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Nigella sativa as an anti-inflammatory and promising remyelinating agent in the cortex and hippocampus of experimental autoimmune encephalomyelitis-induced rats

Heba M. Fahmy [a,*], Neveen A. Noor [b], Faten F. Mohammed [c], Anwar A. Elsayed [a], Nasr M. Radwan [b]

[a] *Department of Biophysics, Faculty of Science, Cairo University, Giza, Egypt*
[b] *Department of Zoology, Faculty of Science, Cairo University, Giza, Egypt*
[c] *Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt*

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Abstract Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model of multiple sclerosis. This study aimed to investigate the protective and therapeutic effects of *Nigella sativa* (*N. sativa*) seeds (2.8 g/kg body weight) in EAE-induced rats. EAE-induced animals were divided into: (1) EAE-induced animals (''EAE'' group). (2) ''*N. sativa* + EAE'' group received a daily oral administration of *N. sativa* 2 weeks prior to EAE induction until the end of the experiment. (3) ''EAE + *N. sativa*'' group received a daily oral administration of *N. sativa* after the appearance of the first clinical signs until the end of the experiment. All animals were sacrificed at the 28th day post EAE-induction. Disease pathogenesis was monitored using a daily clinical scoring, body weight, open field test, histopathological and ultrastructural examination and determination of some oxidative stress parameters in the cortex and hippocampus. *N. sativa* ameliorated the clinical signs and suppressed inflammation observed in EAE-induced rats. In addition, *N. sativa* enhanced remyelination in the hippocampus. However, protection of rats with *N. sativa* administered 2 weeks prior to EAE induction and its continuation until the end of the

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CFA, complete Freund's adjuvant; CNS, central nervous system; DA, dark astrocyte process; DF, demyelinated fiber; DO, dark oligodendrocyte process; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; EAE, experimental autoimmune encephalomyelitis; GSH, reduced glutathione; GST, glutathione-S-transferase; H&E, hematoxylin and eosin; LA, light astrocyte process; LO, light oligodendrocyte process; M, myelinated fiber; MDA, malondialdehyde; MO, medium oligodendrocyte process; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NO, nitric oxide; *N. sativa*, *Nigella sativa*; PBS, phosphate buffered saline; PD, partially demyelinated fiber; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid; OFT, open field test; R, remyelinated fiber; TEM, transmission electron microscope; TQ, thymoquinone; U, unmyelinated fiber

* Corresponding author. Tel.: +20 1223785563 (mobile), +20 3582070 (H).
E-mail address: heba_moh_fahmi@yahoo.com (H.M. Fahmy).

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experiment resulted in a significant increase in the cortical lipid peroxide level with reference to control and "EAE" rats. In conclusion, *N. sativa* seeds could be used as a protective agent or an adjunct treatment for EAE even when the treatment started after the appearance of the first clinical signs. However, the dose and duration of *N. sativa* must be taken into consideration to avoid its probable pro-oxidant effect.

Introduction

Multiple sclerosis (MS) is an immune-mediated, demyelinating neurodegenerative disease of the central nervous system (CNS) of young adults with a female predominance (Compston and Coles, 2002, 2008). It is a chronic progressive, potentially disabling disorder with considerable social impact and economic consequences (Sadovnick and Ebers, 1993). Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model of MS in which the immune system attacks the myelin protein of the CNS and leads to inflammatory demyelination and oligodendrocyte loss (Frohman et al., 2006; Compston and Coles. 2008; Moore, 2010).

Experimental autoimmune encephalomyelitis is induced in laboratory animals by immunization with myelin-derived antigens; and believed to be mediated by activation of myelin-reactive CD4+ T cells. Expression of high levels of proinflammatory cytokines and chemokines (small chemotactic cytokines) in the brain is thought to contribute to the initiation and maintenance of EAE (Godiska et al., 1995; Ransohoff et al., 1996). Myelin oligodendrocyte glycoprotein (MOG) is a strong encephalitogen, comprising less than 0.05% of all myelin proteins and located exclusively on the surface of CNS myelin sheaths. MOG is a unique myelin autoantigen as it induces not only an encephalitogenic T-cell response in susceptible species, but also a demyelinating autoantibody response (Gold et al., 2006).

Zamvil and Steinman (1990) reported that EAE development is characterized by the infiltration of reactive leukocytes into the CNS. Recruited reactive macrophages/microglia are effector cells in EAE impairing oligodendrocyte axon function (Raivich and Banati, 2004). Astrocytes are involved in multiple and complex actions, including the regulation of the production of pro- and anti-inflammatory cytokines (De Groot et al., 1999; Ambrosini et al., 2002, 2005). In addition, a number of studies reported that reactive oxygen species (ROS) play a key role in myelin damage, contributing to several of the processes underlying MS pathogenesis (Miller et al., 2010; Van Horssen et al., 2011).

The use of natural products as drugs has increased substantially over the last decade to treat many pathological conditions instead of the use of synthetic drugs because of their safety, availability, and ease of administration. *Nigella sativa* (*N. sativa*), also known as black seed or black cumin, belongs to the family of the Ranunculaceae and it is native to Mediterranean countries. Many beneficial biological properties of *N. sativa* extracts have been reported such as anti-inflammatory (Houghton et al., 1995; Alemi et al., 2013; Pichette et al., 2012), antioxidative (Burits and Bucar, 2000) and neuroprotective (Kanter et al., 2006). In addition, *N. sativa* has been widely used in neurodegenerative diseases like Parkinson and Alzheimer because of its antioxidant potential (Hajra, 2011). Therefore, the aim of the present study was to investigate the protective and therapeutic effects of *N. sativa* in the cortex and the hippocampus of EAE-induced female Wistar rats.

Methods

Experimental animals, induction of EAE and clinical scoring

Adult female Wistar rats (age between 6 and 8 weeks) were used in this experiment. They were maintained under fixed appropriate conditions of housing and handling and were given food and water *ad libitum*. All animals received humane care in compliance with the guidelines of the Ethics Committee of the National Research Center, Egypt that followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication Nos. 85–23, revised 1985).

The EAE was induced according to Adelmann et al. (1995) with some modifications. Under light Halothane anesthesia, EAE was induced by a single subcutaneous injection in the tail base with an emulsion of 100 μg $MOG_{35–55}$ dissolved in 100 μl phosphate buffered saline (PBS), mixed with 100 μl complete Freund's adjuvant (CFA) containing 1 mg/ml of *Mycobacterium tuberculosis*.

For the evaluation of the disease course, all rats were weighed and examined daily from the sensitization until the 28th day after immunization. An adapted EAE scoring scale was used (Al-Izki et al., 2012). Animals were scored 0 = normal. 0.5 = incomplete flaccid tail. 1 = fully flaccid tail. 1.5 = semi-impaired righting reflex. 2 = impaired righting reflex. 2.5 = weakness of hind limb. 3 = hind limb paresis. 4 = complete hind limb paralysis. 5 = moribund/death. Control rats were handled in the same manner as the rats of other EAE-induced groups to affront the same degree of stress.

Chemicals

Rat synthetic myelin oligodendrocyte glycoprotein $peptide_{35–55}$ ($MOG_{35–55}$) was purchased from Titan Biotech Limited, Bhiwadi, India. Phosphate buffered saline (PBS) was obtained from the Bio Basic Inc., USA. Complete Freund's adjuvant (CFA), Thiobarbituric acid (TBA) and reduced glutathione were purchased from Sigma Aldrich, Germany. 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Sigma Aldrich, St. Louis, USA. Trichloroacetic acid (TCA) was obtained from SDFCL (SD Fine-Chem Limited), Egypt. Potassium phosphate buffer pH 7.4 (50 mM/L, Triton × 0.1%, EDTA 0.5 mμ), potassium phosphate buffer pH 6.5 (100 mM/L) and kits for the determination of oxidative stress parameters were obtained from Bio Diagnostic Co., Giza, Egypt.

Narcotan Halothane was obtained from EIMC (Egyptian International Medical Centre) pharmaceuticals Co., Egypt.

N. sativa seeds

Syrian *N. sativa* seeds were purchased from a local market in Cairo, Egypt. Grinded whole *N. sativa* seeds were suspended in distilled water and given orally to rats (2.8 g/kg body weight) by means of a gastric tube. The LD_{50} of *N. sativa* oil in rats is 28.8 ml/kg (Zaoui et al., 2002). The dose of *N. sativa* seeds used in this study was calculated as 1/10 of LD_{50} of its oil, to be completely safe.

Experimental design

Animals immunized with MOG were divided into 3 groups: (1) EAE-induced animals (''EAE'' group) received a daily oral administration of distilled water until the end of the experiment. (2) The animals of the second group (''*N. sativa* + EAE'' group) received a daily oral administration of *N. sativa* 2 weeks prior to EAE induction until the end of the experiment. (3) The animals of the third group (''EAE + *N. sativa*'' group) received a daily oral administration of *N. sativa* after the appearance of the first clinical signs until the end of the experiment. All animals were sacrificed by sudden decapitation at the 28th day post immunization. Control animals received a single subcutaneous injection of 200 µl PBS in the tail base and then received daily oral injections of distilled water until the end of the experiment. They were sacrificed simultaneously with the treated groups.

Open field (OF) test

The apparatus was a square 66 × 66 cm with surrounding walls of 30 cm height. The base of the OF was divided into 36 squares. The central zone represented 25% of the total arena. The duration of the test was set to be ten min. The OF parameters measured were the number of squares crossed, the time spent in the central zone, the number of rears, the number of grooming, the number of fecal boli and the number of urine patches. The OF test was done once on the 20th day post immunization.

Histopathological assessment

After decapitation, brains were fixed in buffered neutral 10% formalin, embedded in paraffin and sectioned at 8 micrometer thickness. The brain sections were stained with hematoxylin and eosin (H&E) according to Banchroft et al. (1996). Slides were then examined through the light microscope (Zeiss, Germany) to investigate the deep cerebral cortex and white matter and hippocampus.

Transmission electron microscopy (TEM) for brain tissues

Brains from different experimental groups were dissected to obtain the cortex and hippocampus and then were fixed with 1% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, at 4 °C. The samples were then washed with PBS followed by washing in 0.1 M cacodylate buffer, pH 7.2, and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1.5 h at room temperature. The samples were then washed briefly in deionized water, dehydrated by a graded ethanol series; infiltrated using propylene oxide and EPON epoxy resin and finally embedded in epoxy resin. The samples that had been mixed with epoxy resin were loaded into capsules and polymerized at 60 °C for 24 h. Thin sections were cut using a RMC MT-X ultramicrotome and collected on copper grids. Images were collected using a JEOL transmission electron microscope JEM 1400 (Joel, LTD, Tokyo, Japan) operating at 80 kV.

Biochemical analysis

Tissue homogenization

After decapitation, the brains were transferred rapidly to an ice-cold Petri dish. Each brain was dissected to remove the cortex and hippocampus. The brain samples were weighed and kept at −58 °C until analyzed. Each brain sample was then homogenized in 5% w/v 50 mM phosphate buffer, pH 7.6. The homogenates were centrifuged at 8000 rpm (×g = 7012) for 15 min. The supernatants were stored at −58 °C until use.

Determination of lipid peroxidation

Lipid peroxidation was assayed by measuring the levels of malondialdehyde (MDA) in the brain tissues. MDA was determined by measuring thiobarbituric reactive species using the method of Ruiz-Larrea et al. (1994) in which the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a pink colored complex whose absorbance is read at 532 nm in a Helios Alpha Thermospectronic (UVA 111615, England).

Determination of reduced glutathione (GSH) levels

The assay of reduced glutathione (GSH) levels was performed using Biodiagnostic Kit No. GR 25 11, which is based on the spectrophotometric method of Beutler et al. (1963). It depends on the reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) with glutathione to produce yellow color the absorbance of which is measured at 405 nm in a Helios Alpha Thermospectronic (UVA 111615, England).

Determination of nitric oxide (NO) levels

The assay of nitric oxide (NO) was carried out using Biodiagnostic Kit No. NO 25 33. This method is based on the spectrophotometric method of Montgomery and Dymock (1961) which depends on the measurement of endogenous nitrite concentration as an indicator of nitric oxide production. The resulting azo dye has a bright reddish–purple color whose absorbance is read at 540 nm in a Helios Alpha Thermospectronic (UVA 111615, England).

Determination of catalase activity

Catalase activity was measured using Biodiagnostic Kit No. CA 25 17 which is based on the spectrophotometric method described by Aebi (1984). Catalase reacts with a known quantity of hydrogen peroxide and the reaction is stopped after 1 min with catalase inhibitor. In the presence of peroxidase,

the remaining hydrogen peroxide reacts with 3, 5-dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone to form a chromophore with color intensity inversely proportional to the activity of catalase in the sample. The absorbance of chromophore color is read at 510 nm in a Helios Alpha Thermospectronic (UVA 111615, England).

Determination of glutathione-S-transferase (GST) activity

Glutathione-S-transferase (GST) activity was determined according to the method of Habig et al. (1974). 0.4 ml potassium phosphate buffer (50 mmol/l; pH 6.5), 0.1 ml of supernatant, 1.2 ml water and 0.1 ml CDNB (1-chloro-2,4-dinitrobenzene, 30 mmol/l) were added and incubated in a water bath at 37 °C for 10 min. After incubation, 0.1 ml of reduced glutathione (30 mmol/l) was added. The change in absorbance was measured at 340 nm at one min interval.

Statistical analysis

The maximum clinical score, the days of relapse and the days of remission were assessed using non-parametric statistics, Kruskal–Wallis test followed by Mann–Whitney *U* statistics ($P < 0.05$ was considered significant). Data of body weights, open field and oxidative stress parameters were analyzed by analysis of variance (ANOVA) followed by Duncan's post hoc test when the *F*-test was significant ($p < 0.05$). All analyses were performed using SPSS software (version 16.0) for windows.

Results

*Effect of EAE induction by MOG and its treatment with **N. sativa** on the daily clinical scores in Wistar rats*

As shown in Fig. 1 and Tables 1 and 2, relapse and remission episodes alternated, suggesting a relapsing-remitting EAE model. *N. sativa* administration after the disease onset attenuated the severity of the clinical signs from 13th day post immunization and until the end of the experiment. Meanwhile, *N. sativa* in the "*N. sativa* + EAE" group greatly prevented the clinical signs during the whole course of the experiment compared to the "EAE" group. The "*N. sativa* + EAE" group showed a significant decrease in the days of relapses with respect to the "EAE + *N. sativa*" and "EAE" groups.

The cumulative clinical score/rat (sum of scores measured from disease onset/rat, as an index of disease severity) for the EAE-induced groups is demonstrated in Fig. 2. The "*N. sativa* + EAE" group showed a significant decrease in the cumulative score/rat with respect to both the "EAE" and "EAE + *N. sativa*" groups.

Effect of EAE induction by MOG and its treatment with N. sativa on the body weight of EAE in adult female Wistar rats

As shown in Fig. 3, the "EAE" group showed a general decrease in body weight from 4th day post immunization until the end of the experiment. "*N. sativa* + EAE" group showed a

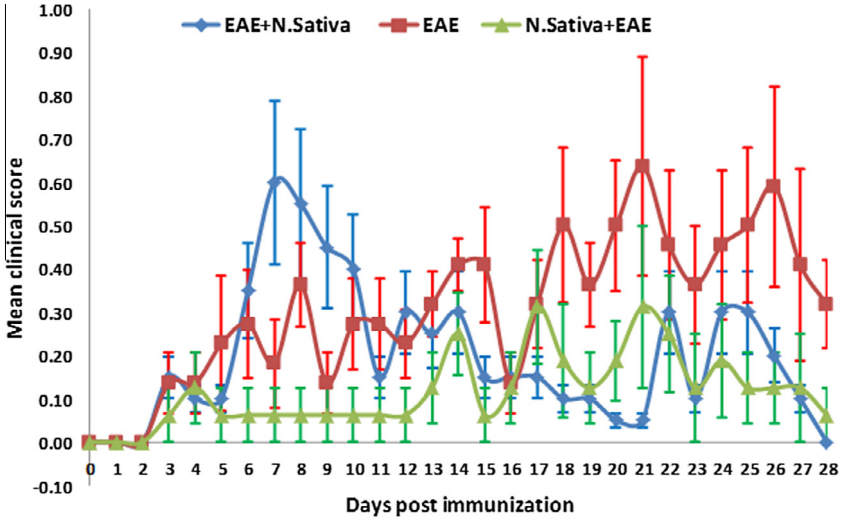

Fig. 1 Effect of EAE induction by MOG and its treatment with *N. sativa* (2.8 g/kg body) on the daily clinical scores in Wistar rats. Values represent mean ± S.E.M.

Table 1 Summary of clinical data obtained from the EAE induced group and *N. sativa* (2.8 g/kg) treated and protected groups.

Groups	Number of rats in each group	Incidence of EAE signs	% of rats recovered from EAE signs until decapitation
"EAE"	12	11/12 (91.67%)	5/11 (45.45%)
"EAE + *N. sativa*"	11	10/11 (91.00%)	10/10 (100.00%)
"*N. sativa* + EAE"	9	8/9 (88.89%)	7/8 (87.50%)

Table 2 Effect of a daily oral administration of *N. sativa* (2.8 g/kg) on the days of relapse and remission in EAE induced adult female Wistar rats.

Experimental groups	Number of animals in each group	Days of relapse			Days of remission		
		Mean rank	Mann–Whitney *U*	Significance	Mean rank	Mann–Whitney *U*	Significance
"EAE"	11	11.77	46.50	n.s.	10.23	46.50	n.s.
"EAE + *N. sativa*"	10	10.15			11.85		
"EAE"	11	12.41	17.50	*	7.73	19.00	*
"*N. sativa* + EAE"	8	6.69			13.12		
"EAE + *N. sativa*"	10	12.15	13.50	*	6.90	14.00	*
"*N. sativa* + EAE"	8	6.19			12.75		

n.s.: non-significant.
* $p < 0.05$ significant.

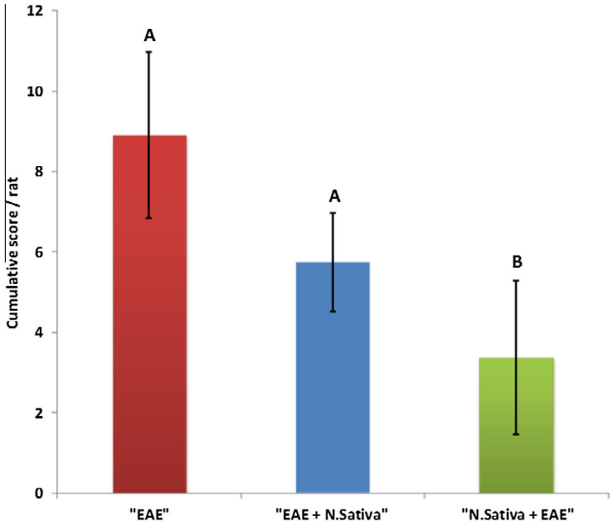

Fig. 2 Cumulative clinical score for each rat in the "EAE", "EAE + *N. sativa*" and "*N. sativa* + EAE" groups. Values represent mean ± S.E.M. A and B: different letters mean significant changes. Significance was tested using the Mann–Whitney *U*-test.

significant increase in body weight in comparison to the control group from 11th to 20th day post immunization. However, treatment of EAE-induced rats with *N. sativa* seeds ("EAE + *N. sativa*") failed to restore the recorded significant decrease of body weight in sick rats to control values.

Effect of EAE induction by MOG and its treatment with N. sativa on open field parameters recorded from adult female Wistar rats

From Table 3 it can be noticed that the rats of the "EAE" group, significantly, spent more time in the center than those of the other groups. The number of rears was significantly increased in the "*N. sativa* + EAE" group with reference to other tested groups.

Effect of EAE induction by MOG and its treatment with N. sativa on the histopathological findings in Wistar rats

Perivascular mononuclear cell aggregation was observed in the deep cerebral gray matter, lesions including glial cell proliferation in the deep gray matter associated with neuronal degeneration and necrosis among variable numbers of motor neurons (Fig. 4c), while lesions in the white matter, including diffuse

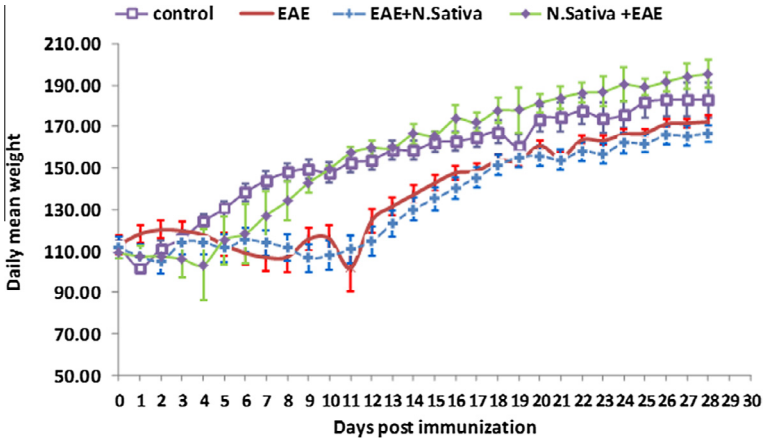

Fig. 3 Effect of EAE induction by MOG and its treatment with *N. sativa* (2.8 g/kg) on the body weight of adult female Wistar rats. Values represent mean ± S.E.M.

Table 3 Effect of EAE induction by MOG and its treatment with *N. sativa* (2.8 g/kg) on some open field parameters recorded from adult female Wistar rats.

Parameters measured	Control group	"EAE" group	% *D*	"EAE + *N. sativa*" group	% *D*	"*N. sativa* + EAE" group	% *D*	*F*-test
Number of squares crossed	161.67 ± 9.34 (6)	146.50 ± 8.50 (6)	−9.38	158.83 ± 13.41 (6)	−1.76	141.17 ± 6.37 (6)	−12.68	n.s.
Number of rears	22.00 ± 2.11 (6) A	24.67 ± 1.58 (9) A	12.14	23.83 ± 1.58 (6) A	8.32	30.00 ± 1.13 (6) B	36.36	*
Time spent in center in seconds	11.17 ± 0.87 (6) A	17.57 ± 2.36 (7) B	57.30	7.57 ± 0.72 (7) A	−32.23	7.50 ± 1.15 (6) A	−32.86	*
Number of grooming	10.36 ± 1.22 (8) AB	7.42 ± 0.74 (11) A	−30.70	11.44 ± 1.18 (6) B	7.62	8.71 ± 1.43 (7) AB	−18.06	*
Number of fecal boli	3.38 ± 1.13 (8)	5.75 ± 0.80 (8)	70.12	3.44 ± 0.78 (9)	1.78	4.86 ± 0.88 (7)	43.79	n.s.
Number of urine patches	1.40 ± 0.43 (10)	1.25 ± 0.41 (11)	−10.71	2.11 ± 0.48 (9)	50.71	1.22 ± 0.36 (8)	−12.86	n.s.

Values represent mean ± S.E.M.
Number of animals is shown between parentheses.
% *D*: percentage difference with reference to control values.
n.s.: non-significant.
A and B: different letters mean significant changes.
* $p < 0.05$ significant.

gliosis with perivascular mononuclear cell aggregation were observed in Fig. 4d. The extent of glial cell proliferation and neuronal damage was reduced in the "EAE + *N. sativa*" group and markedly ameliorated in the "*N. sativa* + EAE" group (Fig. 4 e and h).

Extensive neuronal necrosis comprising the Cornu Ammonis 3 (CA3) (Fig. 5e–h) and vacuolation of granular cells were observed in the Dentate gyrus (DG) region in the "EAE" group compared with the "EAE + *N. sativa*" and "*N. sativa* + EAE" groups that showed normal histological structure of granular cells (Fig. 5i–l).

Effect of EAE induction by MOG and its treatment with N. sativa on the ultrastructure of myelin sheath using transmission electron microscopy (TEM) in Wistar rats

The ultrastructural examination of the "EAE" group revealed slight demyelination of the cerebral cortex and partial to complete demyelination of the hippocampus (Figs. 6 and 7). TEM micrographs of the brain areas of the "EAE + *N. sativa*" group showed slight demyelination of the cerebral cortex, partial incomplete and complete remyelination of most nerve fibers of the hippocampus. Meanwhile, micrographs of the "*N. sativa + EAE*" group showed normal myelin sheath structure of the cerebral cortex. In addition, most of the myelinated fibers were in a state of remyelination in the hippocampus.

Effect of EAE induction by MOG and its treatment with N. sativa on some oxidative stress parameters in different brain areas of Wistar rats

As shown in Table 4, cortical MDA levels showed non-significant changes between the control, "EAE" and "EAE + *N. sativa*" groups. However, a significant increase was recorded in the cortical MDA level of the "*N. sativa* + EAE" group in comparison to both control and "EAE" groups. Cortical GSH levels revealed significant increases in both "EAE" and "EAE + *N. sativa*" groups relative to control value. Meanwhile, a significant decrease was observed in the GSH level in the "*N. sativa* + EAE" group in comparison to the control, "EAE" and "EAE + *N. sativa*" groups. Variations in cortical NO levels demonstrated significant decreases in both "EAE" and "EAE + *N. sativa*" groups with respect to control levels. However, a significant increase in NO level was recorded in the "*N. sativa* + EAE" group in comparison to other experimental groups. Catalase activity in the cortex of the "EAE + *N. sativa*" group showed a significant decrease relative to other experimental groups. The cortical GST activity recorded significant decreases among the experimental groups except for a non-significant change between the "EAE" and "EAE + *N. sativa*" groups.

Variations in the oxidative stress parameters in the hippocampus are presented in Table 5. The hippocampal MDA levels recorded significant decreases among the experimental groups except for a non-significant change between the control and "EAE" groups. A significant increase in NO level in the "EAE + *N. sativa*" group with respect to the control and "*N. sativa* + EAE" groups was also observed. In addition, a significant decrease in catalase activity in the "EAE + *N. sativa*" group with respect to the control, "EAE" and "*N. sativa* + EAE" groups was detected.

Discussion

It has been reported that active induction in which animals were given a CNS antigen results in the initiation of an immune response within the CNS, which leads to either a monophasic EAE, relapsing-remitting (RR) EAE or a chronic EAE depending on the antigen and the species or strain of animal used (Baker et al., 2011; Batoulis et al., 2011; Denic et al., 2011; 't Hart et al., 2011). In the present study, rats of the "EAE" group showed alternative periods of relapses and remissions, suggesting a relapsing remitting (RR) EAE model.

Protective treatment with *N. sativa* showed a significant reduction in the number of days of relapses with respect to the "EAE + *N. sativa*" group. Moreover, the "*N. sativa* + EAE" group showed a significant decrease in the cumulative score/rat with respect to both the "EAE" and "EAE + *N. sativa*" groups. Thus, it could be suggested that *N. sativa* ameliorates the "clinical" manifestations of EAE

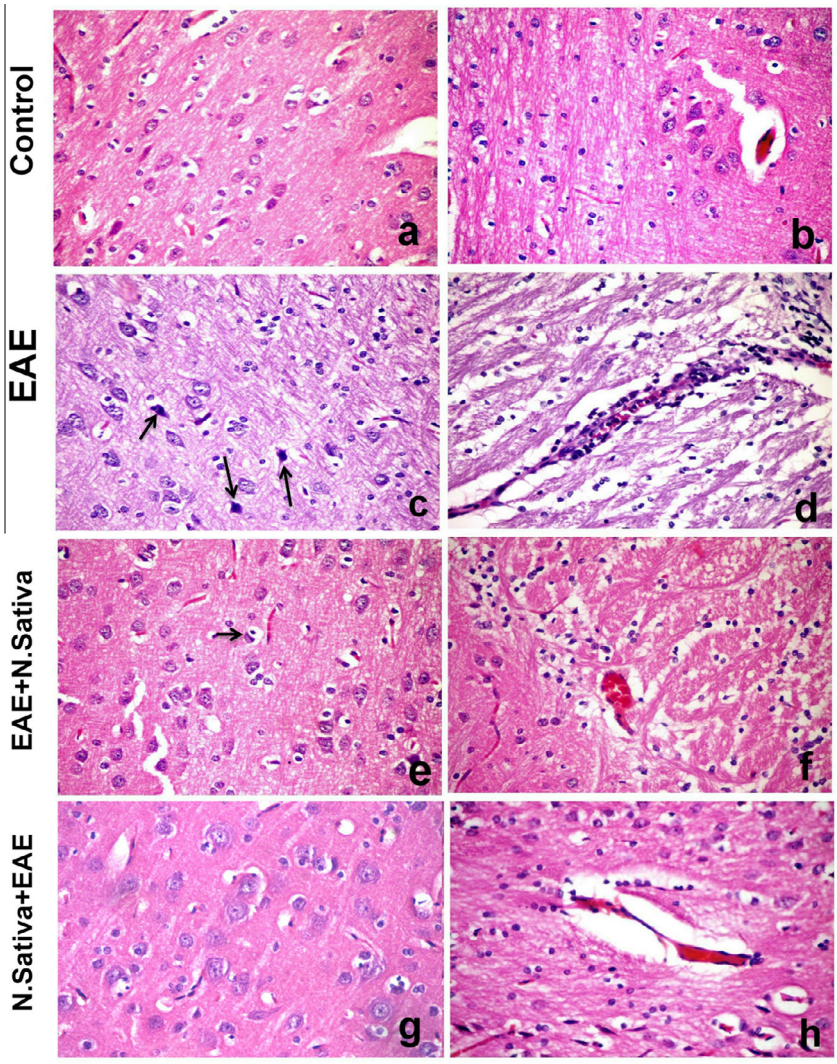

Fig. 4 Cerebrum deep gray matter and white matter (H & E ×400). (a) Control group showing normal stained motor neurons in the deep cerebral gray matter that are surrounded by glial cells. (b) Control group showing small blood vessel with normal clear Virchow Robin space. (c) "EAE" group showing extensive glial cell proliferation associated with necrosis and shrinkage of motor neurons (arrow). (d) "EAE" group showing perivascular lymphocytic aggregation with extensive glial cell proliferation in the cerebral white matter. (e) "EAE + *N. sativa*" group showing mild glial cell proliferation in cerebral gray matter with single neuronal damage. (f) "EAE + *N. sativa*" group showing glial cell proliferation in the cerebral white matter with normal empty Virchow Robin space. (g) "*N. sativa* + EAE" group showing normal motor neuronal structure and normal glial cell distribution. (h) "*N. sativa* + EAE" group showing mild glial cell proliferation in the cerebral white matter.

and decreases the severity of the disease, especially in the protected "*N. sativa* + EAE" group.

Experimental autoimmune encephalomyelitis is accompanied by an impressive loss of body weight (Encinas et al., 2001). Weight loss in EAE is most likely due to side effects of inflammatory mediators (Strassmann et al., 1992). Al-Izki et al. (2012) suggested that losing weight indicates active disease and gaining weight indicates recovery. The observed significant decrease in body weights in the "EAE" group is in line with the results of Pollak et al. (2000) and may indicate active disease and could arise from anorexia (Wekerle and Kurschus, 2006), suppression of appetite or decreasing ability to adequate fluid intake (Encinas et al., 2001).

Traditionally, a tincture prepared from *N. sativa* seeds has been shown to be useful in the cases of loss of appetite (Sharma et al., 2009). In addition, it has been reported that the supplements of medicinal plant mixture including *N. sativa* seeds improved the daily body weight gain for different farm animals (El-Gaafarawy et al., 2003; Hassan, 2009). In parallel with these reports, pre-treatment of rats, in the present study, with *N. sativa* seeds 2 weeks prior to immunization and continuation of *N. sativa* administration till 28th day post immunization succeeded in restoring the body weight of sick animals to control values from 7th to 10th day besides the 21th day post immunization. Moreover, the "*N. sativa* + EAE" group showed significant increase in body weight in comparison to the control group from 11th to 20th day post immunization. The failure in restoring control body weight in the "EAE + *N. sativa*" group may be attributed to the short period of administration of *N. sativa* seeds in comparison to the "*N. sativa* + EAE" group.

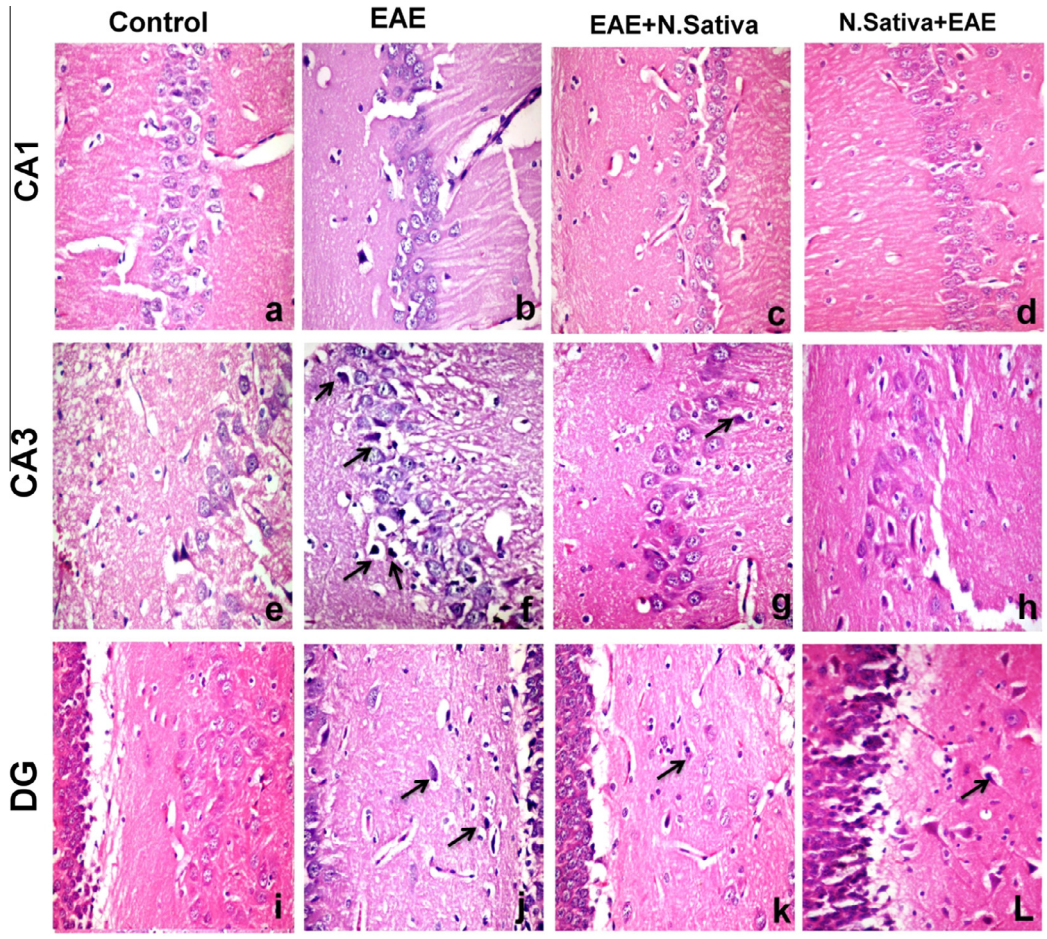

Fig. 5 Hippocampus (H&E ×400). Cornu Ammonis (CA1) (a) Control group showing normal thickness of small pyramidal cells that have vesicular nuclei. (b) "EAE" group showing a decrease in the thickness of small pyramidal cell layer with loss of neurons associated with proliferation of capillary endothelium. (c) "EAE + *N. sativa*" group showing a focal reduction in the small pyramidal cell layer thickness with focal loss neurons. (d) "*N. sativa* + EAE" group showing maintaining of the normal thickness of small pyramidal cell. CA3. (e) Control group showing aggregation of large pyramidal cell with vesicular nuclei. (f) "EAE" group showing extensive necrosis of large pyramidal cells with shrinkage of the cytoplasm and deep basophilic pyknotic nuclei (arrow). (g) "EAE + *N. sativa*" group showing individual necrosis of large pyramidal cell (arrow). (h) "*N. sativa* + EAE" group showing individual necrosis of large pyramidal cell. Dentate gyrus (DG). (i) Control group showing compact aggregation of small granular cells and molecular layer that contained pyramidal and glial cells. (j) "EAE" group showing vacuolation and extensive loss of granular cell associated with neuronal necrosis, neuronophagia (arrow) and glial cell proliferation in the molecular layer. (k) "EAE + *N. sativa*" group showing necrosis of individual neurons (arrow) with glial cell proliferation. (l) "*N. sativa* + EAE" group showing mild glial cell proliferation of the molecular layer associated with necrosis of individual neurons (arrow).

Behavior in the open field is used as a measure of exploration, anxiety, and locomotor behavior (Frye and Rhodes, 2008). The number of central square entries and the duration of time spent in the central square are measures of exploratory behavior and anxiety. A high frequency/duration of these behaviors indicates the high exploratory behavior and low anxiety levels (Walsh and Cummins, 1976). In the present work, rats of the "EAE" group spent significantly more time in the center compared to the other tested groups. It may be suggested that the rats of the "EAE" group showed higher exploratory and lower anxiety behavior levels besides less emotion with respect to other groups, which is inconsistent with the findings of the previous studies (Peruga et al., 2011; Rodrigues et al., 2011).

The number of line crosses and the frequency of rearing in OFT are usually used as measures of locomotor activity, but are also measures of exploration and anxiety. A high frequency of these behaviors indicates increased locomotion and exploration and/or a lower level of anxiety (Walsh and Cummins, 1976). In the present OFT, rats of the "*N. sativa* + EAE" group showed a significant increase in the number of rears with respect to other experimental group. It could be suggested that *N. sativa* when given 2 weeks prior to EAE induction increased the locomotor activity as well as the exploration and decreased the anxiety of rats. These results are in line with the significant amelioration of the clinical signs detected in the "*N. sativa* + EAE" group. Moreover, a previous study showed that *N. sativa seed* constituents (aqueous extract, fixed oil, volatile oil, and major constituents of the volatile oil) produced anti-anxiety effect in different tests used as models for exploration of induced anxiety (Raza et al., 2006).

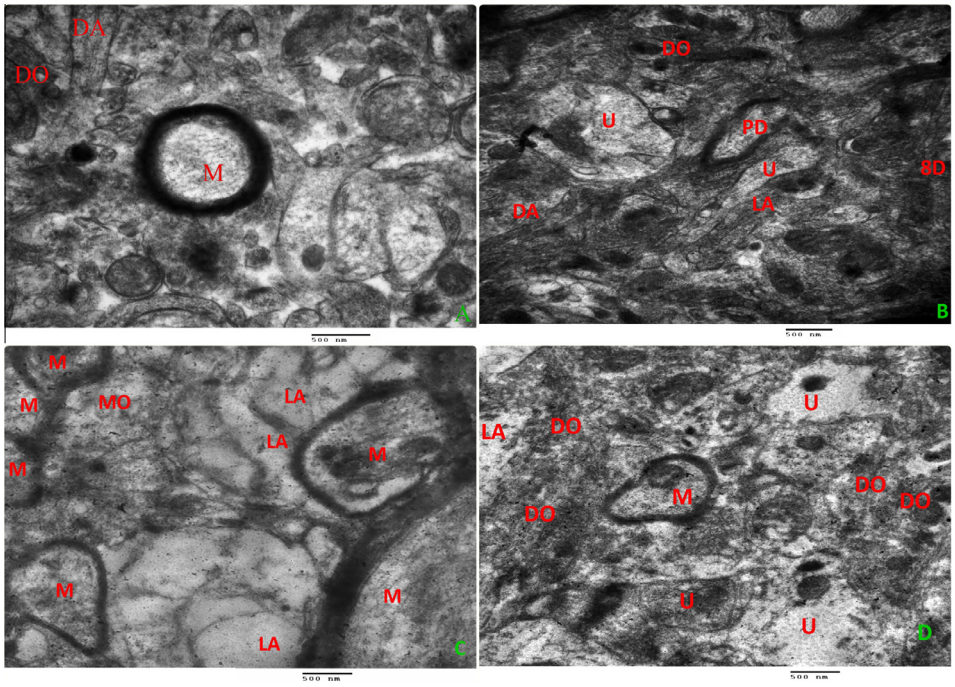

Fig. 6 TEM micrographs of the cortex. (A) Control group, (B) "EAE" group, (C) "EAE + *N. sativa*" group and (D) "*N. sativa* + EAE" group. M, myelinated fiber; U, unmyelinated fiber; PD, partially demyelinated fiber; R, remyelinated fiber; MO, medium oligodendrocyte process; DO, dark oligodendrocyte process; LA, light astrocyte process; DA, dark astrocyte process.

From the above literature and the present results of the OFT, it could be suggested that the number of rears is the preferable OFT parameter to test the EAE induced behavior changes in Wistar rats rather than the time spent in the center.

The present histopathological examination of the rats belonging to the "EAE" group revealed the appearance of perivascular mononuclear cell aggregation in the deep cerebral cortex and white matter, which is in agreement with previous study of Zamvil and Steinman (1990).

Autoimmune encephalomyelitis has originally been believed to be a "white matter disease." However, some reports suggest additional pathologic changes in the gray matter, including loss and/or atrophy of motor neurons also exist (Derfuss et al., 2009; Rudick and Trapp, 2009). These reported alterations in the gray matter of MS patients were evidenced in the present histopathological examination of the brain of rats belonging to the "EAE" group in which perivascular mononuclear cell aggregation and lesions including glial cell proliferation associated with neuronal degeneration and necrosis among variable numbers of motor neurons were seen in the deep cerebral cortex.

Demyelination in EAE as in MS is a result of inflammatory lesions in the white matter responsible for clinical deficits (Trapp et al., 1999). In the present study, the ultrastructural examination of brain areas of rats of the "EAE" group revealed slight demyelination of the cerebral cortex in addition to partial to complete demyelination of the hippocampus.

From the present clinical signs, histopathological findings and ultrastructural examination of rats belonging to the "EAE" group, it could be concluded that a single subcutaneous immunization of Wistar rat with 100 μg MOG in CFA containing 1 mg/ml of *M. tuberculosis* at the base of the tail could provide a successful EAE animal model for MS that fulfills the majority of histopathological characteristics of the well-established EAE models.

A key principle of traditional Chinese medicine is that multiple ingredients in a plant extract are more effective and less toxic than a single purified active ingredient or a purified drug derived from a plant product (Wong et al., 2010). Based on this concept, the whole *N. sativa* seed was the choice in this study to be less toxic, easy to administrate and more effective than its single purified active ingredients.

It has been reported that inflammatory cytokines and mediators are key components in the inflammatory process (Germano et al., 2008). *N. sativa* seeds were used as anti-inflammatory (Houghton et al., 1995; Mutabagani and El-Mahdy, 1997) agent. Several studies revealed that *N. sativa* seed oil contains potent, but non-toxic compounds that suppress excessive inflammatory molecules (Shahzad et al., 2009; Butt and Sultan, 2010).

In the present investigation, the extent of glial cell proliferation and neuronal damage in the deep cerebral cortex was reduced in the "EAE + *N. sativa*" group and markedly ameliorated in the "*N. sativa* + EAE" group as compared to the "EAE" group. Regarding the hippocampus, the "EAE + *N. sativa*" and "*N. sativa* + EAE" groups showed normal histological structure of granular cells.

Thymoquinone (TQ), most active ingredient in *N. sativa*, has been proved experimentally to be an anti-inflammatory substance (Chehl et al., 2009). It significantly reduced the levels of pro-inflammatory mediators {IL-1β, IL-6, TNF-α, IFN-γ and PGE (2)} and increased level of IL-10 in arthritic rats (Umar et al., 2012). Moreover, Mohamed et al. (2005) found that encephalomyelitis could be prevented and ameliorated by TQ treatment. Therefore, the protective and the ameliorative effects of *N. sativa* administration to EAE-induced rats,

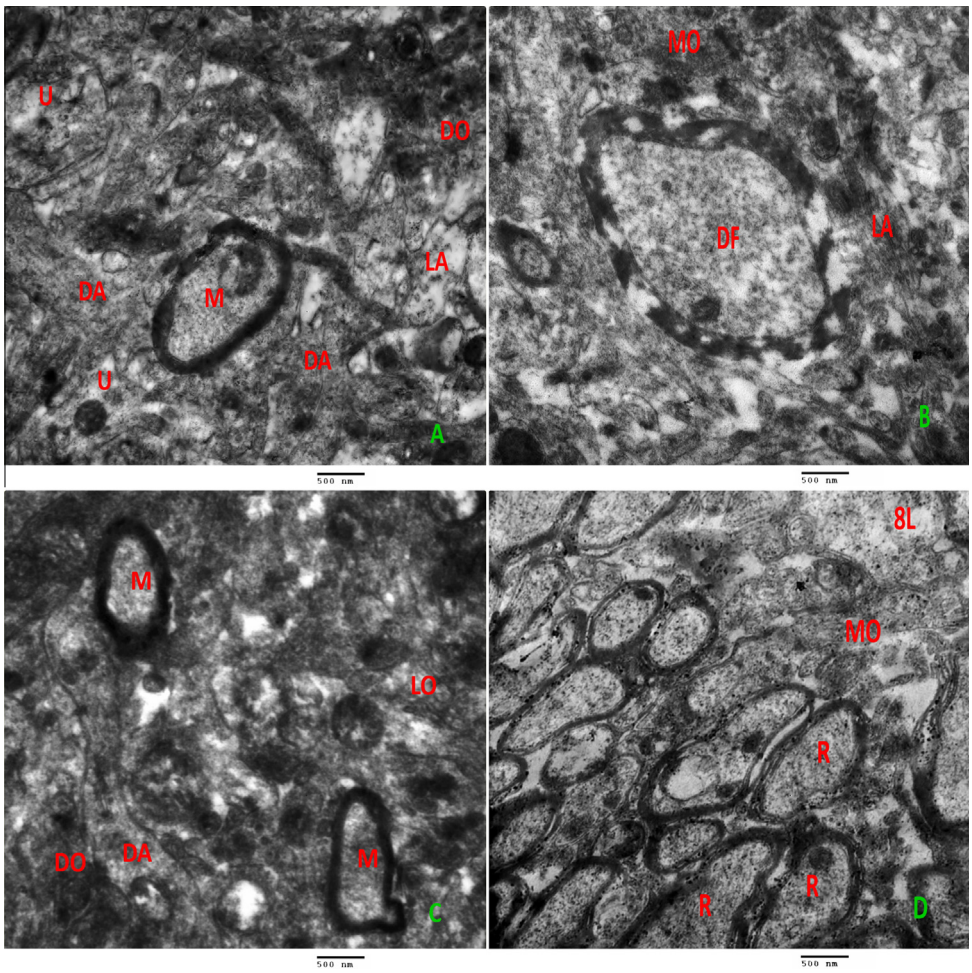

Fig. 7 TEM micrographs of the hippocampus. (A) Control group, (B) "EAE group", (C) "EAE + *N. sativa*" group and (D) "*N. sativa* + EAE" group. M, myelinated fiber; U, unmyelinated fiber; DF, demyelinated fiber; R, remyelinated fiber; LO, light oligodendrocyte process; MO, medium oligodendrocyte process; DO, dark oligodendrocyte process; LA, light astrocyte process; DA: dark astrocyte process.

Table 4 Effect of EAE induction by MOG and its treatment with *N. sativa* (2.8 g/kg) on the levels of MDA, GSH and NO and activities of catalase and GST in the cortex of adult female Wistar rats.

Oxidative stress parameter	Control group	"EAE" group		"EAE + *N.sativa*" group		"*N.sativa* + EAE" group		*F*-test
		Value	% *D*	Value	% *D*	Value	% *D*	
MDA (nmol/g tissue)	2.90 ± 0.26 (6) A	2.33 ± 0.26 (6) A	−19.66	3.61 ± 0.30 (7) AB	24.48	5.11 ± 0.70 (8) B	76.21	*
GSH (mmol/g tissue)	3.60 ± 0.34 (6) A	5.30 ± 0.19 (8) B	47.20	5.11 ± 0.46 (8) B	41.94	1.59 ± 0.16 (6) C	−55.83	*
NO (μmol/g tissue)	0.41 ± 0.04 (6) A	0.25 ± 0.03 (6) B	−39.02	0.23 ± 0.02 (7) B	−43.90	0.99 ± 0.08 (6) C	141.50	*
Catalase (U/g tissue)	13.99 ± 0.91 (5) A	14.01 ± 0.61 (5) A	0.14	7.96 ± 0.79 (6) B	−43.10	13.96 ± 0.95 (6) A	−0.21	*
GST (U/g tissue)	7.47 ± 0.11 (5) A	5.31 ± 0.33 (6) B	−28.92	5.77 ± 0.53 (8) B	−22.76	4.12 ± 0.34 (7) C	−44.85	*

Values represent mean ± S.E.M.
Number of animals is shown between parentheses.
% *D*: percentage difference in comparison to control group.
A, B and C different letters mean significant changes.
* $p < 0.05$ significant.

in the present study, could be due to its anti-inflammatory role through the suppressive effect of its components on the inflammatory cytokines and mediators which are the key components in the inflammatory processes (Germano et al., 2008).

In the present investigation, partial to complete remyelination in the *N. sativa* treated and protected groups were clearly detected by the ultrastructural examination of the brain areas under investigation.

Table 5 Effect of EAE induction by MOG and its treatment with *N. sativa* (2.8 g/kg) on the levels of MDA, GSH and NO and activities of catalase and GST in the hippocampus of adult female Wistar rats.

Oxidative stress parameter	Control group	"EAE" group		"EAE + *N. sativa*" group		"*N. sativa* + EAE" group		*F*-test
		Value	% *D*	Value	% *D*	Value	% *D*	
MDA (nmol/g tissue)	19.51 ± 0.66 (6) A	21.00 ± 1.17 (6) A	7.63	15.69 ± 1.41 (5) B	−19.58	8.47 ± 1.27 (5) C	−56.59	*
GSH (mmol/g tissue)	5.78 ± 0.78 (6)	5.14 ± 0.69 (6)	−11.07	4.26 ± 0.43 (6)	−26.30	6.62 ± 0.49 (6)	14.53	n.s.
NO (μmol/g tissue)	0.39 ± 0.03 (6) A	0.48 ± 0.03 (7) AB	23.08	0.58 ± 0.03 (6) B	48.72	0.45 ± 0.06 (6) A	15.38	*
Catalase (U/g tissue)	6.74 ± 0.36 (6) A	6.36 ± 0.26 (7) A	−5.64	3.53 ± 0.69 (6) B	−47.63	6.62 ± 0.65 (5) A	−1.78	*
GST (U/g tissue)	9.10 ± 0.40 (6)	9.14 ± 0.77 (7)	0.44	9.42 ± 0.96 (6)	3.52	8.30 ± 0.41 (5)	−8.79	n.s.

Values represent mean ± S.E.M.
Number of animals is shown between parentheses.
% *D*: percentage difference in comparison to control group.
n.s.: non-significant.
A, B and C: different letters mean significant changes.
* $p < 0.05$ significant.

It has been suggested that the inactivation of microglia inhibits EAE (Heppner et al., 2005). Previously, it had been reported that reactive microglia/macrophage contribute to demyelination in EAE by killing oligodendrocyte with the release of TNF-α (Zajicek et al., 1992). In addition, the events that occur around the lesion site include the proliferation of astrocytes, which leads to astrocytic hypertrophy and the expression of inhibitory molecules (Silver and Miller, 2004).

As seen in the pathological examination, in the present study, *N. sativa* administration resulted in a decreased number of reactive astrocytes and proliferated microglial cells in the EAE-induced rats. In support of the present role of *N. sativa*, Bano et al. (2009) suggested that *N. sativa* is useful as a potential treatment for neurodegeneration after chronic toluene exposure in rats. Hence, it could be suggested that the detected remyelinating power of *N. sativa* in the EAE-induced rats might be due to its inhibitory effect on astrocytic and microglial proliferation.

Several studies reported that the elimination of macrophages or microglia suppresses clinical and histopathological manifestations in rodent models of MS (Bauer et al., 1995; Polfliet et al., 2002; Heppner et al., 2005). Therefore, the amelioration in clinical symptoms, in the present study, in the *N. sativa* treated groups could be attributed to the suppression of inflammatory cells.

It is well known that the CNS is equipped with an endogenous antioxidant defense mechanism consisting of antioxidant enzymes. Production of these cytoprotective enzymes is induced upon exposure to ROS via a mechanism regulated at the transcriptional level (Itoh et al., 2003, 2004; Motohashi and Yamamoto, 2004).

Reduced glutathione is the major thiol present in the brain tissue, and the most important redox buffer in cells, which has an important role in the protection against oxidative injury due to ROS (Wang and Ballatori, 1998). Therefore, the present significant increases in GSH contents in the cerebral cortex of the "EAE" group were expected in view of the increased production of ROS. Unfortunately, these significant increases in cortical GSH persisted after the treatment of EAE-induced rats with *N. sativa* and reversed to significant decreases in the protected group.

Zheng and Bizzozero (2010) measured the levels of GSH and lipid peroxidation products in the cerebellum during the acute and chronic phases of MOG-induced EAE in C_{57}BL/6 mice. They found that GSH levels were reduced in both acute and chronic phases of EAE and the amount of lipid peroxidation products was similar to that in controls both at the peak of the disease and in the chronic phase. Moreover, in a recent study of Dasgupta et al. (2013), the authors measured the levels of GSH and lipid peroxide in the spinal cord regions during the course of MOG_{35-55} peptide-induced EAE in C_{57}BL/6 mice. The results showed that GSH levels were reduced during acute EAE and returned to normal values in the chronic EAE while the amount of lipid peroxidation products was high in acute EAE and decreased to nearly normal levels in the chronic phase.

The present data of the "EAE" group revealed non-significant changes in MDA levels in the cortex and hippocampus, which is in agreement with the results of Zheng and Bizzozero (2010) and Dasgupta et al. (2013). In addition, non-significant changes in GSH levels in the hippocampus of the "EAE" group was observed, which disagree with the results of Zheng and Bizzozero (2010) but agree with those of Dasgupta et al. (2013). In view of these results and the above-mentioned literature, it could be suggested that the present relapsing-remitting EAE-induced Wistar rat model attained the chronic stage at the time of decapitation (28th day post immunization). Thus, the present EAE model could be considered as a chronic-relapsing EAE model.

Treatment of EAE-induced animals with *N. sativa* after the appearance of the first clinical signs revealed non-significant changes in the cortical levels of MDA, GSH and NO as well as the activity of GST in comparison to the "EAE" group. However, protection of rats with *N. sativa* administered 2 weeks prior EAE induction and continuation of *N. sativa* administration until 28th day post immunization resulted in a significant increase in the cortical MDA level with reference to control and "EAE" rats and reversed the results of GSH and NO recorded in both "EAE" and "*N. sativa*" groups. Unluckily, this increase in the cortical MDA level could be considered as one of the probable drawbacks of *N. sativa* administration and this controversy between treated and protected groups could be due to the longer duration of *N. sativa*

administration in the protected group. In accordance with these results, Alhosin et al. (2010) had found that TQ could produce ROS metabolites, which in turn trigger mitochondrial membrane potential loss in Jurkat cells. Moreover, TQ has been reported to exert anti-oxidant activity at lower concentration (Mansour et al., 2002), but at higher concentration, it showed significant pro-oxidant effects (EL-Najjar et al., 2010; Koka et al., 2010).

Nitric oxide is a reactive molecule that is synthesized by macrophages and microglia during inflammation (Hendriks et al., 2005). It has been found that the levels of $NO/ONOO^-$ stable end products are elevated in MS patients (Giovannoni, 1998). In contrast to these studies, the data of the present investigation revealed a significant decrease in the cortical NO level in the rats of the "EAE" group in comparison to the control level.

It has been reported that NO has contradictory roles in cellular systems such as an oxidant or sometimes a scavenger of superoxide anion (Fadillioglu et al., 2003; Ilhan et al., 2004). Gulati et al. (2007) reported a reversal of function of NO from antioxidant at lower concentration, to pro-oxidant at higher concentration.

El-Mahmoudy et al. (2002) investigated the effect of TQ on NO production by macrophages after lipopolysaccharide stimulation and found that TQ suppresses the production of NO by macrophages. This reveals that TQ mediates its inhibitory effect on NO production via reduction of iNOSmRNA and protein expression. The authors suggested that this might be important in ameliorating the inflammatory and autoimmune conditions. As TQ is the main active component in *N. sativa* seeds, therefore, the present significant decrease in the cortical NO level in the "EAE + *N. sativa*" group is in accordance with the study of El-Mahmoudy et al. (2002).

In the hippocampus, results of both treated and protected groups showed a significant decrease in MDA level accompanied by a significant increase in NO level and decrease in catalase activity in the case of the treated group "EAE + *N. sativa*" only.

There is evidence that serum NO may have protective immunomodulatory effects: NO inhibits Th_1 cell proliferation, and the expression of adhesion molecules and proinflammatory cytokines (Taylor-Robinson et al., 1994; De Caterina et al., 1995). Rats recovering from EAE had significantly increased serum NO production and this seemed to protect them against a second episode of the disease (O'Brien et al., 1999). Moreover, Hummel et al. (2006) described the antioxidant effect of NO. A previous study of Kanner et al. (1992) showed that NO reduced the generation of ROS, such as hydrogen peroxide and superoxide and prevented lipid peroxidation. Therefore, it could be suggested that the increase in NO level in the hippocampus of the "EAE + *N. sativa*" group may provide a protective mechanism against suspected incoming relapse.

It is noticeable from the present oxidative stress analysis that the effect of *N. sativa* is variable in different brain areas, which may be due to the differential uptake of *N. sativa* constituents. In addition, the dose and duration of *N. sativa* administration must be taken into consideration to avoid its probable pro-oxidant effect.

In conclusion, the whole *N. sativa* seeds could attenuate the clinical signs, suppress inflammation and lead to remyelination in EAE-induced Wistar rats. Therefore, *N. sativa* seeds could be used as a protective agent or an adjunct treatment for EAE even when the treatment started after the appearance of the first clinical signs.

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Conflict of interest

No conflict of interest.

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