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In Vivo Protective Role of *Nigella sativa* L. Against $Pb(NO_3)_2$ Induced Toxicity

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ABSTRACT

In this study, the protective role of *Nigella sativa* L. seed extract (NSSE) against $Pb(NO_3)_2$ toxicity in albino mice was investigated. For this purpose, the mice were randomly divided to six groups. In control group, mice were treated with tap water and in the treatment groups 500 mg kg^{-1} bw $Pb(NO_3)_2$, 300 and 600 mg kg^{-1} bw doses of NSSE were exposed to mice. The effects of all treatments on organism development were investigated by determining the changes in body, liver and kidney weights of each group. Genotoxic effects were determined by investigating the chromosomal abnormalities (CAs) in bone marrow cells, micronucleus (MN) frequency in erythrocyte and buccal mucosa cells. As a result, it was observed that $Pb(NO_3)_2$ treatment resulted in a 6.33-fold decrease in body weight gain and 2.08 and 1.65-fold increase in liver and kidney weights compared to control group, respectively. In addition, it was determined that these alterations in weights were positively changed after NSSE treatment. From MN assays it was observed that MN frequencies of erythrocyte and buccal mucosa cells in 500 mg kg^{-1} bw $Pb(NO_3)_2$ treated group were found as 58.17±5.46 and 19.00±2.76 while in 600 mg kg^{-1} bw NSSE+$Pb(NO_3)_2$ treated group, the frequencies were determined as 32.67±3.78 and 6.50±1.87, respectively. A similar protective role was also observed against CAs formation, and 600 mg kg^{-1} NSSE treatment was found to reduce the chromatid breaks by 44%. As a result, it has been determined that NSSE can be used as a protective nutrient against the harmful effects of chemicals such as heavy metals especially Pb.

Keywords: *Albino mice, Body weight, Cytotoxicity, Nigella sativa, Organ weight, $Pb(NO_3)_2$*

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Pb(NO3)2 Toksisitesine Karşı *Nigella sativa* L.'nin *In vivo* Koruyucu Rolü

ÖZET

Bu çalışmada albino farelerde Nigella sativa L. tohum ekstrakt (NSTE) uygulamasının Pb(NO3)2 toksisitesine karşı koruyucu rolü araştırılmıştır. Bu amaçla, fareler rastgele altı gruba ayrılmıştır. Kontrol grubu fareler çeşme suyu, uygulama grubundakiler ise 500 mg kg-1 c.a Pb(NO3)2, 300 ve 600 mg kg-1 c.a NSTE ile muamele edilmiştir. Her bir grupta total vücut ağırlığı, karaciğer ve böbrek ağırlığı belirlenerek organizma gelişimi üzerine etkiler incelenmiştir. Genotoksik etkiler, eritrosit ve yanak mukoza hücrelerinde mikronukleus (MN) sıklığı ve kemik iliği hücrelerinde kromozomal anormallikler (KA) belirlenerek saptanmıştır. Sonuç olarak Pb(NO3)2 uygulamasının vücut ağırlığında kontrol grubuna kıyasla 6.33 katlık bir azalmaya, karaciğer ve böbrek ağırlıklarında ise sırasıyla 2.08 ve 1.65 katlık bir artışa neden olduğu belirlenmiştir. NSTE uygulamasının ağırlıklarda gözlenen bu anormallikleri olumlu yönde değiştirdiği belirlenmiştir. MN analizleri sonucunda, 500 mg kg-1 Pb(NO3)2 uygulanan grupta eritrosit ve yanak mukoza hücrelerinde MN sıklığı sırasıyla 58.17±5.46 ve 19.00±2.76 olarak bulunurke, 600 mg kg-1 NSSE+Pb(NO3)2 uygulanan grupta bu değerler sırasıyla 32.67±3.78 ve 6.50±1.87 olarak bulunmuştur. NSTE'nin benzer bir koruyucu etkisi KA oluşumlarında da gözlenmiş, 600 mg kg-1 NSTE uygulamasının kromozom kırıklarını %44 oranında azalttığı belirlenmiştir. Sonuç olarak NSTE'nin kimyasalların özellikle Pb gibi ağır metallerin zararlı etkilerine karşı koruyucu bir besin olarak kullanılabileceği saptanmıştır.

Anahtar kelimeler: *Albino fare, Canlı ağırlığı, Sitotoksisite, Nigella sativa, Organ ağırlığı, Pb(NO3)2*

I. INTRODUCTION

In recent years, environmental pollution has increased rapidly due to an increase in the world population, irregular urbanization and rapid technological progress. Heavy metal-containing factories are among the leading industrial organizations that increase environmental pollution and play an important role in the deterioration of ecological balance. Metal ions such as mercury, zinc, cobalt, copper, iron, silver, chromium, arsenic, and lead used in these organizations cause unconscious destruction of ecological balance by contaminating the solid or water sources [1]. Lead (Pb) is also one of the toxic heavy metals which cause environmental pollution and widely used in the construction industry as galvanizing agent, isolation of underground communication cables, oxide paint manufacture, fuel octane adjuster, electronic devices and automotive industry [2]. The toxic effects of Pb on living organisms have been studied for many years and the toxic effects have been reported. Pb acts on cells as inducing free radical formation, leading to lipid peroxidation, which disrupts cell membrane structure and causes cell damage. Pb exposure in children and adults has been reported to cause cerebral edema, inhibition of hemoglobin synthesis, sensory disorders, weight loss, headache, anemia, stomach complaints and hypertension [3].

The toxic effects of Pb can be reduced by many natural food supplements [4]. The biological effects of many plant species that are consumed as food are examined and the large number of plant diversity in the world makes such studies inadequate. In literature, Seven et al. [5] used nettle extract against the genotoxic effects of Paraben. Yalcin et al. [6] reported the protective effect of β-carotene against the

genotoxic effects of Ammonium sulfate. In this study, the protective role of NSSE against $Pb(NO_3)_2$ toxicity in Swiss albino mice was investigated. *N. sativa* (Black seed) is an annual herbaceous plant species from the family Ranunculaceae and widely cultivated in many countries. It has been determined that the main components characterized in NSSE were thymoquinone (27.8-57.0%), carvacrol (5.8%-11.6%), ρ-cymene (7.1%-15.5%), 4-terpineol (2.0%-6.6%), longifolene (1.0%-8.0%) and t-anethole (0.25%-2.3%) [7]. In another study, it was reported that NSSE contain 4% ash, 6.4% water, 32% fat, 6.6% crude fiber, 20.2% crude protein and 37.4% carbohydrate, fixed oil consists of 1.2% myristic, 8.4% palmitic, 2.9% stearic, 17.9% oleic, 60.8% linoleic, less arachidic and 1.7% eicosadienoic acids. In the seeds also there is a small amount of vitamins B_1, B_2, B_6, amino acids, minerals such as iron, calcium, magnesium, zinc and selenium [8, 9]. Due to the rich content of NSSE, further studies on the protective effects against toxicity should be made and used alternatively in the treatment of various diseases. There are no studies about *in vivo* protective effects of NSSE against $Pb(NO_3)_2$ induced damages. In this context, this study will provide the first data introduce about the protective effects of NSSE against Pb toxicity into the literature. In this context, protective effects of NSSE against $Pb(NO_3)_2$ toxicity were investigated by using MN analysis in erythrocyte and buccal mucosa cell, chromosomal abnormalities in bone marrow cell, weight changes in body, liver and kidney.

II. Material and Methods

A. ANIMALS AND CHEMICAL

$Pb(NO_3)_2$ and other chemicals were obtained from Sigma-Aldrich Inc. NSSE (grounded black seed powder) was obtained commercially from 1001 Natural herb-store. In this study, a total of 36 adult male *Mus musculus* var. *albinos* mice weighing 25-30 g maintained in Giresun University Experimental Animals Research Laboratory were used. All animals were kept under controlled laboratory conditions of 22±3°C and 55±5% relative humidity with a 12-h light-dark cycle and housed in 26x15x50 cm stainless steel cages throughout the experimental period.

B. EXPERIMENTAL PROTOCOL

Animals were assigned to six groups as one control and five treatment groups and each group contained six animals. During treatment period for 10 weeks, mice in control group were fed with orally tap water and treatment groups treatment groups were fed with different doses of NSSE and $Pb(NO_3)_2$ shown in Table 1.

Table 1. *Groups and grouping principles*

Groups	Treatments
Group I	Tap water
Group II	300 mg kg^{-1} bw NSSE
Group III	600 mg kg^{-1} bw NSSE
Group IV	500 mg kg^{-1} bw $Pb(NO_3)_2$
Group V	500 mg kg^{-1} bw $Pb(NO_3)_2$+300 mg kg^{-1} bw NSSE
Group VI	500 mg kg^{-1} bw $Pb(NO_3)_2$+600 mg kg^{-1} bw NSSE

The methods and techniques applied to mice were carried out according to the guidelines set by the World Health Organization [10] and by the approval of Giresun University Animal Experiments-Local Ethics Committee (Protocol date: 27.04.2016, Decision number: 2016/01) for Animal Experiments. The survival rate of the treated animals was 100% until the end of the experiment.

C. BODY AND ORGAN WEIGHTS

Body weights of the control group and treatment groups of albino mice were determined by using precision balance under anesthesia. Changes in body weight were determined by taking the weight differences before and after application. To determine the effect of $Pb(NO_3)_2$ and NSSE on liver and kidney weight, weights of organs were measured in each group after the mice were sacrificed.

D. ERYTHROCYTE MICRONUCLEUS (MN) ASSAY

For MN assay, the mice were anesthetized and blood samples were collected from tail vein. 5 μL of peripheral blood collected was mixed with 3% EDTA and smeared onto a clean slide. After fixation with 70% methanol the slides dried. Then the slides were stained with 5% May–Grünwald Giemsa for 15 min and washed with distilled water. Usually, three or four slides are prepared for each group and the MN frequency on each slide is counted. A total of 1.000 normochromatic erythrocytes for each group were counted under the research microscope at X100 magnification and the number of cells with MN was determined and photographed at X500 magnification [11].

E. MN ASSAY FOR EXFOLIATED BUCCAL MUCOSA CELLS

The mouth of mice was washed before sampling and the exfoliated cells were collected by right and left buccal mucosa scraping. The scraped cells were transferred to slide surface and allowed to dry. After fixation in methanol:acetic acid the slides were stained with Feulgen and counterstained with Fast Green. 1.000 exfoliated cells were counted for each slide and MN cells were photographed. MN scoring was conducted according to Fenech et al. [12].

F. PREPARATION OF BONE MARROW CELLS FOR CHROMOSOME ANALYSIS

Mice were treated i.p. with 0.025% colcemid before killing and sacrified by cervical dislocation after 2 hours. The femurs dissected out and bone marrow cells were aspirated. After hypnotic treatment with 0.075 M KCl, preparations fixed in Carnoy’s solution and stained with 5% May-Grünwald Giemsa. CAs was classified according to criteria proposed by Savage et al. [13].

G. STATISTICAL ANALYSIS

The statistical analyses were carried out with SPSS for Windows v22.0 (IBM Corp.). Results were analyzed using "one-way" ANOVA and Duncan's test. The data are given as mean ± SD values, and values of $P < 0.05$ are considered statistically significant.

III. RESULTS AND DISCUSSION

Exposure to heavy metals such as Pb has a negative effect on organisms and this exposure causes serious health problems. Consumption of biologically active medicinal foods is very important in reducing the effects of these exposures. In this study, the protective role of NSSE against $Pb(NO_3)_2$ toxicity which people are frequently exposed to in daily life was investigated with multi-parameters.

A. EFFECTS ON BODY AND ORGAN WEIGHT

The first parameter to be examined is the weight changes in body, liver and kidney tissues and the results are given in Table 2. It was observed that only NSSE treatment had no statistically significant effect on weights of 300 and 600 mg kg^{-1} bw NSSE treated groups compared to control group. Interestingly, weight gains were decreased, liver and kidney weights increased after $Pb(NO_3)_2$ treatment. In control and 500 mg kg^{-1} bw $Pb(NO_3)_2$ treated group body weight gain were found as 10.46±1.11 and 1.65±0.45, respectively. It was determined that liver and kidney weights increased after $Pb(NO_3)_2$ treatment and this increase was 2.09 and 1.65 times higher compared to control, respectively. The changes observed in body and organ weights can be associated with the toxic effects of Pb. $Pb(NO_3)_2$ readily dissolves in water to give Pb^{2+} and NO^{-3} ions. The ionic mechanism of Pb^{2+} toxicity originates from the ability to replace other bivalent cations such as Ca and monovalent cations such as Na. Such cations are very important for the survival and continuity of cells, and the replacement of these cations leads to inhibition of many metabolic pathways. Significant disruption occurs in various biological processes such as cell adhesion, intracellular signaling, protein folding, ionic transport, enzyme regulation [14]. Disruptions that occur in vital reactions affect the growth of cells, tissues and ultimately the living organism and change the weight gain of the body [15]. In contrast to the decrease in total body weight, an increase in liver and kidney weights was observed after $Pb(NO_3)_2$ treatment and this increase was associated with hypertrophy. In cases of hypertrophy, the cells in tissue are not increased by number but by volume and the weight increase in related tissues can be associated with excessive work of cells to detoxify chemicals [16]. Similarly, Yagminas et al. [17] reported an increase in spleen and kidney weights and a decrease in live body weights in triethyl lead treated Sprague-Dawley rats. The changes in body and organ weights observed after $Pb(NO_3)_2$ treatment improved after NSSE application. In $Pb(NO_3)_2$ treated group, an increase of 1.65±0.45 g was observed in the body weight, while in 600 mg kg^{-1} bw NSSE+$Pb(NO_3)_2$ treatment, an increase of 6.63±0.78 g was determined. A similar recovery was also found in liver and kidney weights and the weights were found to be close to the control group values. This protective role may be associated with the hepato- and nephroprotective properties of NSSE reported in the literature [18].

Table 2. *Effects of $Pb(NO_3)_2$ and NSSE treatment on body weight gain, liver and kidney weights (g)*

Groups	Weight Gain	Liver Weight	Kidney Weight
Group I	$10.46±1.11^{a}$	$1.34±0.14^{c}$	$0.84±0.13^{c}$
Group II	$10.51±1.39^{a}$	$1.35±0.20^{c}$	$0.83±0.12^{c}$
Group III	$10.66±1.35^{a}$	$1.33±0.14^{c}$	$0.84±0.12^{c}$
Group IV	$1.65±0.45^{d}$	$2.80±0.31^{a}$	$1.39±0.12^{a}$
Group V	$3.93±0.43^{c}$	$2.53±0.34^{a}$	$1.19±0.18^{b}$
Group VI	$6.63±0.78^{b}$	$1.96±0.26^{b}$	$0.99±0.23^{c}$

**Data were shown as mean ± SD (N=6). Statistical significance between the means were determined using "one-way" ANOVA followed by Duncan's test. Means with the different letters in the same column are statistically significant (P < 0.05)*

B. EFFECTS ON MN FREQUENCY

The effects of $Pb(NO_3)_2$ and NSSE treatment on MN frequency in erythrocyte cells and exfoliated cells of buccal mucosa are shown in Table 3, 4 and Fig. 1. MN formation were not observed in control group, Group II and Group III. The highest MN formation among the groups was observed in $Pb(NO_3)_2$ treated group. It was observed that the frequency of MN in erythrocyte cells decreased by 1.18 and 1.78-fold in group V and VI compared to 500 mg kg^{-1} bw $Pb(NO_3)_2$ treated group. In buccal mucosa cells, the formation of MN in Group V and VI was decreased 1.44 and 2.92 times. This result indicates that NSSE reduces MN formation and provides more effective protection at 600 mg kg^{-1} bw. In literature, MN formation especially in erythrocytes induced by metal toxicity was reported. Tapisso et al. [19] reported an increase in MN frequency in polychromatic erythrocytes of male *Mus spretus* treated with different heavy metals including $Pb(C_2H_3O_2)_2$. In another study, Swiss albino rats were treated with 0.625-80 mg kg^{-1} bw $Pb(NO_3)_2$ and a significant increase in MN frequency of polychromatic erythrocyte cells was reported [20]. MN formation is an effective parameter used to evaluate the cytotoxic effects of chemicals and indicates cell division errors, spindle damages and chromosome breaks [21]. As erythrocytes are non-dividing cells, observation of MN in erythrocytes indicates toxicity in bone marrow stem cells. Briefly, bone marrow stem cells continuously produce new erythrocytes and erythrocytes are the only mammalian cell type without nucleus. If a chemical induced toxicity forms in a stem cell and a MN occurs as a result of this damage, MN remains in cytoplasm after the main nucleus is pushed [22]. MN formation observed in the buccal mucosa cell indicates the division disturbances in these cells while MN consisted erythrocytes indicates a toxicity in bone marrow stem cells.

Table 3. *MN frequency in erythrocyte cells*

Groups	The Number of Scored Cells	Minimum	Maximum	Average MN
Group I	1000	0	0	0.00 ± 0.00^{d}
Group II	1000	0	0	0.00 ± 0.00^{d}
Group III	1000	0	0	0.00 ± 0.00^{d}
Group IV	1000	50	65	58.17 ± 5.46^{a}
Group V	1000	45	60	49.00 ± 5.73^{b}
Group VI	1000	28	37	32.67 ± 3.78^{c}

**Data were shown as mean ± SD (N=6). Statistical significance between the means were determined using "one-way" ANOVA followed by Duncan's test. Means with the different letters in the same column are statistically significant (P < 0.05)*

Table 4. *MN frequency in exfoliated cells of buccal mucosa*

Groups	Number of Scored Cell	Minimum	Maximum	Average MN
Group I	1000	0	0	0.00 ± 0.00^{d}
Group II	1000	0	0	0.00 ± 0.00^{d}
Group III	1000	0	0	0.00 ± 0.00^{d}
Group IV	1000	50	65	19.00 ± 2.76^{a}
Group V	1000	45	60	13.17 ± 2.32^{b}
Group VI	1000	28	37	6.50 ± 1.87^{c}

**Data were shown as mean ± SD (N=6). Statistical significance between the means were determined using "one-way" ANOVA followed by Duncan's test. Means with the different letters in the same column are statistically significant (P < 0.05)*

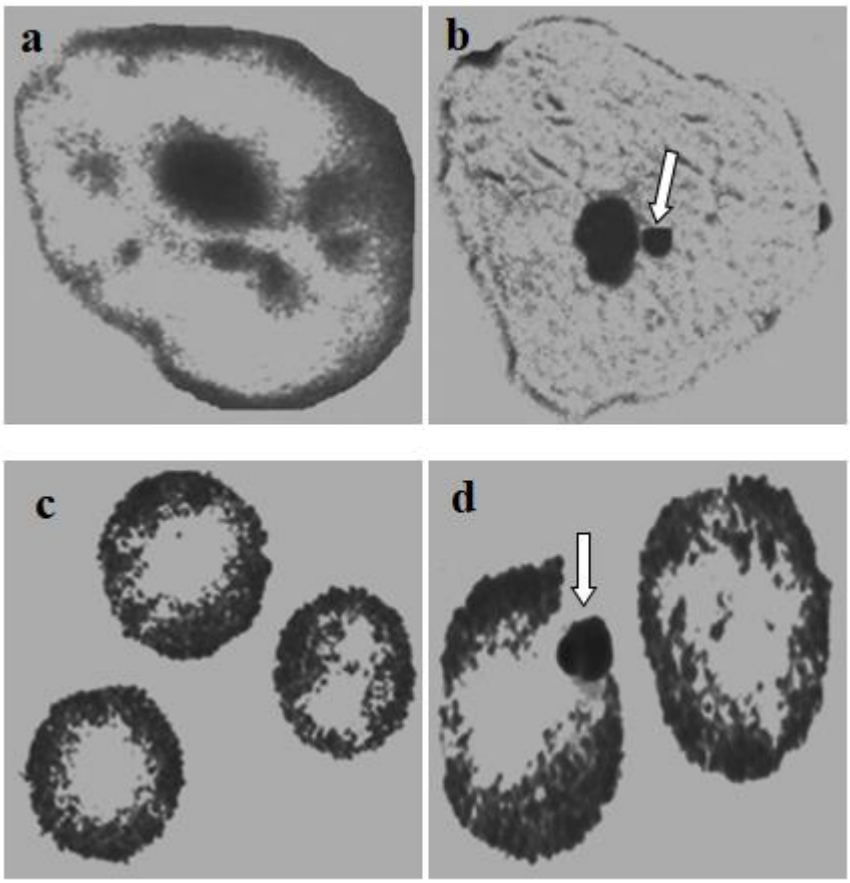

Figure 1. *MN analysis. Buccal mucosa cells in control group* ***(a)****, MN consisted buccal mucosa cells* ***(b)****, erythrocytes in control group* ***(c)****, MN consisted erythrocytes* ***(d)***

C. EFFECTS ON CHROMOSOMAL DAMAGE

CAs frequencies in $Pb(NO_3)_2$ and NSSE treated groups are shown in Table 5. No CAs formation was observed in the control group and only NSSE treated groups except for a few gaps. But there was a significant difference between the statistical parameters of CAs between the control group and $Pb(NO_3)_2$ treated group (*P<0.05*). A very high frequency of CAs was observed in 500 mg kg^{-1}

$Pb(NO_3)_2$ treated group and the order of these formations was chromatid break > fragment > gap > ring > acentric chromosome. It was observed that CAs frequencies importantly decreased in 300 and 600 mg kg^{-1} bw NSSE treatment groups and the most prominent protection effect of NSSE was found against the acentric chromosome. The frequency of acentric chromosome was found to be decreased by 7.9 times in 600 mg kg^{-1} bw NSSE treated group compared to Group IV. Similar, Aboul-Ela [23] demonstrated that extract of *N. sativa* reduced the formation of CAs induced by *Schistosoma mansoni* infection in mice. The CAs observed in this study can be explained by direct or indirect mutagenic effects of Pb. Oxidative stress caused by Pb destroys biological macromolecules such as lipids, proteins and DNA, resulting in cellular damage [24]. The effects of oxidative stress on DNA include base and sugar modifications, single and double chain fractures, formation of a basic region, DNA-protein cross-linking. All these mechanisms cause various CAs and many disruptions in the cell.

Table 5. *The frequency of chromosomal aberrations in bone marrow cells*

Groups	Chromatid Break	Fragment	Gap	Ring	Acentric Chromosome
Group I	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.33 ± 0.52^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
Group II	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
Group III	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
Group IV	45.17 ± 4.31^{a}	40.83 ± 3.49^{a}	18.33±3.27a	15.83 ± 2.86^{a}	10.50 ± 3.21^{a}
Group V	34.83 ± 5.91^{b}	29.17 ± 2.71^{b}	12.67 ± 3.01^{b}	9.50 ± 3.08^{b}	4.67 ± 1.03^{b}
Group VI	24.00 ± 5.66^{c}	18.67 ± 2.50^{c}	5.83 ± 1.17^{c}	4.67 ± 1.21^{c}	1.33 ± 0.52^{c}

**For CAs analysis, a total of 600 cells were counted as 100 cells per animal. Data were shown as mean ± SD (N=6). Statistical significance between the means were determined using "one-way" ANOVA followed by Duncan's test. Means with the different letters in the same column are statistically significant (P < 0.05)*

D. PROTECTIVE ROLE OF NSSE

It can be seen from the results of Group II and III, NSSE treatment in two doses alone does not cause any toxic effects. NSSE treatment with $Pb(NO_3)_2$ in Group V and VI showed marked protective properties in all tested parameters. The observed decrease in body weight gain and the increase in organ weights caused by $Pb(NO_3)_2$ started to improve with NSSE application. And also the frequencies of MN and CAs in $Pb(NO_3)_2$ treated group were significantly reduced. The improvement of abnormalities in tested parameters is more pronounced in 600 mg kg^{-1} NSSE application. Namely, it was shown that NSSE treatment has a dose-dependent protective effect on toxicity induced by $Pb(NO_3)_2$. Many researchers report that environmental pollutants lead to different abnormalities such as oxidative stress, cytogenetic damage, hepatoxic and nephrotoxic effects in organisms [25]. $Pb(NO_3)_2$ also causes damages through oxidative stress and the enhancement in antioxidant defense and the reduction of oxidant molecules provides protection against these damages. The detoxification, antioxidant and protective capacities of NSSE against oxidative damage, hepatoxic effects and nephrotoxic effects has taken place in the literature. Ahmad et al. [18] reported that NSSE has a strong antidiabetic, anticancer, immunomodulator, antimicrobial, antiinflammatory, hepatoprotective, renal protective properties. 1000 mg kg^{-1} *N. sativa* seeds have been reported to show a nephroprotective activity by decreasing the malondialdehyde and increasing the antioxidant enzyme levels against the toxicity of paracetamol [26]. Mabrouk and Cheikh [27] reported that the application of thymokinone, a component of *N. sativa* seed, resulted in an improvement in the oxidative damage parameters induced by lead. It has also been reported that 10 ml kg^{-1} *N. sativa* seed oil exhibits an important hepatoprotective effect against the oxidative damage and histological damages in liver induced by thioacetamide which is an experimental hepatotoxic agent [25]. Salomi et al. [28]

examined the inhibitory effects of NSSE on skin carcinogenesis and found that NSSE prevented the cancer and reduced the cytotoxicity of antineoplastic drugs. The protective activity of NSSE is related to the phytochemical content and studies in literature reported that more than 100 ingredients were found in NSSE. A combination of fatty acids, essential oils and trace elements is believed to contribute to the pharmacological activity [29]. It has also been reported that the seed oil of NSSE contains dihydrothymoquinone, thymo hydroquinone, thymol and mainly thymoquinone [30]. Many studies have shown that thymoquinone increases the activities of antioxidant enzymes such as superoxide dismutase, catalase and the protective effects of NSSE against genotoxicity observed in this study possibly occurs via these cumulative effects [31].

IV. CONCLUSION

Exposure to heavy metals as a result of rapid industrialization has highlighted the use of protective natural foods and accelerated the work on this subject. In this study, the protective role of NSSE against $Pb(NO_3)_2$ toxicity in Swiss albino mice was investigated and a moderately high protective effect was determined. Considering the serious side effects of the drugs used in the treatment of diseases such as cancer, kidney and liver failure, the tendency towards natural and protective herbal sources is increasing. For this purpose, various protective effects of natural plants should be investigated in a dose-dependent manner and these studies should be a guide for the use of natural resources.

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