

Histopathological and Immunohistochemical Study of Antidiabetic Effects of *Heracleum persicum* Extract In Experimentally Diabetic Rats

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ABSTRACT

Objective: This study aims to investigate the antioxidant properties and protective effects of *Heracleum persicum* (HP) extract in streptozotocin (STZ)-induced diabetic rats.

Material and Methods: Forty-two Wistar albino male rats were divided into six groups including Control (C); Diabetes mellitus (DM); DM + Akarboz 20 mg/kg; DM + 100 mg/kg HP extract (HP1); DM + 200 mg/kg HP extract (HP2) and DM + 400 mg/kg HP extract (HP3). Experimental diabetes was established by a single-dose [45 mg/kg, intra-peritoneal (i.p)] STZ injection. Essential dosages of HP extracts and Akarboz were applied with gastric gavage for 21 day. **Results:** In histopathological evaluation of the stained liver and kidney sections of diabetic rats showed degeneration and necrosis of hepatocytes, inflammatory cell infiltration and hydropic degeneration and necrosis in tubulus epithelial cells, disorder of glomerular structure and lymphocyte infiltration. These histopathological changes were ameliorated in the HP-treated rats depending on the dose level. Glutathione peroxidase 1 (GPx-1) immunoreactivity was detected in hepatocytes of liver and tubule epithelial cells of kidney. We have shown that treatment with extracts of HP modulates GPx-1 expression in HP-treated rats. STZ-induced degenerative changes in beta-cells caused decreases in the number of functioning beta-cells and insulin immunoreactivity in the pancreas of the diabetic rats. The pancreas of HP-treated rats were improved and the number of immunoreactive β cells were significantly increased. **Conclusion:** Our data suggests that the STZ-induced immunohistochemical and histopathological alterations could be prevented by HP extract probably due to possess the ability to regenerate β -cells.

Key words: *Heracleum persicum*, Histopathology, Immunohistochemistry, Diabetes, Rat.

INTRODUCTION

Diabetes mellitus (DM) is a rapidly growing disease with a high mortality and morbidity risk.¹ DM is a chronic metabolic disorder characterized with hyperglycemia, which is formed as a result of insulin secretion, activation or defect in both.² Chronic hyperglycemia in the course of DM 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

damage⁴. It is emphasized that this condition causes micro- and macrovascular complications of DM.⁵

Oxidative stress is observed to be increased in the liver, due to DM⁶. Since the liver has a central and critical role in the regulation of carbohydrate metabolism,⁷ various disorders affecting glycogen and lipid metabolism are formed in the liver as a result of DM.⁸ Similarly, the oxidative stress is effective in the pathogenesis of diabetic kidney complications.⁹ In addition, damage of β cells of pancreas, which is known to

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be one of the most susceptible 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) is one of the natural antioxidant defense system against free radicals. It protects lipid peroxidation by catalyzing the reduction of hydrogen peroxide (H₂O₂),¹² and it protects cell membranes against oxidative injury.¹³ GPx-1 is an intracellular selenoprotein with high expression levels in erythrocytes, liver, and kidneys.^{14,15}

Heracleum persicum (HP) has been known as “Suh” in Turkey. In consequence of atherosclerosis antioxidant capacities of HP Desf, species of Apiaceae family were assessed by identifying their effects on radical scavenging and lipid peroxidation inhibition as well as their total phenolic contents.^{16,17} Pimpinellin, bergapten, isopimpinellin, sphondin and isobergapten are furanocoumarins which are reported from roots of this plant. HP fruits are a rich source of furanocoumarins which might act as an immunostimulatory agent.¹⁸ HP has stimulated both humoral as well as cellular arms of the immune system. Major constituents of HP essential oil were reported as Hexyl butyrate (% 56,5), octyl acetate (%16,5), hexyl 2-methylbutanoate (%5,2) and hexyl isobutyrate (%3,4).¹⁹

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

MATERIALS AND METHODS

Plant material extract

Plant samples were collected from Turkey (Hizan/Bitlis) in August 2015. The identity of the plant materials was confirmed by Fevzi Ozgokce, PhD, at the Biological Sciences Department, Science Faculty, Yuzuncu Yil University, Turkey, and a voucher specimen stored at the university's herbarium (*Heracleum persicum* DESF. herbarium No:165061). 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 previously²⁰ with some modifications.

Animals

Forty-two Wistar albino male rats 3–4 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 wheat-soybean-meal-based diet. Water and feed were offered ad libitum. The ethical 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 (02.01.2014/01).

Induction of diabetes

The animals were fasted for 12 h prior to the induction of diabetes. Streptozotocin (STZ) freshly prepared in citrate buffer (0.1 M, pH 4.5) was administered intraperitoneally (i.p.) at a single 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 and used in this study.

Experimental model

The rats were randomly divided into six groups each containing seven (n = 7) rats.

Normal control group (NC): NC rats received citrate buffer (pH 4.5) (1 ml/kg, i.p.).

Diabetes mellitus group (DM): DM rats received STZ in a single dose (45 mg/kg, i.p.).

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

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

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

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

Histopathological examination

At the end of experiments, the rats were anesthetized by injection of ketamine (5 mg/100 g body weight) i.p. Tissue samples were taken from the testicles and fixed in Bouin's 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). Histopathological findings were graded in three ways; slight (*), medium (**), and severe (***)

Immunohistochemistry (IHC) staining

Immunohistochemistry was performed to investigate GPx1 and insulin expressions. The streptavidin-peroxidase method (ABC) was used to stain the sections. Commercial antibodies were visualized on 5- μ m-thick paraffin sections using an indirect streptavidin/biotin immunoperoxidase kit (Histostain Plus Bulk Kit, Zymed, South San Francisco, CA, USA). Tissue sections were incubated with the GPx1 (abcam-ab22604) (1:400) and insulin (abcam ab-181547) (1:1000) primary antibodies overnight at 4° C. Finally, to visualize the reactions, the sections were reacted for 5-15 min with diaminobenzidine (DAB) for GPx1 and insulin staining.

RESULTS

Histological findings

Liver

The normal histological view of the liver was observed in the control group (Figure 1-A). Extensive hydropic degeneration in hepatocytes and necrosis as a single component were observed in liver sections of DM group rats. Large or small, irregularly-edged, partially rounded vacuoles were observed in the cytoplasm of degenerated hepatocytes. Mild fibrosis, bile duct proliferation, 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 (Figure 1-B). These findings were found to be significantly decreased in the liver of rats in DM + Acarbose treatment group as well as degeneration and necrosis was observed locally in hepatocytes (Figure 1-C). Also, findings were found to be significantly reduced in HP1 and HP2 and HP3 (400 mg/kg) group rats treated with HP extract (Figure 1-D).

Kidney

The normal histological view of the kidney was observed in the control group (Figure 2-A). In DM group rats kidney, hydropic degeneration and necrosis were observed significantly in tubulus epithelial cells. Focal inflammatory cells were seen in the intertubular areas. Bowman capsule of glomerulus were observed to be enlarged whereas some parts of it was found to be narrowed. Glomeruli, which were narrowed in bowman capsule, were observed to create adhesion with the basal membrane (Figure 2-B). Mild hydropic degeneration and necrosis were observed in some parts of the tubular epithelial cells in the kidney of rats in DM + Acarbose treatment group. Additionally, adhesions were observed in bowman capsule of some glomerulus (Figure 2-C). The findings were detected to be significantly reduced

in the kidney of rats in HP1 (100 mg/kg), HP2 (200 mg/kg) and HP3 (400 mg/kg) group rats (Figure 2-D).

Pancreas

The islets of Langerhans were observed to be large and regular as well as their limits were identified well. The islet cells of Langerhans were found to be in a normal histologic appearance (Figure 3-A). The most important changes in the group with DM were degenerative and necrotic changes in the islet cells of Langerhans. 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 degeneration 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). Degenerative and necrotic changes were formed in only several cells in DM + Acarbose treatment group (Figure 3-C). A significant improvement was observed in the islet of Langerhans of rats in HP1 (100 mg/kg), HP2 (200 mg/kg) and HP3 (400 mg/kg) treatment group rats (Figure 3-D).

Imunohistochemical findings

Liver

Control group had the most intense staining. GPx-1 immunoreactivity was observed to be formed in the same density in the centrilobular, midzonal and perisiner regions of the liver. GPx-1 immunoreactivity was found to be only cytoplasmic in some of hepatocytes, and observed to be more intense both cytoplasmic and nuclear. No staining was observed in Kupffer cells and sinusoidal vessel endothelium (Figure 4-A). In the DM group, GPx-1 immunoreactivity was detected less in both the nucleus and cytoplasm of the cells compared to the control group (Figure 4-B). Immunoreactivity was observed more intense in DM + Acarbose treatment group rats than DM group whereas it was observed to be lower than control group (Figure 4-C). HP1 (100 mg / kg), HP2 (200 mg / kg) and HP3 (400 mg / kg) treatment groups had higher staining density than DM group (Figure 4-D).

GPx-1 immunoreactivity was observed in the kidney tissue sections which were received from all groups. Control group had the most intense staining. GPx-1 showed immunoreactivity in distal and proximal tubular epithelial cells, particularly in collecting tubules. No immunoreactive reaction was observed in the glomerulus and bowman capsules. GPx-1 immunoreactivity was found to be more intense both cytoplasmically and nuclearly; however, it was found less only cytoplasmically (Figure 5-A). The GPx-1 immunoreactivity was

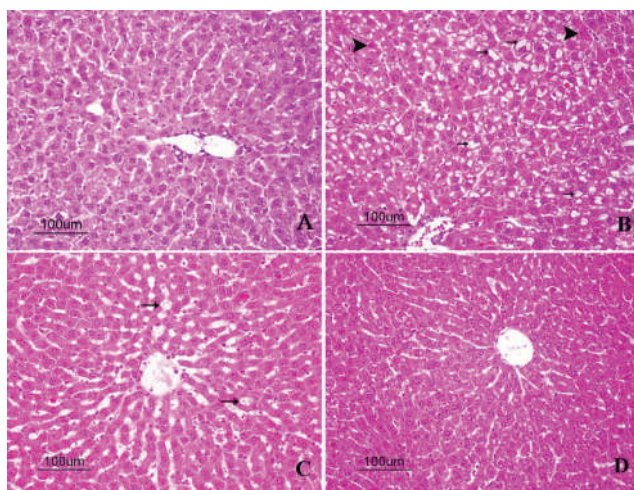


Figure 1 (A-D): Liver sections stained with hematoxylin and eosin. (A): Normal histological view of liver in control group rats. (B): Histologic appearance from the liver tissue of a rat belonged to diabetic group showing vacuolar hydropic degeneration (Arrow) and coagulative necrosis in hepatocytes (arrow heads). (C): Liver from an animal treated with acarbose showing dissociation in remarc cordons (arrow). (D): Liver from an animal treated with 400 mg/kg (DM+HP3 group) showing almost normal morphology.

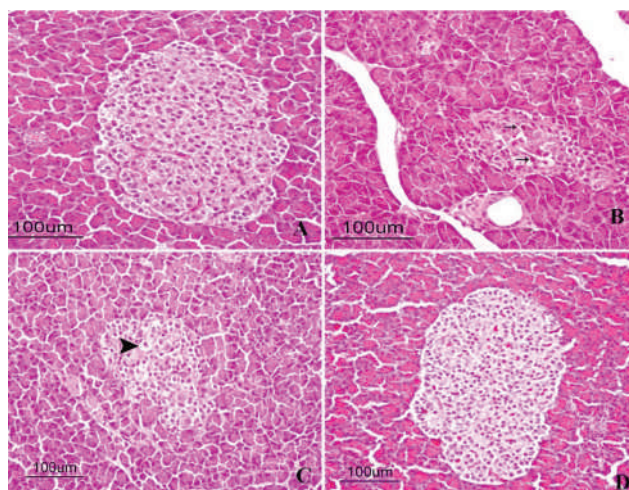


Figure 3 (A-D): Pancreas sections stained with hematoxylin and eosin (A): Normal histological view of pancreas in control group rats. (B): Pancreas section of a rat from DM group showing a shrunken and distorted islet of Langerhans displaying cells with vacuolated cytoplasm and large darkly stained nuclei (arrows). (C): Section of pancreas from acarbose treated group rats showing shrunken and distorted islets of Langerhans (arrows) contained cells with degenerative and necrotic changes (Arrowhead). (D): Pancreas section of a rat from HP3 group (400 mg/kg) showing normal islet of langerhans with numerous β -cells.

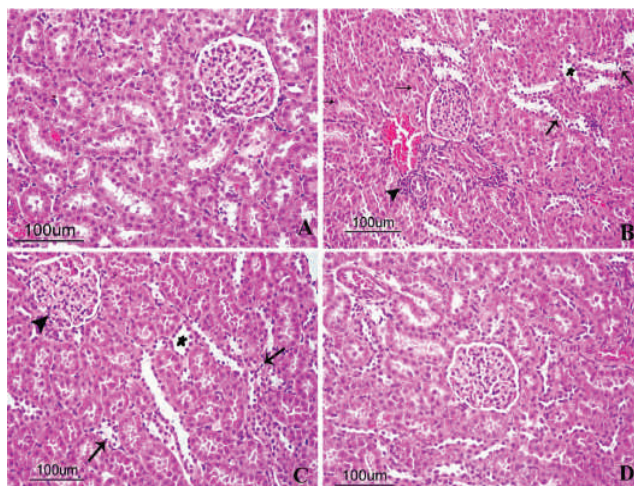


Figure 2 (A-D): Kidney sections stained with hematoxylin and eosin. (A): Normal histological view of kidney in control group rats. (B): Section of kindey from the DM group rats showing necrosis (thin arrows) and vacouler dejeneration (thick arrows) in tubular epithelium cells, inflammatory cell infiltration (arrowheads) and dilatation of tubulus (star). (C): Kidney section of a rats from DM+acarbose group showing hydropic dejeneration in tubular epithelium cells (arrows), dilatation of tubulus (star); and deterioration of the glomerular structur. (D): Kidney section of a rats from HP3 group (400 mg/kg) showing nearly histological view of kidney in control group.

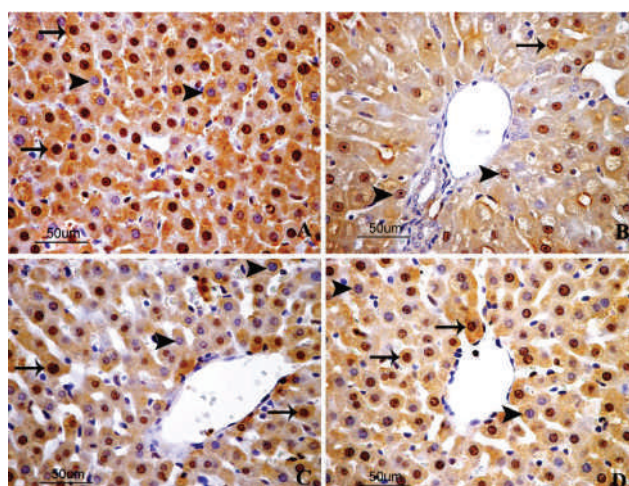


Figure 4 (A-D): Immunolocalization of GPx1 in liver tissue of rats. (A): Cytoplasmic and nuclear expression of GPx1 in liver section of control group rats. The localization of GPx1 was found in the hepatocytes in periacinar and midzonal areas. (B): Liver section of from DM group showing weak nuclear and cytoplasmic staining of GPx1 in comparison with control group. (C): Liver section of rat from DM + acarbose group showing intense cytoplasmic and nuclear GPx1 immunoreactivity in comparison with DM group. (D): Liver section of rat from HP3 group (400 mg/kg) showing strong cytoplasmic and nuclear GPx1 immunoreactivity in hepatocytes in comparison with DM group (D). Arrowhead = cytoplasmic GPx1 immunoreactivity, arrow = cytoplasmic and nuclear GPx1 immunoreactivity.

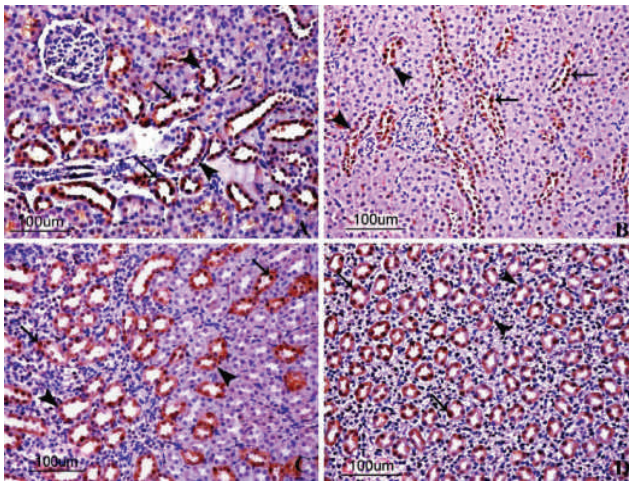


Figure 5 (A-D): Immunolocalization of GPx1 in kidney tissue of rats. (A) Cytoplasmic and nuclear expression of GPx1 in kidney section of control group rats. The localization of GPx1 was found predominantly in distal and proximal convoluted tubules and collecting ducts. (B) Kidney section of diabetic group showing slight cytoplasmic and nuclear expression of GPx1 in distal and proximal tubules. (C) Kidney section of DM + acarbose group rats showing intense cytoplasmic and nuclear GPx1 immunoreactivity in the distal and proximal tubules. (D) Kidney section of diabetic rats treated with 400 mg/kg HP extract showing strong cytoplasmic and nuclear GPx1 immunoreactivity in collecting ducts. (D). Arrowhead ; cytoplasmic GPx1 immunoreactivity, arrow ; cytoplasmic and nuclear GPx1 immunoreactivity.

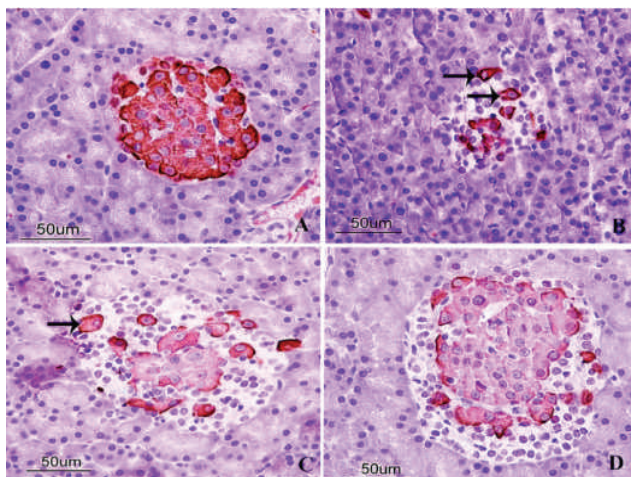


Figure 6 (A-D): Immunohistochemical staining of insulin in pancreatic islets of rats. (A) Pancreas section of rats from control group showing strong immunoreactivity of insulin in beta-cells, which occupy most of the islet. (B) Pancreas section of a rats from DM group showing evident decline in the immunohistochemical expression of insulin in beta-cells (arrows). (C) In the pancreas of DM+acarbose group rats, the apparent marked increase in number and area of beta-cells is evident in comparison with DM group (arrow). (D) Pancreas section of rats from HP3 group (400 mg/kg) showing an evident increase in insulin expressing beta-cells with normal density, compared with DM group.

found to be weaker and fewer in the DM group (Figure 5-B). However, GPx-1 immunoreactivity in the kidney sections of DM + Acarbose and HP treatment groups was less than in the control group, and it was found to be stronger in more cells compared to DM group (Figure 5-C,D).

Intense insulin immunopositive reaction areas were observed in pancreatic tissue sections and the islets of Langerhans of the control group rats (Figure 6-A). The insulin-positive areas in pancreases of rats in DM group were observed to be considerably reduced compared to the control group (Figure 6-B). Acarbose treatment group had more insulin-positive area than the DM group (Figure 6-C). HP1 (100 mg / kg) treatment group showed significant increase compared to DM group whereas the appearance of insulin immunopositive areas in pancreatic sections of HP2 (200 mg / kg) and HP3 (400 mg / kg) treatment groups was almost similar with the control group. This condition was recorded as the most significant result of this study (Figure 6-D).

DISCUSSION

Diabetes mellitus is one of the oxidative stress conditions in which free radicals are increased and/or antioxidant mechanisms are inhibited.⁵ Free radicals induce Oxidative stress and can lead to injury of cellular membrane.^{21,22} Free radical formation has been reported to be a direct consequence of hyperglycemia.²³ Expression of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase are known to be in the lowest level in pancreatic islet cells compared to other tissues such as the liver, kidney and adipose tissue.²⁴ Increased free radicals may lead to degradation of cellular functions and oxidative damage to membranes by consuming antioxidant defense components.²⁵ As a result, prolonged oxidative stress and changes in antioxidant capacity may be related to the occurrence of long-term complications of DM.²⁶ Plants with antioxidant activity and some of the active ingredients obtained from these plants are thought to be useful in the treatment of DM since they provide protection against oxidative stress caused by hyperglycemia and have positive effects on glucose metabolism.²⁷ Studies have revealed that these compounds have a sweeping effect on free radicals and have a strong antioxidant role.^{28,29}

The liver has been reported to be a central metabolic organ and is exposed to reactive oxygen species due to oxidative damage resulting from DM.³⁰ In STZ-induced diabetes studies, necrosis in hepatocytes, inflammatory cell infiltration, lipodosis,³¹ dilation in sinuses³² and impairment in portal intervals³³ are reported histopathological

findings. In this study, similar findings were observed in the DM group rats. These results were observed to be significantly reduced in HP1, HP2 and acarbose treatment groups; however, the liver of HP3 group rats was found to have a similar appearance with the liver of control group rats. These results indicated that HP extract may greatly ameliorated hepatocyte degeneration, necrosis and infiltration of inflammatory cells associated with development of DM.

Thickening in diabetic kidneys and glomerulus basal membranes, mesangial enlargement, hypertrophy, glomerular epithelial cell (podocyte) loss⁴ interstitial cell infiltration, tubular dilatation and atrophy are among the reported histopathological findings.³⁴ 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 HP3 treatment group with the control group was reported as a significant result.

Reduction 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 DM³⁵. GPx depletion may have a major role in the pathogenesis of vascular complications and microalbuminuria with increased oxidative stress.³⁶

In a study by Yoshimura *et al.*³⁷ 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 centrilobular area, and no staining was reported in endothelial cells around the sinusoids and in Kupffer cells. Asayama *et al.*,³⁸ 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.*,³⁹ reported that GPx-1 was stained immunohistochemically at similar concentrations in centrilobular and periportal areas of hepatocytes in the liver of fetal and neonatal rats. In this study, GPx-1 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. GPx-1 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.⁴⁰ In addition to this, poor immunoreactivity was observed in some bile duct epithelial cells.

In this present study, all groups had similar GPx-1 immunolocalization in their livers, and GPx-1 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.^{40, 41} Deprem *et al.*⁴² reported that capsaicin treatment decreased the GPx-1 immunoreactivity in the liver of diabetic rats whereas Taşçı and Gülmez⁴¹ reported that melatonin treatment increased the GPx-1 immunoreactivity in the liver. In the present study, we found that GPx-1 immunoreactivity in the liver of rats treated with HP extract increased significantly compared to rats in the DM group.

In studies, GPx-1 immunoreactivity has been reported to be observed in glomerulus, cytoplasm of proximal and distal tubulus epithelia, transitional epithelium of kidney and smooth muscle cells of kidney arteries.⁴³ GPx had a wide intracellular distribution with its localization in mitochondria, peroxisome, nucleus and cytoplasm in the kidney cells.⁴³ In this study, cytoplasmic and nuclear staining were observed in some of the collecting duct epithelial cells, proximal and distal tubulus epithelia of the GPx-1 immunoreactivity whereas only cytoplasmic staining was observed in less cells. However, no staining was observed in glomerulus and bowman capsule. GPx-1 immunoreactivity was observed to be higher in control group, and less in DM group. HP1 and HP2 treatment groups had more intense staining than DM group whereas HP3 treatment group had an approximate GPx-1 immunoreactivity level with the control 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.⁴⁴ 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.^{45,46} The changes was observed to be significantly decreased as a result of HP extract treatment.

In recent studies, it has been suggested that regeneration of β -cells may be possible after they are damaged severely due to STZ therapy.^{47,48} 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.^{49,50} Our results show that HP 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.

In another study evaluating the biochemical results of this study,⁵¹ HP extract was reported to have biochemical anti-diabetic, anti-hyperglycemic and anti-oxidant effects on blood and tissues, and it has protective effects for tissues against the oxidative damage occurred in DM in terms of blood serum enzyme levels, antioxidant enzymes in tissues and lipid peroxidation (MDA) values.⁵¹ These biochemical results were consistent with the histopathological and immunohistochemical findings of our study.

CONCLUSION

In conclusion, HP 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, HP extract seems to be useful in the treatment of diabetic related complications.

ABBREVIATION USED

The authors thank to Dr. Suleyman Mesut PINAR who confirmed plant materials.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

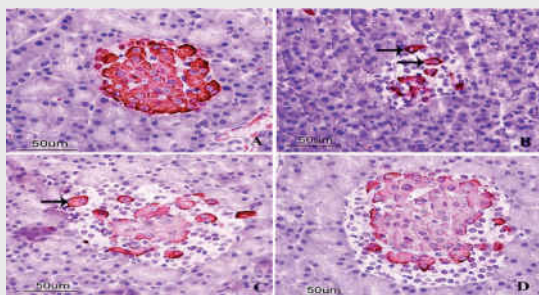
DM: Diabetes mellitus; **HP:** *Heracleum persicum*; **GPx:** Glutathione peroxidase; **STZ:** Streptozotocin.

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PICTORIAL ABSTRACT



SUMMARY

- STZ-induced degenerative changes in beta-cells caused decreases in the number of functioning beta-cells and insulin immunoreactivity in the pancreas of the diabetic rats.
- The pancreas of HP-treated rats were improved and the number of immunoreactive β cells were significantly increased.
- Our data suggests that the STZ-induced immunohistochemical and histopathological alterations could be prevented by HP extract probably due to possess the ability to regenerate β -cells.

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