PROTECTIVE EFFECT OF NIGELLA SATIVA L. EXTRACTS AND THYMOQUINONE, ITS ACTIVE CONSTITUENT, ON RENAL ISCHEMIA-REPERFUSION-INDUCED OXIDATIVE DAMAGE IN RATS

Hossein Hosseinzadeh¹* and Reza Montahaei²

*1-Correspondence author: Pharmaceutical Research Center, Faculty of Pharmacy, Mashhad University of Medical Sciences, P.O. Box: 1365-91775, Mashhad, I. R. Iran, Fax: 98 5118823251, E-mail: hosseinzadehh@mums.ac.ir

2- Faculty of Pharmacy, Mashhad University of Medical Sciences, P.O. Box: 1365-91775, Mashhad, I. R. Iran

Summary

Generation of reactive oxygen species and lipid peroxidation are associated with tissue injury following Ischemia/Reperfusion (I/R). Therefore, this model was used to assess the antioxidant effects of aqueous and ethanolic extracts of Nigella sativa and its active ingredient, thymoquinone, on oxidative stress following renal I/R injury (IRI). Male wistar rats were injected with aqueous and ethanolic extracts (doses of 0.7, 1 and 1.6 g/kg i.p.) and thymoquinone (doses of 2.5, 5 and 10 mg/kg i.p.). Normal saline was injected to control group (10 ml/kg) and one group was selected as a sham that did not have I/R. The markers of oxidative stress including thiobarbituric acid reactive substances (TBARS), total sulphydryl (SH) groups and antioxidant capacity of kidney tissue (using FRAP assay) were measured. The left kidneys were exposed to warm ischemia for 60 min followed by reperfusion for 90 min. Agents were administrated prior to reperfusion. IRI caused a significant increase in TBARS level and decrement in both antioxidant power (FRAP value) and total thiol concentrations in kidney homogenate samples. In the aqueous extract pretreated groups (in all doses) and ethanolic extract groups (with doses of 0.7 and 1 g/kg), there was not found any effective results. In the ethanolic group (1.6 g/kg), a reduction in TBARS level (P<0.001) and elevation in antioxidant power (FRAP value) (P<0.05) and total thiol concentrations was seen (P<0.05). Thymoquinone also reduced lipid peroxidation products (P<0.001; 5 and 10 mg/kg) and increased antioxidant power (P<0.05 with 2.5 mg/kg, P<0.01 with 5 mg/kg and P<0.001 with 10 mg/kg) and total thiol concentrations (P<0.05 with 2.5 mg/kg and 5 mg/kg and P<0.01 with 10 mg/kg) in ischemia-reperfusion injured rat kidneys. This study suggests that ethanolic extract of N. sativa and thymoquinone may be useful agents for the prevention of renal ischemia-reperfusion (IR)-induced oxidative injury in rats.

Keywords: Nigella sativa, thymoquinone, renal ischemia-reperfusion, lipid peroxidation.

Running title: Protective effect of Nigella sativa and thymoquinone, on renal ischemia-reperfusion
Introduction

*Nigella sativa* L., commonly known as black seed or black cumin, is used in folk medicine as a natural remedy for a number of disease and condition such as asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness and gastrointestinal disturbances [1]. Furthermore, modern pharmacological and toxicological studies have demonstrated that crude extracts of the seeds and some of its active constituents (volatile oil, thymoquinone) might have protective effect against nephrotoxicity and hepatotoxicity induced by either disease or chemicals [1]. *Nigella sativa* oil has also antipyretic, analgesic, anti-inflammatory, antimicrobial, and antineoplastic activity [1].

Thymoquinone, the major active constituent of *N. sativa* seeds, is a pharmacologically active quinone, which possesses several properties including analgesic and anti-inflammatory actions [2-3], protection against chemical induced carcinogenesis [4-5], and the inhibition of eicosanoids generation [3]. Moreover, it has been reported that thymoquinone prevents oxidative injury in hepatocytes induced by carbon tetrachloride or tert-butyl hydroperoxide in various *in vitro* [6] and *in vivo* [7] hepatotoxicity models, as well as acetic acid-induced colitis in rats [8]. It has been suggested that thymoquinone may act as an antioxidant agent and prevent the membrane lipid peroxidation in hepatocytes [9].

It has been well documented that ischemia increases lipid peroxidation reactions and reactive oxygen species (ROS), which cause secondary neural tissue damage [10-11]. Lipid peroxidation is an autocatalytic mechanism leading to oxidative destruction of cellular membranes [12]. Their destruction can lead to cell death and to production of toxic and reactive aldehyde metabolites called free radicals. Among these free radicals, malondialdehyde (MDA) is the most important [13]. Therefore, the use of antioxidants, free radical scavengers or trapping agents may be rational in cerebral ischemia-reperfusion injury (IRI) [14-16]. Recently, there is overwhelming attention to plant products and natural agents that can limit free radical-mediated injuries, for better therapeutic management of IRI.

The aim of this study was to investigate the effect of *N. sativa* extracts and thymoquinone, an active constituent of *N. sativa*, on renal IRI induced oxidative stress in rats.

**Material And Methods**

**Animals**

Adult male Wistar rats weighing 220-300 g were used throughout the study. They were kept in the same room, constant temperature (22 ± 2°C) and illuminated 7:00 a.m. to 7:00 p.m., with food pellets and water available ad libitum.
The animals were divided into 11 groups, each of which contained 8-10 rats. Group 1 was the sham group in which only surgery was done without induction of ischemia. Group 2 was the control group in which saline solution (10 ml/kg) was given intraperitoneally. In group 3-11, the aqueous extract (0.7, 1 and 1.6 g/kg i.p.), ethanolic extract (0.7, 1 and 1.6 g/kg i.p.) and thymoquinone (2.5, 5 and 10 mg/kg i.p.) were administrated prior to induction of reperfusion.

**Chemicals**

DTNB (2, 2'-dinitro-5, 5'-dithiobenzonic acid), TPTZ (2, 4, 6-tri 2-pyridyl)-1, 3, 5-triazine), TBA (2-thiobarbituric acid), n-butanol, tris, Na2 EDTA, Sodium acetate, glacial acetic acid, phosphoric acid, potassium chloride, tetramethoxypropane (TMP), ferric chloride (FeCl3.6H2O), ferrous sulfate and hydrochloric acid was obtained from Merck. Thymoquinone was purchased from Aldrich Chem.

**Preparation of aqueous and ethanolic extracts**

*N. sativa* seeds (collected from Bajestan, KRazavi Khorasan, Iran) were authenticated by Pharmacognosy Department, School of Pharmacy, MUMS , Iran. For preparation of aqueous extract, the powdered seed (50 g) was boiled in 500 ml boiling water for 15 min. Subsequently, the mixture was filtered and concentrated under reduced pressure at 40°C (yield: 9% w/w). For preparation of the ethanolic extract, the powdered seed (50 g) was macerated in 500 ml ethanol (80% v/v) for 48 h and subsequently the mixture was filtered and concentrated under reduced pressure at 40°C (yield: 7.5% w/w).

**Induction of renal ischemia-reperfusion injury (IRI)**

The animals were subjected to left renal warm ischemia for 60 min, and reperfusion for 90 min. Briefly, under intraperitoneal ketamin/xylazine anesthesia (60 mg/kg and 10 mg/kg, respectively) and through a midline incision; the abdominal contents were displaced to the night side. The left renal artery and vein were dissected and the perirenal fat was preserved.

The vascular pedicle was temporarily ligated with 2-0 silk before the abdominal contents were replaced and the incision was covered with a moistened pad. At the end of the ischemia period, the abdominal cavity was reentered, the ligature was removed and reperfusion was supplied. Throughout the experiments, body temperature was kept at 36-38°C by placing the rats under light source. At the 90th min of reperfusion, left kidney was removed and maintained at -80°C until analysis [17]. At the day analysis, the kidney tissue was homogenized in cold KCl solution (1.5%) to give a 10% homogeny suspension and used for biochemical assays. The aqueous and ethanolic extracts as well as thymoquinone were dissolved in physiologic saline and injected exactly prior to reperfusion.
**Tiobarbituric acid reactive species (TBARS) measurement**

The lipid peroxidation level of the kidney tissues was measured as malondialdehyde (MDA) which is the end product of lipid peroxidation, and 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 [18]. 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 µM [19].

**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^{II}-tripyridyltriazine compound from the colorless oxidized Fe^{III} from by the action of electron donating antioxidants [20].

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 FeCl$_3$.6H$_2$O in the ratio of 10:1:1. Briefly, 50 µl of kidney 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^{II} in the range of 100 to 1000 mM were prepared from ferrous sulphate (FeSO$_4$.7H$_2$O) in distilled water. The data was expressed as mmol ferric ions reduced to ferrous from per liter (FRAP value) [21].

**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 peak absorbance at 412 nm [22].

Briefly, 1 ml Tris-EDTA buffer (pH=8.6) was added to 50 µl kidney 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: Total thiol concentration (mM) = (A$_2$-A$_1$-B) × 1.07/0.05 × 13.6
**Statistical analysis**

Data are expressed as mean ± 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**

There was an increase (100%) in the MDA levels following IRI as compared with sham-operated animals (28.7 ± 0.1 vs. 14.1 ± 0.1 nmol/g tissue, P<0.001) (Figure 1). The aqueous extract did not show any significant effect on the radical-mediated lipid peroxidation (Figures 1).

The ethanolic extract in dose 1.6 g/kg resulted in a significant reduction in the MDA levels (Figure 1). Thymoquinone in doses 5 and 10 mg/kg reduced free radical-mediated lipid peroxidation significantly, by a decrease in the MDA levels (Figure 1).

The ethanolic extract in upper dose (1.6 g/kg) reduced lipid peroxidation products (from 28.74 ± 0.4 to 20.15 ± 0.2 nmol/g tissue, P<0.001) in IR injured rat kidneys.

In thymoquinone pretreated groups, a reduction in TBARS levels with two upper doses (from 28.81 ± 0.3 to 15.80 ± 0.3 nmol/g tissue, P<0.001; 10 mg/kg) were observed.

Modulation of FRAP value by extracts and thymoquinone IRI caused a significant reduction in FRAP value (42.8%) of kidney homogenate samples as compared with sham-operated animals (from 14.284 ± 0.5 to 8.17 ± 0.1 µmol/g tissue, P<0.001) (Figure 2).

The ethanolic extract only in upper dose increased antioxidant power (from 8.24 ± 0.2 to 10.43 ± 0.3 µmol/g tissue, P<0.05; 1.6 g/kg). The aqueous extract was not able to elevate antioxidant power (Figure 2). Thymoquinone pretreated also increased antioxidant power, dose dependently (from 8.2 ± 0.2 to 13.9 ± 0.2 µmol/g tissue, P<0.001; 10 mg/kg) (Figure 2).

Following IRI, a significant reduction (43.9%) in total SH groups (0.754 ± 0.1 vs. 0.423 ± 0.02 mM, P<0.001) in kidney homogenate samples were observed (Figure 3). The aqueous extracts in all doses and ethanolic extract in two lower doses did not protect kidney cells from IRI and failed to increase total SH groups (Figure 3). The ethanolic extract (1.6 g/kg) significantly elevated total thiol concentration (P<0.01) (Figure 3). Thymoquinone pretreatment caused a significant and dose dependently increase in total thiol concentration as compared with normal saline group (from 0.402 ± 0.01 to 0.799 ± 0.08 mM, P<0.01; 10 mg/kg) (Figure 3).
Figure 1: Effect of aqueous (A), ethanolic (B) extracts of *Nigella sativa* and thymoquinone (C) on lipid peroxidation following renal ischemia-reperfusion injury. MDA level were measured in 10% homogenates of kidney samples from rat subjected to 60 min of ischemia and 90 min of reperfusion. All drugs were administrated intraperitoneally prior to reperfusion. Values are mean ± SEM (n=8). ***P<0.001 as compared with normal saline treated animals (One-way ANOVA followed by Tukey-Kramer test).
Figure 2: Effect of aqueous (A), ethanolic (B) extracts of *Nigella sativa* and thymoquinone (C) on antioxidant power of kidney homogenate samples following renal ischemia-reperfusion injury. FRAP values were measured in 10% homogenate samples from rats subjected to 60 min of ischemia and 90 min of reperfusion. All drugs were administrated intraperitoneally prior to reperfusion. Values are mean ± SEM (n= 10). *P<0.05, **P<0.01, ***P<0.001 as compared with normal saline treated animals (one-way ANOVA followed by Tukey-Kramer test).
Figure 3: Effect of aqueous (A), ethanolic (B) extracts of *Nigella sativa* and thymoquinone (C) on total thiol concentrations following renal ischemia-reperfusion injury. Total sulfhydryl (SH) groups were measured in 10% kidney homogenate samples from rats subjected to 60 min of ischemia and 90 min of reperfusion. All drugs were administrated intraperitoneally prior to reperfusion. Values are mean ± SEM (n= 8). *P<0.05, **P<0.01, ***P<0.001 as compared with normal saline treated animals (one-way ANOVA followed by Tukey-Kramer test).
Discussion

The results obtained in this study suggest that the *N. sativa* ethanolic extract and its active constituent, thymoquinone, have an overall protective effect against kidney IR 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 [23-24]. ROS have been shown to play a major role in IRI [25-26] and also collectively are instrumental in impairing overall renal function [27-28]. ROS can induce damage to endothelial, glomerular mesangial and tubular epithelial cells (especially S3 segment of proximal tubule) [25-26] and induce apoptosis in renal cells [29]. Cellular death following renal ischemia-reperfusion injury is well associated with ROS production and lipid peroxidation and antioxidant therapy has been well documented to help in the improvement of organ functions [30].

To assess the effect of *N. sativa* extracts and thymoquinone, we studied their effects 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.

Sulfhydryl (SH) groups known to be sensitive to oxidative damage and depleted following ischemic insult [31], therefore we studied the effect of these on total thiol concentration during IRI. In our study, total sulfhydryl groups wee decreased following IRI.

Under acute and chronic pathologic conditions such as ischemia, the balance between oxidant and antioxidant systems has been interrupted [32-33]. Therefore we evaluate the antioxidant or reducing potential of kidney homogenate samples following IRI, using FRAP assay. As expected following IRI, a significant reduction in antioxidant power, as indicated by FRAP value, was observed.

Thymoquinone and ethanolic extract (in high dose; 1.6 g/kg) pretreated rats exhibited a reduction in MDA levels that confirmed the antioxidant role of this agents in IRI.
These pretreated animals exhibited higher SH contents that their respective controls. Therefore these agents increased significantly the antioxidant power of kidney homogenate samples.

It has been shown that both the *N. sativa* and thymoquinone inhibit non-enzymatic lipid peroxidation in liposomes [3]. Burits and Bucar showed that *N. sativa* as well as its compounds, especially thymoquinone, have appreciable antioxidant and free radical scavenger properties but no pro-oxidant effect. The antioxidant action of *N. sativa* and/or thymoquinone may explain the protective effect of these agents against various hepatotoxic and nephrotoxic models *in vivo* and *in vitro* [6-8, 34-36], as well as liver fibrosis and cirrhosis [37]. Al-Gharably et al. suggested that the protective effect of thymoquinone against carbon tetrachloride-induced hepatotoxicity might be related to the ability of this agent to inhibit lipid peroxidation [38]. Recently, El-Abhar et al. showed that *N. sativa* oil and thymoquinone have a marked protective action against ischaemia-reperfusion-induced gastric mucosal lesions [39].

*N. sativa* and thymoquinone have also anti-inflammatory and analgesic actions[2, 40-42] 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 [3].

In our study, all doses of aqueous extracts and the two lower doses of ethanolic, slightly increased both total concentration and antioxidant power following insult, but this elevation was not significant as compared with control group. According to some results, the effectiveness of aqueous extract has been less than ethanolic extract [43].

In this study, thymoquinone and ethanolic extract were more potent than aqueous extract. In fact, thymoquinone is one of the most important constituent of *N. sativa* that caused cell protection against IRI by its antioxidant mechanism. This may due to the fact that thymoquinone and the other pharmacologically active agents such as dithymoquinone, thymohydroquinone and thymol, are not enough soluble in water to insert into the aqueous extract.

In conclusion, the present study showed thymoquinone and ethanolic extract of *N. sativa*, have protective effect on IRI-induced oxidative stress in rats kidney that at least partly due to antioxidant properties of these agents.
References


