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Nigella sativa Mitigative Antioxidant Properties against Citral Induced Oxidative Stress and Biochemical changes during Development of Chick Embryo

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ABSTRACT: The aim of this study is to evaluate the effect of different concentrations of citral on glutathione (GSH), nitric oxide (NO), lipid peroxide (LPO) and vitamin C. Also, the protective effect of *N. sativa* was studied. Fertilized eggs of the chick *Gallus domesticus* were divided into control or experimental groups which received three different concentrations of citral (50, 100 and 200 µM), *N. sativa* extract (5 µl) or a combination of *N. sativa* extract with citral. Citral and *N. sativa* groups decreased GSH & vitamin C and NO, while increased LPO. Co-treatment with citral and *N. sativa* increased GSH & vitamin C and decreased LPO levels. Citral induced oxidative stress by inhibiting RA synthesis. The limited mitigative properties of *N. sativa* are attributed to either its antineoplastic properties, the high levels of oxidative stress provoked by citral and above its antioxidative capacity or the low administered dose.

Key words: Citral, *Nigella sativa*, GSH, NO, LPO, Vitamin C, Chick embryo.

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INTRODUCTION

The various components of the avian embryo's antioxidant system may be classified into two categories: The first category includes those components such as α- tocopherol (vitamin E), vitamin A and selenium which are pre-loaded in the initial yolk. The second category is those antioxidant components which are synthesized in the embryonic tissues during development. Typical representatives of such group include vitamin C and the enzymes glutathione peroxidase, superoxide dismutase (SOD) and catalase (CAT) (Surai *et al.,* 1996).

Maintenance of an antioxidant-oxidant balance in the tissues is important during chick embryo development for supporting normal embryonic development and post-hatching viability. Such a balance can be achieved by important natural antioxidants like vitamins A, C, E and catalase, glutathione peroxidase and superoxide dismutase (Yigit *et al.,* 2014).

Cellular malfunction and damage of structural and genetic material is a reflection of oxidative stress due to elevated levels of oxidative stressors. The oxidative impairment of cellular functions is explained as a result of the non equivalence between free radicals and its scavengers, where the dominance of free radical milieu is probably due to the increased generation of the prooxidants, or due to the poor scavenging capacity in the body (Salem, 2005). Vitamin A is a main player in several physiological functions, including vision, red blood cell production, immunity, and reproduction (Zeng *et al.,* 2017).

Retinoic acid (RA) in which the alcohol group has been oxidized, is a natural derivative of vitamin A. RA is a powerful differentiation agent (Chang *et al.,* 2007).

Ahlemeyer & Krieglstein (1998) investigated whether RA could prevent staurosporine-induced apoptotic cell death in chick embryonic neurons and whether inhibition of oxidative stress is involved in the mechanism of action. They suggested that the impairment of ROS production was coincided with the antiapoptotic effect of RA.

Oils extracted from certain kinds of plants are characterized by strong medical importance due to its antibacterial, antifungal or antiparasitic properties. Among the most important active compounds of these essential oils responsible for these activities is citral (Xia *et al.,* 2013). There are evidences that citral can interfere with the embryogenesis and carcinogenesis (Di Renzo *et al.,* 2007). It regulates the synthesis of RA (Yang *et al.,* 2009).

Citral has anticancer properties that can be observed in cancer of different sites such as the endometrium, ovary, cervix, B-lymphoma, and glioblastoma. Such properties can be achieved via aldehyde dehydrogenase 1A3 suppression. This aldehyde affects gene functions through RA signaling and implements a crucial role in the progression and chemotherapy resistance of cancer. Aldehyde dehydrogenase 1A3 is inhibited by citral which is able to suppress its mediated breast tumor growth, potentially via blocking its colony forming and gene expression regulation activity (Thomas *et al.,* 2016).

Nigella sativa L. is an astonishing herb with a rich religious and historical background (Salem *et al.*, 2016). *N. sativa* belongs to family Ranunculacea and is famous as black cumin or black seed. The source of the active constituents of such annual plant is the seeds. The chemical content of the seeds is widely diverse including volatile and fixed oils, alkaloids, carbohydrates, amino acids, saponins, and so many other constituents (Ahmad *et al.,* 2013; Dastan & Aliahmadi, 2015; Duby *et al.,* 2009; Ghafory *et al.,* 2015).

The bioactive ingredients of the seeds showed trustworthy power to scavenge free radicals. These components were reported to possess variable antioxidant properties. The antioxidant action of *N. sativa* may explain its pretended application in public medicine. The protective effect of *N. sativa* which is relied on its antioxidant properties may support its usage against hepatotoxicity, liver fibrosis and cirrhosis, and hepatic damage induced by parasitic *Schistosoma mansoni* infection (Mahmoud *et al.,* 2002) or irradiation injury (Radwan & Mohamed, 2018).

The present study goal is to reveal and get a deep insight on the influence of the RA inhibitor, citral, to investigate some biochemical parameters during development of the chick embryo and to find out whether the extract of *N. sativa* can spend trustable protective properties during development of chick embryo.

MATERIAL AND METHODS

A. 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.

B. 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 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 *N. 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 the age of 8 and 10 days of incubation for obtaining the required embryonic stages. Eggs were carefully opened under physiological saline solution. Embryos were carefully removed from the yolk and membranes after that they were transferred to a new saline solution for washing then stored in -80ºC. For preparation of 10% weight/volume homogenate, 0.5 g of chick embryo tissue was homogenized in 5 ml (0.0067 M) phosphate buffer (pH 7.4) using homogenizer (Unidrive 1000 D, Germany). The homogenates were centrifuged at 5000 rpm for 20 min and the supernatant were kept frozen at -20°C for the subsequent biochemical assays.

For the biochemical analysis, Glutathione (GSH) was determined using the method of Beutler *et al.* (1963), vitamin C was measured as described by Jagota & Dani (1982), nitric oxide (NO) was measured as nitrite concentration using the method of Ding *et al*. (1988) and for lipid peroxidation (LPO) determination, LPO products as thiobarbituric acid reactive substances were determined according to the method of Ohkawa *et al.* (1979).

C. 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

Table 1 and Figure 1 revealed that after eight days of treatment, GSH levels in all treated groups were non significantly lower than control, except the combination between citral (100 µM) & *N. sativa*, where GSH level was non significantly higher than control. Also, combination of *N. sativa* with the medium concentration of citral (100 µM) significantly elevated the GSH level compared to the combination with highest concentration of citral (200 µM).

Compared to control, the biochemical measurements of GSH levels were decreased in all treated groups with the exception of the combination between citral (100 µM) & *N. sativa* group.

Statistically, the level of GSH in all treated groups showed non-significant difference compared to control, where P>0.05. Treatment with *N. sativa* revealed non-significant decrease in GSH level compared to control and citral groups. Combinations of *N. sativa* and citral showed non-significant increase in the level of GSH compared to citral alone treated groups.

Ten days after treatment, GSH levels showed non-significant differences in all treatments. 50 µM and 100 µM citral treatments resulted in GSH level lower than control, while 200 µM treatment resulted in GSH level higher than control. Also, *N. sativa* treatment resulted in non-significantly higher GSH level than control. Combinations of *N. sativa* and citral improved GSH levels compared to control and citral alone treatments (Table 1 and Fig. 2).

Eight days after treatment, the level of vitamin C (Vit C) in all treated groups showed non-significant decrease compared to control, except the combination between citral (100 µM) & *N. sativa*, where the level of Vit C was higher than control. The same treatment, citral (100 µM) & *N. sativa*, resulted in a significant increase compared to citral alone (100 µM) and also compared to the combination of citral (200 µM) & *N. sativa*. (Table 1 and Fig. 3).

Ten days after treatment, there was non-significant decrease in vitamin C level in citral treated groups, with the exception of the highest dose of (200 µM), where the level was non significantly higher than control. Also, *N. sativa* treatment resulted in non-significant increase of vitamin C level compared to control. All combinations of *N. sativa* and citral treatments resulted in a significant increase in vitamin C level compared to control and citral alone where P<0.05, except for the highest dose of citral, where the increase in vitamin C level was non-significant (Table 1 and Fig. 4). Eight days after treatment, citral treated groups exhibited a significant decrease in NO levels except for the highest dose (200 µM) where the decrease was non-significant.

Table 1: Effect of different concentrations of citral, *Nigella sativa* and their co-treatment on GSH, Vitamin C, NO and LPO production. a, b, c: significant difference between groups. Data are presented as means ± SE.

Groups / Parameters		Control	Saline	50µM Citral	100µM Citral	200µM Citral	5 µL Nigella	50µM Citral + 5 µL Nigella	100µM Citral + 5 µL Nigella	200µM Citral + 5 µL Nigella
8 days	GSH	4.684±0.488^{ab}	2.736±0.4240^{a}	3.637±1.528^{ab}	2.964±0.344^{ab}	3.250±0.726^{ab}	2.407±0.4159^{a}	3.856±0.0534^{ab}	5.201±0.8242^{b}	2.765±0.3925^{a}
	Vit C	113.5±18.83^{ab}	55.15±6.006^{a}	108.7±43.44^{ab}	63.43±9.104^{a}	80.04±28.48^{ab}	74.47±25.13^{ab}	89.73±7.975^{ab}	154.9±39.81^{b}	64.69±7.096^{a}
	NO	134.5±14.17^{a}	57.65±8.673^{b}	68.46±23.79^{b}	70.69±6.895^{b}	86.72±28.78^{ab}	66.88±19.12^{b}	119.0±14.82^{ab}	135.2±31.47^{a}	87.09±6.386^{ab}
	LPO	1.582±0.0988^{a}	2.380±0.373^{ab}	2.847±0.234^{ab}	4.012±0.1347^{b}	5.898±0.6922^{c}	3.646±0.493^{b}	3.228±0.829^{ab}	3.039±0.865^{ab}	3.363±0.880^{ab}
10 days	GSH	2.108±0.02890	2.395±0.1041	1.729±0.04132	1.911±0.01082	2.458±0.1285	2.332±0.4659	2.432±0.5456	2.020±0.05530	2.038±0.2538
	Vit C	68.45±11.41^{ab}	67.80±3.867^{ab}	45.93±1.490^{a}	66.97±9.348^{ab}	85.08±11.11^{bc}	84.26±12.78^{bc}	108.9±10.21^{c}	106.0±3.421^{c}	104.2±10.81^{c}
	NO	72.85±0.5067	72.02±15.60	75.57±5.798	87.51±17.37	84.53±4.528	78.11±32.29	71.04±14.85	92.77±20.13	58.83±5.255
	LPO	1.701±0.03501	2.037±0.3168	1.225±0.2015	2.087±0.9683	1.451±0.4511	1.337±0.3250	1.479±0.2016	1.463±0.3175	2.476±0.9625

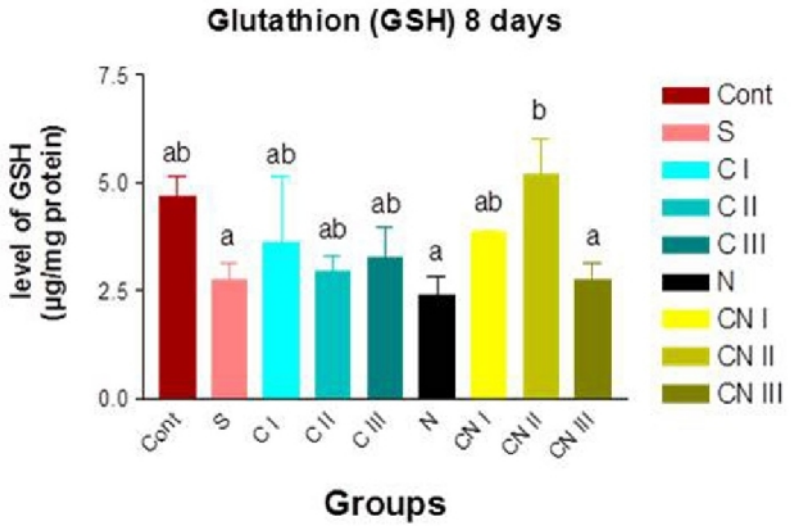

Fig. 1. Levels of GSH (µg/mg protein). A comparison between different treated groups, eight days after treatment. a & b: significant difference between groups. Columns are presented as means ± SE. For all figures Cont: untreated control, S: saline injected group, CI: 50 µM-citral treated group, CII: 100 µM-citral treated group, CIII: 200 µM-citral treated group, N: 5 µl- *N. sativa* extract treated group, CNI: 50 µM-citral+5 µl- *N. sativa* extract treated group; CNII: 100 µM-citral+5 µl- *N. sativa* extract treated group and CNIII: 200 µM citral+5 µl *N. sativa* extract treated group.

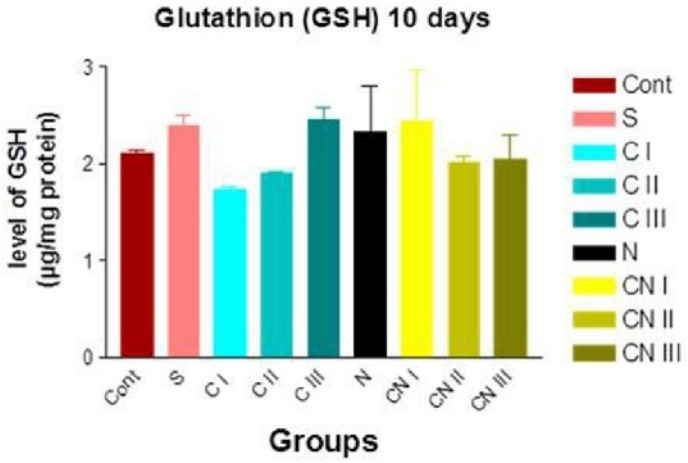

Fig. 2. GSH levels (µg/mg protein). A comparison between different treated groups, ten days after treatment. Columns are presented as means ± SE.

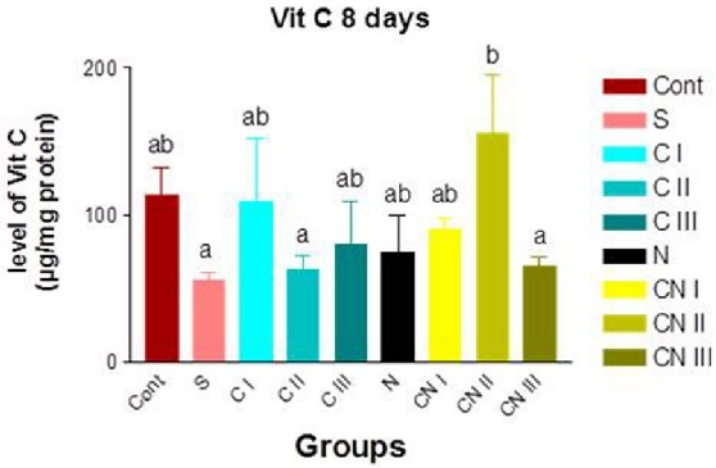

Fig. 3. Vitamin C levels (µg/mg protein). A comparison between different treated groups, eight days after treatment. a & b: significant difference between groups. Columns are presented as means ± SE.

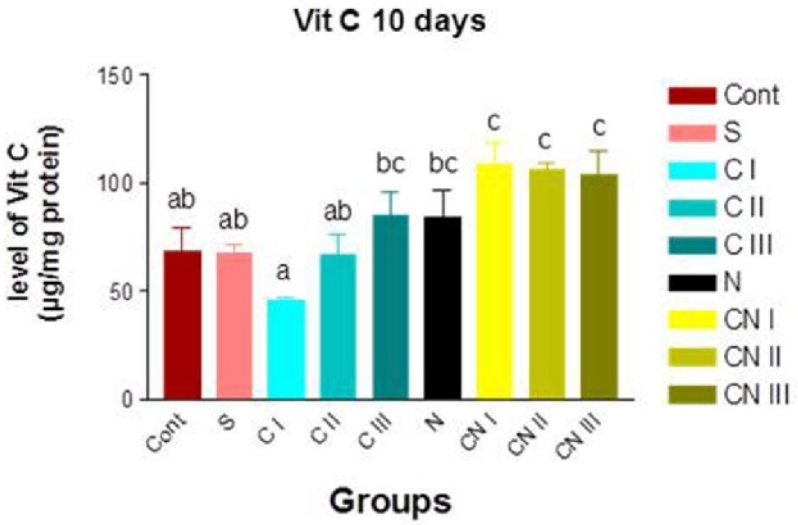

Fig. 4. Vitamin C levels (µg/mg protein). A comparison between different treated groups, ten days after treatment. a, b & c: significant difference between groups. Columns are presented as means ± SE.

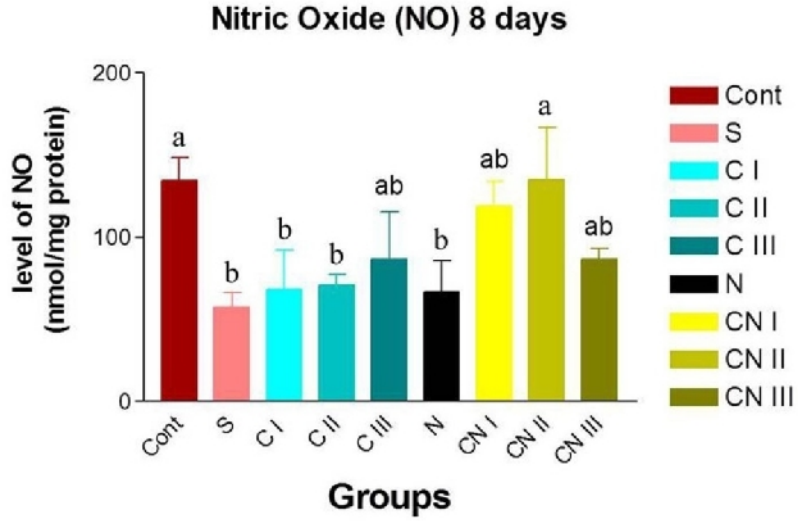

Fig. 5. The level of NO (nmol/mg protein). A comparison between different treated groups, eight days after treatment. a & b: significant difference between groups. Columns are presented as means ± SE.

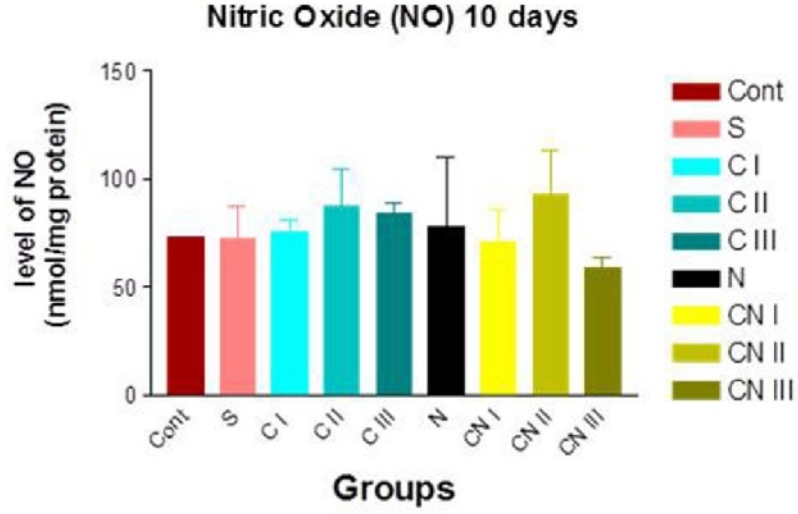

Fig. 6. The level of NO (nmol/mg protein). A comparison between different treated groups, ten days after treatment. Columns are presented as means ± SE.

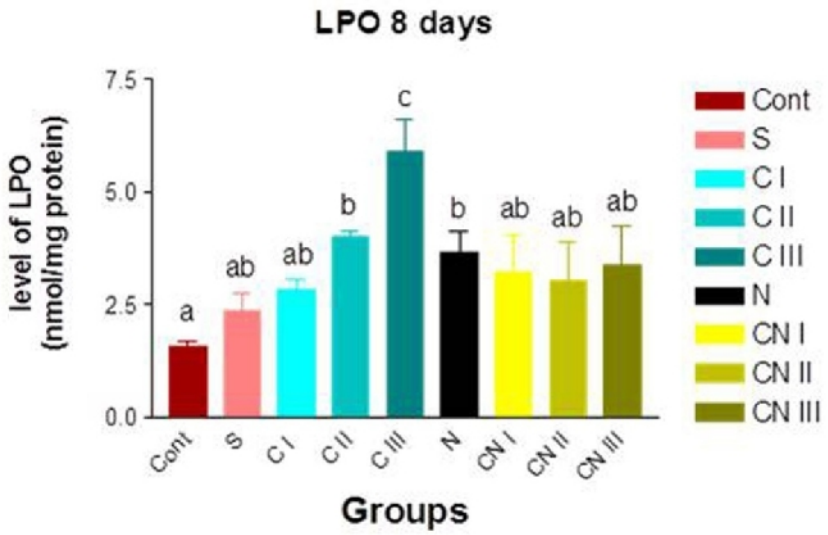

Fig. 7. The level of LPO (nmol/mg protein). A comparison between different treated groups, eight days after treatment. a, b & c: significant difference between groups. Columns are presented as means ± SE.

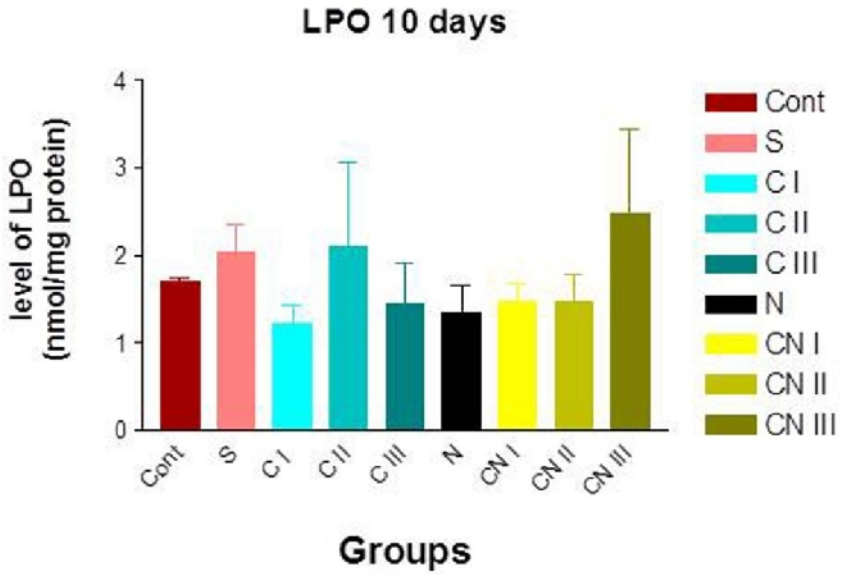

Fig. 8. The level of LPO (nmol/mg protein). A comparison between different treated groups, ten days after treatment. Columns are presented as means ± SE.

N. sativa treatment significantly decreased NO level compared to control. Combinations of *N. sativa* and citral resulted in a non-significant increase in NO level compared to citral, except for the combination of *N. sativa* and citral (100 µM) where the increase was significant compared to the lowest two citral treatments, while the difference was non-significant compared to control (Table 1 and Fig. 5).

Ten days after treatment, all citral treated groups, *N. sativa* treated group, and the combination of *N. sativa* and 100 µM-citral group showed non-significant increase in NO level compared to control, where $P>0.05$.

The other two combinations of *N. sativa* and 50 µM- and 200 µM-citral resulted in non-significant decrease in NO level compared to control (Table 1 and Fig. 6).

Eight days after treatment, LPO levels showed an increase in all treated groups compared to control. Statistical analysis revealed a non-significant difference between control and among treated groups except that the medium and the highest dose of citral (100 µM & 200 µM) and *N. sativa* groups showed significant difference compared to control, where $P<0.05$.

Treatment with *N. sativa* and combinations of *N. sativa* and citral significantly decreased the LPO level compared to the highest dose of citral, where $P<0.05$ while the difference was non-significant compared to the two lower doses of citral where $P>0.05$ (Table 1 and Fig. 7). After ten days of treatment, there was non-significant decrease in all treated groups with the exception of the medium dose of citral (100 µM) group and the combination between the highest dose of citral (200 µM) & *N. sativa* group where they increased compared to control, where $P>0.05$ (Table 1 and Fig. 8).

DISCUSSION

Oxidative stress causes impairment to several important biological macromolecules and thus inducing apoptotic cell death (Chandra *et al.,* 2000). Protection from oxidative damage and rescuing cells from induced apoptosis is the main function of antioxidants. Vitamin C and GSH are antioxidants of non-enzymatic origin that comprise strong blocks against oxidative damage and alteration in metabolic activation of carcinogens (Yeung & Or, 2007).

GSH is found in considerable amounts in the cells, and it protects cells from free radical generation. GSH exerts oxidative suppression power against free radicals by rendering a hydrogen atom resulting in neutralizing the hydroxyl radicals. GSH maintains reduction of protein thiols and causes reduction of peroxides. It is considered the only built-in intramitochondrial defense against excessive ROS production suggesting that the cellular redox state may regulate cell survival and thus, high GSH levels may provide resistance to oxidative stress-induced apoptosis (Nwanjo & Oze, 2007).

Contrasting effects of retinoids on GSH in different cell lines and in a dose and time dependent manner were reported in several studies. Teixeira *et al*. (1996) indicated that chondrocytes possess different enzyme systems that are important for removal of ROS. The presence of these enzymes (CAT, glutathione reductase and SOD) serves to protect cell and extracellular matrix components from oxygen radical attack. RA treated cells showed elevated levels of such protective enzymes. They also found that GSH levels are regulated by RA. GSH synthesis can be prohibited by the presence of physiological doses of RA in a dose-correlated manner. It is probable that the concurrent decrease in both intra and extracellular cysteine concentrations results from the change in intracellular GSH metabolism. The change in GSH metabolism may provide one mechanism to regulate gene expression in cartilage growth. In contrast, Morales *et al*. (2005) suggested that atRA can protect leukemia cells against induced oxidative stress by enhancing their antioxidative capacity. The basal GSH levels are increased after atRA exposure, resulting in lower levels of the generated oxidative stress, reduced apoptosis associated mitochondrial changes and enhanced cell survival by obstructing oxidative stress. Nefedova *et al.* (2007) revealed the importance of RA as a regulator for gene expression. RA increased GSH synthase levels in acute and specific manner in myeloid-derived suppressor cells that are important for tumor escape by suppressing T-cell responses. This resulted in elevation of GSH concentration in these cells.

Retinoids can interfere directly with protein kinase C, the GSH dependent and corner stone enzyme in signal transduction. Also, in tumor cells, retinoids elevate ROS accumulation, which is associated with oxidative DNA damage and apoptosis. GSH enhanced DNA replication in mammalian cells. Thus, GSH synthesis is fundamental for normal growth. There is tight evidence supporting the concept that a decrease in GSH is often correlated with the apoptotic programme through a mitochondrial dependent apoptotic pathway, whereas recovery of GSH content may enable the cells to avoid the apoptotic signal (Palomares *et al.,* 2006).

RA is important for different cellular vital activities including survival, metabolism, growth, reproduction (Chang *et al.,* 2016).

The nuclear factor erythroid 2-related factor 2 (Nrf2) is one of the fundamental players in the cellular reduction oxidation equilibrium. It functions on reduction side. It chiefly modulates the transcription of more than one antioxidant, e.g., glutathione S-transferase (GST) and quinone oxidoreductase. Expression of Nrf2 could be reduced by low concentrations of RA. GST in turn suppressed oxidative stress via initiating GSH synthesis (Jia *et al.,* 2017). Different factors can induce oxidative stress by reducing the presence of antioxidants like reduced GSH with subsequent increase in ROS generation. Absence or reduction of GSH results in many neurodegenerative diseases. Recent studies indicated that RA increases the activity of glutathione synthesis and exhibits neuroprotective functions in brain cells (Crockett *et al.,* 2011).

Mohanty *et al.* (2017) reported the effects of the carcinogenic polycyclic aromatic hydrocarbon, benzo(a)pyrene (B[a]P). They reported that such a carcinogen causes oxidative stress through elevating induction of cytochrome P450 1A1 (CYP1A1) expression. CYP1A1 is the enzyme that is essential for degradation of RA. Any deviation from the required concentration of RA leads to production of ROS. RA can mediate oxidative stress leading to alteration in GSH level and antioxidant enzyme activity in zebrafish brain. In the current investigation, we reported that citral lowered GSH levels. These effects are similar to the action of B[a]P.

GSH sharing through direct non-enzymatic and enzymatic (via Glutathione S-transferase, GST) reactions, is a fundamental step in intracellular citral metabolism (Nakamura *et al.,* 2003). As a result, it is heartening to suggest that citral may be acting as a chemical sink and hence causing a decrease in intracellular GSH levels.

Kapur *et al.* (2016) treated malignant cells with 10-50 µM citral. They suggested a mechanism to explain the rapid fall down of intracellular GSH following citral treatment. Citral is suggested to conjugate with principal thiol groups of enzymes that are important for GSH synthesis and thereby produce an immediate and pronounced effect on the intracellular quantities of this fundamental antioxidant. Iersel *et al.* (1996) studied the effect of citral on GSH depletion in human melanoma cancer cells. They found that the majority of the GSH depleting effect is reached with the lowest treatment level of citral. This finding was obviously reported in the present study after ten days of treatment.

In a recent study (Ali *et al.*, 2018), we revealed an increase in the ROS levels after citral treatment and this was suggested to be a reflection for the decreased levels of endogenous RA. Furthermore, citral induced oxidative stress as indicated by high levels of ROS might be suggested to be due to decreased levels of antioxidant molecules such as GSH. The present study is in agreement with (Jia *et al.*, 2017 and Crockett *et al.*, 2011). Also we support the conclusion of Nakamura *et al.* (2003) and Kapur *et al*. (2016) that citral may act as a chemical sink of GSH and directly conjugate with principal thiol groups of enzymes that are important for GSH synthesis and consequently produce an immediate and pronounced effect on the intracellular levels of GSH.

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 & Blunden (2003) stated that constituents of *N. sativa* including TQ and other bioactive compounds have many potential antitumor effects. Hussain *et al.* (2011) found that TQ is considered to be a powerful apoptosis inducer in different lymphoma cell lines by its power to release ROS. Yu & Kim (2013) found 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.

Hamed *et al.* (2013), Kanter *et al.* (2005), Kushwah *et al.* (2014) and Noor & Mourad (2010) reported that GSH levels increased after *N. sativa* treatment against bromobenzene, ethanol, paracetamol and monosodium glutamate toxicity respectively in rats. *N. sativa* was reported to have a protective effect against irradiation-induced oxidative injury via increasing GSH levels in an experimental model (Cemek *et al.*, 2006).

On the other hand, Develi *et al*. (2014) disagreed the possible protective action of *N. sativa* oil pretreatment against oxidative damage and hepatic dysfunction induced in ethanol treated rats. They found that *N. sativa* did not elevate the protective antioxidant levels as compared to controls, and no changes were observed in GSH levels as compared to ethanol group.

Our data indicated that after eight and ten days of treatment, citral lowered the level of GSH in the developing chick embryo via inhibition of RA by citral. We suggested that the low levels of RA might decrease the level of GSH and also decrease the expression of Nrf2, leading to induction of oxidative stress (Ali *et al.*, 2018) via inhibiting GST expression and consequently GSH production. Also, the present results revealed that *N. sativa* treatment alone resulted in a decreased level of GSH and this might be explained basing on the explanation of Yu & Kim (2013) that the bioactive components of *N. sativa* like TQ possess antineoplastic properties and significantly increased apoptotic cell death in the developing chick embryo via elevating ROS levels leading to the consumption of the antioxidant molecule, GSH. Co-treatments of citral and *N. sativa* results in a relative non-significant increase in the level of GSH compared to the citral treatment alone. The mitigative effect of *N. sativa* here might be due to its protective antioxidative properties exerted against ROS production by citral and its damaging stress. Ten days after treatment, differences in GSH levels were non-significant in all treated groups compared to control. This is explained as an adaptive response (Ahlemeyer *et al*., 2001).

Vitamin C is important antioxidant and unique free radical scavenger in extracellular matrix. Vitamin C is hardly seen in the yolk at the early embryonic development of chick. It is synthesized in the yolk sac and its levels are markedly elevated around days 8 and 13 and are kept at a high level throughout development. It is then transferred to different body parts, especially to the brain. Vitamin C may play a key role in the antioxidant defense system of the avian brain (Surai *et al.*, 1996).

Ramya *et al*. (2012) validated the anti-cancer efficacy of RA against B[a]P -induced lung carcinogenesis in mice. They reported that RA treatment resulted in high levels of vitamin C in RA treated mice. Vitamin C lowers ROS production during the metabolism of carcinogen and thus possibly protects the genetic material at the initiation and promotion stages of carcinogenesis.

Develi *et al.* (2014) found that *N. sativa* did not increase the vitamin C levels as antioxidant against ethanol toxicity; While Cemek *et al*. (2006) found an increase in vitamin C content after *N. sativa* treatment against irradiation injury. Sheikh & Mohamadin (2012) recorded increased levels of non-enzymatic (GSH & vitamin C) and enzymatic antioxidants in the rat brain to normal levels after propoxur toxicity.

The present study revealed that the level of vitamin C in citral treated groups was non-significantly decreased compared to control. This might be explained depending on the fact that the already low level of endogenous vitamin C is consumed in scavenging the oxidative stress induced by citral and the blockade of endogenous RA prevented the enhancing of vitamin C synthesis (Ramya *et al.*, 2012). Co-treatment with citral and *N. sativa* partially elevated the level of vitamin C after eight days of treatment but significantly increased the level after ten days of treatment.

This is suggested as a result of age effect resulting in generation of more vitamin C in addition to the antioxidative properties of *N. sativa*.

Nitric oxide (NO), a highly reactive nitrogen radical, is involved in several vital functions. It is an essential mediator of the host innate immune and inflammation reactions to several pathogens (Wallace, 2005). NO is small neutral molecule having gaseous properties that has a short life span and can withdraw through biological membranes of cells (Brown, 2010). It is a unique gaseous neurotransmitter and modulator that achieves several important functions in the embryology of brain. NO functions a fundamental role in supporting behaviors like learning and memory. NO as a signal molecule might positively contribute to the organism but if it is over produced it can strongly contribute to the cell damage (Brown, 2010).

It has been proven that inducible nitric oxide synthase (iNOS) is expressed in melanoma in high rate. NO and iNOS enhance proliferation of human melanoma cells and its survival. To understand the NO role in melanoma development, it is important to know that NO that produced by iNOS, is able to reduce the expression of CXCL10, a chemokine that prohibits angiogenesis, tumor cell growth, and metastasis (Tanese *et al.*, 2012).

Addition of RA to the medium of neural precursors derived from human embryonic stem cells significantly elevated the levels of NO and nitric oxide synthase-1, demonstrating a prodifferentiation capacity of nitric oxide synthase-1, stimulated by RA coincident with morphological differentiation (Tao Li *et al.*, 2010).

Pretreatment with liarozole, a RA-metabolism inhibitor strongly reduced NO and tumor necrosis factor-α (TNF-α) release. Liarozole is known as RA metabolism blocking agent. Pharmacological inhibition of intracellular RA reduction with liarozole, exerted strong anti-inflammatory effects on activated microglia. Liarozole showed effects coinciding with that of RA on NO and TNF-α release of lipopolysaccharide (LPS) activated microglia. Liarozole inhibited the intracellular RA catabolism via cytochrome P450. Some molecules whose concentration is under tight temporal and spatial control can show fine changes in the biometabolism that may lead to significant effects. RA is such a molecule. Concentrations of RA are under tight spatial and temporal control and this explains the contrasting effects of RA on NO production that observed previously (Hellmann-Regen *et al.*, 2013).

Essential oil from fruits exhibited a strong NO inhibitory properties and cytotoxicity. Citral represents a major effective component of the fruit essential oil. In the LPS stimulated macrophage cell line, citral exerted strong NO inhibitory properties. Lee *et al.* (2008) indicated that citral prohibits LPS induced NO generation. This inhibition coincided its inhibition of the expression and transcriptional activity of iNOS, suggesting that citral could be anti-inflammatory as it inhibits of NO production via the suppression of iNOS expression.

Recent studies revealed powerful suppression in NO generation induced by citral, which could represent a potential therapeutic target in melanoma therapy. Results indicated that citral possesses antiproliferative and cytotoxic properties in murine melanoma cells. Prohibition of cell proliferation might be due to reduced nuclear factor kappa B, and consequently, NO levels. Cytotoxicity could be elucidated as a result of induced oxidative stress. These findings focus on the prospective antineoplastic effects of citral in melanomain vivo models, with a tumor-causing mechanism of action controlling the depletion of NO and interference in signaling pathways pivotal to cell proliferation and survival Sanches *et al*. (2017) and Uchida *et al*. (2017).

Our data demonstrated that treatment with citral exhibited a significant reduction in NO except for the highest dose (200 µM) where the decrease was non-significant after eight days of treatment, synchronized with the finding of Lee *et al*. (2008), Sanches *et al*. (2017) and Uchida *et al*. (2017) that citral decreased NO levels. It is suggested that this decrease might me achieved via suppression of NOS. Co-treatment with *N. sativa* elevated NO levels attributed to the antioxidant and anti-inflammatory properties of *N. sativa*. Ten days after treatment, all citral treated groups showed non-significant increase in NO level. This can be explained as a result of citral degredation and consumption being a substrate for enzymatic activities (Tanaka *et al.*, 1996). On the other hand, combined effect of *N. sativa* and citral resulted in non-significant decrease in NO levels. Cemek *et al.* (2006) recorded similar results where *N. sativa* exerted protective properties against the harming effects of ionizing irradiation while NO and LPO levels were lower than control. Also, Hadi *et al.* (2016), Hossen *et al*. (2017) and Yoruk *et al*. (2017) reported that treatment with *N. sativa* produced a significant reduction of NO and improved inflammation in arthritis and rhinosinusitis patients.

Embryonic development causes consumption of more oxygen and increased energy metabolism which can make embryos susceptible to lipid peroxidation (LPO) caused by ROS. ROS is effective during embryonic development because its production is essential for mediating apoptosis and cell elimination (Salas-Vidal *et al*., 1998). Lipid peroxidation may be mediated by the presence of vitamins, since vitamins C, E and RA have been shown to operate as free radical scavengers (Krajcovicova-Kudlackova *et al.*, 2004). Serbecic & Beutelspacher (2005) showed that RA efficiently prevented lipid-peroxidation in acute renal failure and murine corneal endothelial cells respectively.

Lipid peroxidation is achieved in response to liberation of high levels of ROS, which might cause damage to cell membranes due to oxidative stress with the liberation of cytotoxic aldehydes, e.g. malondialdehyde (MDA) and RA was shown to possess suppressor effect against production of MDA (Wong *et al.*, 2016).

The effects of RA might be caused as a result of high levels of lipid peroxidation. At low concentrations, RA could act as a scavenger for free radicals such as fatty acid peroxyl radicals (Samokyszyn & Marnett, 1990), lowering the oxidative stress of the cell and allowing for increased proliferation. The different effects of RA on cell proliferation may be explained by the interference of cellular oxidative stress. With low doses of RA, there is a slight decrease of lipid peroxidation, synchronized with increased cell proliferation. Conversely, increasing concentrations of RA elevate lipid peroxidation, synchronized with decreased cell proliferation. Cell proliferation is negatively correlated with lipid peroxidation, suggesting that RA may cause liberation of free radicals. Tucci *et al.* (2008) indicated that providing RA caused lipid peroxidation. By extra physiological doses, RA induced damage, since at physiological doses RA did not increase lipid peroxidation and there is no effect on cell viability. RA might motivate the activity of antioxidant enzymes GST, SOD and CAT at ordinary doses, thereby inhibiting oxidative cell damage which is achieved at higher doses of RA. There are at least two potential mechanisms of RA mediated lipid peroxidation: (i) RA can stimulate the activity of Δ-6-desaturase resulting in an increase of polyunsaturated fatty acids (PUFA), which are then readily oxidized. (ii) RA can also directly elevate free radical levels which could result in increased lipid peroxidation.

The present results showed that LPO levels significantly increased in citral treated groups eight days after treatment. This is suggested to be due to inhibitory effect of citral on RA production that was supposed to prevent MDA production, the highly reactive cytotoxic product of LPO (Wong *et al.*, 2016). On the other side, supplementation of *N. sativa* oil alone unexpectedly caused significant elevation of LPO level compared to control. According to Noor & Mourad (2010) and Khader *et al.* (2009) who found increase in MDA level after *N. sativa* treatment, they explained the increase in LPO after treatment with *N. sativa* oil, that TQ like other quinone compounds, can be considered to be a redox-cycler which is metabolized in vivo to hydroquinones or semiquinone radicals by cellular oxidoreductases leading to the production of MDA and consequently ROS. Co-treatment with *N. sativa* resulted in lowering LPO levels. Hamed *et al.* (2013), Develi *et al*. (2014), Hadi *et al*. (2016) and Seif (2014) found that *N. sativa* oil protected against damage induced by MDA to a tremendous level. We suggest that the conflict of the amphoteric behavior of *N. sativa* can be solved as *N. sativa* oil behaved as oxidative stressor when it is alone administered in vivo, where the oxidative stress level is relatively low. When *N. sativa* is administered as a co-treatment, where there is already citral-induced oxidative stress, here at this point, *N. sativa* exerts the antioxidant properties. At the age of 10 days, our result showed non-significant differences in LPO levels. This might be explained as an adaptive response of the treated embryos.

It may be concluded that citral administration prohibited the levels of the non-enzymatic antioxidants (GSH & vitamin C) and NO, while increased the level of LPO. This citral inhibitory effect is interpreted as a result of repression of RA synthesis and consequently resulting in induction of oxidative stress. Treatment with *N. sativa* alone exerted antineoplastic properties against the developing chick embryos via decreasing GSH, vitamin C and NO levels and increasing LPO level. For this reason *N. sativa* administration is not highly recommended for newborns and infants or during pregnancy. Co-treatment of *N. sativa* and citral increased the levels of non-enzymatic antioxidants (GSH & vitamin C) and NO and decreased LPO levels. This was initiated by the antioxidative properties of *N. sativa* in response to citral induced oxidative stress. The limited mitigative antioxidant properties of *N. sativa* are attributed to eitherits antineoplastic properties, the significantly high levels of oxidative stress provoked by citral and above its antioxidative capacity or the low dose administered in the experiment.

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