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Effect of *Gundelia tournefortii* extract on diabetic gastropathy: involvement of inflammation, apoptosis, oxidative stress, and histopathology

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Abstract

In this study, the effect of *Gundelia tournefortii* (GT) extract against diabetic gastropathy was investigated by pathological methods. The animal groups were designed as the control, diabetes, diabetes + GT50, diabetes + GT100, and diabetes + GT200 groups. No treatment was applied to the control group. The other groups received 45.00 mg kg^{-1} streptozotocin intraperitoneally on the experimental day. The treatment groups were also given 50.00, 100, and 200 mg kg^{-1} of GT extract daily by gavage for 21 days. Tissues were stained with Hematoxylin and Eosin for histopathological examination. Immunohistochemical staining was performed to reveal the presence of inflammation (tumor necrosis factor alpha), apoptosis (cysteine aspartate specific proteases-3), and oxidative stress (heat shock protein-27). Histopathological examination revealed no pathological lesion in the control group. In the diabetes group, mucosal tissue damage, and vascular and inflammatory changes were observed. In the treatment groups, GT decreased histopathological findings in parallel with the dose increase. Immunohistochemical examination revealed no immunopositivity in the control group, while severe immunopositivity was observed in the diabetes groups in terms of inflammation, apoptosis, and oxidative stress. In the treatment groups, there was a decrease in the severity of immunopositivity's depending on the dose increase. As a result of this study, which has not been done before, GT was found to have a protective effect against gastropathy, being an important complication of diabetes, and this study is thus an important reference point for future research and promises new hope for the patients.

Introduction

Diabetes mellitus is a metabolic disease characterized by hyperglycemia caused by impaired insulin production, resistance to insulin, or both. This systemic disease with multi-factorial etiology has various complications, such as retinopathy, nephropathy, neuropathy, cardiopathy, myopathy, angiopathy, enteropathy, and gastropathy.[1,2] Diabetic gastropathy is a term used for gastric discomfort due to the complex pathogenesis of diabetes. In diabetic gastropathy, which can occur acutely due to the hyperglycemia, a series of neuromuscular dysfunctions, including abnormalities in gastric tone, contractility and myoactivity can be observed. Gastroparesis may develop in chronic conditions. Altered gastric emptying time and microangiopathy due to the diabetic neuropathy are effective in mucosal destruction.[3,4] Angiopathy, being effective in the pathogenesis of diabetes complications, causes disruption of microcirculation and increase in hypoxia and reactive oxygen species. The increase in free radicals leads to inhibition of cell proliferation and induction of inflammation through the expression of some pro-inflammatory cytokines. The mucosal damage occurring in this sequence of events is difficult to heal and may even turn into hemorrhagic ulceration due to the phylogistic effect of diabetes.[5-7]

Gundelia tournefortii (GT), a tropical plant, is a member of the Astraceae (Compositae) family. It grows as a natural plant flora mostly in the West Asia region, and is consumed by the people of the region believing that it has many benefits. Studies have shown that GT has hepato-protective, hypoglycemic, hypolipemic, anti-diabetic,

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anti-cancer, anti-inflammatory, anti-bacterial, anti-oxidant, anti-septic, anti-parasitic, anti-platelet, and anti-emetic effects. It has been reported to have therapeutic potential especially in digestive system disorders.[8-11]

Tumor necrosis factor alpha (TNF-α), acting as a cell signaling protein in the immune response from induction to termination of inflammation, is widely used as a biomarker of inflammation. Cysteine aspartate specific proteases-3 (CASP-3), a member of the caspase family, is used as an apoptosis biomarker as it has a key role in the execution of cell apoptosis by activation of caspases. Heat shock protein-27 (HSP-27), being encoded by the *HSPB1* gene, is involved in chaperone activity, thermotolerance, apoptosis, inflammatory response, and cell proliferation and differentiation, and is also used as an important oxidative stress biomarker.[12-14]

In this study, the possible effect of GT extract against gastropathy, one of the complications of diabetes, was investigated using pathological methods. For this purpose, changes related to the tissue damage and inflammatory reaction in the stomach were observed histopathologically. Immunohistochemical examination was performed to observe the expressions of inflammation, apoptosis, and oxidative stress biomarkers in the tissue.

Materials and Methods

Animals and groups. In the study, 30 adult male *Wistar* albino rats weighing 240 ± 20.00 g were obtained from Harran University Experimental Medicine Research and Application Center, Şanlıurfa, Türkiye. All rats were housed in a well-ventilated room under standard housing conditions, including constant temperature (25.00 ± 2.00 ˚C), 50.00 ± 10.0 % humidity, and 12 hr light/12 hr dark cycle. All experimental animals were received a standard laboratory balanced commercial diet and drinking water *ad libitum*. The use of animals and the study protocol were ethically approved by the Harran University Animal Experiments Local Ethics Committee, Şanlıurfa, Türkiye (Approval No. 2023/008/10). Thirty male rats were randomly assigned into five equal groups of six rats each. The animal groups were designated as control, diabetes, diabetes + GT50, diabetes + GT100, and diabetes + GT200. The control group was not exposed to any treatment. The other groups (diabetes, diabetes + GT50, diabetes + GT100, and diabetes + GT200) were administered 45.00 mg kg^{-1} streptozotocin (Sigma-Aldrich; St. Louis, USA) intraperitoneally on the experimental day. The *ad libitum* access to water containing 10.00 % sugar was provided for 48 hr to prevent hyperglycemia. Animals in the diabetes group were given 0.25 mL of sterile water by gavage daily for 21 days. In the diabetes + GT50, diabetes + GT100, and diabetes + GT200 groups, 50.00, 100, and 200 mg kg^{-1} of GT extract were administered daily by gavage for 21 days, respectively. At the end of the experiment, the animals were euthanized humanly using an over-dose of ketamine (100 mg kg^{-1}; Alfasan, Woerden, The Netherlands) and xylazine (10.00 mg kg^{-1}; Alfasan), and gastric tissues were removed under appropriate storage conditions.[15,16]

Histopathological examination. Stomach samples from rats were fixed in 10.00% buffered formaldehyde. Formaldehyde was removed in running tap water and tissues were subjected to routine tissue follow-up. Then, 4.00-µm-thick sections were taken from each paraffin blocked tissue using a rotary microtome (RM 2125; Leica, Wetzlar, Germany). The sections were kept in the oven for 1 hr and stained with Hematoxylin and Eosin after deparaffinization-rehydration procedures. The tissue sections were covered with a coverslip dripped with Entellan™ (Sigma-Aldrich), and examined under a light microscope (BX 53; Olympus, Tokyo, Japan). Histopathological lesions including erosive-ulcerative lesions (EUL), degenerative-necrotic changes (DNC), inflammatory cell infiltrates (ICI), and hyperemia were scored as absent (-), mild (+), moderate (++), and severe (+++) according to the severity.[17,18]

Immunohistochemical examination. The tissues were placed on adhesive slides and kept in an oven for 1 hr followed by deparaffinization-rehydration procedures. For endogenous enzyme inactivation, tissues were kept in 3.00% H_2O_2 for 10 min. Washing was performed with phosphate-buffered saline (PBS). To reveal the presence of antigen, the sections were boiled and cooled in the retrieval solution three times in a microwave oven. Washing was done with PBS. Tissues were incubated in protein block (Thermo Fisher Scientific, Waltham, USA) for 20 min to prevent non-specific binding. Then, TNF-α (No.: sc-1348; Santa Cruz Biotechnology, Dallas, USA), CASP-3 (No.: sc-56053; Santa Cruz), and HSP-27 (No.: sc-1048; Santa Cruz) primary antibodies were diluted 1/100 and incubated overnight at 4.00 ˚C. Washing was performed with PBS. Biotinized secondary antibody (Thermo Scientific) compatible with the primary antibody was added to the tissues and incubated for 20 min. Washing was performed with PBS. For avidin-biotin complex, streptoavidin-peroxidase (Thermo Fisher Scientific) was added to the tissues and incubated for 20 min. Washing was performed with PBS. To demonstrate antibody binding, 3,3′-diaminobenzidine (DAB; Thermo Fisher Scientific) chromogen was added to the tissues. Washing was performed with distilled water. The tissues were counterstained with Mayer's Hematoxylin (Sigma-Aldrich), dried in alcohol, cleared in xylol, covered with a coverslip with Entellan™, and examined under a light microscope. Immunopositivity was scored as absent (-), mild (+), moderate (++), and severe (+++) according to the severity.[17,19]

Statistical analysis. The score values of histopathological and innunohistochemical findings were analyzed statistically. The SPSS Software (version 21.0; IBM Corp., Armonk, USA) was used for this purpose.

Parametric One-Way ANOVA test was used to analyze the data obtained. Multiple comparison between groups was performed with *post-hoc* Tukey test. The $p < 0.05$ was considered significant, and results were expressed as mean ± standard deviation.

Results

Histopathological findings. Microscopic examination of the control group did not reveal any pathological findings and normal histological appearance of the stomach was noted. In the histopathological examination of the diabetes group, EUL characterized by mucosal tissues loss and severe degeneration and necrosis around them were observed. Disruption of parietal cell arrangement and desquamation of necrotic cells into the lumen were also seen. Inflammatory cell infiltration was found in the sub-mucosa and mucosa. In addition, edema and hyperemia were obvious in the sub-mucosa in the inflammation area. In the diabetes + GT50 group, tissue loss was mostly in the form of erosion, and ulcers were rarely observed. Degeneration, necrosis, inflammatory cell infiltration, and hyperemia in epithelial cells decreased compared to the diabetes group. Pathological lesions in the diabetes + GT100 group decreased compared to the diabetes + GT50 group. In the diabetes + GT200 group, there were fewer pathological lesions, and the tissue was close to the normal appearance (Fig. 1). Pathological lesions (EUL, DNC, ICI, and hyperemia) increased in the diabetic group compared to the control group ($p < 0.0001$ and $p < 0.05$). When the diabetes + GT200 group was compared with the diabetic group, pathological lesions regressed to a level close to normal (Table 1).

Immunohistochemical findings. Immunohistochemical examination monitored TNF-α (inflammation biomarker), CASP-3 (apoptosis biomarker), and HSP-27 (oxidative stress biomarker) expressions. No immunopositivity was found in the immunohistochemical examinations of the control group. In the diabetes group, TNF-α, CASP-3, and HSP-27 expressions in the mucosal tissue were severe. In the diabetes + GT50 group, there were fewer immunopositivity's than diabetes group. There were also fewer immunopositivity's in the diabetes + GT100 group than diabetes + GT50 group. In the diabetes + GT200 group, the immunopositivity's were almost the same as the control group and were very rare (Fig. 2). There was an increase in immunopositivity (TNF-α, CASP-3, and HSP-27) in the diabetes group compared to the control group ($p < 0.0001$ and $p < 0.05$). When the diabetes + GT200 group was compared with the diabetes group, immunopositivity's decreased to a level close to normal (Table 1).

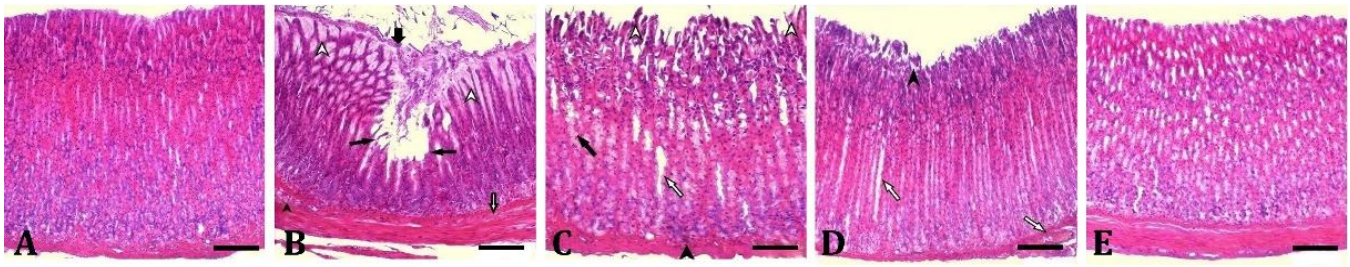

Fig. 1. Histopathological findings of the study groups (Hematoxylin and Eosin staining; Bars = 200 µm). **A)** Control group with normal histological appearance; **B)** Diabetes group exhibiting degenerative-necrotic changes (hollow arrowheads), desquamation (thick arrow), erosive-ulcerative lesions (thin arrows), sub-mucosal edema (hollow arrow), and inflammatory cell infiltration (arrowhead); **C)** Diabetes + GT50 group showing necrosis and desquamation of mucosal epithelium (hollow arrowheads), degeneration of epithelial cells (arrow), inter-cellular edema in mucosa (hollow arrow), and inflammatory cell infiltration in sub-mucosa (arrowhead); **D)** Diabetes + GT100 group having degenerative and necrotic changes in mucosal epithelium (arrowhead) and edema in mucosa and sub-mucosa (hollow arrows); **E)** Diabetes + GT200 group with histological appearance close to the control group. GT: *Gundelia tournefortii.*

Table 1. Statistical analysis of the histopathological and immunohistochemical findings.

Groups	Histopathological findings				Immunopositivity		
	EUL	DNC	ICI	H	TNF-α	CASP-3	HSP-27
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Diabetes	2.00 ± 0.89[a]	2.50 ± 0.55[ab]	2.50 ± 0.55[a]	2.17 ± 0.75[ab]	2.67 ± 0.52[a]	1.83 ± 0.75[a]	2.33 ± 0.82[a]
Diabetes+GT50	1.67 ± 0.82[a]	2.00 ± 0.63[a]	1.83 ± 0.75[a]	1.67 ± 0.82[a]	2.17 ± 0.75[ad]	1.67 ± 0.82[a]	1.83 ± 0.75[a]
Diabetes+GT100	1.33 ± 1.03	1.67 ± 0.82[a]	1.33 ± 1.21	1.17 ± 0.75	1.67 ± 0.82[a]	1.33 ± 1.03[c]	1.50 ± 0.55[a]
Diabetes+GT200	0.83 ± 0.75	1.00 ± 0.63	1.00 ± 0.89	0.83 ± 0.75	1.17 ± 0.75[c]	0.67 ± 0.82	0.83 ± 0.75

GT: *Gundelia tournefortii*; EUL: Erosive-ulcerative lesions; DNC: Degenerative-necrotic changes; ICI: Inflammatory cell infiltrates; H: Hyperemia; TNF-α: Tumor necrosis factor alpha; CASP-3: Cysteine aspartate specific proteases-3; HSP-27: heat shock protein-27.
[a] $p < 0.001$ compared to the control; [b] $p < 0.05$ compared to the Diabetes + GT200; [c] $p < 0.05$ compared to the control; [d] $p < 0.01$ compared to the Diabetes + GT200.

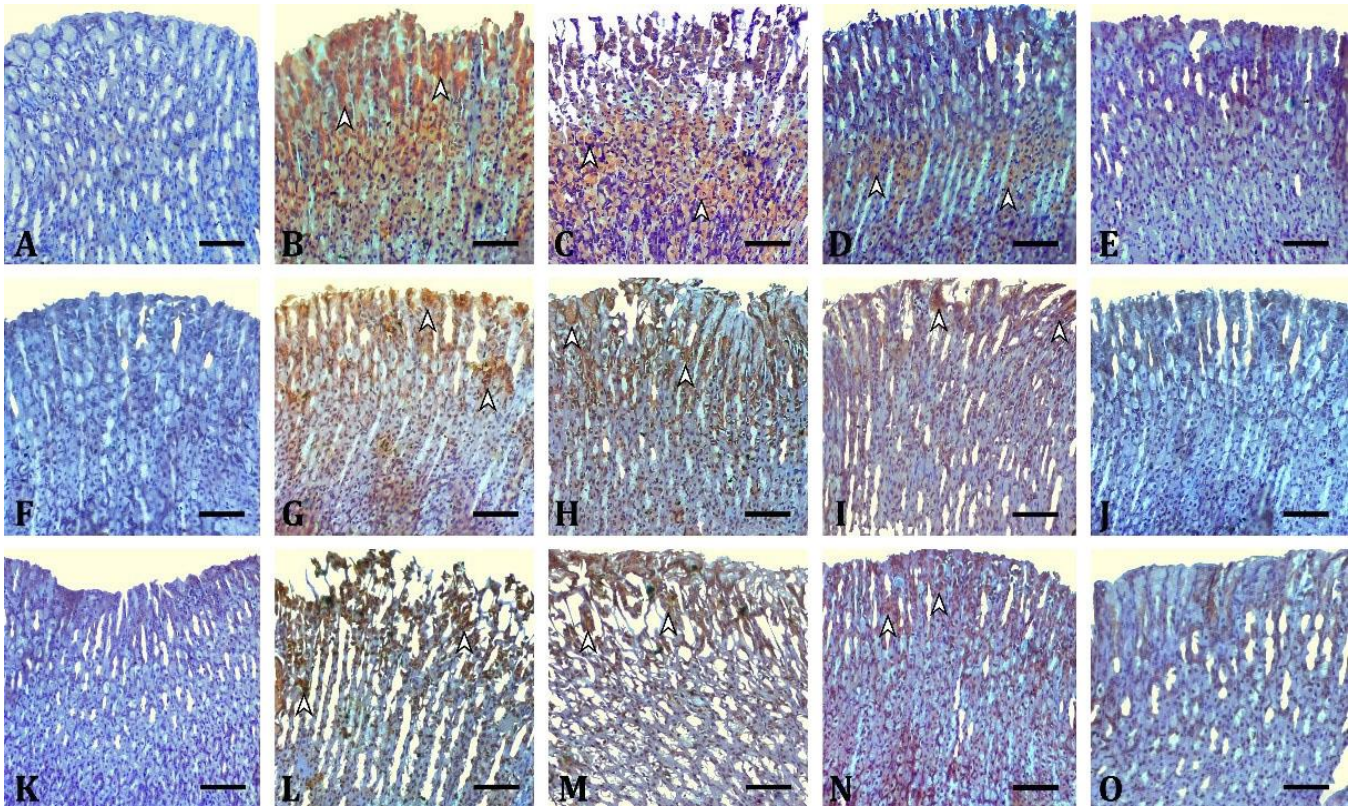

Fig. 2. Immunohistochemical findings of the study groups (Immunohistochemistry staining; Bars = 100 μm). **A)** Control group being negative for tumor necrosis factor alpha (TNF-α) expression; **B)** Diabetes group having severe TNF-α expression (hollow arrowheads); **C)** Diabetes + GT50 group with moderate TNF-α expression (hollow arrowheads); **D)** Diabetes + GT100 group with mild TNF-α expression (hollow arrowheads); **E)** Diabetes + GT200 group having very low TNF-α expression; **F)** Control group being negative for cysteine aspartate specific proteases-3 (CASP-3) expression; **G)** Diabetes group having severe CASP-3 expression (hollow arrowheads); **H)** Diabetes + GT50 group with moderate CASP-3 expression (hollow arrowheads); **I)** Diabetes + GT100 group with mild CASP-3 expression (hollow arrowheads); **J)** Diabetes + GT200 group having very little CASP-3 expression; **K)** Control group being negative for heat shock protein-27 (HSP-27) expression; **L)** Diabetes group having severe HSP-27 expression (hollow arrowheads); **M)** Diabetes + GT50 group with moderate HSP-27 expression (hollow arrowheads); **N)** Diabetes + GT100 group with mild HSP-27 expression (hollow arrowheads); **O)** Diabetes + GT200 group having very little HSP-27 expression, Immunohistochemistry. GT: *Gundelia tournefortii.*

Discussion

Diabetes has become a major focus of medical science as an important health problem that has affected humanity from the past to the present day. Complications of diabetes, which have at least as serious consequences as diabetes itself, have been the subject of several studies. One of the important complications of diabetes is gastropathy. Unfortunately, studies on this problem, negatively affecting the standard of living of many patients, are insufficient.[1,4] Diabetic gastropathy, being viewed as a diabetes-related stomach disorder, causes early satiety, indigestion, nausea, vomiting, abdominal pain, and epigastric discomfort in affected patients. The prevalence of gastric symptoms related to the delayed gastric emptying in patients with diabetes has been reported to be 30.00 - 50.00% and can be as high as 70.00%.[20,21] Although diabetic gastropathy has a multi-factorial etiopathogenesis, diabetes-induced neuropathy and microangiopathy are among the primary causes. Gastric discomfort is caused by dysfunction of capsaicin-sensitive neurons playing a role in gastric movements and protection of the gastric mucosa due to the neuropathy. Angiopathy causes hypoxia and subsequent cell damage (degeneration and necrosis) due to the insufficient microcirculation. Hypoxia alters the mitochondrial redox state and causes oxidative stress with an increase in reactive oxygen species. High oxidant elements, such as hydroxyl radicals and superoxide ions cause damage to the gastric mucosa. Hyperglycemia also increases oxidative stress by disrupting the anti-oxidative mechanisms.[6,22,23] Cell injury due to the oxidative stress leads to pro-inflammatory cytokine expression and induction of apoptosis. The TNF-α in particular has been reported to induce an inflammatory reaction leading to the gastric damage. The TNF-α-related apoptosis-inducing ligand induces apoptosis by binding to the receptors. In addition, superoxide production in mitochondria induces apoptosis through caspases *via* mitogen-activated protein

kinase pathways.[5,24-26] In the light of this information, oxidative stress, inflammation, and apoptosis, being involved in the pathogenesis of diabetic gastropathy, were evaluated in this study.

Studies on the importance of gastropathy in diabetes complications are not sufficient. In most of the studies, gastric ulcer was induced by various applications in experimental diabetes.[27-29] In this study, a natural and more realistic approach was taken and gastric tissue with experimental diabetes was examined without induction of gastric ulcer. There are few similar studies. Turkyilmaz *et al.*, have reported an increase in oxidized protein products in diabetic rat stomachs.[30] Yilmaz-Ozden *et al.*, found that oxidative stress parameters increased in diabetic rat stomachs.[31] There are very few studies in which histopathological examination was performed without induction of gastric ulcer. Vador *et al.*, have reported severe mucosal destruction, inflammatory cell infiltrations, and vascular changes with various severities in diabetic rat stomachs.[25] Owu *et al.*, found disorganization, mucosal lesions, necrosis, and parietal cells disarrangement in the stomach of diabetic rats.[32] In this study, lesions related to the mucosal damage, inflammatory cell infiltration, and hyperemia were observed in the diabetes group in accordance with the data from the literature and previous studies attributing these to the pathogenesis of diabetic gastropathy. The severity of the lesions decreased with the dose of GT extract.

Studies have been conducted to prevent or treat the development of complications being at least as important as diabetes.[33-35] In various studies on diabetic gastropathy, several agents were found to have a protective effect, including *Cuminum cyminum* extract, hesperidin, telmisartan, quercetin, coenzyme Q10, zinc, vanadium, and vitamin C.[25,27-32] In this study, it was observed that diabetes-induced gastric mucosal damage decreased with the use of GT extract.

In addition to histopathological examination, immunopathological examination was performed in terms of inflammation, apoptosis, and oxidative stress, considering the pathogenesis of diabetes complications. The TNF-α was used of for inflammation, CASP-3 for apoptosis, and HSP-27 for oxidative stress biomarkers. Elshazly *et al.*, have used TNF-α for inflammation and inducible nitric oxide synthase (iNOS) for oxidative stress in their diabetic gastropathy study and reported that the use of hesperidin decreases TNF-α and iNOS values in diabetic stomach.[27] Fouad *et al.*, used TNF-α for inflammation and CASP-3 for apoptosis in diabetic gastropathy studies and reported that telmisartan reduced TNF-α and CASP-3 levels in diabetic stomachs.[28] Khaleel *et al.*, used TNF-α for inflammation, iNOS for oxidative stress, and Bax and p53 for apoptosis and reported that quercetin and coenzyme Q10 administrations decreased TNF-α, iNOS, Bax, and p53 levels in diabetic gastropathy.[29] In the literature, no diabetic gastropathy study was found reporting the expression of inflammation, apoptosis, and oxidative stress biomarkers in tissue using the immunohistochemical method. In addition, no diabetic gastropathy study was found using the HSP-27 as an oxidative stress biomarker. In this study, it was found that the expression of inflammation, apoptosis, and oxidative stress biomarkers increased in diabetic stomachs and these expressions decreased with the use of GT extract.

There are very few studies investigating the protective effect of GT extract against diabetes complications. Demircioğlu and Dörtbudak have reported that GT extract exerts a protective effect against diabetes-induced spleen tissue damage[16], while Mohammadi *et al.*, have reported a protective effect against diabetic nephropathy.[36] However, no study was found investigating the possible effect of GT extract against diabetic gastropathy. In this study, the possible effect of GT extract against diabetic gastropathy was investigated through considering its anti-inflammatory, anti-oxidant, and anti-diabetic effects, and its protective activity was determined.

It was observed histopathologically that lesions and inflammatory reaction occurred in diabetic gastric mucosa. In addition, TNF-α, CASP-3, and HSP-27, being the biomarkers of inflammation, apoptosis, and oxidative stress, were observed to have a strong expression in diabetic gastric tissue. However, it was determined that these findings decreased with the application of GT extract. In this study, the protective effect of GT extract against diabetic gastropathy, which had not previously been established, was revealed.

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Conflict of interest

The authors declare no competing interests.

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