

EFFECT OF *NIGELLA SATIVA* SEED EXTRACTS ON ISCHEMIA-REPERFUSION IN RAT SKELETAL MUSCLE

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Summary

In this study, the effect of *Nigella sativa* seed extracts was evaluated on an animal model of I/R injury in the rat hind limb. Hind limb ischemia was induced using clamping the common femoral artery and vein. After 2 h ischemia, the clamp of the femoral vessels of animals was taken off and the animal underwent 1h reperfusion. Muscle injuries were evaluated by recording of the electromyographic (EMG) potentials and performing some biochemical analysis including thiobarbituric acid reactive substances (TBARS), total sulfhydryl (SH) groups and antioxidant capacity of muscle (using FRAP assay). Ischemia was induced using free-flap surgery in skeletal muscle. The aqueous (1, 1.5 and 2 g/kg) and ethanolic extracts (1.6, 2.4 and 3.2 g/kg) of *N. sativa* as well as normal saline (10 ml/kg) were administered intraperitoneally 1 h prior reperfusion. The average peak-to-peak amplitude during ischemic-reperfusion was significantly increased in extracts groups in comparison with control group. Following the extracts administration, the total SH contents and antioxidant capacity were elevated in muscle flap. The MDA level was declined significantly in test groups. It is concluded that *N. sativa* extracts have some protective effects against muscle tissue injury caused by lower limb ischemia-reperfusion.

Keywords: *Nigella sativa*, Oxidative stress, Lower limb ischemia, Reperfusion, Electromyography.

Introduction

A vast amount of circumstantial evidence implicates oxygen-derived free radicals (especially superoxide and hydroxyl radical) and high-energy oxidants (such as peroxynitrite) are as mediators of inflammation, shock, and ischemia/reperfusion injury (Cuzzocrea et al., 2001). The oxidant injury can potentially occur during ischemia and reperfusion due to an excess production of oxygen free radicals, a decrease in antioxidant defenses, or both. Because antioxidants function by removing the toxic oxygen metabolites, they are generally highly effective in reducing ischemia-reperfusion injury (Das and Maulik, et al., 1994). Skeletal muscle

ischemia and reperfusion injury remains an issue of concern because of the morbidity and mortality that follows revascularization of an acutely ischemic limb (Ascher et al., 2001). Many studies have suggested the beneficial antioxidant effects of antioxidant nutrients such as vitamin E, green tea extract, ginkgo biloba extract, resveratrol and niacin in reducing or preventing cerebral or muscle injury during ischemia-reperfusion (Ikeda et al., 2003). *Nigella sativa* Linn. is indigenous to the Mediterranean region but has been cultivated into other parts of the world including Saudi Arabia, northern Africa and parts of Asia. The plant is known by names, such as black cumin (English), black-caraway seeds (USA) and shonaiz (Persian) (Khan, 1999). Different

pharmacological effects such as isulintropic (Fararh et al., 2002), hypoglycemic (El-Dakhakhny et al., 2002), anticancer (Salomi et al., 1999; Mabrouk et al., 2002), antinociceptive, anti-inflammatory (Abdel-Fattah et al., 2000; El-Dakhakhny et al., 2002; . Ghannadi et al., 2005), hepatoprotective (Mahmoud et al., 2002), neuroprotective (Kanter et al., 2006), antihistamine, antiulcer (Kanter et al., 2006) and bronchodilator (Gilani et al., 2001) activities have been reported for this plant. Black cumin has been traditionally used in the Indian continent, Arabian countries and Europe for culinary and medicinal purposes as a natural remedy for a number of illnesses and conditions that include asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness and influenza. The seeds or its oil are used as a carminative, diuretic, lactagogue and vermifuge (Ali and Blunden et al., 2003).

N. sativa seeds contain 36%–38% fixed oils, proteins, alkaloids, saponin and 0.4%–2.5% essential oil. The fixed oil is composed mainly of unsaturated fatty acids. The essential oil was analyzed using GC-MS. Many components were characterized, but the major ones were thymoquinone (27.8%–57.0%), *p*-cymene (7.1%–15.5%), carvacrol (5.8%–11.6%), *t*-anethole (0.25%–2.3%), 4-terpineol (2.0%–6.6%) and longifoline (1.0%–8.0%). Thymoquinone readily dimerizes to form dithymoquinone. Four alkaloids have been reported as constituents of *N. sativa* seeds. Two, nigellicine and nigellidine have an indazole nucleus, whereas nigellimine and its N-oxide are isoquinolines (Ali and Blunden et al., 2003). *N. sativa* and thymoquinone have been shown to decrease ischemia-reperfusion (I/R) injury in hippocampus (Hosseinzadeh et al., 2007) or gastric mucosal tissues (El-Abhar et al., 2003). Thus, in this study the effect *N. sativa* extracts was evaluated during ischemia-reperfusion on an animal model of

ischemia-reperfusion injury in hind limb. Ischemia damages were evaluated using recording of EMG potentials (to show the viability of nerve) and measuring oxidative markers. Malondialdehyde and SH-groups were measured as parameters for lipid peroxidation and oxidative-stress markers (Huang et al., 2005). Antioxidant capacity of muscle was evaluated using FRAP assay (Huang et al., 2005).

Methods and Materials

Animals: Wistar male rats, 200-230 g were housed in colony rooms with 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 in accordance with Mashhad University of Medical Sciences, Ethical Committee Acts.

Plant material: *N. sativa* seeds were purchased from a local market in Mashhad, Iran, that collected from the south of Khorassan. The seed was cultivated and the plant was identified by Herbarium Center of Mashhad Ferdowsi University, Mr Joharchi (247-1419-01). The aqueous extract was decocted with hot water for 15 min (yield: 11.5 w/w). The ethanolic extract was macerated with ethanol (80% v/v) for 72 hour (yield: 9.6 w/w). The extracts were then concentrated under reduced pressure to volume desired. The residual water was evaporated to dryness at 30°C on a water bath.

Chemicals: DTNB (2, 2'-dinitro-5, 5'-dithiodibenzoic acid), TPTZ (2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine), TBA (2-thiobarbituric acid), n-butanol, Tris, Na_2EDTA , sodium acetate, glacial acetic acid, phosphoric acid, potassium chloride, tetramethoxypropane (TMP), ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ferrous sulfate and hydrochloric acid was obtained from Merck. Xylazine and ketamine were obtained from

Loughrea, Co. (Galway, Ireland) and Rotexmedica (GmbH, Germany), respectively.

Induction of ischemia: The rats were anesthetized with intraperitoneal injection of ketamine/xylazine (60 mg/kg and 6mg/kg, respectively). Additional doses of these agents were used if anesthesia lightened during experiment.

An incision in the inner side of the hind leg from the inguinal ligament to the tendon calcaneus insertion was made. Then it was divided up and the triceps surae was dissected as a muscle flap, after that insertions to femur was cut. Previously dissected femoral vessels, the artery and vein were clamped with a single clamp of microsurgery. The absence of bleeding was verified in the muscle flap. Then the incision was closed to prevent desiccation. For reperfusion periods, the clamp of the femoral vessels of animals was taken off and then the bleeding of the muscle flap was verified (Hosseinzadeh et al., 2005). The muscle tissues was homogenized in cold KCl solution (1.5%) to give a 10% homogeny suspension and used for biochemical assays. The results were expressed by nM or μ M/g tissue (1ml of homogenate = 0.1 g of tissue). Eight groups of animals were used, each of which contained 8 rats: Group 1 including sham operated animals; group 2 served as ischemic control to which saline (10 ml/kg) was injected intraperitoneally (i.p.); groups 3-5 were received the aqueous extract (1, 1.5 and 2 g/kg) and groups 6-8 were received the ethanolic extract and ethanolic extracts (1.6, 2.4 and 3.2 g/kg). Except group 1, other groups underwent 2h ischemia and 1h reperfusion. All agents were administrated 1h before reperfusion.

Electromyography Data Collection: To determine the muscles activities during ischemia-reperfusion, intramuscular

electromyograph (EMG) signals were recorded with PowerLab data acquisition systems. Two pairs of pin electrodes in terminating alligator clips were inserted into the triceps surae (muscle flap) in hind leg, and adductor muscles. The distance between the two electrodes of a pair in each muscle was 5 mm. A grounding electrode was gently attached to the rat tail (Ossowska et al., 1996). The EMG signals were collected with sampling frequency of 12 PPM (MacLab/4SP). Duration for each stimulation was 20 ms. The raw EMG signals were low-pass filtered at 50 Hz and EMG signal is expressed as average peak-to-peak amplitude for a 10 min recording periods. The electromyography (EMG) signals were recorded 10 min before ischemia, 10 min before reperfusion and 10 min at end of reperfusion phase.

Biochemical assays

Thiobarbituric acid reactive substances (TBARS) measurement. Malondialdehyde (MDA) levels, as an index of lipid peroxidation, were measured. MDA reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS) to produce a red colored complex which has peak absorbance at 532 nm (Fernandez et al., 1997).

3 ml phosphoric acid (1%) and 1 ml TBA (0.6%) was added to 0.5 ml of homogenate in a centrifuge tube and the mixture was heated for 45 min in a boiling water bath. After cooling, 4 ml of n-butanol was added the mixture and vortex-mixed for 1 min followed by centrifugation at 20000 rpm for 20 min. The organic layer was transferred to a fresh tube and its absorbance was measured at 532 nm. The standard curve of MDA was constructed over the concentration range of 0-40 μ M (Uchiama and Miahara, 1978).

Ferric Reducing / Antioxidant Power (FRAP) assay. The FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{2+} -tripirydyltriazine compound from the colorless oxidized Fe^{3+} form by the action of electron donating antioxidants (Benzie and Strain, 1996). The FRAP reagent consist of 300 mM acetate buffer (3.1 g sodium acetate + 16 ml glacial acetic acid, made up to 1 liter with distilled water; pH=3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in the ratio of 10:1:1.

Briefly, 50 μl of muscle homogenate was added to 1.5 ml freshly prepared and prewarmed (37 °C) FRAP reagent in a test tube and incubated at 37 °C for 10 min. The absorbance of the blue colored complex was read against reagent blank (1.5 ml FRAP reagent + 50 μl distilled water) at 593 nm. Standard solutions of Fe^{2+} in the range of 100 to 1000 mM were prepared from ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) in distilled water. The data was expressed as mM ferric ions reduced to ferrous form per liter (FRAP value) (Benzie and Strain, 1999).

Total sulfhydryl (SH) groups assay. Total SH groups were measured using DTNB (2, 2'-dinitro-5, 5'-dithiodibenzoic acid) as the reagent. This reagent reacts with the SH groups to produce a yellow colored complex which has a peak absorbance at 412 nm (Ellman, 1959). Briefly, 1 ml Tris-EDTA buffer (pH=8.6) was added to 50 μl muscle homogenate in 2 ml cuvettes and sample absorbance was read at 412 nm against Tris-EDTA buffer alone (A_1). Then 20 μl DTNB reagent (10 mM in methanol) was added to the mixture and after 15 min (stored in laboratory temperature) the sample absorbance was read again (A_2). The absorbance of DTNB reagent was also read as a blank (B). Total thiol concentration (mM) was calculated from the following equation:

$$\text{Total thiol concentration (mM)} = (A_2 - A_1 - B) \times 1.07 / 0.05 \times 13.6$$

Statistical analysis. Data are expressed as mean \pm SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey-Kramer *post-hoc* test for multiple comparisons. The p-values less than 0.05 were considered to be statistically significant.

Results

Electromyography Data

The average peak-to-peak amplitudes in the control-ischemic group were decreased during ischemia and reperfusion compare with sham group. Both extracts increased the amplitude compare with ischemia and reperfusion control-ischemic group (Table 1).

Thiobarbituric acid reactive species (TBARS) measurement

There was an increase in the MDA levels following ischemia reperfusion as compared with sham-operated animals (Figure 1). The extracts with higher doses resulted in a significant reduction in the free radical-mediated lipid peroxidation as indicated by a decrease in the MDA levels, at various dose levels (Figures 1 and 2). A reduction in TBARS levels were very prominent in the higher doses (Figures 1 and 2).

Modulation of FRAP value

Ischemia reperfusion caused a significant reduction in FRAP value of muscle homogenate samples as compared with sham-operated animals (Figure 3). The aqueous (1.5 and 2 g/kg) and ethanolic (2.4 and 3.2 g/kg) extract pretreatment increased antioxidant power (FRAP value) of muscle homogenate samples (Figures 3 and 4).

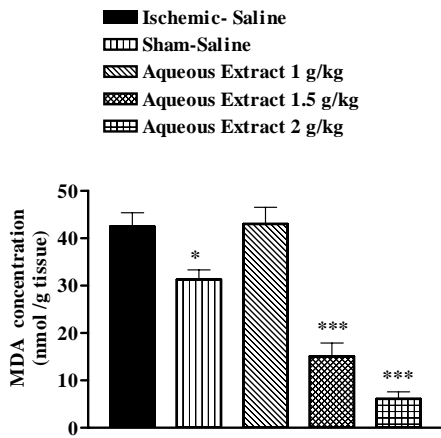


Figure 1. Effect of aqueous extract of *N. sativa* seeds on lipid peroxidation following muscle ischemia reperfusion injury. MDA levels were measured in 10% homogenates of muscle samples from rats subjected to 120 min of ischemia and 60 min of reperfusion. All drugs were administrated intraperitoneally 60 min prior to reperfusion. Values are mean \pm SEM (n=8). **p<0.01, ***p<0.001 as compared with ischemic-saline treated animals (One-way ANOVA followed by Tukey-Kramer test).

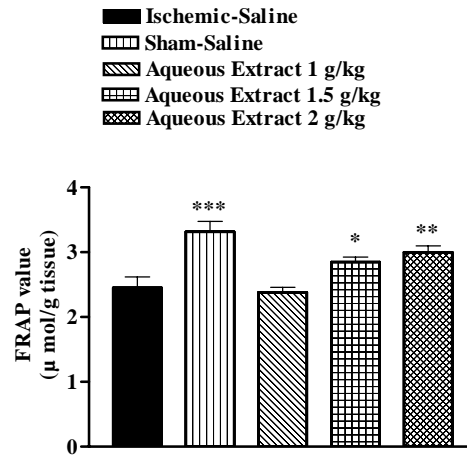


Figure 3. Effect of aqueous extract of *N. sativa* seeds on antioxidant power of muscle homogenate samples following muscle ischemia reperfusion injury. FRAP values were measured in 10% homogenate samples from rats subjected to 120 min of ischemia and 60 min of reperfusion. All drugs were administrated intraperitoneally 60 min prior to reperfusion. Values are mean \pm SEM (n=8). **p<0.01, ***p<0.001 as compared with ischemic-saline treated animals (One-way ANOVA followed by Tukey-Kramer test).

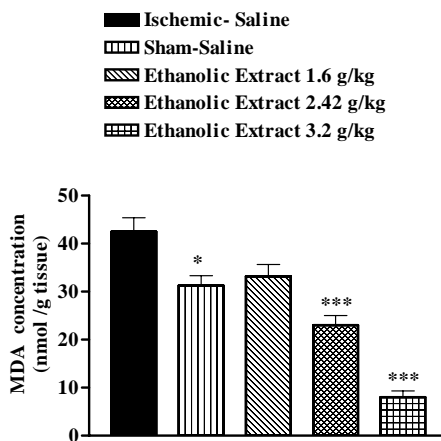


Figure 2. Effect of ethanolic extract of *N. sativa* seeds on lipid peroxidation following muscle ischemia reperfusion injury. MDA levels were measured in 10% homogenates of muscle samples from rats subjected to 120 min of ischemia and 60 min of reperfusion. All drugs were administrated intraperitoneally 60 min prior to reperfusion. Values are mean \pm SEM (n=8). **p<0.01, ***p<0.001 as compared with ischemic-saline treated animals (One-way ANOVA followed by Tukey-Kramer test).

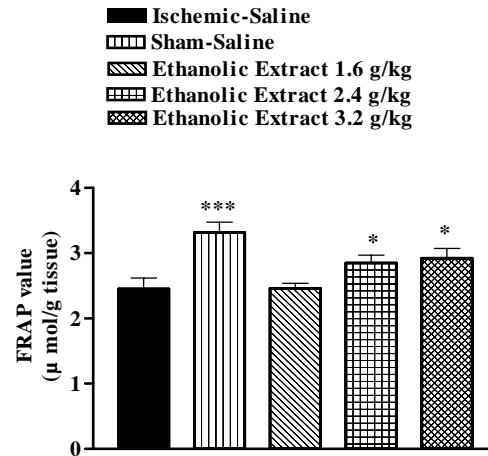


Figure 4. Effect of ethanolic extract of *N. sativa* seeds on antioxidant power of muscle homogenate samples following muscle ischemia reperfusion injury. FRAP values were measured in 10% homogenate samples from rats subjected to 120 min of ischemia and 60 min of reperfusion. All drugs were administrated intraperitoneally 60 min prior to reperfusion. Values are mean \pm SEM (n=8). **p<0.01, ***p<0.001 as compared with ischemic-saline treated animals (One-way ANOVA followed by Tukey-Kramer test).

Total sulfhydryl concentration

Following ischemia-reperfusion injury, a significant reduction in total SH groups in muscle homogenate samples was observed (Figure 5). The aqueous extract (1.5 and 2

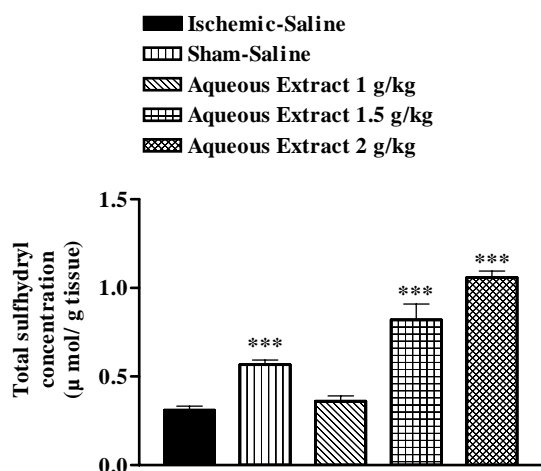


Figure 5. Effect of aqueous extract of *N. sativa* seeds on total thiol concentrations following following muscle ischemia reperfusion injury. Total sulfhydryl (SH) groups were measured in 10% muscle homogenate samples from rats subjected to 120 min of ischemia and 60 min of reperfusion. All drugs were administrated intraperitoneally 60 min prior to reperfusion. Values are mean \pm SEM (n=8). *p<0.05, ***p<0.001 as compared with ischemic-saline treated animals (One-way ANOVA followed by Tukey-Kramer test).

Discussion

The results obtained in the present investigation suggest that *N. sativa* extracts have an overall protective effect against muscle I/R injury in a rat model.

A number of processes have been implicated in the pathogenesis of oxygen deprivation-induced cell injury. These include disturbance of cell calcium homeostasis, depletion of adenine nucleotides, activation of enzymes like phospholipases with production of toxic lipid metabolites, proteases and endonucleases and generation of free radicals (ROS) that can cause oxidative damage to cellular macromolecules

(g/kg) and all doses of the ethanolic extract pretreatment caused a significant elevation in total sulfhydryl concentration, as compared with control-ischemic group (Figures 5 and 6).

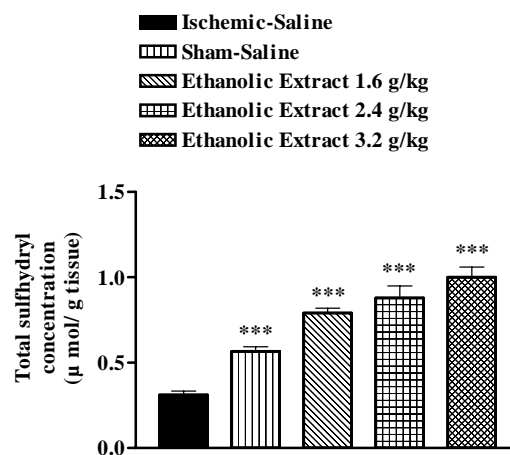


Figure 6. Effect of ethanolic extract of *N. sativa* seeds on total thiol concentrations following following muscle ischemia reperfusion injury. Total sulfhydryl (SH) groups were measured in 10% muscle homogenate samples from rats subjected to 120 min of ischemia and 60 min of reperfusion. All drugs were administrated intraperitoneally 60 min prior to reperfusion. Values are mean \pm SEM (n=8). *p<0.05, ***p<0.001 as compared with ischemic-saline treated animals (One-way ANOVA followed by Tukey-Kramer test).

(Montagna et al., 1998; Rhodena et al., 2002). ROS have been shown to play a major role in IRI (Greene and Paller et al., 1992; Zager and Gmur, 1989) and also collectively are instrumental in impairing overall renal function (Yoshioka. And Ichikawa, 1989; Paller, 1988). ROS can induce damage to endothelial, glomerular mesangial and tubular epithelial cells (especially S3 segment of proximal tubule) (Greene and Paller et al., 1992; Zager and Gmur, 1989) and induce apoptosis in renal cells (Burns et al., 1998). Cellular death following renal ischemia-reperfusion injury is well associated with ROS production and lipid peroxidation and antioxidant therapy has been

Table1. Effects of aqueous and ethanolic extracts of *N. sativa* seeds prior, during and after ischemia-reperfusion in rat skeletal muscle.

Group (n=8)	Preischemia (v)	Ischemia (v)	Reperfusion (v)
Ischemic-saline group (10 ml/kg)	2.80 ± 0.04	1.31 ± 0.03	1.50 ± 0.03
Sham-saline group	2.59 ± 0.02	2.67 ± 0.02***	2.59 ± 0.03***
Aqueous extract 1 g/kg	2.04 ± 0.02	2.11 ± 0.03***	1.86 ± 0.07**
Aqueous extract 1.5 g/kg	2.21 ± 0.03	2.29 ± 0.02***	2.30 ± 0.03***
Aqueous extract 2 g/kg	2.41 ± 0.03	2.42 ± 0.01***	2.13 ± 0.02***
Ethanolic extract 1.6 g/kg	2.02 ± 0.03	2.10 ± 0.03***	1.85 ± 0.08**
Ethanolic extract 2.4 g/kg	2.15 ± 0.04	2.28 ± 0.02***	2.31 ± 0.04***
Ethanolic extract 3.2 g/kg	2.41 ± 0.04	2.42 ± 0.01***	2.10 ± 0.02***

Values are mean ± S.E.M. ** P<0.01 and ***P<0.001 when compared with control group with Tukey-Kramer test.

well documented to help in the improvement of organ functions (Lee et al., 1997).

We studied the effect of the extracts on lipid peroxidation and measured the MDA, a stable metabolite of the free radical-mediated lipid peroxidation cascade. MDA levels increased significantly following renal IRI. The extracts pretreated rats exhibited a reduction in MDA levels that confirmed the antioxidant role of these agents in IRI.

Sulfhydryl (SH) groups known to be sensitive to oxidative damage and depleted following ischemic insult (Soszynski and Bartosz, 1997), therefore we studied the effect of these on total thiol concentration during IRI. In our study, total sulfhydryl groups were decreased following IRI. The pretreated animals with extracts exhibited higher SH contents than their respective controls. Therefore extracts increased significantly the antioxidant power of muscle homogenate samples.

Under acute and chronic pathologic conditions such as ischemia, the balance between oxidant and antioxidant systems has been interrupted (Abdollahi et al., 2004; Parihar and Hemnani, 2003). Therefore we evaluate the antioxidant or reducing potential of muscle homogenate samples following IRI, using FRAP assay. As expected following IRI, a significant reduction in antioxidant power, as indicated by FRAP value, was observed.

Burits and Bucar (2002) showed that *N. sativa* has appreciable antioxidant and free radical scavenger properties but no pro-oxidant effect. Recently, El-Abhar et al. (2003) showed that *N. sativa* oil and thymoquinone have a marked protective action against ischaemia-reperfusion-induced gastric mucosal lesions. It has been also shown that *N. sativa* and thymoquinone may have protective effects on lipid peroxidation process during IRI in rat hippocampus (Hosseinzadeh et al., 2007).

N. sativa and thymoquinone have also anti-inflammatory and analgesic actions (Abdel-Fattah et al., 2000; El-Dakhkhny et al., 2002; Ghannadi et al., 2005; Houghton et al., 1995) and it seems these effects may be related to inhibition of eicosanoid generation, namely thromboxane B₂ and leucotrienes B₄ (by inhibiting cyclooxygenase and 5-lipoxygenase, respectively), and membrane lipid peroxidation (Houghton et al., 1995).

In this study, the extracts maintained the nerve conductivity. Nerve conduction is decreased during ischemia-reperfusion (Oguzhanoglu et al., 2000; Schmelzer et al., 1989). Mild muscle necrotic changes occur after 2-3 h ischemia (Korthals et al., 1985; Iida et al., 2003). Oxidative stress and the production of oxygen free radicals during ischemia-reperfusion is one mechanism of ischemic fiber degeneration, causing a breakdown of the blood-nerve barrier,

endoneurial edema and lipid peroxidation (Nagamatsu et al., 1996). The extracts prevented lipid peroxidation and showed antioxidant activity in this study. These effects and other activities such as anti-inflammatory effect (Houghton et al., 1995) may preserve viability of nerve conductivity. Present study showed that the extracts of *N. sativa* suppressed the increase of MDA levels in the rat skeletal muscle and therefore inhibited lipid peroxidation following ischemia-reperfusion injury. The inhibition of lipid peroxidation and anti-ischemic

effects are probably related to the antioxidant properties and free radical scavenging activity of the extracts.

Conclusions

In conclusion, the present study showed that the extracts of *N. sativa* seeds have protective effect on ischemia reperfusion injury-induced oxidative stress in rat muscle that at least partly is due to antioxidant properties of the extracts.

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