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RESEARCH ARTICLE

# *Nigella sativa* Ameliorates Diethyl Phthalate – Induced Hepatotoxicity

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## ABSTRACT

The present study was undertaken to evaluate the dose – dependent toxic effect of diethyl phthalate (DEP) in liver of mice and its amelioration by extract of *Nigella sativa*. Healthy adult Swiss strain female albino mice were orally administered with DEP (310, 620 and 1240 mg/kg body weight/day) and DEP (1240 mg/kg body weight/day) plus *Nigella sativa* seed extract (150 and 300 mg/kg body weight/day) for 30 days. Various parameters were evaluated. The results revealed significant ($p < 0.05$) elevation in hepatic lipid peroxidation, which could be due to significant ($p < 0.05$) reduction in non-enzymatic (glutathione and total ascorbic acid contents) and enzymatic (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione transferase activities) antioxidants. The effect was dose – dependent. Oral administration of *Nigella sativa* seed extract (150 and 300 mg/kg body weight/day) with DEP (High dose) for 30 days resulted in dose – dependent and significant ($p<0.05$) reduction in lipid peroxidation as compared to DEP alone treated group. Similarly, all doses of *Nigella sativa* seeds extract ameliorated DEP- induced changes in non – enzymatic and enzymatic antioxidants. Amelioration of DEP- induced changes were maximum by 300 mg/kg body weight/day extract. Results of present study indicate that DEP – caused hepatotoxicity by inducing oxidative stress, whereas antihepatotoxic effect of *Nigella sativa* seeds extract was mainly due to its antioxidative potency.

## KEYWORDS

Diethyl Phthalate, *Nigella Sativa*, Oxidative Stress, Hepatotoxicity

## INTRODUCTION

Over the last decades there has been an increase in global concern over public health impacts attributed to environmental pollution. World Health Organization estimated that about a quarter of the diseases facing mankind today occur due to prolonged exposure to environmental pollution. The suspected chemicals originate from a variety of compounds of anthropogenic origin, such as pesticides, detergents and plasticizers. Phthalate acid esters are a class of organic compounds most widely used in the industrial production and mainly serve as plasticizers for polyvinyl chloride resins, adhesive, and cellulose film coatings (about 85% of the whole production)[1]. Owing to the large scale production and utilization; these compounds are leached out by water thus turning into ubiquitous aqueous persistent organic pollutant in the environment[2].

Diethyl phthalate (DEP) is widely used as a plasticizer and softener, coating materials and cosmetic additives[3]. Human beings are frequently in direct contact with DEP as the chemical is included in numerous cosmetic formulations such as bath products, hair sprays, nail polish solvents,

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shampoos, perfumes, after – shave lotions and detergents[4]. In Indian subcontinent, DEP is also extensively used in the manufacture of incense sticks and as a perfume binder[5]. DEP has been found to have diverse acute and chronic toxic effects on several species at different trophic levels, as well as endocrine disrupting properties[6,7,8]. Inhalation of DEP to cats at concentration of 356 ppm for 6 hours/day (corresponding to 3,289 mg/kg b.w./day) for 7 days caused decreased activity, vomiting, suppression of central nervous system, thirst and decreased appetite[9]. Diethyl phthalate affects the apoptotic system in PC12 cells and may enhance oxidative stress such as that induced by reactive oxygen species[10]. According to Jones *et al*[11], DEP produced mitochondrial swelling, focal dilation and vesiculation of smooth endoplasmic reticulum, and increased interstitial macrophage activity associated with the surface of the Leyding cells of rats. DEP also cause mitochondrial proliferation and peroxisomal proliferation[12]. Monoethyl phthalate, a main metabolite of DEP, is reported to cause increased DNA damage and also significantly associated with increased incidence of breast cancer in premenopausal women[13].

Medicinal plants are used in the preparation of herbal medicines as they are considered to be safe as compared to modern allopathic medicines. Many researchers are focusing on medicinal plants since only a few plant species have been thoroughly investigated for their medicinal properties, potential mechanism of action, safety evaluation and toxicological studies. The seed of *Nigella sativa* (NS), an annual *Ranunculaceae* herbaceous plant, has been used traditionally for centuries in the Middle East, Northern Africa, Far East and Asia for the treatment of asthma. Recently conducted clinical and experimental researches have shown many therapeutic effects of NS extracts such as immunomodulator[14], anti- inflammatory[15] and anti-tumour agents[16].

Present study deals with the evaluation of protective effect of *Nigella sativa* extract on DEP-induced hepatotoxicity under *in vivo* conditions.

## MATERIAL AND METHODS

### Chemicals

Diethyl phthalate (CAS No. 84-66-2) was procured from Sisco Research Laboratories, Mumbai, India. All other chemicals used in present study were of analytical grade.

### *Nigella Sativa* Extracts Preparation

*Nigella sativa* was purchased from LBG, Ahmedabad and hydro - alcoholic extract was prepared according to Bhargava and Singh with slight modification[17]. 5 gm finely ground *Nigella sativa* seeds powder was dissolved in 50% aqueous methanol and allowed to stand overnight for maximum extraction of polyphenols. Percolation of the extract was performed at room temperature. Collected filtrate was evaporated below 50˚C to obtain a final product in the form of residues which were stored under refrigerated conditions. Extract was redissolved in water and used for treatment.

### Experimental Animals

All the animal experiments were performed on inbred healthy adult Swiss strain female albino mice (weighing 30-35gm). They were maintained at a temperature of 25 ± 2˚C and relative humidity of 50 – 55% and 12 h light/dark cycle. They were fed with certified pelleted rodent feed supplied by Amrut Feeds, Pranav Agro Industries Ltd., Pune, India and potable water *ad libitum*. The experimental protocols were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg-167/1999/CPCSEA), New Delhi, India. Animals were handled according to the guidelines published by the Indian National Science Academy, New Delhi, India (1991).

### Experimental Design and Treatment Schedule

Eighty animals were randomly divided into eight groups. Animals of group 1 (untreated control) were without any treatment. Animals of Group 2 received 0.2 ml olive oil/animal/day (olive oil was used to dissolve DEP) and marked as vehicle control. Antidote control group (group 3) animals were given oral treatment of *Nigella sativa* seeds extract (300 mg /kg body weight/day). Groups 4,

5 and 6 animals were given oral treatment of low dose (310 mg /kg body weight/day; 1/20$^{th}$ of LD $_{50}$ value), mid dose (620 mg /kg body weight/day; 1/10$^{th}$ of LD $_{50}$ value), and high dose (1240 mg /kg body weight/day; 1/5$^{th}$ of LD $_{50}$ value) of DEP. Based on the results, the high dose of DEP (1240 mg /kg body weight/day) was chosen further to evaluate hepatoprotective effect of *Nigella sativa*. Further animals of groups 7 and 8 were treated with DEP (1240 mg /kg body weight/day – HD DEP) along with 150 and 300 mg /kg body weight/day of aqueous *Nigella sativa* seed extract.

All treatment was carried out for 30 days. After completion of the treatment, animals were humanely sacrificed and the liver was isolated, blotted free of blood, quickly weighed and used for determination of biochemical parameters.

**Lipid Peroxidation**

Lipid peroxidation (LPO) in liver was measured by estimating malondialdehyde (MDA) - an intermediary product of lipid peroxidation - by thiobarbituric acid reactive substances (TBARS) by the method as described by Ohkhawa*et al.*[18]. The formed MDA was measured spectrophotometrically at 532 nm. The level of lipid peroxidation was expressed as nmoles of MDA formed/mg protein/60 min.

**Enzymatic Antioxidants**

Activity of superoxide dismutase (SOD) (EC 1.11.1.6) was measured by the method of Marklund and Marklund[19]. Superoxide radical formed reacts with pyrogallol dye causing its autooxidation resulting in blue colour which is read at 470 nm. Superoxide dismutase activity was expressed as U/mg protein. Catalase (CAT) (EC 1.15.1.1) activity in tissue was measured by the method of Sinha*et al.*[20] using hydrogen peroxide as standard substrate. Potassium dichromate-acetic acid reagent was added at the interval of 0, 15, 30 and 60 s to terminate the reaction. Resulting orange-yellow colour was read at 590 nm. Catalase activity was expressed as µ moles $H_2O_2$ consumed/mg protein/min. The glutathione peroxidase (GPx) (EC 1.11.1.9) activity in the liver was assayed by modified method of Pagila and Valentine[21]. The enzyme activity was expressed as units/mg protein/min, where 1 unit of GPx equals to nmoles NADPH consumed/mg protein/min. The glutathione reductase (GR) (EC 1.11.1.9) activity in liver was assayed by the method of Mavis and Stellwagen[22]. The enzyme catalyzes the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). The decrease in absorbance was recorded for 5 min at 340 nm. The enzyme activity was calculated as nmoles NADPH consumed/mg protein/min. The liver glutathione S-transferase (GST) (EC 2.5.1.18) activity was assayed by the method of Habig*et al.*[23]. The increase in absorbance was noted at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB). The enzyme activity was calculated as µmoles CDNB conjugates formed/mg protein/min.

**Non-Enzymatic Antioxidants**

Glutathione content (GSH) was determined by Ellman's reaction using 5'5'-dithio-bis-2-nitrobenzoic acid (DTNB) as described by Moron *et al.*[24]. The amount of reduced glutathione was measured at 412 nm on spectrophotometer and was expressed as µg GSH/100 mg tissue weight. Total ascorbic acid (TAA) content in the liver was estimated by the method of Roe and Kuether[25]. TAA is oxidized to dehydroascorbic acid (DHA) by Noritreagent in the presence of TCA. This couples with 2,4-dinitro phenyl hydrazine in the presence of thiourea and sulfuric acid to yield a red colored complex which was read at 540 nm against blank. The TAA content was expressed as mg/gm of tissue weight.

**Protein Content**

Protein content in liver was measured by the method of Lowry *et al.*[26] using bovine serum albumin as a standard. Resulting blue colour was measured at 540 nm. The protein content was expressed as mg/100 mg tissue weight.

**Statistical Analysis**

All the data were expressed as means ± standard error mean (SEM). Statistical analysis and linear regression were performed using Graphpad Instat

 

software version 5.03. The data were statistically analyzed using One - way Analysis of Variance (ANOVA) followed by Tukey's test. The level of significance was accepted with $p < 0.05$.

## RESULTS AND DISCUSSION

The results shown in Figure 1 revealed no significant changes between different control groups (Groups 1, 2 and 3). In the present study, DEP caused hepatotoxicity by inducing oxidative stress. Lipid peroxidation is probably the most extensively investigated free radical induced process[27,28]. Lipid peroxidation is a major harmful consequence of reactive oxygen species (ROS) formation[29,30]. Oral administration of DEP for 30 days caused significant ($p<0.05$) and dose- dependent ($r^2 = 1$) elevation in lipid peroxidation. It could be due to significant reduction in enzymatic and non enzymatic antioxidants.

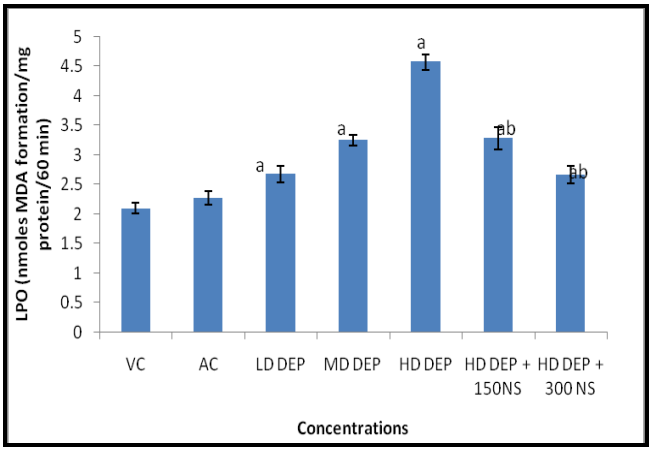


Figure 1: Effect of *Nigella sativa* seed extract on lipid peroxidation in liver induced by diethyl phthalate

Table 1: Effect of *Nigella sativa* seedsextract on DEP - induced changes in enzymatic antioxidants in mice liver

| Experimental Groups | Superoxide dismutase (Units/mg protein) | Catalase (µmoles $H_2O_2$ consumed/m protein/min) | Glutathione peroxidase (nmoles NADPH/mg protein/min) | Glutathione reductase (nmoles NADPH consumed/mg protein/min) | Glutathione transferase (µmoles of CDNB conjugate formed/mg protein/min) |
|---|---|---|---|---|---|
| **(I)Control** | | | | | |
| 1. Untreated | 1.68 ± 0.03 | 10.45 ± 0.28 | 6.27 ± 0.25 | 3.13 ± 0.17 | 4.82 ± 0.26 |
| 2. Vehicle | 1.65 ± 0.02 | 10.19 ± 0.21 | 6.70 ± 0.21 | 3.15 ± 0.05 | 4.76 ± 0.21 |
| 3. Antidote (NS300) | 1.65 ± 0.08 | 10.93 ± 0.38 | 6.35 ± 0.27 | 3.13 ± 0.16 | 4.01 ± 0.21 |
| **(II) Diethylphthalate(DEP) - Treated** | | | | | |
| 4. DEP310 ; LD | 1.23 ± 0.05 [a] | 8.58 ± 0.35 [a] | 5.59 ± 0.17 [a] | 2.54 ± 0.07 [a] | 3.85 ± 0.18 [a] |
| 5 .DEP620 ; MD | 0.81 ± 0.04 [a] | 6.55 ± 0.24 [a] | 3.13 ± 0.17 [a] | 2.02 ± 0.11 [a] | 2.34 ± 0.16 [a] |
| 6 .DEP1240 ; HD | 0.51 ± 0.02 [a] | 3.58 ± 0.18 [a] | 1.73 ± 0.08 [a] | 1.86 ± 0.15 [a] | 1.43 ± 0.13 [a] |
| **(III) DEPHD+ Nigella sativa (NS)extract - Treated** | | | | | |
| 7. HD DEP + NS150 | 0.89 ± 0.04[ab] (33.4) | 7.45 ± 0.38[ab] (58.6) | 3.76 ± 0.18[ab] (40.9) | 2.28 ± 0.13[ab] (31.5) | 2.70 ± 0.15[ab] (38.2) |
| 8. HD DEP + NS300 | 1.36 ± 0.06[ab] (74.6) | 9.09 ± 0.63 [b] (83.4) | 5.63 ± 0.28[ab] (78.5) | 2.94 ± 0.15 [b] (83.5) | 3.52 ± 0.24[ab] (62.8) |

Values are mean ± S.E.M.; n=10, No significance difference was noted between groups 1, 2 and 3. Significant at the level

[a] $p<0.05$ as compared to vehicle control (group 2) [b]$p<0.05$ as compared to toxin treated (group 6)

Values in parenthesis indicate hepatoprotective index (HPI).

 

Table 2: Effect of *Nigella sativa* seeds extract on DEP - induced changes in non-enzymatic antioxidants in mice liver

| Experimental Groups | Glutathione (µg/100mg tissue weight) | Total ascorbic acid (mg/gm tissue weight) |
|---|---|---|
| **(I)Control** | | |
| 1. Untreated | 54.57 ± 2.00 | 6.68 ± 0.32 |
| 2. Vehicle | 54.38 ± 1.38 | 6.41 ± 0.27 |
| 3. Antidote (NS300) | 54.49 ± 1.18 | 6.41 ± 0.19 |
| **(II) Diethyl phthalate (DEP)- Treated** | | |
| 4. DEP310 ; LD | 47.22 ± 1.32[a] | 5.22 ± 0.14 [a] |
| 5 .DEP620 ; MD | 36.93 ± 0.56 [a] | 4.44 ± 0.06 [a] |
| 6 .DEP1240 ; HD | 25.43 ± 0.68 [a] | 3.06 ± 0.08 [a] |
| **(III) DEPHD+ Nigella sativa (NS) extract - Treated** | | |
| 7. HD DEP + NS150 | 38.44 ± 1.32[ab] (45) | 4.48 ± 0.08[ab] (42.4) |
| 8. HD DEP + NS300 | 50.91 ± 0.20 [b] (85.6) | 5.83 ± 0.17[ab] (82.7) |

Values are mean ± S.E.M.; n=10
No significance difference was noted between groups 1, 2 and 3. Significant at the level [a] p <0.05 as compared to vehicle control (group 2) [b]p<0.05 as compared to toxin treated (group 6)
Values in parenthesis indicate hepatoprotective index (HPI).

Increased lipid peroxidation might be due to reduction in enzymatic and non-enzymatic antioxidants. As compared to vehicle control, activities of SOD and CAT were significantly ($p<0.05$) reduced in DEP – treated animals (Groups 4, 5 and 6) (Table 1). The effect was dose – dependent ($r^2$= 0.990, 0.990 respectively). Similarly, oral administration of DEP caused significant ($p<0.05$) and dose-dependent reduction in the activities of GPx ($r^2$ = 0.964), GR ($r^2$ = 0.888) and GST ($r^2$ = 0.979) as compared with vehicle control (Table 1). GSH is an important tripeptide found in most mammalian cells. It has numerous roles in cell function, which include removal of peroxides via selenium-dependent GSH peroxidase[31], non enzymatic reduction of free radicals[32] and conjunction of exogenous reactive intermediates either non-enzymatically or catalyzed by glutathione S-transferase[33]. Significant decrease in liver GSH and TAA of three treated groups indicates that increased toxic injury to the liver. Pereira *et al*[34] also reported depletion of GSH and GR in female Wistar rats.

Table 2 revealed that oral administration of DEP caused significant ($p<0.05$), dose-dependent reduction in GSH ($r^2$ = 0.999) and TAA ($r^2$ =0.974) contents in liver of mice. Significant decrease in liver GSH and TAA of three treated groups indicates that increased toxic injury to the liver. Pereira *et al*[34] also reported depletion of GSH and GR in female Wistar rats. Activities of enzymatic antioxidants (SOD, CAT, GPx, GR and GST) were found to reduce with DEP treatment for 30 days, which could be due to increased production of free radicals characterized by increased MDA content. These enzymes are known to scavenge free radicals such as superoxide, hydroxyl and hydrogen peroxide, thus preventing damage caused by oxidative stress to the tissue[35].

 

The coefficient of regression $r^2$ was obtained by linear regression. All result exhibited coefficient of regression $r^2 > 0.9$ ($p < 0.05$). The Pearson correlation revealed strong correlation between LPO versus GSH (r = -1), LPO versus TAA (r = -1), LPO versus SOD (r = -1), LPO versus CAT (r = -1), LPO versus GPx (r = -1), LPO versus GR (r = -1), LPO versus GST (r = -1).

Protection denoted by *Nigella sativa* against DEP – induced lipid peroxidation was 52.01% (NS 150) and 77.01% (NS 300) as calculated by hepatoprotective index. Activities of enzymatic and non enzymatic antioxidants were severely affected by DEP treatments which were brought back to normal by co treatment of *Nigella sativa* seeds extract shown in Table 1 and 2. Activities of hepatic SOD and CAT were reduced by DEP treatment and were found to increase significantly ($p < 0.05$) by various doses of *Nigella sativa* seeds extract in a dose-dependent manner. Hepatic protection shown in Table 1 by two doses of *Nigella sativa* for SOD and CAT was 47.4%, 59.3% (NS150) and 65.8%, 93.9% (NS300) respectively. Similarly, the protective effect of *Nigella sativa* seeds extract on the activities of GPx, GST and GR were also significant ($p < 0.05$) and dose-dependent as compared to high dose DEP intoxication. Hepatoprotective index calculated for GPx and GST activity was 22.2%, 26.2% (NS150) and 28.6%, 55.3% (NS300) respectively, which was 58% (NS150) and 95.6% (NS300) in case of GR activity (Table 1). Treatment of Nigella *sativa* also increased the level of GSH and TAA in DEP intoxicated animals. Hepatoprotective index calculated for GSH and TAA content was 65.4%, 42.4% (NS200) and 95%, 56.2% (NS300) respectively as shown in Table 2.

*Nigella sativa* doses significantly reduced levels of lipid peroxidation in DEP treated animals which could be due to increased enzymatic and non-enzymatic antioxidants (Table 1 and 2). Free radical scavenging activity of *Nigella sativa* polyphenols were well correlated in our earlier *in vitro* studies[36]. The antioxidant effect of *Nigella sativa* seed seems to be due to its flavonoids and also antioxidant vitamins like ascorbic acid. In addition flavonoids are a class of polyphenolic compounds that seem to have antioxidant properties by suppressing reactive oxygen and nitrogen species formation, scavenging reactive oxygen and nitrogen species and protecting the antioxidant defence system[37,38].

## CONCLUSION

In conclusion, DEP oral administration caused alteration in oxidative stress marker lipid peroxidatopn as well as enzymatic and non-enzymatic antioxidants in mice liver, which could be a principal mechanism responsible for its hepatotoxicity. Hydro – alcoholic extract if *Nigella sativa* seeds reduced DEP induced hepatic changes mainly due to its phytochemicals having antioxidative properties.

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