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Nigella sativa improves the carbon tetrachloride-induced lung damage in rats through repression of erk/akt pathway

***Nigella sativa* improves the carbon tetrachloride-induced lung damage in rats through repression of erk/akt pathway**

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Abstract

The objective of this study was to examine whether *Nigella sativa* plays a protective role against the damage in the lung by administering carbon tetrachloride (CCl₄) to rats. Male Wistar albino (n=28, 8 weeks old) rats were divided into 4 groups: a) negative control: Normal water consuming group to which no CCl₄ and *N. sativa* was administered; b) Positive control: Normal water consuming group to which no CCl₄ was administered but *N. sativa* was administered; c) CCl₄ Group: Normal water consuming and group to which CCl₄ was administered (1.5 mL/kg, ip); d) *N. sativa* plus CCl₄ group: CCl₄ and *N. sativa* administered group (1.5 mL/kg, ip). Caspase-3, caspase -9, erk, akt protein syntheses were examined via Western blotting. Malondialdehyde determination in lung tissue was made using spectrophotometer. As a results, malondialdehyde amount was decreased in the CCl₄ plus *N. sativa* group in comparison to CCl₄ group whereas caspase-3, caspase-9 was increased and erk, akt had decreased. These results show that *N. sativa* protects the lung against oxidative damage.

Introduction

Many clinical studies carried out today on experimental animals put forth the toxic effects of free radicals on the cells as well as the therapeutic effects of antioxidant systems on these toxic effects (Li et al., 2011; Aslan et al., 2014; Aslan, 2015). Carbon tetrachloride (CCl₄) is one of the chemicals that can cause severe toxic effects on the body and is frequently used to create experimental damage (Vuda et al., 2012). It can easily enter the body via respiration, ingestion or dermal absorption and is rapidly absorbed via the gastrointestinal channel. A significant portion of CCl₄ in humans settles on the fat tissue. It then continues to move towards the lungs. About 4% of the metabolized CCl₄ is thrown out via respiration and the remainder interacts with the protein and intracellular molecules (Al-Dbass et al., 2012; Aslan and Can, 2014).

Some plants known as medicinal plants have been widely used in the treatment of many diseases since ancient times. *Nigella sativa* is one of these plants and it has been used for medicinal purposes for centuries with protective effects especially on the lungs which have been put forth in light of recent experimental studies (Sahin et al., 2003). Caspase-3, caspase-9, erk and akt proteins are important for the apoptosis mechanism (Korsmeyer, 1999; Aslan and Can, 2014; Lu and Xu, 2006). In this study, rats were administered with carbon tetrachloride and it was examined whether *N. sativa* plays a protective role against damages in the lungs or not.

Materials and Methods

Chemical substances and *N. sativa*

The primary antibodies used in the Western blotting



stage were obtained from BioVision (USA) whereas secondary antibody was obtained from Santa Cruz Biotechnology (USA). All other chemicals used in experimental work were acquired from Sigma-Aldrich (Germany), Bio-Rad (USA), Bio Shop (Canada) and Merck (USA). Dust form NS seeds to be boiled and mixed in the daily drinking waters of rats were acquired from oz gida organik and yoresel urunler Ltd. Sti. (Elazığ).

Animal material and research groups

All of the animal experimentation work of our study was carried out at the Firat University Experimental Animals Research Institute (FUDAM) with consent number 129 of Firat University Animal Experiments Ethics Council during the meeting on 27/11/2013. 28 male Wistar albino (n=28, 8 weeks) rats were used in the study. Light and dark periods of 12 hours were applied to rats. The rats were distributed into 4 groups according to their live weights. The groups were: (i) Negative control: Normal water consuming group to which no CCl₄ and *N. sativa* was administered; b) Positive control: Normal water consuming group to which no CCl₄ is administered but *N. sativa* was administered; c) CCl₄ Group: Normal water consuming group to which CCl₄ was administered (1.5 mL/kg live weight, i.p); d) CCl₄ + *N. sativa* group: CCl₄ and *N. sativa* administered group (1.5 mL/kg live weight, i.p). The extract of boiled *N. sativa* seeds was mixed into the drinking water of animals to be used as *N. sativa* source (10% w/v). The initial live weights of the animals were arranged to be equal. Live weights were recorded 3 times weekly throughout the study.

CCl₄ application

CCl₄ application was carried out intraperitoneally twice per week for four weeks at 1.5 mL/kg live weight injected together with olive oil at 1:3 ratio (Bahcecioglu et al., 2008; Shaker et al., 2010).

Preparation of N. sativa extract

The extract prepared by mixing the ground *N. sativa* seeds in water and boiling them was added to the drinking water of rats at a ratio of 10%. The water consumption of rats was monitored and recorded regularly.

Lung tissue homogenization

Lung tissue samples were divided into small parts and were broken down inside lysis buffer (0.5 M Tris; pH 8; EDTA, β-mercaptoethanol, phenyl methyl sulphonyl fluoride [PMSF]) in mechanical homogenizer. These broken down tissue samples were centrifuged at 15,000 rpm for 45 min. Supernatant was taken and stored at -80°C until usage time (Aslan and Can, 2014).

Analysis of proteins via western blotting method

The primary antibodies used in this study (Bio vision)

were diluted at a ratio of 1/250, whereas the secondary antibodies were diluted at a ratio of 1/2000 (Santa Cruz Biotechnology). Protein densities were measured using Lowry kit and 35 μg protein was loaded to each well. The protein samples of the tissues were run on 12% gel via SDS-PAGE method. Afterwards, these proteins (caspase-3, caspase-9, erk, akt) were transferred to nitrocellulose membrane with western blotting method and their synthesis ratios were examined (Laemmli, 1970). The protein levels were then measured via density measurement analysis system (Image J; National Institute of Health, Bethesda, USA).

Lung tissue malondialdehyde measurement

Lung tissue samples were divided into small parts after which they were broken down in 4.5 mL 1.15% KCl per 0.5 g tissue. Malondialdehyde determination which is the final product of lipid peroxidation was made from this prepared homogenate (Ohkawa et al., 1979; Aslan and Can, 2014; Ustundag et al., 2005). This method is based on malondialdehyde which is one of the aldehyde products of lipid peroxidation and TBA (thiobarbituric acid) reaction. 0.1 mL 8.1% sodium dodecyl sulphate (SDS), 750 μL 20% (pH 3.5) acetic acid solution was added to 0.1 mL tissue homogenate during measurement process. Afterwards, 750 μL of 0.8% (pH 3.5) TBA solution was added along with distilled water to make the final volume 4 mL. It was then left to wait in a 95°C boiling water bath for 45 min after which it was vortexed following the addition of 1 mL distilled water and 15:1 (v/v) ratio 5 mL n-butanol-pyridine mixture. Following centrifugation at 5,000 rpm for 10 min, the organic layer at the top was removed and measured spectrophotometrically at a wavelength of 532 nm. The results were recorded as nmol/g.

Statistical analyses

All data were evaluated via SPSS 20 package software using variance analysis. One-Way ANOVA *Post Hoc* Tukey test was applied to determine the differences inside the groups. The measurements were repeated at least 3 times to ensure the reliability of the statistical analyses after which they were recorded in the SPSS 20 package software and evaluation process was started.

Results

Live weight change and water consumption

When the statistical data in Table I are examined, it is observed that there is a statistically significant difference among groups in terms of water consumption (p<0.05). Water consumption in CCl₄ administered groups is lower in comparison with the control groups which show that the damage that occurs over time due to CCl₄ has negative effects on water

Table I			
Body weight and water consumption in rat			
Groups	First live weight (g)	Last live weight (g)	Daily water consumption (mL)
Negative control	252.9 (25.1)	358.4 (32.3) ^a	317.3 (1.5) ^a
Positive control	247.9 (23.9)	309.0 (20.9) ^b	260.4 (1.5) ^b
CCl ₄	237.3 (19.3)	281.3 (15.7) ^c	180.3 (1.5) ^c
CCl ₄ plus <i>N. sativa</i>	243.8 (18.3)	300.6 (19.0) ^d	213.3 (1.5) ^d

a-d: Differences between groups in the same column with different letters are statistically significant ($p < 0.05$); Data within the parenthesis are SD; One-way ANOVA post hoc Tukey test

consumption. When the initial and final live weights of the study were compared, no statistically significant difference was determined ($p > 0.05$); whereas it is observed that there is a statistically significant difference among the final live weights ($p < 0.05$) and that the lowest live weight ratio is observed in the CCl₄ group.

Malondialdehyde measurement results

When the malondialdehyde levels in the lung tissue shown in Table II are examined, no statistically significant difference is determined between the control groups ($p > 0.05$) and it was also observed that the highest malondialdehyde level is observed in the CCl₄ group and that there is a statistically significant difference between other groups ($p < 0.05$). Severe decrease in the malondialdehyde levels was observed in the CCl₄ + *N. sativa* groups in comparison with the CCl₄ group.

Expression levels of caspase-3, caspase-9, erk, akt proteins

The expression levels of proteins present in cell death mechanism stages are observed in the results section. When Figure 1A is examined, the caspase-3 protein expression level differed at a statistically significant level between groups ($p < 0.05$). The highest value was observed in the negative control group and CCl₄ + *N. sativa* group, whereas the lowest value was observed in the CCl₄ group. It is observed in Figure 1B that the caspase-9 protein expression level shows statistically significant differences among groups ($p < 0.05$). The caspase-9 protein expression level of CCl₄ + *N. sativa* group was determined to be higher in comparison with those of positive control and CCl₄ groups. When the protein expression level of signal transmitting erk protein is examined in Figure 1C, no statistically significant difference was determined between the negative control and CCl₄ + *N. sativa* groups. A statistically significant difference has occurred between

Table II	
Lung tissue malondialdehyde level	
Groups	Lung tissue malondialdehyde (nmol/g)
Negative control	3.6 (0.5) ^a
Positive control	3.7 (0.6) ^a
CCl ₄	12.5 (1.6) ^b
CCl ₄ plus <i>N. sativa</i>	4.0 (1.5) ^a

a-b: Differences between groups in the same column with different letters are statistically significant ($p < 0.05$); Data within the parenthesis are SD; One-way ANOVA post hoc Tukey test

CCl₄ and CCl₄ + *N. sativa* groups ($p < 0.05$). The highest value was observed in the CCl₄ group; whereas the lowest value was observed in the CCl₄ + *N. sativa* group. Accordingly, we can state that *N. sativa* has an effect that decreases the synthesis of these proteins. Finally, when the protein expression level of the signal transmitting protein akt is examined in Figure 1D, a statistically significant difference was observed between the groups ($p < 0.05$). The akt value in the CCl₄ group was determined to be greater than that of the CCl₄ + *N. sativa* group.

Discussion

The role that plants with antioxidant features play in preventing cellular and tissue damage has been clearly understood thanks to many studies carried out in recent years. Since the number of studies related with the effects of *N. sativa* on lung damage is relatively low, we are of the opinion that the results of this study will make significant contributions to literature. It was shown that *N. sativa* extract with its antitumor, bronchodilator, antidiabetic etc. effects displays antioxidant features with its free radical inhibition attribute as well as its treatment effects (Aqel and Shaheen, 1996; Harzallah et al., 2011). Ahmad et al (2013) define *N. sativa* as a miraculous plant and mention it as one that eases respiration while also having lung protective attributes thanks to its treatment effects on the trachea and respiration tract. Kanter et al (2005) examined the effects of *N. sativa* on cadmium induced oxidative stress in rat blood thus putting forth that the increase in malondialdehyde levels of plasma and erythrocytes can be decreased with *N. sativa* treatment. Sahin et al (2003) carried out a study examining the effects of *N. sativa* application on CCl₄ and liver necrosis, thus putting forth that *N. sativa* has significant treatment effects on the malondialdehyde levels as well as various biochemical parameters such as ALT, AST. Krishnan and Muthukrishnan (2012)

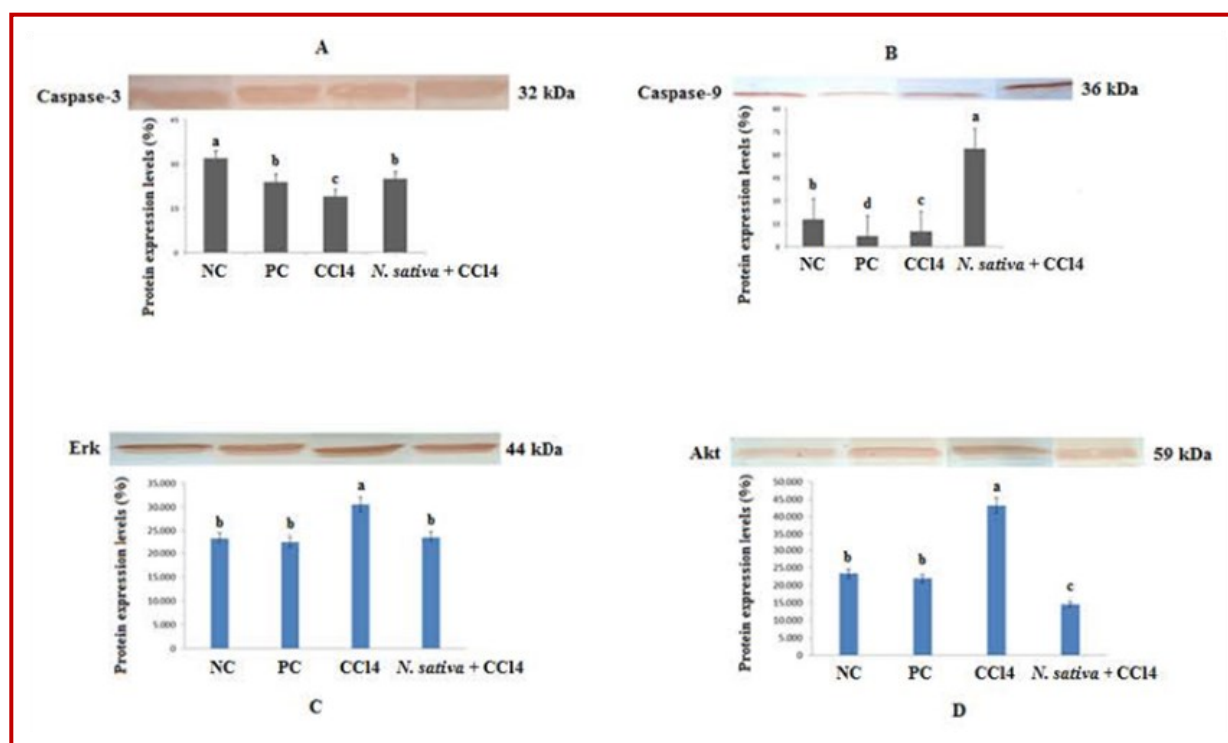


Figure 1: Caspase-3 (A), caspase-9 (B), Erk (C) and Akt (D) protein expression levels. Differences between groups in the same column with different letters are statistically significant ($p < 0.05$). One-way ANOVA post hoc Tukey test

carried out a study examining the effects of *N. sativa* seed extract on carbon tetrachloride-induced hepatotoxicity in rats thus putting forth that carbon tetrachloride causes damage on cells and tissues while disrupting many biochemical activities; while also stating that *N. sativa* seed extract administered to rats at a ratio of 10% via drinking water is quite effective on the decrease of damages on the antioxidant defense system as well as hepato-oxidative damage. Taking into account the regular rat weight determination that we conducted in our study (Table I), comparison of the first day weights and the weights 24 hours prior to slaughtering; a constant and high level of increase is observed in the control groups, whereas a lower amount of increase is observed in CCl₄ administered groups. Even though the weight increase in the CCl₄ + *N. sativa* group is lower in comparison with that of the control groups, a higher weight increase has been observed in comparison with the CCl₄ group. In conclusion, the lowest weight increase has occurred in the rats in the CCl₄ group. It can be said just by taking into consideration the differences in the increase in weight that *N. sativa* provides a protective effect on rats in comparison with CCl₄ which is a strong xenobiotic and that this effect has played an important role in many factors such as the feeding of the animals. Tayman et al (2013) carried out a study examining the protective effect of *N. sativa* on the hyperoxia induced lung damage in which they divided the rats into three groups, namely, control, hyperoxia and hyperoxia + *N.*

sativa thus subjecting the infant rats in the hyperoxia group to 95 % O₂. Whereas they used 100 % natural *N. sativa* oil in the hyperoxia + *N. sativa* group. As a result of the examination of the malondialdehyde level which is an indicator of lipid peroxidation, they reported that malondialdehyde level is much lower ($p < 0.05$) in the hyperoxia + *N. sativa* group in comparison with the hyperoxia group. When the malondialdehyde levels in the lung tissue in our results are compared (Table II), no statistically significant difference was determined between the control groups and CCl₄ + *N. sativa* group ($p > 0.05$). However, when the malondialdehyde level of the CCl₄ group is examined, it is observed that it has a higher ratio in comparison with the other three groups and that there is a statistically significant difference ($p < 0.05$). Based on this result, it can be stated that *N. sativa* plant is an effective antioxidant for decreasing the lipid peroxidation related malondialdehyde ratio. Jayaraman et al (2012) examined the effects of thymoquinone, a bioactive compound acquired from *N. sativa*, together with various apoptotic markers on cancer. They observed in their studies the effects of thymoquinone compound at various concentrations (0, 20, 40 μ M) on the expression of caspase-3 protein in the rat neuroblastoma and have put forth that caspase-3 expression ratio increases with increasing thymoquinone concentrations while also determining a statistically significant difference ($p < 0.05$). According to our results, the caspase-3 activity in the CCl₄ was lower at a statistically significant level ($p < 0.05$) in comparison

with other groups. Whereas a statistically significant difference was not observed between the positive control and CCl₄ + *N. sativa* groups in terms of caspase-3 protein expression, ($p > 0.05$) a greater caspase-3 activity was observed in the CCl₄ + *N. sativa* group in comparison with the CCl₄ group (Figure 1A). In addition, caspase-9 expression increased in the CCl₄ + *N. sativa* group in comparison with the CCl₄ group (Figure 1B). Hsu et al (2009) reported in their study carried out to examine the post-trauma lung damage decrease by way of ERK signal in female rats that ERK protein expression ratio is different at a statistically significant level ($p < 0.05$) in all groups with trauma and especially the vehicle group thereby putting forth that ERK signal path damage triggers various cancer types. When the ERK protein expression is examined in our study; it is observed that ERK synthesis has decreased at a statistically significant level in the CCl₄ + *N. sativa* group (Figure 1C) in comparison with the CCl₄ group ($p < 0.05$). Khalife et al (2014) carried out a study examining the antiproliferative (preventing cell proliferation) and proapoptotic effects of the active substance of *N. sativa* on acute myelogenous leukemia and put forth as a result of the various western blot analyses of some proteins that *N. sativa* extract prevents tumor angiogenesis (formation of blood veins) and tumor growth by way of suppressing Akt and ERK activation and that it can be used as a potential drug for cancer treatment. Tuzcu et al (2012) indicated that tomato powder has positive effects on caspase activity in colon cancer. Sahin et al (2010) indicated that Epigallocatechin-3-gallate activates Nrf2/HO-1 signaling pathway in rats. Sahin et al (2012) carried out a study that chromium picolinate, phosphatidylserine, docosahexaenoic acid and boron activates the antioxidant pathway Nrf2/HO-1 and protects the brain against oxidative stress in high-fat-fed rats. In our study, Akt protein expression level was lower in the CCl₄ + NS group (Figure 1D) in comparison with the control groups at a statistically significant level. The highest expression ratio was observed in the CCl₄ group and the Akt expression ratio in this group was different in comparison with those of the other groups at a statistically significant level ($p < 0.05$). Thus, we think that *N. sativa* extract decreased the synthesis of these proteins by affecting the erk/akt signal path mechanism.

Conclusion

In accordance with the results we acquired, it can be stated that *N. sativa* which is a strong antioxidant plant has protective effects against the possible lung damages in rats and that similarly it can be used in humans for treating the damages in the lungs due to these therapeutic effects.

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Conflict of Interest

The authors have declared that there are no conflict of interest among the authors.

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