

Therapeutic potential of *Juglans regia* L. leaf extract against diabetic retinopathy in rat

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ARTICLE INFO	ABSTRACT
<p>Article type: Short communication</p> <hr/> <p>Article history: Received: Nov 21, 2016 Accepted: Aug 10, 2017</p> <hr/> <p>Keywords: Antioxidant Apoptosis Diabetic retinopathy Hyperglycemia Inflammation <i>Juglans regia</i> L. leaf</p>	<p>Objective(s): Oxidative stress has a pivotal role in the pathogenesis of diabetic retinopathy (DR). <i>Juglans regia</i> L. (JRL) leaf extract has hypoglycemic and antioxidative properties. This study aimed to determine the ameliorative effects of JRL against diabetic retinopathy.</p> <p>Materials and Methods: The DR rat model was generated by injection of streptozotocin (STZ). A subset of the diabetic rats received JRL or metformin after the onset of hyperglycemia. Histopathology and immunohistochemistry of apoptotic and inflammatory factors were assessed along with biochemical assessments of lipid peroxidation and antioxidant status.</p> <p>Results: Lipid peroxidation level and catalase activity significantly improved after JRL consumption ($P < 0.001$). Degeneration of the retina attenuated after JRL consumption. Attenuation of the caspase-3, COX-2, PARP, and S100B expression could be detected significantly ($P < 0.001$) in the JRL-treated rats. While, blood glucose level decreased after JRL consumption ($P < 0.001$).</p> <p>Conclusion: JRL leaf extract exert protective effects against diabetic retinopathy.</p>
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Introduction

Diabetic retinopathy is one of the most common long term complications of diabetes mellitus which is leading to blindness in working-age adults (1). In this regard, several mechanisms account for the development of diabetic retinopathy such as microvascular damages, inflammation, metabolic disorders, haemodynamic changes, and oxidative stress (2-4). Among these factors, oxidative stress has a pivotal role in the development of diabetic retinopathy. Increased oxidative stress after hyperglycemia is caused mainly due to autooxidative glycosylation, advanced glycation end products (AGEs) formation, and increasing polyol pathway activity (5). Therefore, it has been postulated that the use of antioxidant supplements may offer some protection against this complication through free radical scavenging actions. Within the previous decades, a rapidly growing number of natural phenolic compounds, secondary metabolites of plants, with antioxidant effects have been described. The seeds, green husks, and leaves of the Persian or common walnut (*Juglans regia* L.), the best-known member of the Juglans genus (6, 7), are a rich source of these molecules.

They have been traditionally used in Iranian folk medicine for treatment of several diseases such as infections, inflammations, and diabetes and its complications. Flavonoids, phenolic acids, and naphthoquinones are considered as major phenolic compounds in *J. regia* leaves (8-10). There is accumulating evidence that attributed the beneficial effects of *J. regia* leaf extract to a variety of biological activities, including anti-oxidative (10, 11), anti-inflammatory (12), anti-carcinogenic (13), anti-microbial (14), and anti-fungal properties (15). Recently, there are a few experimental studies on the hypoglycemic effect of *J. regia* leaf extract in diabetes mellitus. These studies documented that administration of *J. regia* leaf extract significantly reduced fast blood sugar (FBS) and glycated hemoglobin (HbA1c) compared to control groups (16-19). Moreover, results of two clinical trial studies have shown that FBS and HbA1c significantly decreased after consumption of 100 mg *J. regia* leaf extract for 3 months (20) and 200 mg *J. regia* leaf extract for 2 months (21) compared to placebo groups. An *in vitro* study also reported that walnut leaf extract inhibits protein tyrosine phosphatase 1B (PTP1B) and enhances glucose-uptake (22).

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Due to the potent antioxidant and hypoglycemic properties of *J. regia* leaf extract, we investigated for the first time the protective effects of the extract against diabetic retinopathy as a common serious complication of diabetes.

Materials and Methods

Extract preparation and GC-MS

Fresh leaves of *J. regia* were collected during July-August 2014 from cultivated trees in Khorramabad (Lorestan Province, Western Iran) and authenticated by Natural Resources Research Center of Lorestan Province. Briefly, the leaves were dried and pulverized and then stored in dark at room temperature. Methanol was added to the pulverized leaves for 72 hr and then filtered through filter paper. The obtained extracts were concentrated at 40 °C. Gas chromatography-mass spectrometry (GC-MS) was carried out using a Hewlett-Packard 6859 with a quadrupole detector, on a HP-5 column, operating at 70 eV ionization energy, using the same temperature program and carrier gas as above. Retention indices were measured by retention times of n-alkanes that were injected after the extract (23).

Animals

Male adult Sprague-Dawley rats were used (250-275 g) (Laboratory Animal Research Center, Sari, Iran). They were kept in the laboratory under constant conditions of temperature (23±2 °C) and light/dark cycle (12 hr/12 hr) for at least one week before and through the experimental work. All procedures were done according to the guidelines of the university's animal care codes (IR.MAZUMS.REC.95.S171) to minimize the animal's suffering and were fed a standard rat chow and drinking water *ad libitum* throughout the study period.

Induction of diabetes and experimental design

Diabetes was induced by a 55-mg/kg single dose of streptozotocin (Santa Cruz Biotechnology) diluted in 0.1 M citrate buffer with pH-4.5. Blood samples were collected from tail vein 48 hr after streptozotocin (STZ) administration and estimated plasma glucose levels using a commercial glucometer and test strips (Accu-Chek® Active test meter). Rats with plasma glucose level more than 250 mg/dl were considered as diabetics and were further considered for study. *J. regia* leaf extract (200 mg/kg/day) and metformin (350 mg/kg/day) was administered by oral gavages. The doses and treatment schedules were based on previous studies (16, 18, 24, 25) and pilot experiments in our laboratory.

Animal groups

The animals were randomly allocated to five groups, each containing 7 rats: (I) Control group, which received citrate buffer intraperitoneally and isotonic saline orally for the duration of the study; (II) Control+ *J. regia* leaf extract (JRL) group, which received JRL orally (200 mg/kg/day) for a period of two months; (III) Diabetic group, which received single injection of

STZ (55 mg/kg) intraperitoneally and were also given isotonic saline orally for the duration of the study; (IV) Diabetic+JRL group, which received JRL (200 mg/kg/day) starting one week after STZ injection for a period of two months; (V) Diabetic+Metformin group (positive control group), which received Metformin (350 mg/kg/day) starting one week after STZ injection for a period of two months. Blood glucose levels were measured weekly until the end of the experiment.

Biochemistry

The retina samples (the left side retina) were thoroughly cleaned of blood and then were immediately frozen and stored in a -80 °C freezer for assays of tissue malondialdehyde (MDA) levels (26) and catalase (CAT) activities (27). The absorbance of the supernatant was measured by spectrophotometry. MDA levels and CAT activities were expressed as micromoles per milligram of protein and as nanomoles trolox equivalent per milligram of protein, respectively.

Histopathology

The retina samples (the right side retina) were obtained and then were immediately fixed in 10% buffered formaldehyde and embedded in paraffin. Five-micrometer serial sections were prepared from the paraffin-embedded blocks using microtome. For histopathological assessment, some tissue sections were deparaffinized with xylene, stained with hematoxylin and eosin (H&E), and studied by light microscopy. Morphometric computer-assisted image analysis of the retina was done using Image_J software (MacBiophotonics ImageJ 1.41a) using methods previously described (28). All the histological assessments were performed in a blinded fashion.

Immunohistochemistry

For immunohistochemistry, some sections were incubated with anti-caspase 3 (1:100, Abcam), anti-COX 2 (1:100, Abcam), anti-S100B (1:1000, Abcam) and anti-PARP (1:100, Abcam) overnight at 4 °C. Then followed by incubation with secondary antibody conjugated with horseradish peroxidase (Abcam) and detected by DAB for 10 min. Afterwards, they were dehydrated and mounted. Quantitative analysis was assessed by densitometry using Image_J software (five immunohistochemical photographs from each sample). Data are expressed as a percentage of total tissue surface area.

Statistical analysis

Statistical analysis was carried out in SPSS (Version 15, Chicago, IL, USA). Results were presented as mean values (±SD). The K-S test was used in order to evaluate the normality of the data. Also, the Tukey's multiple comparison tests and the analysis of the variance were used to compare each two groups and data among the groups, respectively. A value of $P < 0.05$ was considered significant.

Table 1. Effect of the *J. regia* (JRL) leaf extract on fasting blood sugar (FBS) level

Experimental Groups	FBS (mg/dl) 0d	FBS (mg/dl) 7d	FBS (mg/dl) 30d	FBS (mg/dl) 60d
Control	86.00±8.57	98.60±9.12	94.60±16.43	97.20±15.07
Control+JRL	92.00±11.11	90.40±9.96	94.80±12.09	93.80±11.65
Diabetic	86.40±8.08	408.00±99.88*	442.60±62.03*	430.00±70.81*
Diabetic+JRL	85.20±8.70	373.80±45.35*	271.40±67.01**	227.80±65.59**
Diabetic+Metformin	89.20±6.70	359.40±49.36*	268.40±47.09**	237.70±21.56**

Data are represented in Mean±SD. * $P<0.001$ versus Control and Control+JRL groups; ** $P<0.001$ versus Diabetic group. Time (d) after STZ induction

Results

GC-MS analysis

The principal components identified in *J. regia* leaf extract were 2-β-pinene (17.09%), α-pinene (13.29%), trans-caryophyllene (10.58%), and germacrene D (8.90%). Other minor identified constituents were dl-limonene (3.85%), terpine-4-ol (3.70%), β-selinene (3.25%), and methyl salicylate (3.07%).

Blood glucose levels

Fasting blood sugar (FBS) levels for all groups are shown in Table 1. Administration of STZ in the diabetic group produced a significant elevation ($P<0.001$) in FBS level and the hyperglycemia was maintained throughout experimental period compared to the control and control+ JRL groups. At the end of the experiment, the FBS levels in the diabetic+JRL group were significantly lower than that in the diabetic group ($P<0.001$), While the differences between diabetic+JRL and diabetic+ metformin group were not significant ($P>0.05$).

Biochemical analysis

MDA levels for all groups at the end of the experiment are shown in Table 2. Administration of STZ in the diabetic group produced a significant elevation ($P<0.001$) in lipid peroxidation level compared to Control and Control+JRL groups. The MDA levels in the Diabetic+JRL group were significantly lower than that those in the Diabetic group ($P<0.001$), While the differences between diabetic+JRL and diabetic+ Metformin group were significant ($P<0.01$).

Catalase (CAT) activity levels for all groups at the end of the experiment are shown in Table 2. Administration

Table 2. Effect of the *J. regia* (JRL) leaf extract on biochemical markers of rat retina affected by STZ-induced diabetic retinopathy

Experimental Groups	MDA μmol/mg-protein	CAT unit/mg-protein
Control	99.31±0.44	8.66±0.2
Control+JRL	94.02±0.02	7.41±0.25
Diabetic	147.30±0.84*	2.36±0.50##
Diabetic+JRL	94.29±4.90**	8.11±2.11***
Diabetic+Metformin	113.60±3.79**,#	8.87±0.53***

Data are represented in Mean±SD. * $P<0.001$ versus Control and Control+JRL groups; ** $P<0.001$ versus Diabetic group; ## $P<0.01$ versus Diabetic+JRL group; # $P<0.01$ versus Control and Control+JRL groups; *** $P<0.01$ versus Diabetic group

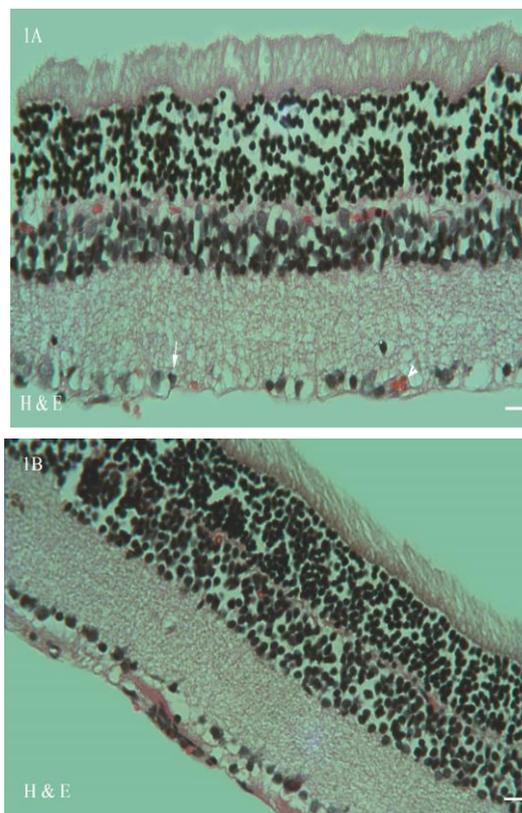


Figure 1. Photomicrographs of the retina in diabetic group (1A) revealed histopathological changes such as ganglion cell layer shrinkage (arrow) and new vessel formation (arrowhead). Treatment with JRL and metformin ameliorated the dramatic histological alterations (1B) (stained with hematoxylin and eosin; original magnification: ×400, bar: 100μm)

of STZ in the diabetic group produced a significant ($P<0.01$) decrease in CAT activity compared to the control and control+JRL groups. The CAT activities in the diabetic+JRL group were significantly ($P<0.01$) higher than that in the diabetic group, While the differences between diabetic+JRL and diabetic+metformin group were not significant ($P>0.05$).

Histopathologic changes

Histological examination of the retina in the diabetic animals (Figure 1A) revealed histopathological changes including, ganglion cell layer shrinkage, new vessel formation in the junction between ganglion cell layer and inner plexiform layer. Furthermore, the retinas of diabetic rats somewhat disorganized and thinner than

Table 3. Effect of the *J. regia* (JRL) leaf extract on morphometric analysis of the diabetic retina

Experimental Groups	Retinal thickness (µm)	Number of cells in GCL/100µm
Control	790±64.97	6.2±1.20
Control+JRL	806±51.68	6.15±1.40
Diabetic	584.1±29.18*	3.13±1.30*
Diabetic+JRL	736.5±56.63**	5.66±1.23**
Diabetic+Metformin	686.5±46.53**,#	4.13±2.45**,#

Data are represented in mean±SD. *P<0.001 versus control and control+JRL groups; **P<0.001 versus diabetic group; #P<0.001 versus diabetic+JRL group

those of normal rats. Treatment with *J. regia* leaf extract (Figure 1B) and Metformin ameliorated the dramatic histological alternations but did not reach the normal structural pattern. No detectable injury was shown in control and control+JRL groups. Computer-aided morphometric analyses of the retina in the experimental groups are shown in Table 3.

Immunohistochemical assessment

Figures 2-5 show the immunohistochemical staining of caspase-3, COX-2, PARP, and S100B, respectively. Administration of STZ in the diabetic group increased the expression of caspase-3 (Figure 2A), COX-2 (Figure 3A), PARP (Figure 4A), and S100B (Figure 5A), while *J. regia* leaf extract treatment in the diabetic+JRL (Figure B) and metformin in the diabetic+ metformin groups reduced the

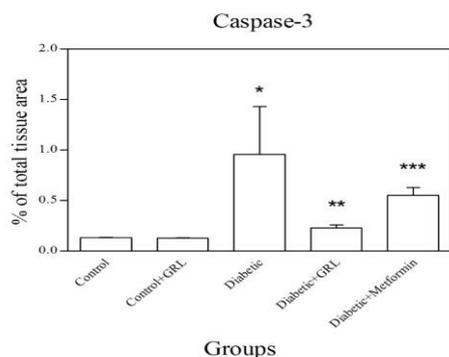
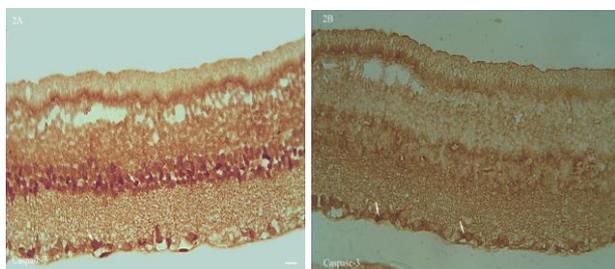


Figure 2. Light photomicrographs show immunohistochemical expression of caspase-3 in diabetic (2A) and diabetic+JRL (2B) group. The positive staining of caspase-3 is presented by a brown color of cytoplasm (arrows) (original magnification: ×400, bar: 100 µm). Densitometry analysis of immunohistochemical photomicrographs for caspase-3 was assessed. Data are expressed as a percentage of total tissue area (2C). *P<0.001 versus control and control+JRL groups; **P<0.001 versus diabetic group; ***P<0.05 versus diabetic group

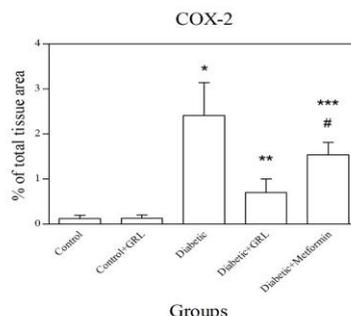
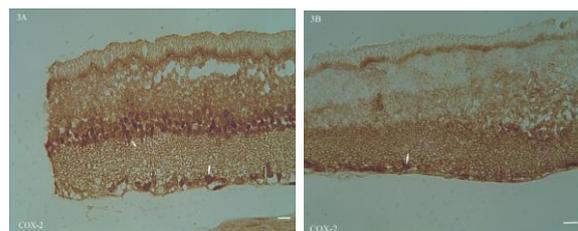


Figure 3. Light Photomicrographs show immunohistochemical expression of COX-2 in Diabetic (3A) and Diabetic + JRL (3B) groups. The positive staining of COX-2 is presented by a brown color of cytoplasm (arrows) (original magnification: ×400, bar: 100µm). Densitometry analysis of immunohistochemical photomicrographs for COX-2 was assessed. Data are expressed as a percentage of total tissue area (3C). *P<0.001 versus Control and Control+JRL groups; **P<0.001 versus Diabetic group; ***P<0.05 versus Diabetic group; #P<0.05 versus Diabetic+JRL group

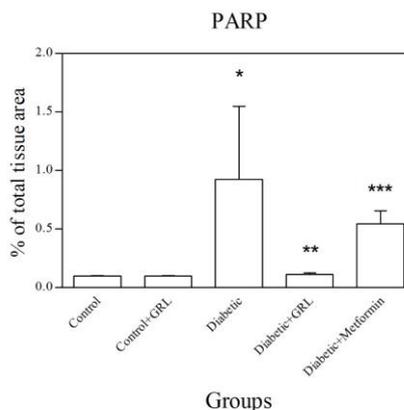
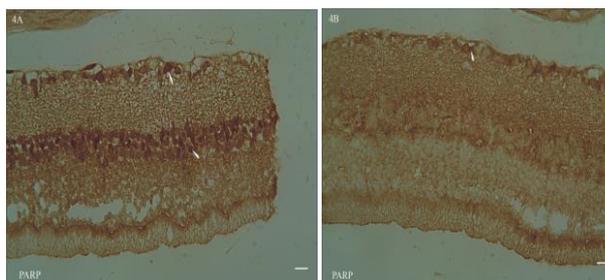


Figure 4. Light photomicrographs show immunohistochemical expression of PARP in diabetic (4A) and diabetic+JRL (4B) groups. The positive staining of PARP is presented by a brown color of cytoplasm (arrows) (original magnification: ×400, bar: 100 µm). Densitometry analysis of immunohistochemical photomicrographs for PARP was assessed. Data are expressed as a percentage of total tissue area (4C). *P<0.01 versus control and control+JRL groups; **P<0.001 versus diabetic group; ***P<0.05 versus diabetic group

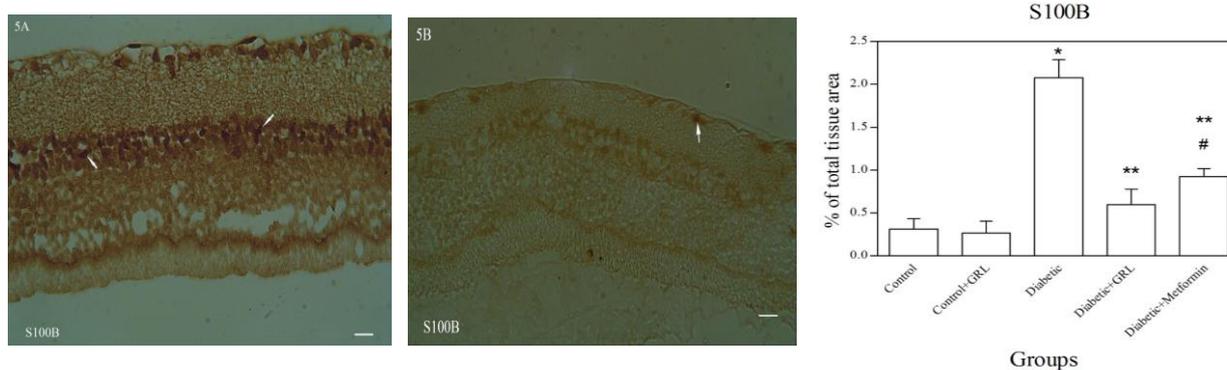


Figure 5. Light photomicrographs show immunohistochemical expression of S100B in diabetic (5A) and diabetic+JRL (5B) groups. The positive staining of S100B is presented by a brown color of cytoplasm (arrows) (original magnification: $\times 400$, bar: $100\mu\text{m}$). Densitometry analysis of immunohistochemical photomicrographs for S100B was assessed. Data are expressed as a percentage of total tissue area (5C). * $P < 0.001$ versus control and control+JRL groups; ** $P < 0.001$ versus diabetic group; # $P < 0.05$ versus diabetic + JRL group

degree of positive staining for caspase-3 (Figure 2B), COX-2 (Figure 3B), PARP (Figure 4B), and S100B (Figure 5B) compared to the diabetic group. The histograms of the quantitative analysis of caspase-3, COX-2, PARP, and S100B positive staining in the experimental groups are shown in Figures 2C, 3C, 4C, and 5C respectively.

Discussion

The main findings of the current study showed that administration of *J. regia* leaf extract attenuates criteria of retinopathy in STZ-induced diabetic rats, in addition to hypoglycemic effects. The hypoglycemic effects of *J. regia* leaf extract in this study, have been previously proven by the experimental (18, 19) and human clinical trial studies (20, 21). In this regard, the activity was attributed to the antioxidant capacity of the polyphenols present in walnut leaves (29), its effects on glucose-uptake due to inhibition of protein tyrosine phosphatase 1B (22), and its effects on beta cells regeneration and its anti-inflammatory properties (30). One of the most common complications of diabetes mellitus is retinopathy. Free radical induced oxidative stress has been implicated to play an important role in the pathogenesis of diabetic retinopathy (31). Meanwhile, the retina is vulnerable to oxidative stress due to polyunsaturated fatty acids high content, high oxygen uptake, and high glucose oxidation (32). In this regard, we observed a significant increase in lipid peroxidation and a significant reduction of catalase activity in the retina of diabetic rats. Treatment with *J. regia* leaf extract ameliorated lipid peroxidation and improved antioxidant status in the retina of diabetic rats. Free radicals reactive oxygen species and nitrogen species have been implicated as a potential contributor to the pathogenesis of diabetic complications (32-34) which seems appropriate for therapeutic interventions such as the use of free radical scavengers. *J. regia* leaves contain a large amount of phenolic compounds, well-known free radicals scavengers. Phenolic acids, naphthoquinones, and flavonoids are the main phenolic compounds in fresh *J. regia* leaves (10, 29, 35, 36). In this regard, a study of antioxidant activity of *J. regia* leaf

extract by the reducing power assay and the scavenging effect on DPPH radicals revealed that walnut leaves cultivars have high antioxidant properties (9). *In vitro* study indicated that flavonoids from *Juglans regia* leaves could reduce the reactive oxygen species level in RAW264.7 cells (10). Carvalho *et al.* documented that *J. regia* leaf extract significantly protected AAPH-induced oxidative hemolysis of human erythrocytes in a time- and concentration-dependent manner (13). In another study, the antioxidant potential of ethanolic extract of *Juglans regia* leaves was measured and the highest ability to chelate Fe^{2+} , high reducing power, high antiradical activity, and relatively low prevention of lipid oxidation documented (37). Results of an *in vivo* study demonstrated that administration of walnut leaf extract increased the antioxidant enzymes superoxide dismutase and catalase against CCl_4 -induced oxidative damage in rat liver (38).

Several lines of evidence obtained in experimental and clinical studies demonstrated that inflammatory-like processes play a critical role in the development of the early and late stages of the retinopathy. In this regard, it was well documented that the enhanced inflammatory response in the early stages of diabetic retinopathy was mediated by NF- κB axis activation; so that selective inhibition of the NF- κB reduces retinal degeneration and expression of proinflammatory proteins such as COX-2, TNF α , PARP, iNOS, and IL-1 β (39-41). On the other hand, microglial activation which is observed in diabetic retinopathy causing the release of inflammatory factors (42). Our immunohistochemical assessments showed that increased COX-2 and PARP expression in diabetic rats significantly attenuated after treatment with *J. regia* leaf extract. Meanwhile, S100B expression, as a marker of glial cell activation, which is upregulated in diabetic retina attenuated after treatment with *J. regia* leaf extract. Hosseinzadeh *et al.* documented that the aqueous and ethanolic extracts of *J. regia* leaves have anti-inflammatory effect against xylene-induced ear swelling in mice, which is mediated by a membrane-stabilizing effect that reduces capillary permeability and/or release of inflammatory mediators

(30). In the other study, it was shown that *J. regia* leaf extract exhibited anti-inflammatory activity against carrageenan-induced hind paw edema model in mice. However, the mechanism underlying this phenomenon is not clear (12).

Apoptosis is a key mechanism of degenerative diseases, which is triggered by some factors such as hyperglycemia toxicity. *In vivo* studies revealed that hyperglycemia affected the cell survival and induced apoptotic changes within the inner layers of the retina including inner plexiform layer and ganglion cell layer secondarily to the caspase-dependent pathway (43, 44). These findings were confirmed after administration of a specific inhibitor of caspase-3 which was significantly reduced the intensity of apoptosis in the diabetic retina (44). Our immunohistochemical results showed that administration of STZ considerably increased the expression of caspase-3, which plays a critical role in apoptosis. Also, our results showed that these up- and down-regulations significantly attenuated after *J. regia* leaf extract consumption. Javidanpour *et al.* documented the proliferative effects of *J. regia* leaf extract on pancreatic β -cells in STZ-induced diabetic rats (16). Results of another study demonstrated that walnut leaf extract has a hepatoprotective effect against carbon tetrachloride-induced cell death (38). On the contrary, some studies demonstrated that walnut leaf extract showed a higher antiproliferative efficiency than green husk and seed extracts against various cancer cell line such as human renal, oral, breast and colon cancer cell lines (13, 45), which is more likely related to its phenolic constituents. Also, it was found that walnut extracts suppressed proliferation and induced apoptosis of cancer cells in a dose- and time-dependent manner by modulating expression of apoptosis-related genes namely Bax, caspase-3, Bcl2, and tp53 (46, 47).

Conclusion

Results of this study suggest that JRL leaf extract exert protective effects against STZ-induced diabetic retinopathy in rats which might be associated with its anti-oxidative, anti-inflammatory, and anti-apoptotic properties, in addition to hypoglycemic effects.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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