

Anti-Aging Effect of Nigella Sativa Fixed Oil on D-Galactose-Induced Aging in Mice

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Key Words

aging, apoptosis, black seed oil, D-galactose, oxidative stress

Abstract

Objectives: Aging is an unconscious and gradual process that can lead to changes in biological systems. Induction of oxidative stress and apoptosis, hepatotoxicity and neurotoxicity are involved in the aging process. Regarding the antioxidant property of black seed oil, the aim of this study was to evaluate the anti-aging effect of *Nigella sativa* (*N. sativa*) oil on d-galactose-induced aging in mice.

Methods: For induction of aging, D-galactose (500 mg/kg, subcutaneously SC) was administrated to male mice for 42 days. Animals were treated with D-galactose alone or with black seed oil (0.1, 0.2, 0.5 mL/kg, intraperitoneally (ip)). Additionally, vitamin E (200 mg/kg) was used as a positive control. At the end of treatment, the malondialdehyde (MDA) and the glutathione (GSH) contents in brain and liver tissues were measured. Also, enzymes in serum, including aspartate aminotransferase (AST) and alanine amino transferase (ALT), were determined. The levels of the proteins Bax, Bcl2, caspase-3 (pro and cleaved) in brain and liver tissues were evaluated.

Results: Administration of D-galactose (500 mg/kg, SC) for 42 days increased serum levels of ALT and AST, as well as the MDA content, in brain and liver tissues, but decreased

the GSH content. Additionally, the levels of apoptotic proteins, including Bax, procaspase-3 and caspase-3 cleaved, were markedly increased. *N. sativa* oil (0.1 and 0.2 mL/kg) diminished the levels of the biochemical markers ALT and AST. Administration of black seed oil (0.1, 0.2 and 0.5 mL/kg) reduced lipid peroxidation and at doses 0.1 and 0.2 mL/kg significantly recovered the GSH content. The oil decreased Bax/Bcl2 levels and at 0.1 mL/kg down-regulated the expressions of caspase-3 (pro and cleaved) proteins in brain and liver tissues.

Conclusion: Through its antioxidant and anti-apoptosis properties, black seed oil exhibited an anti-aging effect in a model of aging induced with D-galactose.

1. Introduction

Aging involves progressive, destructive changes in one or more organs, which over time leads to disease and death. The aging process is associated with immune system impairment, nervous system dysfunction, and apoptosis. In various studies, the induction of apoptosis in aging is accompanied by a reduction in the glutathione (GSH) content and amplifications of oxidative stress in liver and brain tissues [1]. Oxidative stress causes an imbalance between oxidants and the antioxidant defense system in the body [2]; it can also damage cell membranes and may lead to death and/or a worsening of age-related chronic diseases, including cancer and Alzheimer's, Parkinson's and heart disease [3]. Oxidative stress disrupts the normal function of mitochondria, as well [4]. Moreover, apoptosis, which is a form of programmed cell death and plays an important role in various physiological and pathological conditions, is one of the effective factors in the aging

Received: Jan 07, 2017 Reviewed: Jan 18, 2017 Accepted: Feb 23, 2017

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process [5]. D-galactose, which completely metabolized at normal concentrations, is a natural agent in the body. At higher concentrations, it converts to aldose, hydrogen peroxide, and galactose oxidase; the productions of superoxide anions and oxygen reactive radicals increase, leading to disruptions in the activities of macromolecules and cells [6]. Furthermore, in animal studies, a D-galactose overload has been shown to cause changes that resemble the aging process [7-9].

Nigella sativa (black seed), which is an annual plant that belongs to the Ranunculaceae family, is widely grown in many countries [10, 11]. The chemical compounds that make up black seed vary, but its major components are alkaloids, as well as fixed and volatile oils. The fixed oils include linoleic acid, oleic acid and palmitic acid. Thymoquinone, a volatile oil, is the most active constituent of black seed [12]. Black seed has many medicinal properties, including neuroprotective [13-16], hepatoprotective [17, 18], hypotensive [19], renal protective [18, 20], antidiabetic [10, 19], bronchodilatory [10], antibacterial [10, 21], anti-tumor [10], anti-inflammatory [10, 19, 22] and immunomodulative [10, 19, 23] properties. Black seed prevents the neuronal damage caused in the frontal cortex and brain stem by exposure to toluene [11]. Because of its antioxidant mechanisms, black seed oil can be used to reduce the side effects of gentamicin, which include hepatic and renal toxicity [20]. Also, the neuroprotective activity of *Nigella sativa* on neurotransmitters creates an anti-epileptic effect [13, 19]. Because oxidative stress plays a crucial role in aging and antioxidant herbs can effectively prevent diseases related to aging, this study investigated the antioxidant and the anti-aging effects of black-seed fixed oil on D-galactose-induced aging in a mouse model, with a focus on evaluating oxidative stress and apoptosis.

2. Materials and Methods

Acrylamide was purchased from Sigma. Malondialdehyde tetrabutylammonium, DTNB (5-5-dithiobis-(2-nitrobenzoic acid)) and D-galactose were also obtained from Sigma. Rabbit polyclonal Bax, rabbit monoclonal Bcl2, caspase-3 antibodies and anti-rabbit IgG labeled with horseradish peroxidase were purchased from Cell Signaling, as were mouse monoclonal beta actin and anti-mouse IgG labeled with horseradish peroxidase. The black seeds used in this study were harvested from Shahreza City, Esfahan Province, Iran; the oil was extracted by using the cold press method.

Male Razi mice with weights of 25 - 27 g were used in this study. The rodents were housed in colony rooms with a 12-/12-h light/dark cycle at $21 \pm 2^\circ\text{C}$ and had free access to food and water. All animal experiments were carried out according to Mashhad University of Medical Sciences, Ethical Committee's guidelines. All animals were provided by the Faculty of Pharmacy, Mashhad University of Medical Sciences.

D-galactose (500 mg/kg, SC) was administered each day for 42 days to induce aging. This daily dose, as well as the route and the duration of administration, has been well characterized with respect to the induction of aging [24]. For our experiment, the mice were divided at random into 7 groups with 10 mice in each group, and treatment was done as follows: Group 1 was the control group and received daily doses of the normal saline as vehicle for 42 days. The mice in Group 2 were

administered daily doses of D-galactose (500 mg/kg) for 42 days. The mice in Groups 3 to 5 were administered daily doses of D-galactose (500 mg/kg) + black-seed oil (0.1 mL/kg [25], 0.2 mL/kg [25, 26], and 0.5 mL/kg [25]), respectively, for 42 days. The mice in Group 6 received daily doses of black-seed oil (0.5 mL/kg) for 42 days, and those in Group 7 received daily doses of D-galactose (500 mg/kg) + vitamin E (200 mg/kg) [4, 24, 27] three times a week for 42 days. Both the black-seed oil and Vitamin E were administered intraperitoneally (ip).

At the end of the 42-day treatment, blood samples were collected under anesthesia by using cardiac puncture and were kept for 1 h. Serum samples were separated by centrifuging blood at 4°C for 10 min. Liver and brain tissues were separated, snap-frozen in liquid nitrogen, and transferred to freezers at a temperature of -80°C . Malondialdehyde (MDA) as the final product of lipid peroxidation was measured in both brain and liver tissues. MDA reacts with thiobarbituric acid (TBA) and produces a pink-color complex with maximum absorbance at 532 nm. For this test, 3 mL of phosphoric acid (1%) and 1 mL of TBA (0.6%) were added to 0.5 mL of brain or liver tissue homogenate 10% in KCl; then, the mixture was heated for 45 min in a boiling-water bath. After cooling, 4 mL of n-butanol were added, and this complex was vortex-mixed for 1 min, followed by centrifugation at 3,000 g for 10 min. The absorbance of the organic layer was measured at 532 nm. The quantity of MDA was reported as nmol/g tissue [28].

The procedure of Moron *et al* [29] with some changes, was used to measure the GSH content. For this test, 500 μL of 10% tricolor acetic acid (TCA) was added to 500 μL of homogenate tissue. This mixture was vortexed and centrifuged at 2,500 g for 10 min. After that, the supernatant were mixed with 2 mL of phosphate buffer (pH: 8) and 500 μL of DTNB. The absorbance was measured at 412 nm [29] by using a spectrophotometer (Jenway 6105 UV/vis, UK). GSH levels were calculated from a standard curve produced using commercially available GSH. GSH levels were reported as nmol/g tissue.

The levels of alanine amino transferase (ALT) and aminotransferase (AST) were determined using a Mindary auto analyzer (BS 800) according to the kit's protocols. Data were expressed as IU/mL.

At the first step in the Western blot analysis, brain and liver tissue samples were lysed in lysis buffer containing 50-mM Tris-HCl (pH: 7.4), 2-mM EDTA, 2-mM EGTA, 10-mM NaF, 1-mM sodium orthovanadate (Na_3VO_4), 10-mM β -glycerophosphate, 0.2% W/V sodium deoxycholate, 1-mM phenylmethylsulfonyl fluoride (PMSF), and a complete protease inhibitor cocktail (Sigma P8340). Then, the total proteins were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes. After that, blots were blocked with 5% skim milk for 2 h at room temperature. After blocking, blots were incubated with antibodies, including Bax (Cell Signaling #2772), Bcl-2 (Cell Signaling #2870), caspase-3 (8G10) (Cell Signaling #9665) and Beta-actin (Signaling #3700) at 1:1000 dilution, for 2 h at room temperature. After incubation with primary antibodies, membranes were washed three times with 0.1% Tween 20 and TBST and then incubated with horseradish-peroxidase-conjugated anti-rabbit antibody (Cell Signaling #7074) or horseradish-peroxidase-conjugated anti-mouse antibody (Cell Signaling #7076) at 1:3000 dilutions for 1 h at room temperature. Finally, pro-

tein bands were detected using enhanced chemiluminescence (ECL) reagent and Alliance 4.7 Geldoc (UK). Protein bands were analysed using UVtec software (UK). The protein levels were normalized against the Beta-actin intensities.

Statistical analyses were performed using the one-way analysis of variance (ANOVA), followed by the Tukey-Kramer test to determine the discrepancy in the data. Differences were considered statistically significant when $P < 0.05$.

3. Results

Exposure to D-galactose markedly elevated the levels of ALT ($P < 0.001$) and AST ($P < 0.01$) in comparison to the control group while treatment with black-seed oil (0.1 and 0.2 mL/kg) diminished the levels of these biochemical markers (Figs. 1A, 1B). The levels of MDA were determined as a marker of lipid peroxidation in both brain and liver tissues. Significant ($P < 0.001$) elevations, when compared to the control group, in the levels of MDA from 90.67 ± 14.69 to 154.5 ± 33.6 nmol/g tissue in the brain and from 62.10 ± 12.45 to 132.7 ± 19.04 nmol/g tissue in the liver were observed following 42 days of exposure to D-galactose (Figs. 2A, 2B). Interestingly, treatment of mice with black-seed oil at doses of 0.1, 0.2 and 0.5 mL/kg significantly decreased the levels of MDA in brain and liver tissues ($P < 0.001$).

As Fig. 3A shows, administration of D-galactose reduced the GSH content in brain tissue ($P < 0.01$ vs. control) while black-seed oil at doses 0.1 and 0.2 mL/kg significantly recovered the GSH content ($P < 0.05$ and $P < 0.01$, respectively) when compared to D-galactose-treated animals. In liver tissue, a significant depletion of the GSH content was observed in the D-galactose-treated group, but black-seed oil at different doses did not show any protective effect (Fig. 3B).

The levels of proteins, including Bax, Bcl2 and caspase-3 (pro and cleaved forms), which are involved in the apoptosis pathway, were determined using Western blot analyses. According to the results (Figs. 4A, 4B), the level of Bax protein increased in the D-galactose-treated group while no significant change in the level of Bcl2 protein was observed; therefore, the Bax/Bcl2 ratio markedly increased ($P < 0.05$ vs. control). Interestingly, treatment with black-seed oil (0.1 mL/kg) decreased the Bax/Bcl2 ratio from 1.34 ± 0.15 to 0.75 ± 0.19 when compared with the D-galactose-treated group ($P < 0.001$). Caspase-3, as an executioner caspase activated by both intrinsic and extrinsic apoptosis pathways, was determined. D-galactose administration induced apoptosis in the brain and elevated expressions of caspase-3 (pro and cleaved forms) compared to the control group ($P < 0.05$). The caspase-3 level was also significantly reduced ($P < 0.05$) in animals exposed to black-seed oil (0.1 mL/kg) (Figs. 5A, 5B).

According to the obtained results, the increase in the Bax/Bcl2 ratio triggered with D-galactose was attenuated following treatment with black-seed oil. Additionally, the level of caspase-3 (pro and cleaved forms) rose, which indicated D-galactose-induced apoptosis in liver tissue. Black-seed oil showed anti-apoptotic properties and diminished the procaspase-3 protein level from 139 ± 14 to 58 ± 5 and the caspase-3 cleaved level from 116 ± 4 to 63 ± 0.5 (Figs. 6A, 6B

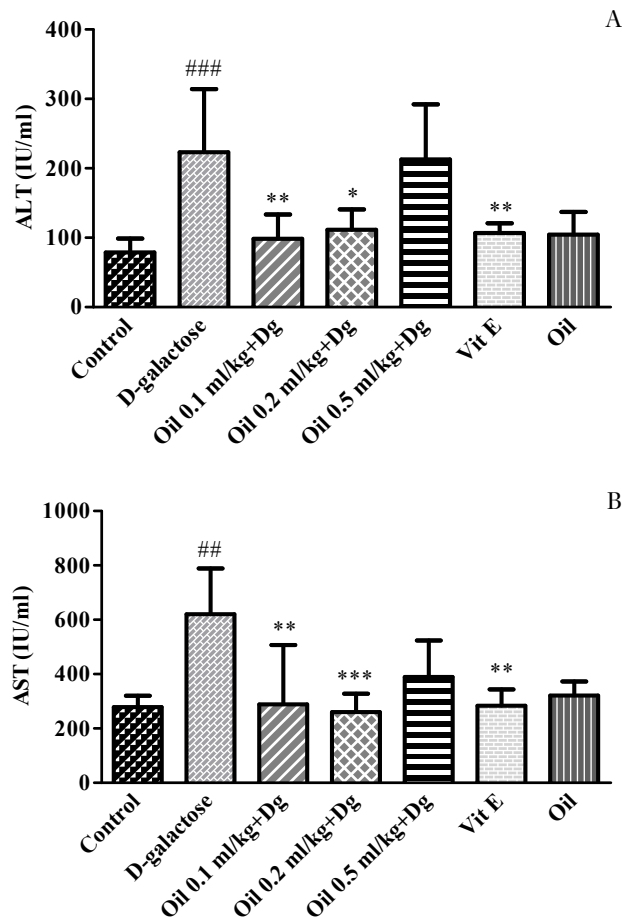
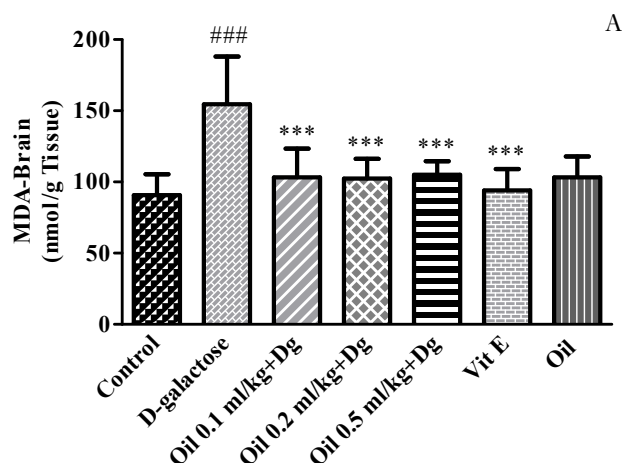


Figure 1 Effect of black seed oil on (A) ALT and (B) AST levels in the serum of Dg-induced aged mice. Data are expressed as means \pm SDs ($n = 7$). $^{##}P < 0.01$ and $^{###}P < 0.001$ vs. control, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. Dg-treated animals.

ALT, alanine amino transferase; AST, aspartate aminotransferase; Dg, D-galactose; SDs, standard deviations.



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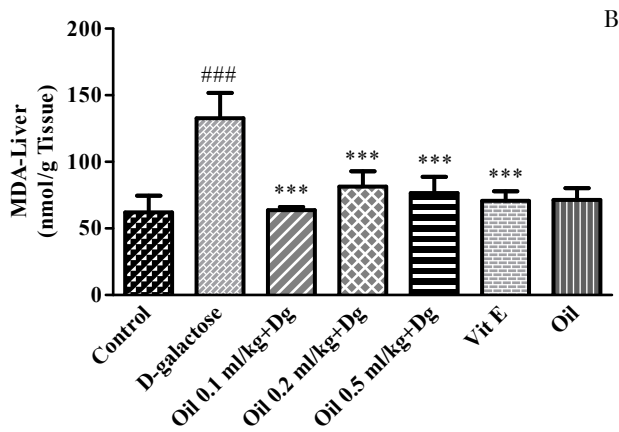


Figure 2 Effect of black seed oil on lipid peroxidation induced by Dg in the (A) brain and the (B) liver. Data are expressed as means \pm SDs (n = 7). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control; $^{***}P < 0.001$ vs. Dg-treated animals.

MDA, malondialdehyde; Dg, D-galactose; SDs, standard deviations.

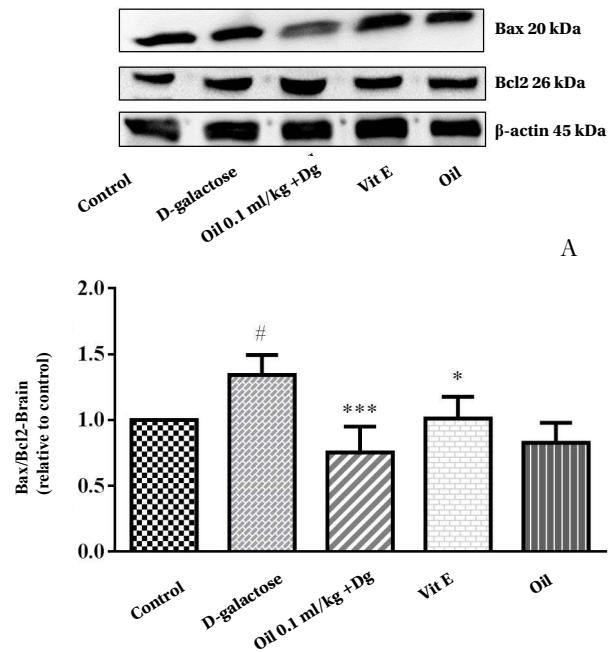


Figure 4 Effect of black seed oil on the protein expressions of Bcl2 and Bax induced in the brain by Dg treatment. (A) Western blot analyses were performed to determine the Bax and the Bcl2 protein levels. (B) The Bax/Bcl2 ratio was determined using a densitometric analysis. Data are expressed as means \pm SDs of four separate experiments. $^{*}P < 0.05$ vs. control; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. Dg-treated animals.

Dg, D-galactose; SDs, standard deviations.

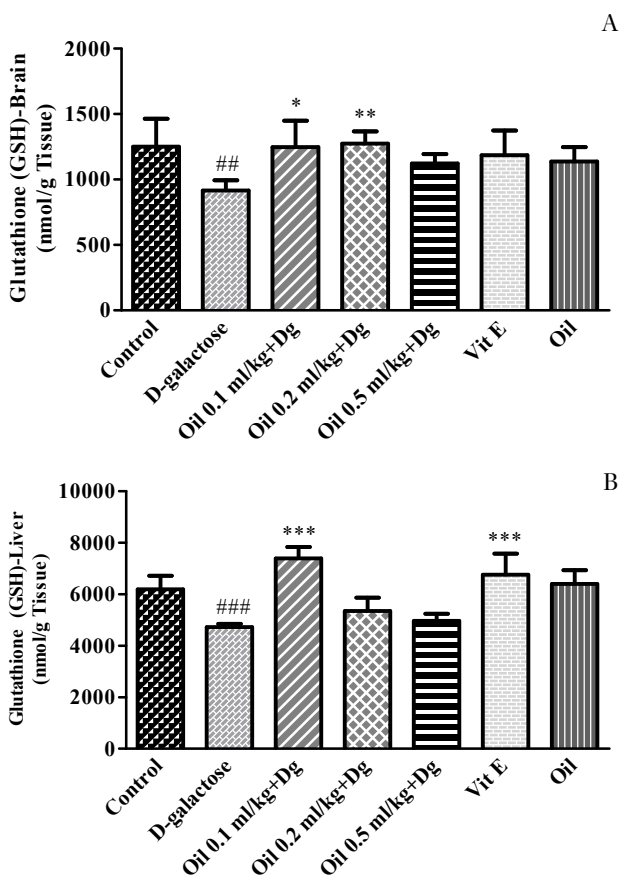
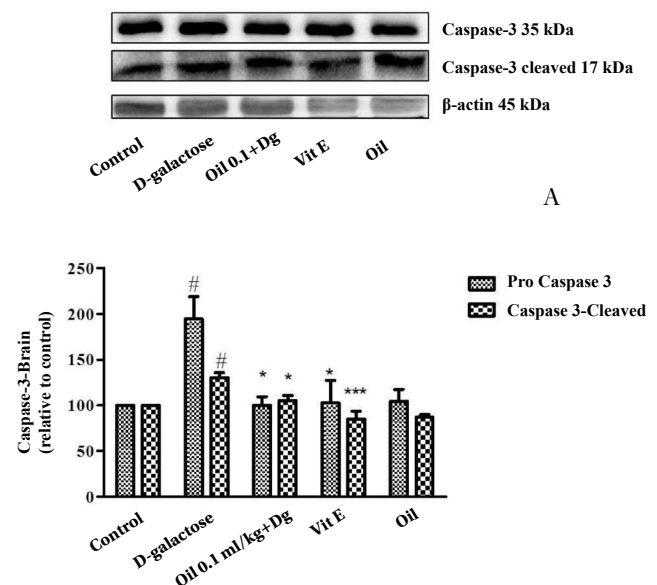


Figure 3 Effect of black seed oil on the GSH contents in the (A) brain and the (B) liver following treatment for the Dg-treated animals. Data are expressed as means \pm SDs (n = 7). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. Dg-treated animals.

GSH, glutathione; Dg, D-galactose; SDs, standard deviations.



B

(Continued)

Figure 5 Effect of black seed oil on the protein levels of procaspase-3 and caspase-3 cleaved induced in the brain by Dg treatment. (A) Western blot analyses were performed to determine the procaspase-3 and caspase-3 cleaved protein levels. (B) The procaspase-3 and caspase-3 cleaved levels relative to those of the control were determined using a densitometric analysis. Data are expressed as means \pm SDs of four separate experiments. $^{\#}P < 0.05$ vs. control; $^*P < 0.05$, $^{***}P < 0.001$ vs. Dg-treated animals.

Dg, D-galactose; SDs, standard deviations.

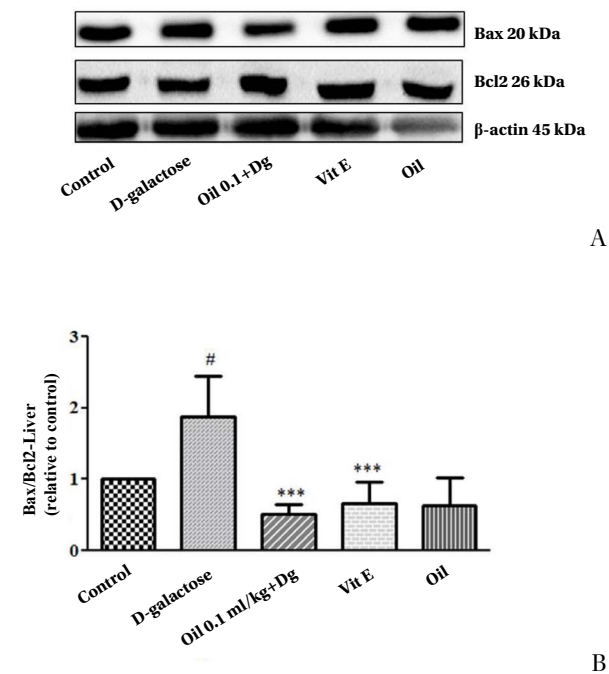


Figure 6 Effect of black seed oil on the expressions of the proteins Bcl2 and Bax induced in the liver by Dg treatment. (A) Western blot analyses were performed to determine the Bax and the Bcl2 protein levels. (B) The Bax/Bcl2 ratio was determined using a densitometric analysis. Data are expressed as means \pm SDs of four separate experiments. $^{\#}P < 0.05$ vs. control; $^{***}P < 0.001$ vs. Dg-treated animals.

Dg, D-galactose; SDs, standard deviations.

and 7A, 7B).

4. Discussion

In the present study, the anti-aging effect of black-seed oil in a mouse model of aging induced with D-galactose (SC, for 42 days) was evaluated. During aging, significant oxidative stress and apoptosis were induced, as characterized by a reduction in the GSH content in brain and liver tissues and by enhancements in the MDA level (marker of lipid peroxidation), the Bax/Bcl2 ratio, and the caspase-3 protein level. Biochemical markers, including ALT and AST, increased with aging. Ad-

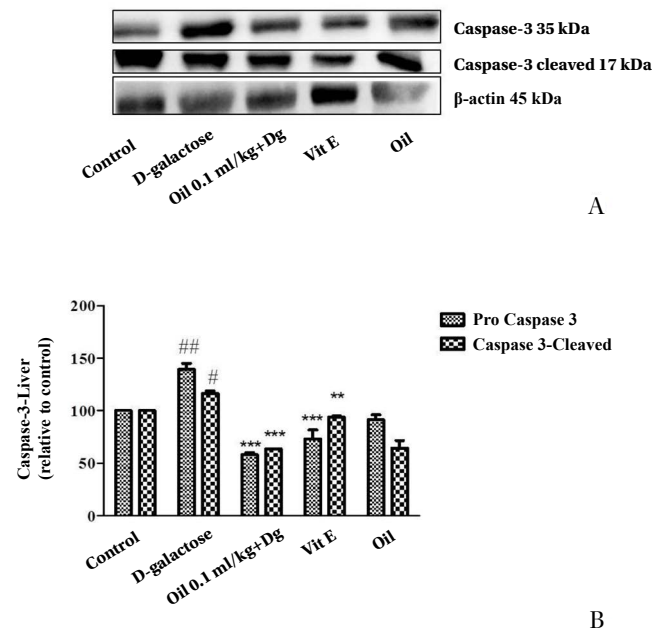


Figure 7 Effect of black seed oil on the levels of the proteins procaspase-3 and caspase-3cleaved induced in the liver by Dg treatment. (A) Western blot analyses were performed to determine the levels of the proteins procaspase-3 and caspase-3 cleaved. (B) The levels of the proteins procaspase-3 and caspase-3 cleaved relative to those of the control were determined using a densitometric analysis. Data are expressed as means \pm SDs of four separate experiments. $^{\#}P < 0.05$ and $^{##}P < 0.01$ vs. control; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. Dg-treated animals.

Dg, D-galactose; SDs, standard deviations.

ministration of black-seed oil (0.1 mL/kg) significantly diminished brain and liver injury through inhibition of oxidative stress and apoptosis.

Aging is a complex biological phenomenon that manifests itself as harmful changes over time [1]. Antioxidants compounds, including IMOD (s a combination of ethanolic extracts of *Rosa canina*, *Tanacetum vulgare* and *Urtica dioica* that are combined with selenium and urea and then exposed to a pulsed electromagnetic field), angipars and rutin, reduce oxidative stress induced by oxidant agents in the body. Thus, such compounds can be used to treat patients with diseases associated with oxidative stress and aging [24, 30]. Thus, in this study, we investigated the effects of *N. sativa* oil on D-galactose-induced aging in mice.

The results of our study showed that the administration of D-galactose (500 mg/kg, SC) for 6 weeks created liver and brain toxicity. Analyses of biochemical parameters, including the levels of liver enzymes in the serum (ALT, AST), indicated significant damage to the livers of mice receiving D-galactose, with D-galactose inducing oxidative stress through an increase in lipid peroxidation (MDA) and decreases in the glutathione contents in brain and liver tissues. The results of the current study are in agreement with those of different experiments which revealed that, in aged animals, the level of MDA was markedly increased while the GSH content and the

levels of antioxidant enzymes, such as glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase, were decreased in liver and brain tissues [6, 31-33].

In our experiment, administration of black-seed oil had effective anti-aging effects; the GSH depletion in brain and liver tissues recovered and lipid peroxidation was inhibited. Also, the levels of ALT and AST returned to normal following exposure to black-seed oil. The hepatoprotective and neuroprotective effects of black-seed oil have been mentioned in other studies [11, 17, 18, 25, 34, 35]. Black-seed oil showed antioxidant effect against schistosomiasis in mice and decreased oxidative stress in the liver [25]. Additionally, administration of black-seed oil led to antioxidant and anti-epileptic properties in mice and could modulate the fatal effects of pentylene-tetrazole (PTZ)-induced seizures in mice through regulation of oxidative stress in the brain [35]. The antioxidant effect of black-seed oil observed in this study was not dose dependent. As the results show, black-seed oil in a dose of 0.1 mL/kg exhibited the best protective effect on different tests.

In different studies, apoptosis has been considered to be one of the mechanisms involved in D-galactose-induced neurotoxicity and hepatotoxicity [5, 36]. Apoptosis is modulated by many proteins, which are expressed or activated in response to apoptotic signals. The proteins in the Bcl2 family of proteins located at mitochondrial and endoplasmic reticulum sites control apoptosis in the intrinsic mitochondrial apoptosis pathway. The balance between the pro and the anti-apoptotic proteins of the Bcl2 family is important in apoptosis progression [37]. Therefore, the Bax/Bcl2 ratio is a crucial predictor of apoptosis [38]. Caspase-3 is known as the executioner caspase and is triggered by both intrinsic and extrinsic apoptosis pathways. Once activated, the executioner caspases cleave a broad range of cellular proteins [39]. Analyses of the proteins involved in apoptosis pathways (Bax, Bcl2, procaspase-3, and caspase-3 cleaved) showed that the administration of D-galactose increased the Bax/Bcl2 ratio and the level of caspase-3 in both liver and brain tissues, and intensified apoptosis was noted in these tissues. Additionally, the administration of D-galactose led to a shrinkage of the cell's nucleus, to dense chromatin cells, and to apoptosis in the hippocampal neurons [33]. Enhancements of caspase-3 and poly-(ADP-ribose) polymerase PARP levels and a reduction in the level of the Bcl2 protein in brain and liver tissues have been reported following exposure to D-galactose [5, 6, 40].

Our study clearly exhibited that black-seed oil (0.1 mL/kg), because of its anti-apoptotic properties, reduced the expressions of procaspase-3 and caspase-3 cleaved, as well as the Bax/Bcl2 ratio, in liver and brain tissues. A similar study on the anti-apoptotic effects of black-seed oil on rats reported that black-seed oil reduced the expressions of caspase-3 and Bax, which had been elevated after exposure to STZ [26]. Also, another study reported that, in rats, black-seed oil regulated the expression of caspase-3 following exposure to sodium nitrite [41].

5. Conclusion

In conclusion, the results of the current study demonstrated the anti-aging effect of black seed oil in a mouse model of aging induced by D-galactose. An elevation of the GSH content,

a reduction in lipid peroxidation, and regulation of the apoptosis pathway can be considered as mechanisms involved the anti-aging effect.

Acknowledgement

The authors are thankful to the Vice Chancellor of Research, Mashhad University of Medical Sciences, for financial support. The results of current study are part of a MSc thesis.

Conflict of interest

The authors declare that there are no conflicts of interest.

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