) and insulin (abcam ab-181547) (1:1000) primary antibodys overnight at 4[0]C. Finally, to visualize the reactions, the sections were reacted for 5-15 min with diaminobenzidine (DAB) for GPx-1 and insulin staining. After the development of the DAB reactions, the sections were counterstained with Gill's Hematoxylin. The sections then were passed through alcohol and xylene and mounted directly with Entellan mounting medium. We used negative controls to verify staining. The slides were reacted with PBS instead of primer antibodies as negative controls. The slides were examined and photographed using a light microscope (E-400; Nikon, Tokyo, Japan) equipped with a video camera (DXM1200F, Nikon, Tokyo, Japan).

FINDINGS

Histopathological findings

The normal histological structure of the liver was observed in the control group (Figure 1-A). Parenchyma and hydropic degeneration in hepatocytes and necrosis as a single component were observed in liver sections of DM group rats. Slight fibrosis and inflammatory cell infiltration were detected in portal areas. The normal arrangement of hepatocyte cords was observed to be impaired and became an irregular cell community. The sinusoidal structure was sharply decreased and lost its continuity in several areas due to the formation of these lesions (dissociation) (Figure 1-B). These findings were found to be significantly reduced in the liver of rats in DM + Acarbose treatment group as well as degeneration and necrosis was observed locally in hepatocytes (Figure
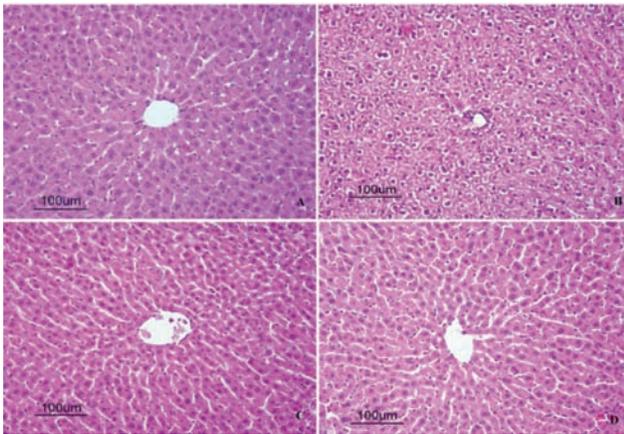
Figure 1 (A-D): Photomicrographs of H&E stained histological slides of the liver. A)Normal histological appearance of liver in the control group. B) Appearence of focal dissemine hydropic degeneration, destructed portal trial and disarranged hepatocytes of liver parenchyma in the DM group. C) Appearence of liver section of rat administered with Acarbose showing normal structures along with hepatocytes in the DM + AC group. D) Nearly normal histological appearence of the liver in the DM+BE group.

Figure 3 (A-D): Photomicrographs of H&E stained histological slides of the pancreas. A) Normal histological appearance of the islets of Langerhans of pancreas in the control group. B) Appearence of shrunken and distorted islet of Langerhans displaying cells with degenerative and necrotic changes in pancreas in the DM group. C) Appearence of pancreas section of rat administered with Acarbose showing normal structures in the DM + AC group. D) Almost normal histological appearance of the islets of Langerhans of pancreas in the DM+BE group.
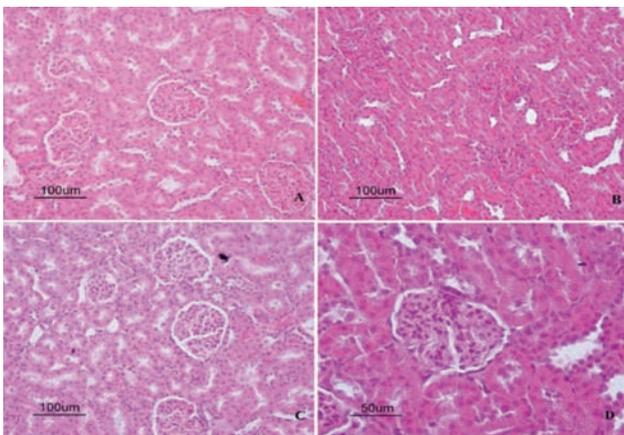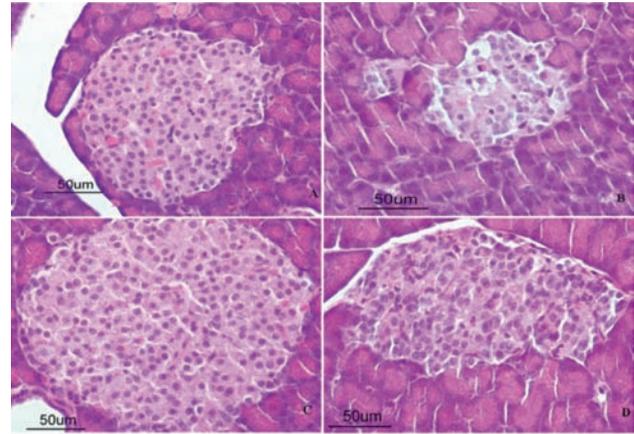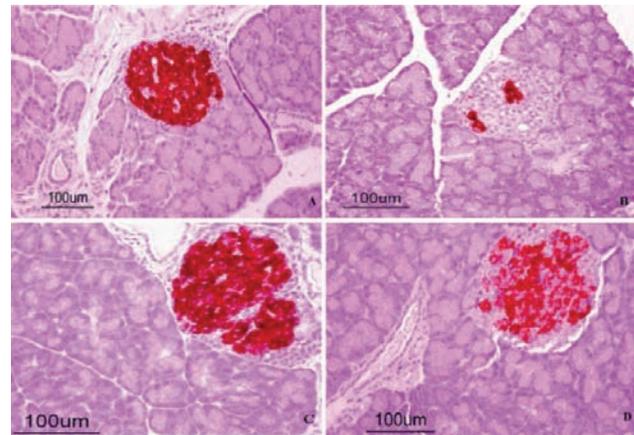

Figure 2 (A-D): Photomicrographs of H&E stained histological slides of the kidney. A) Normal histological appearance of kidney in the control group. B) Appearence of degeneration / necrosis of tubuli epithelium and glomerular mesengial cells, and adesion of bowman capsula in kidney in the DM group. C) Appearence of kidney section of rat administered with Acarbose showing normal structures in the DM + AC group. D) Nearly normal histological appearence of the kidney in the DM+BE group.

Figure 4 (A-D): Photomicrographs of immunohistochemical stained by streptavidin-peroxidase method of the pancreas. A) Appearance of strong immunreactivity of insulin in beta-cells of the islets of Langerhans of pancreas in the control group. B) Appearance of very weak immunreactivity of insulin in beta-cells in the DM group. C) Appearance of almost strong immunreactivity of insulin in beta-cells in the DM + AC group. D) Appearance of almost strong immunreactivity of insulin in beta-cells in the DM+BE group.

1C). These findings were found to be significantly reduced in BE1 and BE2 group rats treated with BE whereas liver sections were found to have similar histological appearance with the control group in BE3 group (Figure 1D).

Kidney was in a normal histological structure in the control group (Figure 2-A). In the kidneys of rats in DM group, degeneration and necrosis were observed significantly in tubulus epithelial cells and glomerular mesengial cells. Focal inflammatory cells were seen in the intertubular areas. Glomeruli, which were narrowed in bowman capsule, were observed to create adhesion with the basal membrane (Figure 2-B). Slight hydropic degeneration and necrosis were detected in some parts of the tubular epithelial cells in the kidney of rats in DM + Acarbose treatment
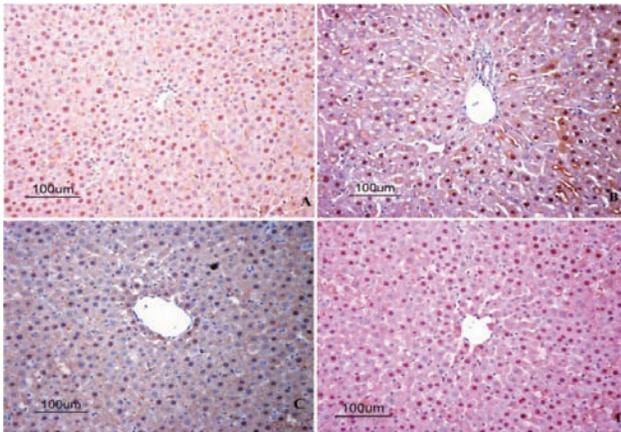
Figure 5 (A-D): Photomicrographs of immunohistochemical stained by streptavidin-peroxidase method of the liver. A) Appearance of strong immunreactivity of GPx1 in the control group. B) Appearance of very weak immunreactivity of GPx1 in the DM group. C) Appearance of almost strong immunreactivity of GPx1 in the DM + AC group. D) Appearance of almost strong immunreactivity of GPx1 in the DM+BE group.
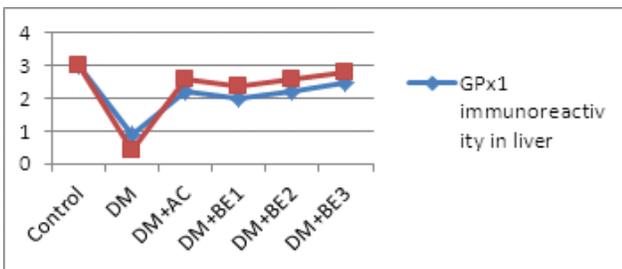

Figure 6: Distribution scores of GPx1 and insulin immunoreactivity in liver and pancreatic tissue sections according to groups are summarized.

group. Additionally, adhesions were observed in bowman capsule of some glomerulus (Figure 2-C). The findings, which were formed as a result of DM, were observed to be significantly reduced in the kidney of rats in BE1 and BE2 groups, and slight degeneration and necrosis were observed in the tubulus epithelial cells as well. However, tubulus and glomerulus of the kidney of rats in BE3 group were found to be in an approximate status as control group in general (Figure 2-D).

The islets of Langerhans were observed to be large and regular as well as their limits were identified well in the control group. The islet cells of Langerhans were found to be in a normal appearance (Figure 3-A). The most significant findings in the group with DM were degenerative and necrotic changes in the islet cells of Langerhans. As a result, significant cell loss was observed in the islets as well as the cellular order was disrupted, the islets were atrophied and the structure was deteriorated. Hydropic degen-

eration and degranulation were observed in the cytoplasm of degenerative cells, and nucleus and cytoplasm were observed to be stained with a dark color (Figure 3-B). The islet of Langerhans were observed to be preserved partially in rats in DM + Acarbose treatment group whereas degenerative and necrotic changes were formed in several cells (Figure 3-C). A significant recovery was observed in the islet of Langerhans of rats in BE1 and BE2 treatment groups thanks to BE extract, and the islets of Langerhans was found to be preserved against the toxic effects of STZ. The islet of Langerhans was found to have same characteristics with control group in BE3 group (Figure 3-D). Distribution of histopathological findings in liver, kidney and pancreas according to groups has been summarized in Table-1.

Immunohistochemical findings

Strong insulin immunopositive reaction were observed in pancreatic tissue sections and the islets of Langerhans of the control group rats (Figure 4-A). The insulin-positive areas in pancreases of rats in DM group were observed to be considerably reduced compared to the control group (Figure 4-B). Acarbose treatment group had more insulin-positive area than the DM group (Figure 4-C). BE1 treatment group showed significant increase compared to DM group whereas the appearance of insulin immunopositive areas in pancreatic sections of BE2 and BE3 treatment groups was almost similar with the control group. This condition was recorded as the most significant result of this study (Figure 4-D).

GPx1 immunoreactivity was observed in the liver tissue sections received from all groups. Control group had the most intense staining. GPx1 immunoreactivity was observed to be formed in the same density in the centrilobular, midzonal and periasiner regions of the liver. GPx1 immunoreactivity was found to be only cytoplasmic in some of hepatocytes, and observed to be more intense both cytoplasmic and nuclear. GPx1 immunoreactivity was not observed in vena sentralis, endothelium and connective tissue of vessels located in portal area whereas mild immunoreaction was detected in bile duct epithelial cells. No staining was observed in Kupffer cells and sinusoidal vessel endothelium (Figure 5-A). In the DM group, GPx1 immunoreactivity was detected less in both the nucleus and cytoplasm of the cells compared to the control group. (Figure 5-B). Staining density was observed higher in DM + Acarbose treatment group rats than DM group whereas it was observed

to be lower than control group (Figure 5-C). BE1 and BE2 treatment groups had higher staining density than DM group, GPx1 immunoreactivity in the BE3 group was similar to that of the control group (Figure 5-D). The difference between groups of GPx1 immunoreactivity was found statistically significant (p<0.05).

DISCUSSION

The prevalence of diabetes for all age roups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030.[1]

Diabetes mellitus is describe as metabolic syndrome that is also associated with overproduction of reactive oxygen species (ROS).[27] Free radical formation has been reported to be a direct consequence of hyperglycemia.[28] Expression of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase, and antioxidant capacity of the liver, kidney, skeletal muscle and adipose tissue is known to be in the lowest level in pancreatic islet cells compared to other tissues such as the liver, kidney, skeletal muscle and adipose tissue.[29] Increased free radicals may lead to degradation of cellular functions and oxidative damage to membranes by consuming antioxidant defense components, and may also increase the susceptibility to lipid peroxidation.[30] As a result, prolonged oxidative stress and changes in antioxidant capacity may be related to the occurrence of long-term complications of diabetes.[31]

Plants with antioxidant activity and some of the active ingredients obtained from these plants are thought to be useful in the treatment of diabetes since they provide protection against oxidative stress caused by hyperglycemia and have positive effects on glucose metabolism.[32] In particular, phenolic compounds have important effects in the treatment and prevention of diseases.[33] Studies have revealed that these compounds have a sweeping effect on free radicals and have a strong antioxidant role.[34,35] Antioxidants have been observed to have significant effects on oxidative stress, protein glycation and glucose metabolism, which are most likely to be impaired in diabetes.[36] The liver has been reported to be a central metabolic organ and is exposed to reactive oxygen species due to oxidative damage resulting from diabetes.[37] In STZ-induced diabetes studies, necrosis in hepatocytes, inflammatory cell infiltration,[38] lipidosis and impairment in portal intervals are reported

histopathological findings.[39,40] In this study, similar findings were observed in the DM group rats. These results were observed to be significantly reduced in BE1, BE2 and Acarbose treatment groups; however, the liver of BE3 group rats was found to have a similar appearance with the liver of control group rats. These results indicated that *Bryonia multiflora* may greatly ameliorated hepatocyte degeneration, necrosis and infiltration of inflammatory cells associated with development of diabetes.

Thickening glomerulus basal membranes, degeneration and necrosis of tubuli, glomerular epithelial cell (podocyte) loss,[4] interstitial cell infiltration, tubular dilatation and atrophy of diabetic kidneys are among the reported histopathological findings.[41] In this study, degeneration and necrosis of the tubular epithelial cells in the DM group and deterioration of the glomerulus structures were at the forefront. The findings of the treatment groups were found to be decreased significantly; however, the similar appearance of the kidneys of rats in BE3 treatment group with the control group was reported as a significant result.

Decrease of GPx levels, which is among antioxidant enzymes, is thought to be occurred due to inactivation of antioxidant enzymes as a result of glycation caused by hyperglycemia in diabetes.[42] GPx depletion may have a major role in the pathogenesis of vascular complications and microalbuminuria with increased oxidative stress.[43] In a study by Yoshimura *et al*.[44] GPx was found to be localized diffusely in the cytoplasm of hepatocytes in the liver of rats. Additionally, periportal area had more intense staining than the centriobular area, and no staining was reported in endothelial cells around the sinusoids and in Kupffer cells. Asayama *et al*.[45] observed staining in cytoplasm, nucleus, mitochondria and other cell organelles in a study on the intracellular distribution of cellular GPx in rat hepatocytes. Asayama *et al*.[46] reported that GPx1 was stained immunohistochemically at similar concentrations in centrilobular and periportal areas of hepatocytes in the liver of fetal and neonatal rats. In this study, GPx1 immunoreactivity was found to be cytoplasmic in some hepatocytes, and cytoplasmic and nuclear in common. Staining in hepatocytes was similar in centrilobular, midzonal and periportal areas. GPx1 immunoreactivity was not observed in the endothelial cells and connective tissue of the vessels in the Kupffer cells, central venous and portal vein similar to other studies.[47] In addition to this, poor immunoreactivity was observed in some bile duct epithelial cells. In this

present study, all groups had similar GPx1 immunolocalization in their livers, and GPx1 immunoreactivity levels of DM group was found to be in a few cells and weaker compared to the control group in accordance with previous studies.[47,48] Deprem *et al.*[49] reported that capsaicin treatment decreased the GPx1 immunoreactivity in the liver of diabetic rats whereas Taşçı and Gülmez reported that melatonin treatment increased the GPx1 immunoreactivity in the liver. [48] In the present study, we found that GPx1 immunoreactivity in the liver of rats treated with *Bryonia multiflora* increased significantly compared to rats in the DM group.

In experimental studies, toxicity of STZ-induced pancreatic β-cells was investigated, and these cells were thought to be damaged due to production of reactive oxygen species.[49] In this study, the morphology of the islets of Langerhans was observed to be deteriorated and become atrophic in the pancreases of DM group rats, and endocrine cells showed significant cytoplasmic vacuolization and picnotic nucleus formation as a result of STZ application. These histological findings were found to be consistent with the conducted studies.[50,51] The findings was observed to be significantly decreased as a result of STZ application and BE treatment, and the islets of Langerhans of BE3 treatment group were found to be in a similar histological appearance with the control group.

In recent studies, it has been suggested that regeneration of β-cells may be possible after they are damaged severely due to STZ therapy.[52,53] Studies of STZ-induced diabetes have shown that the number of immunoreactive cells was decreased against the insulin antibodies in the pancreatic islets of rats. Pancreatic islets were found to be protected morphologically thanks to the treatment with plant extracts, and the immune response was reported to be enhanced for the insulin antibodies.[54,55] In this present study, DM group rats had a very weak insulin immunopositive reaction in the pancreatic islets compared to the control group. In acarbose and *Bryonia multiflora* extract groups, insulin antibody immunopositive areas were found to be increased significantly. Similar immunopositive areas were observed to be formed in BE3 treatment group as the control group. These results show that BE extracts at a dose of 100, 200 and 400 mg/kg BW/day can induce significant amelioration in the functioning of beta-cells. These effects may be a consequence of the protection of β-cells from oxidative stress.

CONCLUSION

In conclusion, *Bryonia multiflora* extract has been shown to prevent damage, which is formed in liver, kidney and pancreas due to possible antioxidant activity, in the experimental diabetes model developed with STZ in accordance with the results of this study. Therefore, *Bryonia multiflora* extract seems to be useful in the treatment of diabetic related complications.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

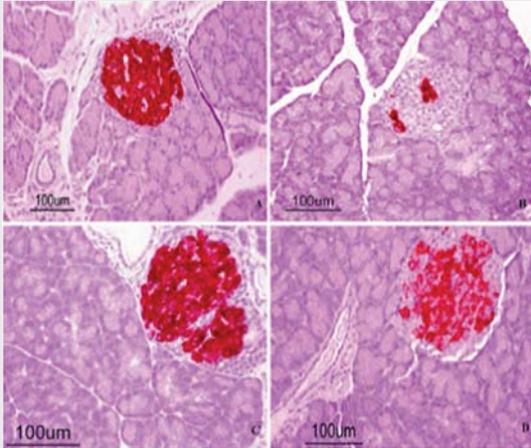The authors declare no conflict of interest.

ABBREVIATIONS

DM: Diabetes mellitus; **GPx:** Glutathione peroxidase; **STZ:** Streptozotocin; **SOD:** Superoxide dismutase; **CAT:** Catalase.

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PICTORIAL ABSTRACT

SUMMARY

- STZ-induced degenerative changes in beta-cells in the pancreas of the diabetic rats has reduced insulin immunoreactivity.
- On the other hand, insulin immunoreactivity in the β cells of pancreas of BE –treated diabetic rats has significantly increased.
- Additionally, Glutathione peroxidase 1 (GPx1) immunoreactivity was lower in the tissues of diabetic rats (DM group) compared to the other groups.
- In conclusion, BE extract has a protective effect on tissue damage probably due to its antioxidant activity and possess the ability to regenerate β-cell in STZ-induced diabetic rats.

ABOUT AUTHORS

Dr. Ahmet UYAR: Working as an assistant professor in the Department of Pathology, Faculty of Veterinary Medicine, Yuzuncu Yil University, Van, TURKEY. He has research experience in the field of Toxicopathology.

Dr. Turan YAMAN: Working as an assistant professor in the Department of Pathology, Faculty of Veterinary Medicine, Yuzuncu Yil University, Van, TURKEY. He has research experience in the field of Toxicopathology.