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ROS GENERATION IN THE DEVELOPING CHICK EMBRYO BY CITRAL AND THE MITIGATIVE EFFECT OF *NIGELLA SATIVA*

Reda Abdel-Rahman Ali*, Hanem Saad Abdel-Tawab, Dalia Elzahraa Farouk Mostafa

Zoology Department, Faculty of Science, Assiut University, Assiut 71516, Egypt.

***Corresponding Author: Reda Abdel-Rahman Ali**
Zoology Department, Faculty of Science, Assiut University, Assiut 71516, Egypt. www.aun.edu.eg
Mail ID: reda@aun.edu.eg

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ABSTRACT
Impairment of retinoic acid results in different malformations during embryonic development. *Nigella sativa* is used in public medicine for treatment of a wide range of diseases. The present study aimed to evaluate the *in vivo* antioxidant effect of *N. sativa* L. seed extracts during development of the chick embryo. The embryos were treated with citral to inhibit endogenous retinoic or treated with a combination of citral and *N. sativa* extract. ROS (The reactive oxygen species), was measured as indicator for oxidative stress. ROS significantly increase in the embryo after treatment with citral. Treatment with *N. sativa* extract only exerted antineoplastic properties through elevating the ROS to significantly higher levels compared to control. Co-treatment with citral and *N. sativa* partly mitigated the oxidative stress levels by reducing the ROS levels compared to citral alone. The partial mitigation of *N. sativa* is suggested to be due to its antineoplastic properties, the high levels of ROS produced by citral above its antioxidative capacity, mode of extraction or the low concentration used in the experiment. Also, *N. sativa* is not recommended as a food supplement for infants and newborns.

KEY WORDS: ROS, oxidative stress, citral, *Nigella sativa*, chick embryo.

INTRODUCTION
Retinoids, the derivatives of vitamin A are critical for many aspects of embryonic development as clearly indicated in the embryos suffered vitamin A-deficieny (Clagett-Dame & DeLuca, 2002). RA (retinoic acid) is an active regulator of cell growth and differentiation (Love & Gudas, 1994).

Treatments with exogenous RA induced teratogenic effects during morphogenesis of developing chick embryos. The defects included malformations in the brain, head, eyes, nostrils, beak, neck, trunk region fore- and hind limbs and tail region (Ali *et al*., 2007). On the other side, endogenous RA is critical for morphogenesis and patterning of different aspects of the embryo including the nervous elements, limbs, branchial arches and front and nose regions of the developing face (Song *et al*., 2004). Vitamin A is locally converted to active metabolites in the tissues of the embryo. The enzymes required to convert retinol to RA include 4 members of the retinaldehyde dehydrogenase enzyme family, RALDH1-4 (ALDH1a1-4) (Lin *et al*., 2003). Once metabolized in the cytosol, RA becomes potentially active in patterning the developing structure through binding to three types of nuclear RA receptors (RARα, β, γ). The RARs may dimerize with RXR receptors resulting in provocation of downstream genes, however, unliganded RA receptors can repress transcription by recruitment of other cofactors (Weston *et al*., 2003). In the experiments of Song *et al*. (2004) using citral, the RALDH antagonist, to evaluate the role of RA signaling from the nasal pit *in vivo*, when beads soaked with citral were implanted into the nasal pit of the developing chicken embryos, there was a specific loss of derivatives from the lateral nasal prominences. Providing exogenous RA enhanced development of the beak demonstrating that most citral-induced defects were produced by the specific blocking of RA synthesis. Citral prevented production of RA through inhibition of retinaldehyde dehydrogenases (Kikonyogo *et al*., 1999).

ROS are produced during normal aerobic metabolism. Increased levels of ROS are known to be involved in the cell apoptosis during embryonic development (Kane *et al*., 1993). ROS were found to be connected with neurodegenerative diseases like amyotrophic lateral sclerosis, Alzheimer's, and Parkinson's disease (Delanty & Dichter, 1998). Consistently, antioxidants promote survival of neural cells in apoptotic models both *in vitro* and *in vivo* (Krohn *et al*., 1998).

It is well known that redox status is an important controlling factor during embryonic development, and oxidative stress, which occurs as a consequence of

increased contents of ROS, plays a pivotal role in cell death (Colton *et al*., 1995).

RA has been shown to reduce susceptibility to oxidative stress in PC12 cells (Jackson *et al*., 1991). Ahlemeyer & Krieglstein (1998) demonstrated that RA reduced staurosporine-induced apoptotic cell death in chick embryonic neurons and that suppression of mitochondrial ROS production may be responsible for the mechanism of action. The same authors found that exogenous treatment with RA protected against oxidative stress through partial reduction of the amount of mitochondrial ROS in cultured neurons of chick embryo (Ahlemeyer & Krieglstein, 2000).

Citral which is an important constituent of the essential oils from lemon grass *Cymbopogon citratus* has antioxidant and free radical scavenging activities in mice (Shah *et al*., 2011).

N. sativa is a plant belongs to the family Ranunculaceae (Ali & Blunden, 2003). Its common name is the black seed or black cumin (Amin & Hosseinzadeh, 2016). The seeds, which are used for medicinal purposes are the most important parts of this plant. They contain fixed oils, volatile oils, alkaloids, proteins, and saponins (Ali & Blunden, 2003). Investigations indicated that thymoquinone (TQ) and other active components of *N. sativa* have been shown to possess several potential therapeutic properties including antioxidant, anticancer, antimicrobial, antidiabetic, anti-inflammatory, analgesic, and immunomodulator ones (Ali & Blunden, 2003). Also, it has been evaluated in clinical trials with various health conditions such as, hyperlipidemia, diabetes mellitus, hypertension, asthma, allergy, cough, bronchitis, fever, headache, infertility, rheumatoid arthritis and gastrointestinal diseases (Khazdair, 2015).

The important constituents of *N. sativa*, mainly TQ, have effective therapeutic properties; they exerted high anti-inflammatory effects during treatment of many disorders that are characterized by inflammatory effects. Such disorders include encephalomyelitis, colitis, Edema, and arthritis. The anti-inflammatory effects were achieved through suppression of the inflammatory mediators such as prostaglandins and leukotrienes (Hajhashemi *et al*., 2004).

N. sativa is characterized by its effective antioxidative properties. Seeds of *N. sativa* could preserve clearly the spatial cognitive in rats suffered from chronic cerebral hypoperfusion (Azzubaidi *et al*., 2012). Also, Abbasnezhad *et al*. (2015) found that *N. sativa* extract reduced the oxidative stress in diabetic rats.

The present study aimed to study the effect of citral, the retinoic acid inhibitor, on production of ROS during development of the chick embryo and to find out whether the extract of *N. sativa* has a protective role to play against the oxidative stress induced by ROS.

MATERIAL AND METHODS

Chick embryos: Fertilized eggs of the chick *Gallus domesticus* (*Dandrawi* strain), obtained from the farm of Faculty of Agriculture, Assiut University, were used in the experiments of the present investigation. All embryological materials needed for the experiments were obtained by artificial incubation using an electrical thermostatically controlled incubator. The incubator was located in a well-ventilated place and was accurately adjusted at 37.5± 0.1°C before use. Both the trays of the eggs and inside of the incubator were thoroughly cleaned using dettol and ethyl alcohol. Sterilization was carried out using Biocidal ZF reagents from Wak-Chimie Germany. Labeled fertile eggs were placed vertically in the trays inside the incubator. Ventilation was allowed in the incubating chamber. Relative humidity was automatically adjusted at 52%. Incubated eggs were automatically turned approximately bihourly from side to another until their operation time. The incubator used in the present study belongs to PTO, Egypt, model C5.

Experimental design

The incubated eggs were divided into nine groups,
The first group: was left untreated as a control one.
The second group: was injected with 100 µl of saline solution as a solvent.
The third, fourth and fifth groups: each of these groups received one injection per embryo each one was 100 µl of different doses of citral (Sigma-Aldrich Inc., USA), i.e. 50 µM, 100 µM, and 200 µM respectively, in saline solution.
The sixth group: received 5 µl cold pressed *N. sativa* extract (Imtenan Health Shop, Egypt), suspended in saline solution in a total volume of 100 µl.
The seventh, eighth and ninth groups: received both the three doses of citral and *Nigella sativa* together respectively.

All the injections were carried out just before incubation. Eggs were thoroughly cleaned with alcohol. A hole was done at the blunt area of the egg. Injection was carried out by means of a Hamilton microsyringe. The needle was inserted vertically for a suitable distance into the yolk sac. The hole was then sealed with a sealing tape. The eggs were incubated until they were taken out at intervals after 4 and 6 days of injection and incubation for obtaining the required embryonic stages.

Specimens' preparation

The eggs were carefully opened under physiological saline solution. Embryos were carefully removed from the yolk and membranes and they were transferred to a new saline solution for washing and then fixed in Kal's solution or 10% neutral formalin. After fixation, the specimens were preserved in 70% ethyl alcohol. Specimens were dehydrated in a series of ethanol, cleared in cedar wood oil, washed in chloroform, embedded in paraffin wax and transverse serial sections were prepared at 7 microns thick (Drury & Wallington, 1980). DCFH-DA (2′7′-dichlorofluorescein diacetate) is

one of the well-known probes for the detection of intracellular ROS formation (Wang & Joseph, 1999). DCFH-DA is cell permeable, and, after being intracellular, it is cleaved by intracellular esterases to 2′, 7′-dichlorofluorescin (DCFH), confined to the cells, and oxidized to the fluorescent molecule 2′, 7′-dichlorofluorescein (DCF) by different ROS. DCFH-DA is considered a general indicator of ROS, reacting with H_2O_2, $ONOO^-$, lipid hydro peroxides, and, to a lesser extent, $O_2^{\bullet-}$. Since H_2O_2 is a secondary product of $O_2^{\bullet-}$, DCF fluorescence has been used to detect $O_2^{\bullet-}$ production. Fixed sections of chick embryos are incubated with DCFH-DA (5 µmol/L; Molecular Probes) (Khatri *et al*., 2004) for 30 minutes at 37°C, for *in situ* detection of H_2O_2. Sections incubated with vehicle can serve as negative controls. All of the images are acquired by Fluorescence microscope at identical settings. Optical densities of the chemiluminescence (fluorescence) were measured in certain areas of the micrographs of the diencephalon wall, eye and spinal cord. Analysis of the micrographs was carried out using the software Image J (the JAVA SE Runtime Environment, version 6).

Statistical analysis

The data were expressed as mean ± SE. The results were analyzed statistically using column statistics and one-way analysis of variance with the Newman–Keuls multiple comparison test as a posttest. These analyses were carried out using Prism software for windows, version 5.0 (Graph pad software Inc., San Diego, California, USA), SPSS (version 22) and Excel (Microsoft office 10). Differences between and among the groups were considered significant if $P < 0.05$, 0.01, or 0.001.

RESULTS

Figures 1& 2 and table 1 revealed that after four days of treatment, the fluorescence density (reflecting ROS generation) in the brain wall of all the treated groups was higher than the untreated control group. Statistical analysis revealed a significant difference between control and treated groups and among treated groups. Treatment with *N. sativa* significantly elevated the level of ROS production compared to control. Combinations of *N. sativa* and citral significantly increased ROS levels compared to control but it was significantly lower than that of the highest citral treatment.

Six days after treatment, the brain wall showed that citral treatments resulted in non-significant increase in ROS levels compared to control, while ROS levels was significantly lower than control after *N. sativa* treatment. Combinations of *N. sativa* and citral none significantly changed ROS levels compared to control (Figs. 3& 4 and Table 1).

Four days after treatment, spinal cord showed a significant decrease of ROS levels due to treatment with the lowest dose of citral compared to control, while there was a significant increase due to the other two citral treatments. *N. sativa* treatment resulted in no change compare to control. Combinations of *N. sativa* and citral resulted in non-significant increase of ROS levels compared to control, except for the highest dose of citral where there was a significant increase of ROS compared to control (Figs. 5& 6 and Table 1).

After six days of treatment, spinal cord showed highly significant increase in the levels of ROS after citral treatments compared to control. *N. sativa* treatments none significantly increased ROS levels compared to control. Combinations of *N. sativa* and citral treatments resulted in significant increase in ROS levels compared to control, except for the medium dose of citral, where the increase in ROS levels was non-significant. In the same time such treatments resulted in significant decrease in ROS levels compared to the highest two doses of only citral treatments (Figs. 7& 8 and Table 1).

Four days after treatment, eye tissues exhibited a significant increase in ROS levels due to citral treatments except for the lowest dose, where the increase was non-significant. *N. sativa* treatment and combinations of *N. sativa* and citral resulted in a significant increase in ROS levels compared to control, except for the highest dose of citral where the increase was non-significant. In the same time, all the combinations of *N. sativa* and citral produced significant decrease in ROS levels compared to the treatment with the highest dose of citral alone (Figs. 9& 10 and Table 1).

Six days after treatment, eye tissues exhibited non-significant change in ROS levels compared to control (Figs. 11& 12 and Table 1).

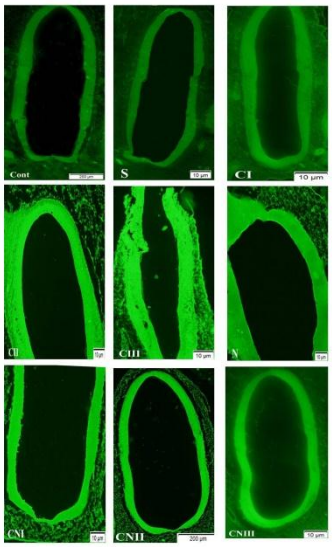
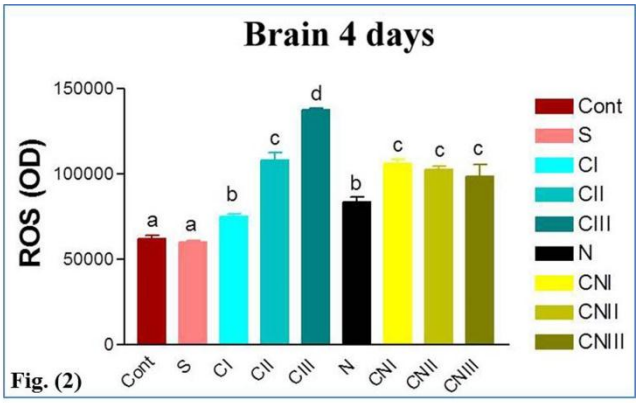


Fig. (1)



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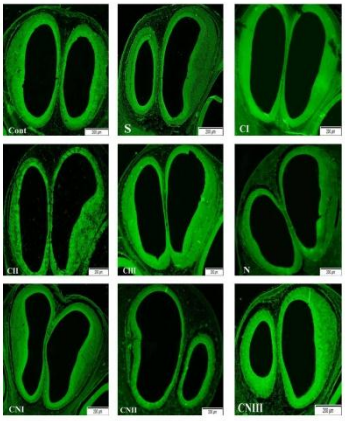

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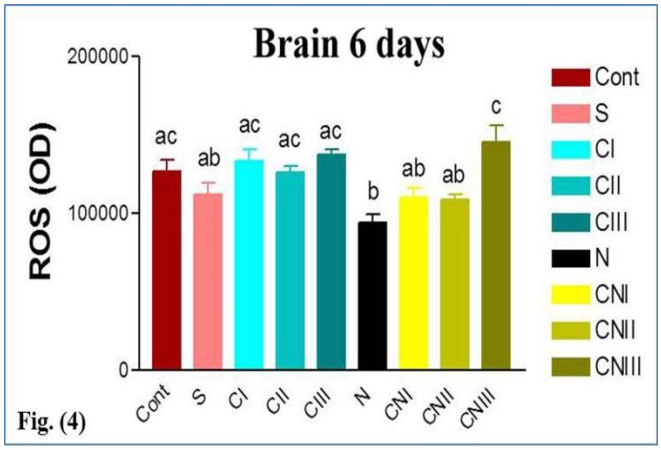

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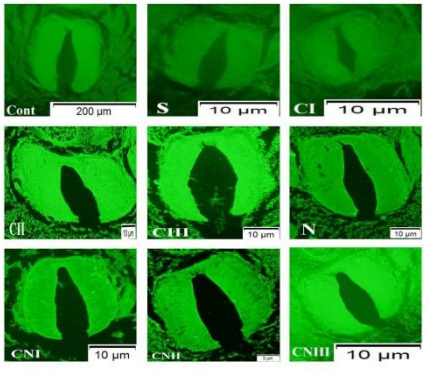

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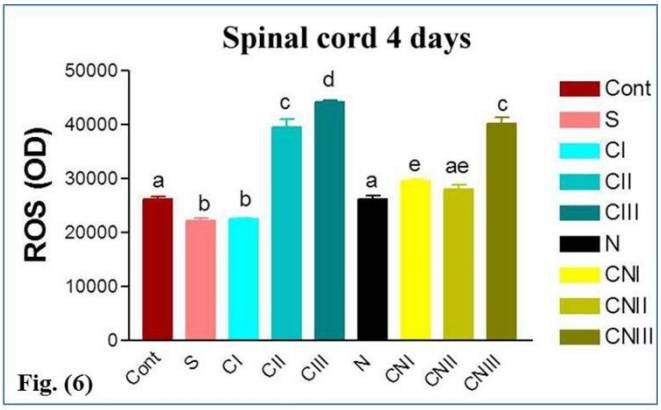

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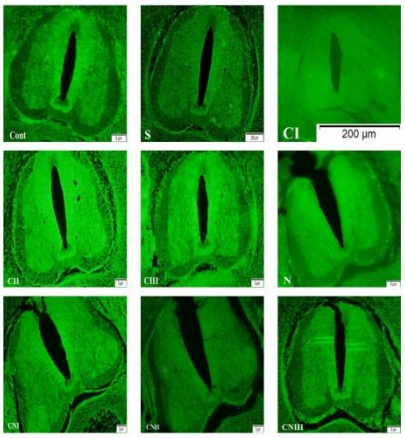

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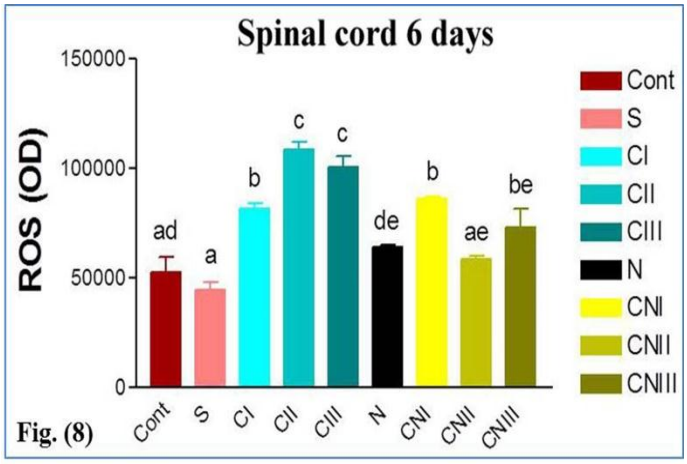

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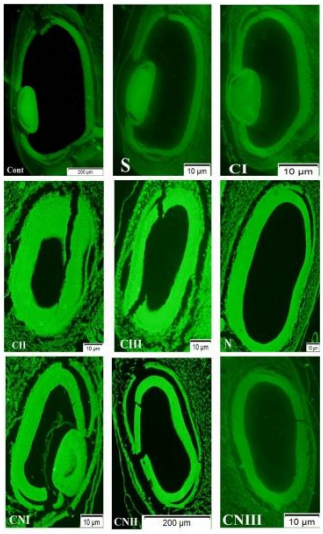

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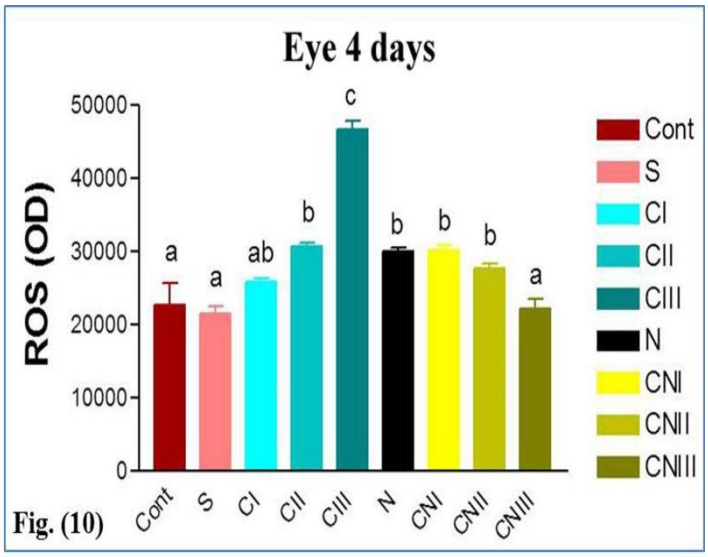

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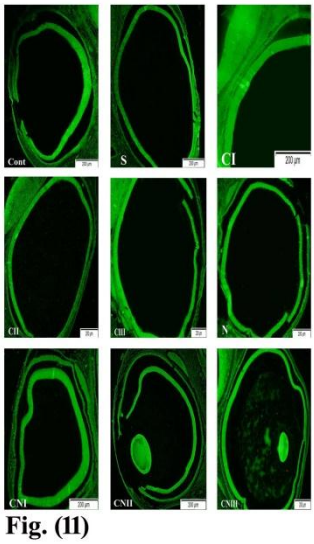


Fig. (11)

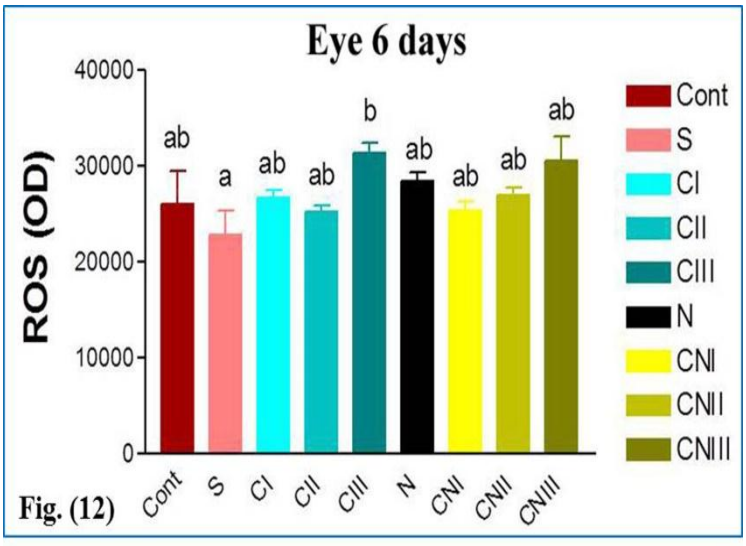


Fig. (12)

FIGURES LEGENDS

Fig. (1): Fluorescent photomicrographs of the brain wall of chicks of different treated groups, reflecting production of ROS. A comparison between groups, four days after treatment.

Fig. (2): Production of ROS at the brain wall. A comparison between different treated groups, four days after treatment. A quantitative image analysis. For all figures: a, b, c& d: significant difference between groups.

Columns are presented as means ± SE. Cont: control, S: saline, CI: 50 µM-citral, CII: 100 µM-citral, CIII: 200 µM-citral, N: 5 µl- *N. sativa*, CNI: 50 µM-citral+5 µl- *N. sativa*, CNII: 100 µM-citral+5 µl- *N. sativa*, CNIII: 200 µM-citral+5 µl- *N. sativa*.

Fig. (3): Fluorescent photomicrographs of the brain wall of chicks of different treated groups, reflecting production of ROS. A comparison between groups, six days after treatment.

Fig. (4): Production of ROS at the brain wall. A comparison between different treated groups, six days after treatment. A quantitative image analysis.

Fig. (5): Fluorescent photomicrographs of the spinal cords of chicks of different treated groups, reflecting production of ROS. A comparison between groups, four days after treatment.

Fig. (6): Production of ROS at the spinal cord. A comparison between different treated groups, four days after treatment. A quantitative image analysis.

Fig. (7): Fluorescent photomicrographs of the spinal cords of chicks of different treated groups, reflecting production of ROS. A comparison between groups, six days after treatment.

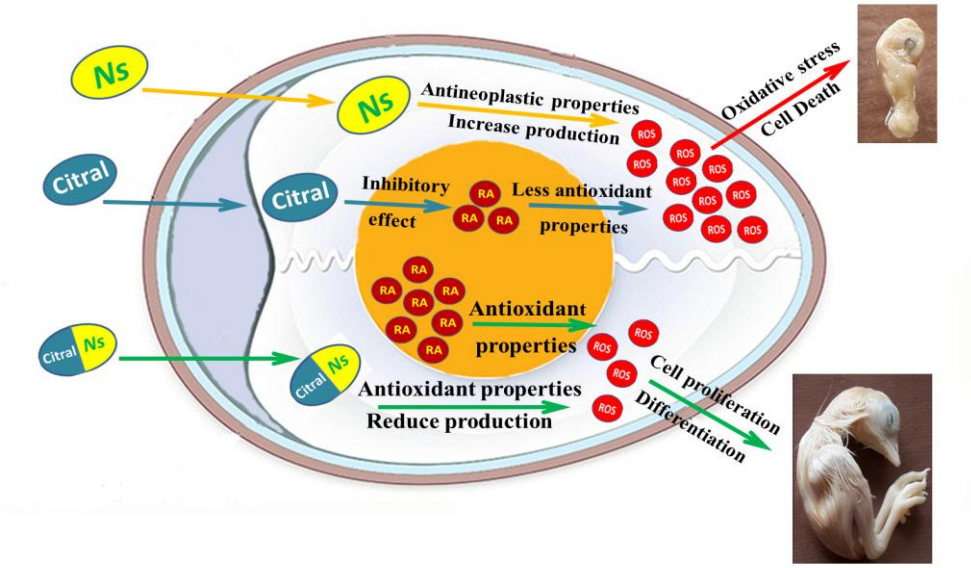
Fig. (8): Production of ROS at the spinal cord. A comparison between different treated groups, six days after treatment. A quantitative image analysis.

Fig. (9): Fluorescent photomicrographs of the eyes of chicks of different treated groups, reflecting production of ROS at the retinal wall. A comparison between groups, four days after treatment.

Fig. (10): Production of ROS at the retinal wall. A comparison between different treated groups, four days after treatment. A quantitative image analysis.

Fig. (11): Fluorescent photomicrographs of the eyes of chicks of different treated groups, reflecting production of ROS at the retinal wall. A comparison between groups, six days after treatment.

Fig. (12): Production of ROS at the retinal wall. A comparison between different treated groups, six days after treatment. A quantitative image analysis.



Antineoplastic & antioxidant properties of *Nigella sativa*

Table (1): Effect of different treatments on optical density (reflecting the amount of ROS production. Quantitative image analysis. a, b, c, d, e: A significant difference between groups.

Groups / Parameters		Control	Saline	50µM Citral	100µM Citral	200µM Citral	5 µL *N. sativa*	50µM Citral + 5 µL *N. sativa*	100µM Citral + 5 µL *N. sativa*	200µM Citral + 5 µL *N. sativa*
4 days	Brain	62290±1918^{a}	60350±1027^{a}	75100±1605^{b**}	108500±4195^{c***}	138000±804.9^{d***}	83820±3061^{b***}	106000±2903^{c***}	102800±2070^{c***}	98810±7008^{c***}
	Spinal cord	26230±478.5^{a}	22130±462.4^{b**}	22470±192.7^{b**}	39580±1342^{c***}	44090±460.2^{d***}	26150±638.0^{a}	29480±391.6^{e**}	28070±710.6^{ae}	40140±1111^{c***}
	Eye	22770±2946^{a}	21500±1053^{a}	25830±603.7^{ab}	30620±488.4^{b**}	46730±1119^{c***}	30010±461.2^{b**}	30220±593.3^{b**}	27780±643.9^{b*}	22200±1327^{a}
6 days	Brain	126900±7284^{ac}	112400±6846^{ab}	133600±6818^{ac}	126300±3896^{ac}	137000±3928^{ac}	94160±5367^{b*}	110000±5910^{ab}	108800±3495^{ab}	145200±11080^{c}
	Spinal cord	52690±6755^{ad}	44420±3425^{a}	81600±2452^{b***}	108500±3267^{c***}	100600±4873^{c***}	63940±1006de	85840±977.0^{b***}	58360±1636^{ae}	72960±8499^{be*}
	Eye	25960±3487^{ab}	22840±2487^{a}	26720±741.6^{ab}	25230±655.4^{ab}	31300±1088^{b}	28460±869.7^{ab}	25340±899.6^{ab}	26980±798.2^{ab}	30550±2516^{ab}

Data are presented as means ± SE. *: Significance when P<0.05. **: Significance when P<0.01. *: Significance when P<0.001.**

DISCUSSION

The present result revealed that citral treatment induced oxidative stress in the developing chick embryo via elevating the ROS production levels. *N. sativa* treatment partially reduced the high levels of oxidative stress produced due to citral induction of ROS levels.

Ahlemeyer & Krieglstein (1998) suggested that reduction of ROS levels by treatment with exogenous RA to staurosporine induced apoptosis in chick neurons is thought to be a causative factor for the antiapoptotic effect of RA. This means in the present study that, blocking or reduction of endogenous RA by citral, which is mediated by inhibition of retinaldehyde dehydrogenases (Kikonyogo *et al*., 1999) might lead to high levels of ROS due to the inhibition of RA synthesis. These citral induced increased levels of ROS were concentration dependent.

Hyatt *et al*. (1996) found a dose dependent increase in cell death in retinas and other brain regions of zebra fish embryos exposed to increased citral concentrations. Embryos showed smaller sizes and retinal tissues were below normal development. These increased levels of cell death should be through increased levels of ROS production which was observed in the present study. Our results support and confirm the findings of Hyatt *et al*. (1996).

Patel *et al*. (2015) studied the effect of citral on breast cancer cells. They found that citral increased ROS production and elevated p53 activity resulting in induction of apoptosis in breast cancer cells. Sheikh *et al*. (2017) examined the ability of citral to induce apoptosis and cytotoxic effects in colorectal cancer cell lines. They found that citral increased intracellular ROS level while glutathione levels were decreased in HCT116 and HT29 cells which were reversed with N-acetycysteine indicating that citral induced mitochondrial-mediated apoptosis via augmentation of intracellular ROS. They suggested that citral induced p53 and ROS-mediated mitochondrial-mediated apoptosis in human colorectal cancer HCT116 and HT29 cells.

Citral (180 μM) inhibited the breast cancer cells growth, arrested the cell cycle arrest at G2/M phase, suppressed cell proliferation and promoted apoptosis through increased ROS production (Ghosh, 2013).

Citral (3,7-dimethyl-2,6-octadienal) was found to prevent formation of RA via inhibition of the oxidation of retinol in mouse epidermis, thereby interfering with the biological effects of retinol in the mouse skin (Connor, 1988). Citral can act as a substrate for both the alcohol and aldehyde dehydrogenases resulting in inhibition of both steps in RA synthesis from retinol. Also, in *Xenopus laevis* embryos citral inhibited the formation of RA and thus citral treatment can rescue the embryos from the teratogenic effects of exogenous retinol (Schuh *et al*., 1993). Because the retinoids are metabolized independently in the limb bud (Thaller & Eichele, 1990), citral applied to the limb bud can inhibit RA formation there.

One of the most important explanations for the degraded effect of citral in generating ROS after six days of treatment comparing with early stages (four days after treatment) is the possibility that citral acted as a substrate for aldehyde dehydrogenases and being metabolized and no longer acting as dehydrogenase inhibitor and thus no allowing production of endogenous RA resulting in antioxidant activity and lowering ROS generation. Kikonyogo *et al*. (1999) explored the biochemical behavior of citral. They indicated that a mixture of two geometric isomers, geranial and neral exists in both natural and synthetic citral. They also mentioned that citral was found to inhibit all the isozymes of aldehyde dehydrogenase. They found that regaining of aldehyde dehydrogenase activity after long incubation in the presence of NAD and formation of NADH from citral suggested that citral was a substrate. Identification of reaction products indicated the formation of geranic acid thus establishing the suggestion that citral acted as a substrate.

ROS production might be in the mitochondria through disruption of electron transport chain resulting in increase of superoxide anions ($O_2^{\bullet-}$) due to induction of apoptosis in retinal cells. Also, there are extra mitochondrial sources of intracellular ROS such nitric oxide ($NO^{\cdot}$) through nitric oxide synthase or through production of ($O_2^{\bullet-}$) through pathways including xanthine oxidase or phospholipase A2 (Gil *et al*., 2003).

One of the suggested mechanisms that organisms respond to oxidative stress is by adaptation reactions like inducing antioxidant molecules such as glutathione, heme oxygenase, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. These adaptive responses to increased levels of ROS are suggested to be participating in the process of tolerance. Ahlemeyer *et al*. (2001) suggested that RA supports the endogenous antioxidant system as an adaptive response and might be more successful than radical scavengers with regard to a long term protection.

The present study revealed an increase in the ROS levels after treatment with citral and this might be suggested to be a reflection for the decreased levels of endogenous RA. Also, we found a decrease in the amounts of glutathione measured after citral treatments (data not published yet). Furthermore, the oxidative stress induced by citral as indicated by high levels of ROS might be suggested to be due to decreased levels of antioxidant molecules. It is suggested here that decreased effect of citral in inducing ROS production after six days of treatment comparing with the earlier (four days after treatment), especially in the brain wall and eye, might be due to either consumption of citral itself as a substrate for retinaldehyde dehydrogenases or due to adaptive

response. Our results coincide and confirm the findings of the previous studies (Ahlemeyer *et al*., 2001; Kikonyogo *et al*., 1999; Patel *et al*., 2015 and Sheikh *et al*., 2017).

Treatment with *N. sativa* extract in the present study revealed a significant increase in ROS levels after four days of treatment in the eye and brain wall, while the increase was non-significant in case of spinal cord.

N. sativa extract is known for its anticancer therapeutic properties due to presence of TQ, one of bioactive compounds in the black seed extract. Ali and Blunden (2003) stated that TQ and other active components of *N. sativa* have many potential anticancer therapeutic properties. Hussain *et al*. (2011) found that by releasing of ROS, TQ is considered to be a potent inducer of apoptosis in different primary effusion lymphoma cell lines. Yu & Kim (2013) found that that TQ possesses antineoplastic properties and significantly increased apoptosis in chondrocytes via increasing ROS generation. Apoptosis was expressed depending on concentration and duration of TQ.

According to Zubair *et al*. (2013), TQ has antioxidative power at lower concentrations and most of the studies that tried to explain the mechanism of action have focused on the antioxidant property. However, other researchers have indicated that TQ may exert prooxidant properties at higher concentrations (Koka *et al*., 2010). Many articles also have repotred that in spite of its low concentrations, antioxidants with plant origin might exert prooxidant features if any transition metal ions were found around. Copper and iron are examples of such ions in this condition. Preneoplastic cells and neoplastic cells such as cancer cells or cells of developing embryos have been reported to possess significantly increased levels of copper when compared with normal cells (Gupte & Mumper, 2008), and may be more critically sensitive to electron transfer with antioxidants to generate molecules of ROS. Therefore, impairment and damage of DNA induced by antioxidants in the presence of redox-active metal Cu may be suggested as an important pathway through which preneoplatic cells and neoplastic cells can be diminished or killed (Ullah *et al*., 2009). It is suggested here that *N. sativa* reacted to the developing chick embryo as an antineoplastic agent and increased the levels of ROS, regardless of the concentration of its bioactive components. For this reason *N. sativa* is not recommended as a food supplement for infants and newborns. The present results are in synchronization with and coincide the conclusions and findings of the earlier studies (Ali & Blunden, 2003; Hussain *et al*., 2011; Koka *et al*., 2010; Ullah *et al*., 2009; Yu & Kim, 2013 and Zubair *et al*., 2013).

The present results revealed that treatment of the developing chick embryos with a combination of different concentrations of citral and *N. sativa* extract mitigated the oxidative stress levels by reducing the ROS levels in all combinations compared to the treatments with citral alone. The mitigating effect was partial because no combined treatment was able to lower the ROS level to a level similar to that of the control embryos.

Farah (2005) indicated that the mode of extraction and concentration of *N. sativa* oil are controlling factors in determination of and deciding the pharmaceutical properties of *N. sativa* extracts including the antineoplastic, anticancer or antioxidant ones. Kanter *et al*. (2005) concluded that *N. sativa* extract partly protected and rescued gastric mucosa from acute alcohol-induced mucosal damage, and these gastroprotective effects might be induced, at least partly by their radical scavenging activity.

Mousavi *et al*. (2010) indicated that pretreatment with *N. sativa* extract and/or TQ ameliorated cell toxicity and rescued cells from injuries resulted from absence of glucose in the media of cultured PC12 cells via reducing the ROS high levels, and its protective effects might be based on the impairment of production of the intracellular ROS. They suggested that pretreatment with *N. sativa* extract and/or TQ protects the PC12 cells against cell toxicity resulted from absence of glucose through exerting antioxidant properties. Their conclusion on the ameliorative and protective effects of *N. sativa* extract and/or TQ on neural cells indicated the possible application of *N. sativa* extract and/or TQ in clinical setting to rescue and treat the common neurological insults.

Adam *et al*. (2016), Cobourne-Duval *et al*. (2016) and Shao *et al*. (2017) concluded that *N. sativa* extract or TQ was able to attenuate acetaminophen-induced oxidative stress and brain injury induced by microglia-derived neurodegeneration or Status epilepticus respectively which result in initiating generation of considerably increased high amounts of the stressor ROS. The therapeutic effect of *N. sativa* extract or TQ was achieved via an anti-oxidative route that decreases ROS production. Coinciding with the present results, all the previous reports (Adam *et al*., 2016; Cobourne-Duval *et al*., 2016; Farah, 2005; Kanter *et al*., 2005 and Mousavi *et al*., 2010) agreed that *N. sativa* extract when used in combination with citral was able to reduce the levels of ROS generation to levels lower than that produced by citral alone. It is clear evident, in the present work, that *N. sativa* extract exerted antioxidant properties, where the ROS levels were reduced after combination treatments comparing to citral treatments. The question here is why the antioxidative properties of *N. sativa* extract was limited and did not reduce the ROS levels compared to control one. Actually, the differences between ROS levels in control and combinations of citral and *N. sativa* were not significant in several cases. This might be explained depending on the conclusion of Kokten *et al*. (2016). They stated that in regards to disadvantage of use of free radical scavengers, although

free radicals are considered prooxidants, antioxidants can also have prooxidant attitude. In spite of being considered as a powerful antioxidant, vitamin C can behave as a prooxidant when it combines with copper and iron, reducing Cu or Fe, which in turn reduce hydrogen peroxide to hydroxyl radicals. α-Tocopherol (vitamin E) is also a powerful antioxidant, but when its concentrations increases, it can behave as a prooxidant because of its antioxidant mechanism. Carotenoids (vitamin A) can also behave as powerful oxidative stressors especially through autoxidative pathways in the presence of high concentrations of hydroxyl radicals that forms oxygen. Even flavonoids can act as oxidative stressors, although each flavonoid responds independantly to the environment in which it is inserted.

Here in the present study it might be suggested that the restricted antioxidative power of *N. sativa* may be due to its antineoplastic properties, behaving as a oxidative stressors, the significantly high levels of ROS produced by citral and above its antioxidative capacity, mode of extraction or the low concentration used in the experiment. Since we used only one dose of *N. sativa* extract in the present experiment, we were not able to determine if *N. sativa* extract is more powerful as an antioxidant if is applied in higher concentrations or not. Further investigations are suggested in this aspect.

It is concluded that treatment of the developing chick embryos with citral significantly induced oxidative stress via elevation the ROS levels. Such elevation was concentration dependent. Treatment with *N. sativa* extract also induced oxidative stress where the bioactive components of the extract exerted prooxidant properties and behaved as an antineoplastic agent toward the developing chick embryo. Combined treatment with citral and *N. sativa* extract reduced the oxidative stress. Such reduction was significant in some cases. The partial mitigation of *N. sativa* as an antioxidant is suggested to be due to its antineoplastic properties, the significantly high levels of ROS produced by citral and above its antioxidative capacity, mode of extraction or the low concentration used in the experiment.

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Conflict of interest

The authors declare that they don't have any conflict of interest.

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