### Original Article Nigella sativa amliorates inflammation and demyelination in the experimental autoimmune encephalomyelitis-induced Wistar rats

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**Abstract:** Multiple sclerosis (MS) is the major, immune-mediated, demyelinating neurodegenerative disease of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model of MS. The aim of the present study was to investigate the protective and ameliorative effects of *N. sativa* seeds (2.8 g/kg body weight) in EAE-induced Wistar rats. EAE-induced rats were divided into: 1- EAE-induced rats ("EAE" group). 2- "*N. sativa* + EAE" group received daily oral administration of *N. sativa* 2 weeks prior EAE induction until the end of the experiment. 3- "EAE + *N. sativa*" group received daily oral administration of *N. sativa* after the appearance of first clinical signs until the end of the experiment. All animals were decapitated at the 28th day post EAE-induction. EAE was investigated using histopathological, immunohistochemical and ultrastructural examinations in addition to determination of some oxidative stress parameters in the cerebellum and medulla. *N. sativa* suppressed inflammation observed in EAE-induced rats. In addition, *N. sativa* enhanced remyelination in the cerebellum. Moreover, *N. sativa* reduced the expression of transforming growth factor beta 1 (TGF  $\beta$ 1). *N. sativa* seeds could provide a promising agent effective in both the protection and treatment of EAE.

**Keywords:** Experimental autoimmune encephalomyelitis (EAE), multiple sclerosis (MS), *Nigella sativa* (*N. sativa*), transforming growth factor beta 1 (TGF-β1), oxidative stress

#### Introduction

Multiple sclerosis (MS) is the major, immunemediated, demyelinating neurodegenerative disease of the central nervous system (CNS) of young adults [1, 2]. MS is considered as one of the most important causes of disability in adults. It is a chronic progressive, potentially disabling disorder with considerable social impact and economic consequences [3]. The peak age of onset of MS is between 20 and 30 years [4]. According to the National MS Society of USA, MS affects 400,000 Americans and over 2 million individuals all over the world [5].

MS usually progresses in two phases; the earlier phase starts with an autoimmune inflammatory attack against myelin sheath components followed by a chronic phase of neurodegeneration in which both the myelin sheath and the underlying axons are damaged [6]. There are several theories of MS pathogenesis implicate infiltrating T cells, pro-inflammatory cytokines, chemokines, antibody-mediated toxicity, activated macrophages, microglia and astrocytes [7-11].

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 [2, 12, 13]. EAE is used in drug development to help identify new therapeutic agents that may be beneficial in MS and other similar disorders [14].

One of the markers for MS is astrocyte activation and hyperproliferation resulting in astrogliosis [15]. Reactive astrocytes in EAE and MS lesions have been found to express pro-inflammatory chemokines [16]. Astrocytes have been found to form a glial scar around EAE and MS lesions [17, 18] which can act as a physical barrier that prevents axonal regeneration.

The transforming growth factor - beta (TGF- $\beta$ ) is a multifunctional cytokine that has been implicated in the control of inflammation and immune responses, cellular proliferation and differentiation, extracellular matrix production and degradation, angiogenesis, cell adhesion, tissue repair and scarring, and cell migration and maturation [19-25].

Oxidative stress is implicated in numerous pathologies including MS [26-30]. It has been also suggested that oxidative stress not only causes destruction of myelin but also inhibits the process of its healing [31]. Some studies have demonstrated lower levels of non-enzymatic antioxidants, including glutathione (GSH), uric acid, bilirubin, vitamin C and changes in antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in MS [26, 32-35].

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), commonly known as black cumin, belongs to the botanical family of Ranunculaceae. N. sativa seeds have been used in Middle Eastern folk medicine as a natural remedy for various diseases [36, 37]. Clinical and animal studies have shown that the extracts of the black seeds have many therapeutic effects such as neuroprotective [38]. antioxidative [39] and anti-inflammatory [40] effects. N. sativa has been widely used in neurodegenerative diseases like Parkinson and Alzheimer because of its antioxidant potential [41]. The biological activity of *N*. sativa seeds is attributed to its essential oil components [40]. The effect of *N. sativa* in EAE-induced rats was investigated in previous studies and results showed that treatment of the rats with N. sativa inhibited reactive oxygen species (ROS) production induced by EAE, showing diminished levels of malondialdehyde (MDA) of both brain and medulla spinalis tissues besides decrease in brain NO level [42-44]. In addition, Mohamed et al. [42] showed that EAE could be ameliorated by thymoquinone treatment, possibly through the increase in glutathione level.

Unfortunately, it is not currently possible to cure MS, the treatments available up to now have centered their action on the anti-inflammatory and disease course-modifying effect, and the purpose of treatment is to prevent recurrence of relapses and accumulation of disability [45]. In addition, all the approved drugs for MS treatment have potentially serious side effects and can suffer response failure during prolonged treatment [46, 47]. Previously, we studied the protective and ameliorative effects of N. sativa seeds in the cortex and hippocampus of EAE-induced rats [48]. The present study was extended to investigate these effects of N. sativa in the cerebellum and medulla of EAE-induced rats besides the role of TGF- $\beta$  in the disease course.

#### Materials and methods

#### Experimental animals

The experimental animals used in this study were adult female Wistar rats (age between 6-8 weeks) at the beginning of the study. The animals were kept under fixed appropriate conditions of housing. The water and chow were provided *ad libitum*. All animals received humane care in compliance with the guidelines of the Ethical Committee of National Research Center, Egypt and that followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

#### Chemicals

Rat synthetic myelin oligodendrocyte glycoprotein peptide 35-55 (MOG<sub>35-55</sub>) was purchased from Titan Biotech Limited, Bhiwadi, India. Complete Freund's adjuvant (CFA), Thiobarbituric acid (TBA) and 98% reduced glutathione were purchased from Sigma Aldrich, Germany. Phosphate buffered saline (PBS) was obtained from the Bio Basic Inc. USA. 1-chloro-2, 4 dinitrobenzene (CDNB) was purchased from Sigma Aldrich, St. Louis, USA. 98% Trichloroacetic acid (TCA) was obtained from SDFCL, Egypt. Triology (cat # 920P-06) was obtained from Cell Margue, CA-USA. Power stain TM 1.0 Poly HRP DAB kit (cat # 54-0017) was purchased from Genemed Biotechnologies, CA-USA. The concentrated rabbit polyclonal TGF \$1 antibody (cat #



**Figure 1.** Effect of *N. sativa* administration on the cerebellar histopathological findings (H&E ×400). A. Control group showing the molecular layer containing a number of glia cells. B. Control group showing cerebellar medullary area containing glia cells. C. "EAE" group showing few lymphocytic infiltrations in the meningeal tissue associated with gliosis of the cerebellar molecular layer. D. "EAE" group showing reactive astrogliosis {note the astrocytes with eosin-ophilic increased cytoplasmic size (arrow head) and the appearance of axonal spheroid (arrow)}. E. "EAE + *N. sativa*" group showing mild gliosis of the cerebellar molecular layer with normal meningeal histology. F. "EAE + *N. sativa*" group showing mild reactive astrogliosis of the cerebellar medulla (arrow head). G. *N. sativa* + EAE" group showing mild gliosis of the cerebellar molecular layer with normal meningeal histology. H. "*N. sativa* + EAE" group showing mild astrogliosis in the cerebellar medulla that is only characterized by astrocytes hyperplasia not hypertrophy.



**Figure 2.** Effect of *N. sativa* administration on the brain stem histopathological findings (H&E ×400). A. Control group showing clusters of nerve nuclei surrounded by glia cells. B. Control group showing normal clear Virchow Robbin space. C. "EAE" group showing chromatolysis and necrosis of nerve nuclei with reactive astrogliosis (arrow). D. "EAE" group showing perivascular lymphocytic cuffing (arrow) with diffuse gliosis. E. "EAE + *N. sativa*" group showing necrosis of individual nerve nuclei (arrow). F. "EAE + *N. sativa*" group showing mild perivascular mononuclear cell aggregation. G. "*N. sativa* + EAE" group showing gliosis with chromatolysis of individual nerve nuclei. H. "*N. sativa* + EAE" group showing focal gliosis of neuropill with scarce perivascular mononuclear cell aggregation.

orb11468) was obtained from Biorbyt Ltd, Cambridge, UK. Phosphate buffer pH 7.4 (50

mM/L, Triton x 0.1%, EDTA 0.5 mµ), potassium phosphate buffer pH 6.5 (100 mM/L) and kits

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**Figure 3.** Effect of *N. sativa* administration on the TGF- $\beta$ 1 expression in the rat brain. A. Control group showing negative immunolabeling astrocytes for TGF- $\beta$ 1 (×400). B. "EAE" group showing wide positive immunolabelling of astrocytes with strong positive brown color staining reaction (arrow) (×200), inserted box with higher magnification (×400). C. "EAE + *N. sativa*" group showing less and weak brown immunolabelling of astrocytes (×200). D. "*N. sativa* + EAE" group showing very few immunolabelling of astrocytes with very faint brown staining reaction (arrow) (×200), inserted box with higher magnification (×400).

for the determination of oxidative stress parameters were purchased from Bio diagnostic Co., Giza, Egypt. Narcotan Halothane was acquired from EIMC pharmaceuticals Co. Egypt.

### Experimental autoimmune encephalomyelitis (EAE) induction

According to Adelmann et al. [49], the EAE was induced with some modifications. EAE was induced by a single subcutaneous injection in the tail base with an emulsion of 100  $\mu$ g MOG<sub>35-55</sub> dissolved in 100  $\mu$ l phosphate buffered saline (PBS), mixed with 100  $\mu$ l complete Freund's adjuvant (CFA) containing 1 mg/ml of Mycobacterium tuberculosis. All rats were immunized under light halothane anesthesia.

#### Preparation of Nigella sativa (N. sativa) seeds

*N. sativa* seeds were purchased from a local market in Cairo, Egypt. Whole *N. sativa* seeds were grinded and suspended in distilled water and administered orally to rats (2.8 g/kg body

weight) by means of a gastric tube. Zaoui et al. [50] reported that the  $LD_{50}$  of *N. sativa* oil in rats is 28.8 ml/kg. Therefore, to be completely safe, the dose of *N. sativa* seeds used in this study was calculated as 1/10 of  $LD_{50}$  of its oil.

As known in the traditional Chinese medicine, the 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 [51]. According to this concept, the whole *N. sativa* seeds were used in this study to be less toxic, easy to administrate and more effective than a single purified active ingredient.

#### Experimental design

Animals immunized with MOG were divided into 3 groups: 1- EAE-induced animals ("EAE" group) received daily oral administration of distilled water (2 ml for a rat weighing 200 g) until the end of the experiment. 2- The animals of the second group ("*N. sativa* + EAE" group) received



**Figure 4.** Effect of *N. sativa* administration on the ultrastructure of myelin sheath in the rat medulla. (A) Control group, (B) "EAE" group, (C) "EAE + *N. sativa*" group and (D) "*N. sativa* + EAE" group. M: Myelinated fiber; T: Mitochondria; DF: Demyelinated fiber; PD: Partially demyelinated fiber; R: Remyelinated fiber; MO: Medium oligodendrocyte process; DO: Dark oligodendrocyte process; LA: Light astrocyte process and DA: Dark astrocyte process.

daily oral administration of N. sativa (2.8 g/kg) 2 weeks prior EAE induction till the end of the experiment. 3- Animals of the third group ("EAE + N. sativa" group) received daily oral administration of N. sativa (2.8 g/kg) after the appearance of first clinical signs until the end of the experiment. 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. Rats were observed for 28 days following immunization and were weighed and scored daily. In each group, animals were identified individually by marking lines around the tail with indelible ink pens. All animals were sacrificed by sudden decapitation at the 28th day post immunization with MOG. Control animals were sacrificed simultaneously with the treated groups.

#### Clinical scoring (grades of symptoms)

The evaluation of clinical scores was done and published in our previous study. For detail, see Fahmy et al. [48].

#### Handling of tissue samples

The animals were sacrificed by sudden decapitation at 28th day post immunization. The control animals were sacrificed simultaneously with the treated groups. For biochemical analysis, the brain of each animal was quickly removed and rapidly transferred to an ice-cold Petri dish and dissected to obtain the cerebellum and medulla. Each brain area was weighed and kept at -58 °C until analysis.

#### Preparation of histopathological samples

The brains, from each experimental group, were fixed in 10% buffered formalin for twenty four hours. Washing was done with tap water, and then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56°C hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 8 microns by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized and



**Figure 5.** Effect of *N. sativa* administration on the ultrastructure of myelin sheath in the rat cerebellum. (A) Control group, (B) "EAE" group, (C) "EAE + *N. sativa*" group and (D) "*N. sativa* + EAE" group. M: Myelinated fiber; U: Unmyelinated fiber; T: Mitochondria; DF: Demyelinated fiber; R: Remyelinated fiber; MO: Medium oligodendrocyte process; DO: Dark oligodendrocyte process; LA: Light astrocyte process; DA: Dark astrocyte process and V: focal area of myelinolysis in the myelin sheath.

stained with hematoxylin and eosin (H & E) stain. Slides were then examined through the light microscope (Zeiss, Germany) to investigate the brain stem and cerebellum.

# Immunohistochemistry (IHC) protocol: transforming growth factor beta-1 (TGF $\beta$ 1) expression by astrocytes

The investigated brain sections were prepared according to the procedures described by Ahmed et al. [52]. The brains from different experimental groups were cut into 4  $\mu$ m sections, then fixed in a 65°C hot oven for 1 hour. Triology is a product that combines the three pre-treatment steps: deparaffinization, rehydration and antigen unmasking. This product enhances standardization of the pre-treatment procedure, thereby producing more consistent, more reliable results. Slides were placed in a Coplin jar filled with 200 ml of the triology work-

ing solution, then: the jar was securely positioned in the autoclave. The autoclave was adjusted so that the temperature reached 120°C and was maintained stable for 15 minutes, after which pressure was released and the Coplin jar was removed to allow slides to cool for 30 minutes. Brain sections were then washed and immersed in Tris Buffer Saline (TBS) to adjust the pH; this was repeated between each step of the IHC procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. Power Stain TM 1.0 Poly HRP (Horseradish Peroxidase) DAB Kit was used to visualize any antigen-antibody reaction in the tissues. The concentrated rabbit polyclonal TGF *B*1 antibody was diluted according to manufacturer specification at 1:500. 2-3 drops from each antibody were applied, and then slides were incubated in the humidity chamber overnight at 4°C. Henceforward, poly

Oxidative stress	Control group "EAE" group		up	"EAE + N. sativa" group		"N. sativa + EAE" group		E toot
parameter		Value	% D	value	% D	Value	% D	F-lest
MDA (nmol/g tissue)	15.00 ± 0.70 (6) A	11.06 ± 0.80 (7) B	-26.27	12.95 ± 0.87 (6) AB	-13.67	12.15 ± 0.56 (5) B	-19.00	*
GSH (mmol/g tissue)	2.98 ± 0.35 (8) AB	2.41 ± 0.11 (7) A	-19.13	3.41 ± 0.21 (6) B	14.43	3.24 ± 0.24 (6) B	8.72	*
NO (µmol/g tissue)	0.26 ± 0.04 (7)	0.47 ± 0.04 (7)	80.77	0.44 ± 0.07 (7)	69.23	0.27 ± 0.04 (6)	3.85	n.s.
Catalase (U/g tissue)	14.01 ± 0.73 (5) A	17.49 ± 0.75 (5) B	24.84	10.63 ± 0.74 (6) C	-24.13	16.60 ± 1.93 (5) AB	18.49	*
GST U/g tissue)	4.19 ± 0.14 (6)	5.01 ± 0.38 (8)	19.57	5.19 ± 0.43 (9)	23.87	5.09 ± 0.32 (7)	23.54	n.s.

 Table 1. Effect of N. sativa administration on the oxidative stress parameters in the cerebellum of rats

Values represent the mean  $\pm$  S.E.M. The number of animals is shown between parentheses. % D: Percentage difference in comparison to control group. n.s.: Non-significant. \*P < 0.05 significant. A, B and C: Different letters mean significant changes.

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Oxidative stress	Control group	"EAE" group		"EAE + N. sativa" group		"N. sativa + EAE" group		- E toot
parameter		Value	% D	Value	% D	Value	% D	F-lest
MDA (nmol/g tissue)	9.45 ± 0.96 (5) A	9.56 ± 0.79 (6) A	1.16	4.40 ± 0.49 (6) B	-53.44	8.48 ± 0.82 (6) A	-10.26	*
GSH (mmol/g tissue)	3.37 ± 0.21 (7) A	4.34 ± 0.22 (7) B	28.78	4.29 ± 0.29 (9) B	27.30	1.99 ± 0.16 (7) C	-40.95	*
NO (µmol/g tissue)	0.47 ± 0.05 (6) A	0.41 ± 0.03 (7) AB	-12.77	0.32 ± 0.02 (6) B	-31.91	0.43 ± 0.02 (6) AB	-8.5	*
Catalase (U/g tissue)	20.62 ± 0.89 (5) A	21.06 ± 1.03 (5) A	2.13	11.26 ± 0.85 (7) B	-45.39	12.16 ± 0.69 (6) B	-41.03	*
GST (U/g tissue)	5.29 ± 0.55 (6)	5.52 ± 0.44 (6)	4.35	5.24 ± 0.37 (6)	-0.95	4.78 ± 0.15 (6)	-9.64	n.s.

Table 2. Effect of *N*. sativa administration on the oxidative stress parameters in the medulla of rats

Values represent the mean  $\pm$  S.E.M. The number of animals is shown between parentheses. % D: Percentage difference in comparison to control group. n.s.: Non-significant. \**P* < 0.05 significant. A, B and C: Different letters mean significant changes.

HRP enzyme conjugate was applied to each slide for 20 minutes. DAB chromogen was prepared and 2-3 drops were applied to each slide for 2 min. DAB was rinsed, after which counterstaining with Mayer Hematoxylin and cover slipping were performed as the final steps before slides were examined under the light microscope (Zeiss, Germany).

#### Transmission electron microscopy (TEM)

The cerebellum and medulla from each experimental group, were primarily fixed in 3% glutaraldehyde and phosphate buffer (pH 7.4) at 4 °C for 4 hours. The samples were then rinsed with phosphate buffer isotonic- pH 7.4 and postfixed in 1% osmium tetraoxide ( $OsO_4$ ) phosphate buffer pH 7.4 for one hour and half at room temperature. The samples were then washed briefly in phosphate buffer pH 7.4 for 10 minutes and this was done three times. The brain samples were then dehydrated by a graded ethanol series (50% ethanol for 10 minutes, 70% ethanol for 10 minutes, 95% ethanol for 10 minutes and another 10 minutes in 100% ethanol). Then,

brain samples were infiltrated using propylene oxide (2 times, 15 minutes for each at room temperature). The samples were then infiltrated with solvent/resin mixture (propylene oxide. Araldite for 15-60 minutes) and then (propylene oxide. Araldite overnight). The specimen vials were rotated during all infiltration steps. The samples were then infiltrated with 100% resin (Araldite or Epson) and this was done 2 times, one in the early AM and the other in the late PM. Samples in Araldite were placed at the apex or in inverted Polyphone BEEM capsule filled with liquid resin. Capsules were then heated at 60°C overnight for 24 hours. After that, blocks can be stored after cutting out capsule. Thin sections were cut using an RMC MT-X ultra-microtome and collected on copper grids. Images were collected using a JEOL transmission electron microscope JEM 1400 (Jeol, LTD, Tokyo, Japan) operating at 80 KV.

#### Tissue homogenization

Oxidative stress parameters were estimated in the cerebellum, and medulla. Each brain area was homogenized in 2 ml phosphate buffer (50 mM, pH 7.4) and 2 ml were used to rinse the homogenizer (Heidolph, Germany). The homogenates were then centrifuged at 8000 r.p.m. (x g = 7012) for 15 minutes. The supernatants were stored at -58°C until use.

#### Determination of lipid peroxidation

Lipid peroxidation was determined by measuring the levels of thiobarbituric reactive species (expressed by malondialdehyde, MDA) according to the spectrophotometric method of Ruiz-Larrea et al. [53]. The thiobarbituric acid reactive substances react with thiobarbituric acid. The absorbance of the produced pink colored complex is read at 532 nm in a Helios Alpha Thermospectronic (UVA 111615, England).

### Determination of reduced glutathione (GSH) levels

Reduced glutathione (GSH) levels were assayed by using Biodiagnostic kit No. GR 25 11 according to the spectrophotometric method described by Beutler et al. [54]. It is based on the reduction of 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) with glutathione to produce a yellow color whose absorbance is measured at 405 nm.

#### Determination of Nitric oxide (NO) levels

According to the spectrophotometric assay of Montgomery and Dymock [55], NO was measured by using Biodiagnostic kit No. NO 25 33. This method depends on the measurement of endogenous nitrite concentration as an indicator of nitric oxide production. The produced azo dye has a bright reddish-purple color whose absorbance is read at 540 nm.

#### Determination of catalase activity

Catalase activity was assayed using Biodiagnostic Kit No. CA 25 17 which is based on the spectrophotometric method of Aebi [56]. Catalase reacts with a known quantity of hydrogen peroxide. After 1 min, the reaction is stopped with catalase inhibitor. The remaining hydrogen peroxide reacts with 3,5-Dichloro-2hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone in the presence of peroxidase to form a chromophore whose absorbance is read at 510 nm. The color intensity is inversely proportional to the activity of catalase in the sample.

## Determination of glutathione-S-transferase (GST) activity

Glutathione-S-transferase activity was determined according to the method of Habig et al. [57]. 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 minutes. After incubation, 0.1 ml of reduced glutathione (30 mmol/l) was added. At one-minute interval, the change in absorbance was measured at 340 nm.

#### Statistical analysis

Data of oxidative stress parameters were analyzed by analysis of variance (ANOVA) followed by the 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. Percentage difference representing the percent of variation in the value with respect to the control was also calculated.

%Difference (%D) =  $(\frac{treated value-control value}{control value}) \times 100\%$ 

#### Results

## Effect of EAE induction and its treatment with *N*. sativa on the histopathological findings in the brain of Wistar rats

The histopathological alterations in the cerebellum of the "EAE" group were characterized by focal degeneration of Purkinje cells, which was associated with less lymphocytic infiltration in the meninges of cerebellar folia (Figure 1C). Diffuse gliosis of molecular layer and little perivascular mononuclear cell aggregation were detected in the cerebellar medullary center, while severe astrogliosis (hyperplasia & hypertrophy of astrocytes) with the appearance of axonal spheroids were evident in the cerebellar medulla (Figure 1D). The absence of meningeal inflammatory cell infiltration and perivascular mononuclear cell aggregation in the cerebellar medullary was noticed in the "EAE + N. sativa" and "N. sativa + EAE" groups. On the other hand, reactive astrogliosis of cerebellar medulla was moderately reduced in the "EAE + N. sativa" (Figure 1E & 1F) and markedly reduced in the "N. sativa + EAE" group (Figure 1G & 1H).

Regarding the brain stem, especially the pons, the typical histopathological picture of the "EAE" group was seen and was moderately reduced in the "EAE + *N. sativa*" group and markedly ameliorated in "*N. sativa* + EAE" group (**Figure 2A-H**).

Effect of EAE induction and its treatment with N. sativa on the expression of TGF- $\beta$ 1 by astrocytes in Wistar rats.

Immunohistochemical analysis of the brain sections for the expression of TGF- $\beta$ 1 by astrocytes in different experimental groups is illustrated in **Figure 3.** The immunohistochemical examination revealed wide and strong expression of TGF- $\beta$ 1 and strong positive staining reaction of astrocytes in the brain of the "EAE" group compared to the "EAE + *N. sativa*" group which showed relatively low expressions of the astrocytes for TGF- $\beta$ 1. In addition, the group "*N. sativa* + EAE" showed very faint expression and weak positive staining of astrocytes.

Effect of EAE induction and its treatment with *N*. sativa on the myelin sheath ultrastructure in Wistar rats

The ultrastructural examination of the "EAE" group revealed partial to complete demyelin-

ation in the medulla (**Figure 4**) in addition to thin and marked myelin destruction with severe myelinolysis of nerve fibers in the cerebellum (**Figure 5**). These changes were alleviated in *N*. *sativa* treated and protected groups. TEM micrographs of the brain areas of "EAE + *N*. *sativa*" group showed partial to complete remyelination in the medulla besides complete remyelination of most nerve fibers in the cerebellumwithwell-developed myelinsheath.Inaddition, most of the myelinated fibers in the "*N*. *sativa* + EAE" group were in a state of remyelination in the cerebellum and medulla.

Effect of EAE induction by MOG and its treatment with N. sativa on some oxidative stress parameters in the cerebellum and medulla of Wistar rats

As indicated in **Table 1**, significant decreases in cerebellar MDA levels were detected in "EAE" and "*N. sativa* + EAE" groups with reference to control values. GSH levels in the cerebellum showed significant increases in the *N. sativa* treated and protected groups with respect to "EAE" group. In addition, a significant increase in the catalase activity of the "EAE" group was obvious when compared to the control group. Nevertheless, a significant decrease in catalase activity was noticed in the cerebellum of "EAE + *N. sativa*" group with respect to control (-24.13%), "EAE" and "*N. sativa* + EAE" groups.

As seen in **Table 2**, MDA levels in the medulla of "EAE + *N. sativa*" group showed a significant decrease relative to other experimental groups. Significant increases in the GSH levels in the medulla of both "EAE" and "EAE + *N. sativa*" groups in comparison to control levels were observed. However, a significant decrease in GSH level was recorded in the "*N. sativa* + EAE" group with reference to other experimental groups. A significant decrease in NO level in the medulla of "EAE + *N. sativa*" in comparison to control value was recorded. Significant decreases in catalase activity in the medulla in both *N. sativa* treated and protected groups with regard to control and "EAE" groups were detected.

#### Discussion

The present histopathological examination of EAE-induced animals revealed the appearance of perivascular mononuclear cell aggregation in the cerebellum besides little lymphocytic infiltration in the meninges. In addition, perivascu-

lar lymphocytic cuffing with diffuse gliosis were seen in the brain stem, especially the pons. These histopathological alterations are in agreement with the findings of Zamvil and Steinman [58]. They showed that EAE development is characterized by the infiltration of reactive leukocytes into the CNS. Moreover, severe astrogliosis with the appearance of axonal spheroids in the cerebellar medulla was observed in EAE-induced rats. Furthermore, focal to diffuse gliosis with microglial proliferation was observed in different areas of the cerebellar medullary center. Given that one of the markers for MS is astrocyte activation and hyperproliferation resulting in astrogliosis [15], therefore, it is obvious that histopathological findings observed in the "EAE" group, in the present work, are in harmony with the reported findings of the previously documented EAE models.

The demyelination in EAE as in MS is a result of inflammatory lesions in the white matter which is responsible for clinical deficits [59]. Neuropathology of the active lesion in the CNS of mice with relapsing EAE shows a severe inflammatory response, primary demyelination with moderate remyelination, and some axonal loss [60]. These lesions become more gliotic and depleted of axons, but less severely demyelinated as the disease becomes chronic [61].

In the present study, the ultrastructural examination of brain areas of rats of the "EAE" group revealed partial to complete demyelination in the medulla. In addition, marked myelin destruction with severe myelinolysis of nerve fibers was shown in the cerebellum. Therefore, the present ultrastructural findings provide a direct support for the success of the present EAE model induction in Wistar rats. The severe myelinolysis observed in the present study in the cerebellum is consistent with the previous results that showed that the cerebellum is the CNS region, which is commonly affected in MS [62] and in MOG peptide-induced EAE [63].

*Nigella sativa (N. sativa)* seeds were used as anti-inflammatory agent [64, 65]. Recently, in vitro study of Alemi et al. [66] indicated that extracts of *N. sativa* seeds and callus have antiinflammatory effect on primary mix glial cells prepared from the cerebellum of Wistar rat. The chemical analysis of *N. sativa* total oil revealed the presence of both a fixed oil and a volatile oil. Thymoquinone (TQ) that may attain up to 27.8% of the volatile oil (w/w) is one of the pharmacologically active constituents of N. sativa [64, 67]. It has been reported that inflammatory cytokines and mediators are key components in the inflammatory process [68]. Several studies revealed that N. sativa seed oil contains potent, but non-toxic, compounds that suppress excessive inflammatory molecules [69-71].

In the present investigation, the severe inflammation detected by histopathological examination were markedly ameliorated in the N. sativa treated groups. The meningeal inflammatory cell infiltration and perivascular mononuclear cell aggregation were absent in the cerebellar medulla of the "EAE + *N. sativa*" and "*N.* sativa + EAE" groups. Moreover, reactive astrogliosis of cerebellar medulla was moderately reduced in the "EAE + N. sativa" and markedly reduced in the "N. sativa + EAE" group with comparison to the "EAE" group. In addition, lesions that were found in the brain stem of "EAE" group, especially the pons was moderately ameliorated in the "EAE + N. sativa" group and markedly ameliorated in "N. sativa + EAE" group.

Thymoquinone (TQ) has been proved experimentally to be an anti-inflammatory substance [72]. It significantly reduced the levels of proinflammatory mediators {IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IFN- $\gamma$  and Prostaglandin E2} and increased level of IL-10 in arthritic rats [73]. Moreover, Mohamed et al. [74] found that encephalomyelitis could be prevented and ameliorated by TQ treatment, possibly through the inhibition of NF-<sub> $\kappa$ </sub>B {nuclear factor kappa-light-chain enhancer of activated B cells, a transcription factor that regulates the expression of many genes, including enzymes, cytokines, cell cycle regulatory molecules and angiogenic factors [75].

Therefore, the protective and the ameliorative effects of *N. sativa* administration on EAE-induced rats, 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 [68].

In the present investigation, partial to complete remyelination in the *N. sativa* treated and pro-

tected groups were clearly detected by the ultrastructural examination of the brain areas under investigation.

It has been reported that glial scar formation is one of the major impediments that hinders axon regeneration after CNS injury. The events that occur around the lesion site include the proliferation of astrocytes, which leads to astrocytic hypertrophy and the expression of inhibitory molecules [76]. In addition, the activation of microglia and recruitment of peripheral macrophages from the blood stream are also involved in glial scar formation. The latter ones have been proposed to function as another significant barrier for axon regeneration [77, 78]. Also, it has been suggested that inactivation of microglia inhibits EAE [79]. Previously, it has been reported that reactive microglia/macrophage contribute to demyelination in EAE by killing oligodendrocytes with the release of TNF-α [80]. However, Nikić et al. [81] suggested that inflammatory axonal damage might be spontaneously reversible.

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. [82] 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.

It has been reported that oxidative stress is a major player in the pathogenesis of both MS and EAE [83]. Excessive production of ROS, primarily by activated microglia/macrophages and astrocytes, leads to severe oxidative stress, which contributes significantly to tissue damage [26].

Data of the present investigation showed significant increase in cerebellar catalase activity in animals belonging to the "EAE" group when compared to the control group. It is well known that catalase is expressed in the CNS where it catalyzes the conversion of hydrogen peroxide into water and molecular oxygen [84]. Van Horssen et al. [85] reported that catalase immunostaining is observed in myelin-laden macrophages and astrocytes of MS brain tissue. Therefore, the present significant increase in cerebellar catalase activity in the "EAE" group could be considered as a result of certain regulatory mechanism to counteract the liberated ROS in this brain area which showed severe demyelination in the present data of histopathological examination. However, treatment of EAE-induced rats with N. sativa ("EAE + N. sativa" group) reversed the recorded significant increase in cerebellar catalase activity to a significant decrease while non-significant change in cerebellar catalase activity in comparison to control value was observed in the "N. sativa + EAE" group.

The present data revealed significant increases in the GSH levels in the medulla of rats of the "EAE" group with reference to control values. GSH is the major thiol present in brain tissue, and the most important redox buffer in cells, which has an important role in the protection against oxidative injury due to ROS [86]. Therefore, the present significant increases in GSH contents in the medulla of the "EAE" group were expected in view of the increased production of ROS. Unfortunately, these significant increases in the medullary GSH persisted after the treatment of EAE-induced rats with *N. sativa* and reversed to significant decreases in the protected group "*N. sativa* + EAE" group.

In the cerebellum, the significant increase in the GSH level in both treated and protected groups with respect to the "EAE" group confined to a previous study done by Mohamed et al. [42] that have found that when EAE animals received TQ, they showed higher GSH levels compared with EAE untreated animals.

Zheng and Bizzozero [83] 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. [87], 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. Their 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 were high in acute EAE and decreased to nearly normal levels in the chronic phase.

In comparison to the control group, the present data of the "EAE" group revealed non-significant change in MDA level in the medulla which is in agreement with the results of Zheng and Bizzozero [83] and Dasgupta et al. [87]. In addition, non-significant change in the GSH level in the cerebellum of the "EAE" group was observed, a result which disagrees with the findings of Zheng and Bizzozero [83] but agree with that of Dasgupta et al. [87].

In view of these results and the above mentioned literature, it could be suggested that the present relapsing-remitting EAE-induced Wistar rat model, as revealed by Fahmy et al. [48], 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.

Analysis of the oxidative stress parameters in the present investigation revealed a significant decrease in MDA level in the cerebellum of the animals belonging to the "EAE" group in comparison to control level. This significant decrease in the cerebellar MDA level could be due to the severe myelinolysis in this brain region of the "EAE" group. It is worth mentioning that the cerebellar MDA level recorded nonsignificant change compared with control level in the "EAE + N. sativa" group which may be attributed to the remyelination (according to the present TEM results) which occurred after the treatment with N. sativa. However, protection by N. sativa failed to prevent the significant decrease in MDA level that was observed in the "EAE" group.

Data of the "EAE + *N. sativa*" group showed a significant decrease in the medullary NO level in comparison to control level. This decrease was accompanied by a significant decrease in MDA level with reference to control, "EAE" and "*N. sativa* + EAE" groups. It has been reported that NO has contradictory roles in cellular systems such as an oxidant or sometimes a scavenger of superoxide anion [88, 89]. Gulati et al. [90] reported a reversal of function of NO from antioxidant at lower concentration, to pro-oxidant at higher concentration.

El-Mahmoudy et al. [91] investigated the effect of TQ on the 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 iNOS mRNA and protein expression. The authors suggested that this may 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 NO level in the "EAE + *N. sativa*" group could meet the results obtained by El-Mahmoudy et al. [91].

Astrocytes are involved in multiple and complex actions, including the production of pro- and anti- inflammatory cytokines [92-96]. TGF- $\beta$ 1 and its receptors are expressed in the CNS inflammatory lesions of MS patients and in various animal models of the disease [93, 97, 98]. It has been suggested that after neuronal injury, TGF- $\beta$ 1 is expressed in most CNS cell types and is rapidly upmodulated, mainly in astrocytes and microglia [99-101].

In the present study, the immunohistochemical examination of brain tissue of the "EAE" group showed very strong expression of astrocytes for TGF- $\beta$ 1 in the brain tissue of the "EAE" group. This result is in parallel with that of Link et al. [102] who found that TGF- $\beta$ 1 is produced by myelin-specific T-lymphocytes isolated from peripheral blood and cerebrospinal fluid of patients with MS.

Although several studies suggested a role for TGF- $\beta$ 1 in CNS injury, it is still unclear whether overproduction of TGF- $\beta$ 1 in these conditions represents a strictly protective compensatory response or somehow contributes to the disease process [103-104]. Unfortunately, these studies on the role of TGF- $\beta$ 1 in MS and EAE models didn't give univocal reports. The study of Luo et al. [105] demonstrated that TGF- $\beta$ 1 in the CNS may be critical in promoting disease. They proposed that CNS-produced TGF-B1 creates a permissive environment for the accumulation of pathogenic T cells and propagation of an autoimmune response. In addition, results of an earlier study of Wyss-Coray et al. [106] indicated that, at least in certain pathological conditions, overexpression of TGF-B1 by resident CNS cells can promote inflammation and immune-mediated CNS disease.

In contrast, it has been reported that TGF- $\beta$ 1deficient mice develop a multifocal autoimmune-type inflammatory disease [109] reported that the lack of TGF- $\beta$ 1 expression in the brain of neonatal mice results in a widespread increase in degenerating neurons accompanied by prominent microgliosis. In addition, several studies have demonstrated that systemic administration of exogenous TGF- $\beta$ 1 inhibits EAE [110, 111] and that administration of TGF- $\beta$ -neutralizing antibodies enhances disease severity [112].

In the present study, immunohistochemical analysis of brain sections of *N. sativa* treated and protected groups indicated relatively few expressions of the astrocytes for TGF- $\beta$ 1 in the "EAE + *N. sativa*" group and very faint expression with weak positive staining of astrocytes in the "*N. sativa* + EAE" group with reference to the "EAE" group. Since the present histopathological investigation showed decreased number of reactive astrocytes as a result of *N. sativa* administration, thus, it is expected to detect little expression of the *N. sativa* treated and protected groups.

The strong expression of astrocytes for TGF- $\beta$ 1 which was accompanied by inflammation and demyelination in the brain sections of the "EAE" group may indicate the inflammatory role of TGF- $\beta$ 1 in the EAE model. This indication is supported by the relatively few expressions of the astrocytes for TGF- $\beta$ 1 with decreased inflammation and remyelination in the *N. sativa* treated and protected groups. In addition, the present reported anti-inflammatory role of *N. sativa* could be due to its inhibitory effect on the expression of astrocytes for TGF- $\beta$ 1.

In conclusion, the whole *N. sativa* seeds could provide a promising agent effective in both the protection and treatment of EAE.

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#### **Disclosure of conflict of interest**

None.

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