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NIGELLA SATIVA AMELIORATES OXIDATIVE STRESS INDUCED BY DIETHYL PHTHALATE – AN *IN VITRO* STUDY

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Keywords:

Diethyl phthalate, Lipid peroxidation, *Nigella sativa*, Oxidative stress, Protein content

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ABSTRACT: The present experiment was designed to assess oxidative stress, if any, caused by diethyl phthalate and its amelioration by *Nigella sativa* seed extract. For this, the liver and kidney homogenates were treated with different concentrations (10-40 µg/mL) of DEP with or without *Nigella sativa* seed extract. Lipid peroxidation, a marker for oxidative stress along with total protein content was analyzed. The results revealed that DEP (10-40 µg/mL) caused, significant and concentration - dependent increase in lipid peroxidation, whereas protein content reduced significantly. Maximum effect on lipid peroxidation was obtained with 40 µg/mL DEP concentration and hence used for further studies. On addition of *Nigella sativa* seed extract (25-200 µg/mL) along with DEP (40 µg/mL) significantly reduced DEP - induced lipid peroxidation in a concentration-dependent manner. However, protein content was higher as compared to toxin alone treated ones. The effect of both DEP alone and DEP along with *Nigella sativa* seed extract were almost similar in liver and kidney homogenates. It is concluded from the present study that supplementation of *Nigella sativa* extract can be beneficial in positively modulating DEP – induced alterations in liver and kidney

INTRODUCTION: Diethyl phthalate is used in pharmaceutical coating, as a fixative in cosmetics, in the manufacture of varnishes and ropes, in the denaturation of alcohol and as perfume binders [1]. Among potential sources of DEP contamination and accumulation in human beings, one is cosmetic products and other is dietary meat of fish particularly from unknown contaminated sources [2, 3].

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DEP, an endocrine disrupter chemical, has been found to have diverse acute and chronic toxic effects in several species at different trophic levels [4]. It caused mitochondrial swelling, focal dilation and vesiculation of smooth endoplasmic reticulum and increased interstitial macrophage activity associated with the surface of Leyding cells of rats [5]. According to Hayashi *et al* [6], DEP affects sperm count in the mammals. It was also reported as neurotoxicant and enhances production of reactive oxygen species (ROS) and lipid peroxidation [7, 8].

Oxidative stress is a condition in which the generation of reactive oxygen species (ROS), a ubiquitous by - products of aerobic metabolism, overwhelm the cellular antioxidant defence

mechanism. Lipid peroxidation has been taken as an indicator of cellular oxidative damage [9]. Lipid peroxidation – derived free radical could attack back-bone of protein and side chains of specific amino acid residues [10].

Complementary and Alternative Medicine (CAM), including herbal medicine, is popular in the general population worldwide [11]. *Nigella sativa* belongs to family Ranunculaceae. It is an annual, erect herb, distributed in South West Asia, South Europe and North Africa [12]. Studies on *Nigella sativa* seed extract have provided scientific support for the treatment of rheumatism, immune stimulation, diabetes, cancer and related inflammatory diseases [13].

The aim of the present study was to evaluate oxidative stress; if any caused by DEP *in vitro* and its amelioration by *Nigella sativa* seed extract *in vitro* condition.

MATERIALS AND METHODS:

Chemicals: Analytical grade diethyl phthalate (DEP) (CAS No. 84-66-2) was purchased from Sisco Research Laboratories, Mumbai, India. All other chemicals used in the present study were of analytical grade.

***Nigella sativa* extract preparation:** Seeds of *Nigella sativa* purchased from local market was used for hydro - alcoholic extract preparation by the method as described by Bhargava and Singh with slight modification [14]. Finely ground *Nigella sativa* seeds powder was mixed with 50% methanol and allowed to stand overnight for maximum extraction of polyphenols. Percolation of the extract was performed at room temperature in two stages. Collected filtrate was evaporated below 50˚C to obtain a final product in the form of residues which was stored under refrigerated condition. Extract was dissolved in double distilled water and used for further studies.

Liver and kidney homogenates: Inbred adult healthy Swiss strain female albino mice weighing 30-35 gm were obtained from Zydus Research Centre, Ahmedabad, India. Animals were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India under controlled conditions (temperature 25 ± 2OC, 12 h light/dark cycle and relative humidity 50-55%). They were fed with certified pelleted rodent feed supplied by Amrut Feeds, Pranav Agro Industries Ltd., Pune, India and potable water *ad libitum*. All 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 Indian National Science Academy, New Delhi, India (1991).

Mice were humanely sacrificed liver and kidneys were isolated, blotted free of blood and used for biochemical studies.

Lipid peroxidation: Liver and kidney homogenates prepared in phosphate buffered saline were used for various treatments followed by estimation of lipid peroxidation.

Following sets of tubes were prepared:

1. Control tubes: These tubes contained tissues homogenates and phosphate buffered saline.

2. DMSO control tubes: These tubes contained tissues homogenates and DMSO.

3. Antidote control tubes: These tubes contained tissues homogenates and *Nigella sativa* seed extracts.

4. DEP - treated tubes: These tubes contained tissues homogenates with different concentrations of DEP (10-40 μg/ml) in DMSO.

5. DEP and *Nigella sativa* seed extract tubes: These tubes contained tissue homogenates with different concentrations of *Nigella sativa* seed extract (25 – 200 μg/ml) along with DEP (40 μg/ml).

The final volume of each tube was made up to 1 ml with phosphate buffered saline. Each tube was added with 0.1 ml H_2O_2 to induce lipid peroxidation. All the tubes were subjected to incubation for 30 min at 37°C.

The lipid peroxidation in control, DMSO control, toxin and toxin plus antidote treated samples were measured by quantification of thiobarbituric acid reactive substance (TBARS) determined by the method of Ohkawa *et al* [15] with slight modifications.

Protein: Liver and kidney were homogenized in chilled water and used for various treatment followed by estimation of protein content.

Following sets of tubes were prepared.

1. Control tubes: These tubes contained tissue homogenates and distilled water.
2. DMSO control tubes: These tubes contained tissue homogenates and DMSO.
3. Antidote control tubes: These tubes contained tissue homogenates and *Nigella sativa* seed extracts.
4. DEP - treated tubes: These tubes contained tissues homogenates with different concentrations of DEP (10-40 μg/ml).
5. DEP plus *Nigella sativa* seed extract – treated tubes: These tubes contained tissue homogenates with different concentrations of *Nigella sativa* seed extract (25 – 200 μg/ml) along with DEP (40 μg/ml).

The final volume of each tube was made up to 1 ml with distilled water. All the tubes were subjected to incubation for 30 min at 37°C.

The protein content in control, DMSO control, toxin and toxin plus antidote treated samples were estimated by the method of Lowry *et al* [16] with slight modifications using bovine serum albumin as a standard.

Statistical analysis:

All the data are expressed as the 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: From **Tables 1 and 2** we could see that addition of different concentrations (10-40 μg/ml) of DEP to liver homogenates of mice significantly ($p<0.05$) increased H_2O_2 – induced lipid peroxidation *in vitro* condition. At 40 μg/ml DEP concentration maximum lipid peroxidation was observed in both liver and kidney homogenates. The effect was significant ($p<0.05$) and concentration dependent in both liver and kidney homogenates (r^2= 0.993, 0.961 respectively) up to 40 μg/ml DEP as indicated in **Figure 1 and 2**.

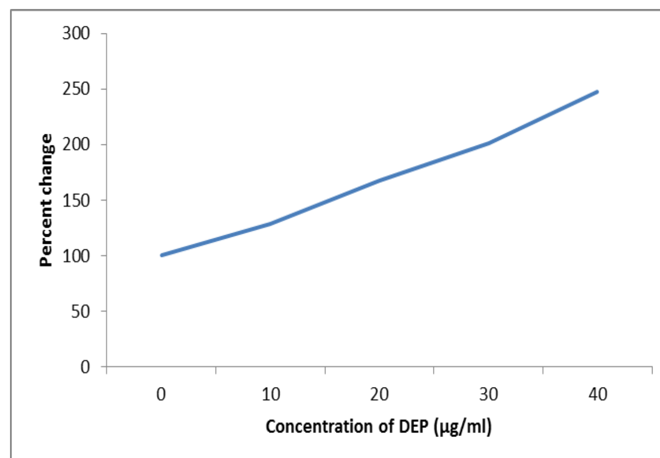

FIGURE 1: SHOWING DEP- INDUCED PERCENT CHANGE IN LIPID PEROXIDATION IN LIVER HOMOGENATES

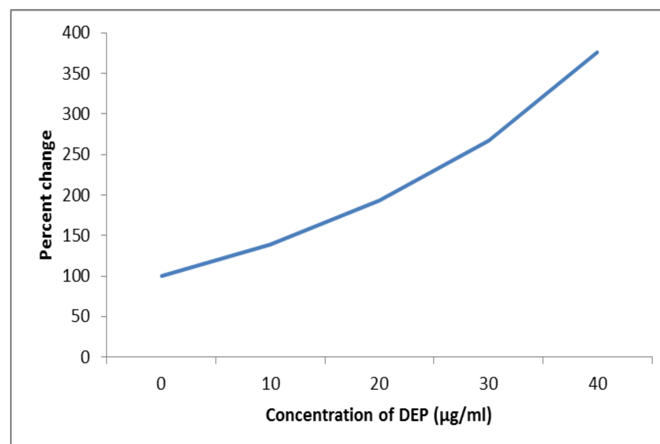

FIGURE 2: SHOWING DEP- INDUCED PERCENT CHANGE IN LIPID PEROXIDATION IN KIDNEY HOMOGENATES

In the present study, it was found that DEP induce – oxidative stress by increasing thiobarbituric acid reactive substances (TBARS) in mice liver and kidney homogenates. Lipid peroxidation is a major harmful consequence of reactive oxygen species (ROS) formation. Increased lipid peroxidation could lead to severe cell organelle damage leading

to impairment in the various metabolic functions of the cell [17]. Elevation of oxidative stress in liver and kidney homogenates indicates high level of ROS production due to damage by exposure of DEP. The higher level of TBARS after DEP treatment was also reported in Wistar rat and olive flounder *Paralichthys olivaceus*, a marine culture fish by Pereira *et al* [18] and Kang *et al* [19] respectively.

Results shown in tables 1 and 2 revealed significant ($p<0.05$), concentration- dependent decrease in protein content in liver and kidney homogenates (r^2= 0.849, 0.948) (**Figure 3 and 4**). Highest decrease in protein content was obtained at the 40 µg/ml DEP concentration. The protein content was significantly decreased in DEP treated liver and kidney homogenates. This could be due to oxidative stress. The decreased protein content in liver of DEP treated mice was also reported by Prajapati and Verma [20].

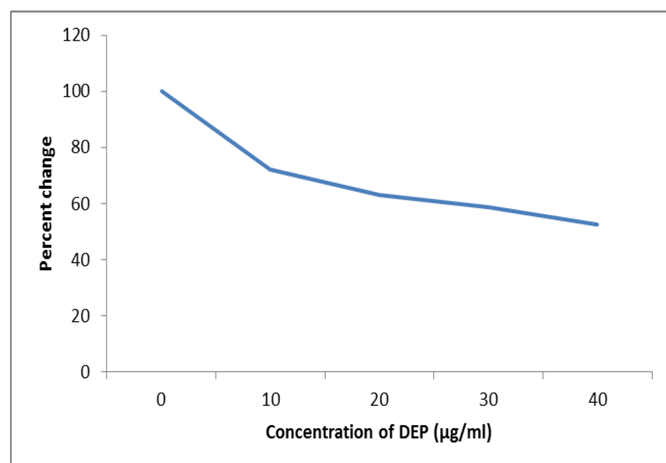

FIGURE 3: SHOWING DEP- INDUCED PERCENT CHANGE IN PROTEIN CONTENT IN LIVER HOMOGENATES

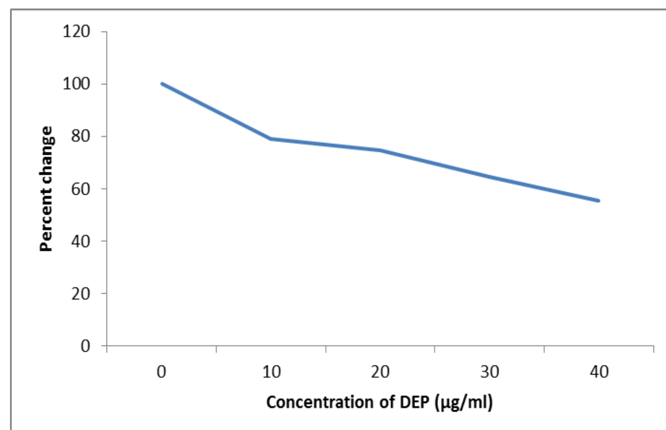

FIGURE 4: SHOWING DEP- INDUCED PERCENT CHANGE IN PROTEIN CONTENT IN KIDNEY HOMOGENATES

Table 1 and 2 shows that cotreatment of *Nigella sativa* seed extract (25 to 200 µg/ml) along with DEP (40 µg/ml) significantly ($p<0.05$) mitigated DEP – induced lipid peroxidation in both liver and kidney homogenates. The effect was concentration – dependent in liver and kidney homogenates (r^2=0.986, 0.984 respectively) with maximum mitigation at 200 µg/ml of *Nigella sativa* seed extract (**Figure 5 and 6**).

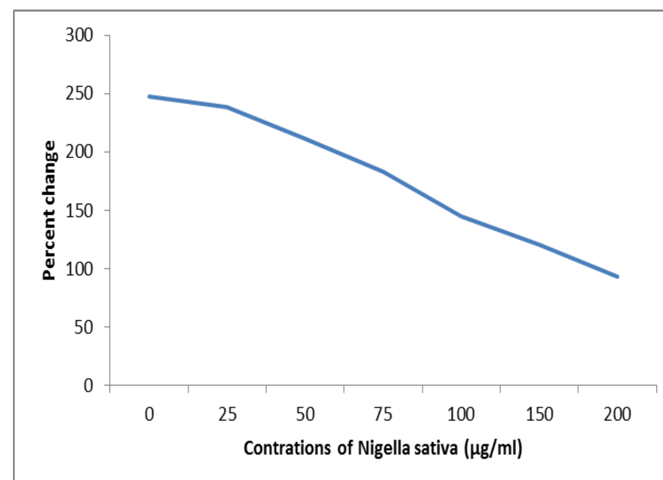

FIGURE 5: SHOWING EFFECT OF *NIGELLA SATIVA* SEED EXTRACT IN AMELIORATING (PERCENT CHANGE) DEP – INDUCED ALTERATION IN LIPID PEROXIDATION IN LIVER HOMOGENATE (Please note that each tube contained 40 µg/ml DEP).

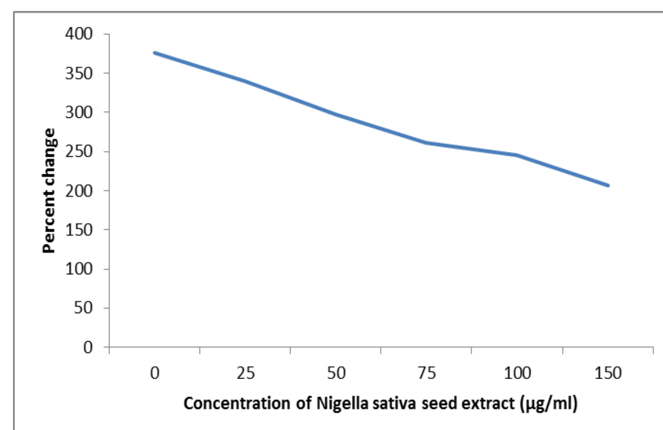

FIGURE 6: SHOWING EFFECT OF *NIGELLA SATIVA* SEED EXTRACT IN AMELIORATING (PERCENT CHANGE) DEP – INDUCED ALTERATION IN LIPID PEROXIDATION IN KIDNEY HOMOGENATE (Please note that each tube contained 40 µg/ml DEP).

The results shown in **Figure 7 and 8** reveal mitigation in concentration of protein content in liver and kidney homogenates treated with DEP along with *Nigella sativa* seed extract. The effect was concentration – dependent in both liver and kidney homogenates (r^2=0.982, 0.903 respectively).

Concurrent treatment of *Nigella sativa* seed extract along with DEP caused significant amelioration in lipid peroxidation and protein content in both liver and kidney homogenates. It could be due to antioxidative properties of *Nigella sativa* seed extract. Prajapati and Verma [21] have reported a strong DPPH radical scavenging activity of *Nigella sativa* seed extract. Free radical scavenging activity of *Nigella sativa* was also reported [22].

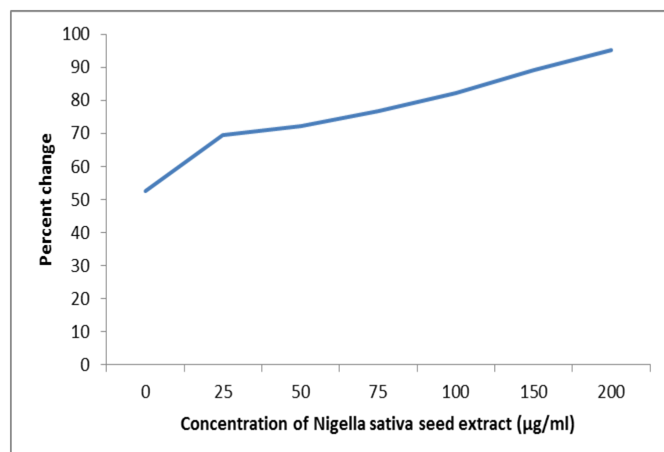

FIGURE 7: SHOWING EFFECT OF *NIGELLA SATIVA* SEED EXTRACT IN AMELIORATING (PERCENT CHANGE) DEP – INDUCED ALTERATION IN PROTEIN CONTENT IN LIVER HOMOGENATE (Please note that each tube contained 40 μg/ml DEP).

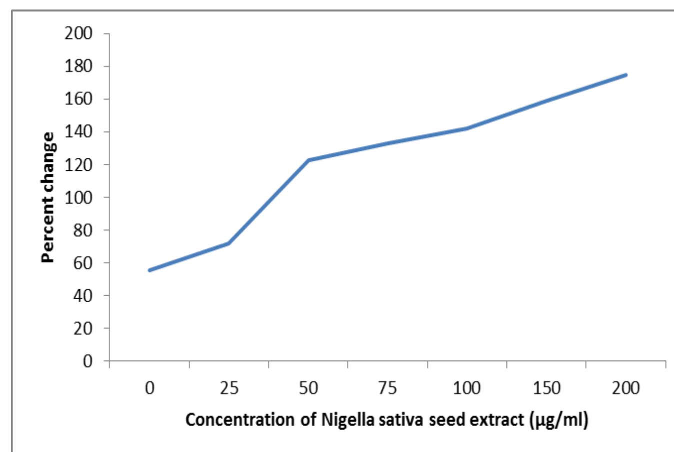

FIGURE 8: SHOWING EFFECT OF *NIGELLA SATIVA* SEED EXTRACT IN AMELIORATING (PERCENT CHANGE) DEP – INDUCED ALTERATION IN PROTEIN CONTENT IN KIDNEY HOMOGENATE (Please note that each tube contained 40 μg/ml DEP).

TABLE 1: SHOWING EFFECT OF *NIGELLA SATIVA* SEED EXTRACT ON DEP -INDUCED ALTERATIONS IN LIPID PEROXIDATION AND PROTEIN CONTENT IN LIVER HOMOGENATES OF MICE

Treatment	Liver homogenate	
	Lipid peroxidation (n moles MDA formed/mg protein/60 min)	**Protein content (mg/100 mg tissue weight)**
(I) Control		
1.Untreated control	20.90 ± 0.27	12.61 ± 0.71
2. DMSO control	21.17 ± 0.39	12.36 ± 0.54
3. Antidote (200 μg/ml)	20.78 ± 0.33	12.57 ± 0.54
(II) Diethyl phthalate - Treated		
4. DEP (10 μg/ml)	27.18 ± 1.87 [a]	8.93 ± 0.65 [a]
5. DEP (20 μg/ml)	35.39 ± 1.00 [a]	7.79 ± 0.68 [a]
6. DEP (30 μg/ml)	42.56 ± 1.65 [a]	7.27 ± 0.63 [a]
7. DEP (40 μg/ml)	52.37 ± 1.99 [a]	6.50 ± 0.57 [a]
(III) DEP1240(HD) + *Nigella sativa* extract - Treated		
8. DEP (40 μg/ml) + NS (25 μg/ml)	50.37 ± 0.46 [a]	8.58 ± 0.42 [ab]
9. DEP (40 μg/ml) + NS (50 μg/ml)	44.69 ± 1.13 [ab]	8.92 ± 0.28 [ab]
10. DEP (40 μg/ml) + NS (75 μg/ml)	38.68 ± 0.52 [ab]	9.49 ± 0.17 [ab]
11.DEP (40 μg/ml) + NS (100μg/ml)	30.71 ± 0.48 [ab]	10.17 ± 0.15 [b]
12. DEP (40 μg/ml) + NS (150 μg/ml)	25.41 ± 0.57 [b]	11.01 ± 0.19 [b]
13. DEP (40 μg/ml) + NS (200 μg/ml)	19.77 ± 0.74 [b]	11.77 ± 0.20 [b]

Results are expressed as Mean ± S.E.M., n = 10. No significant difference was noted between control tubes. a $p<0.05$ as compared to Control. [b] $p<0.05$ as compared to Toxin treated. Level of significance $p < 0.05$

TABLE 2: SHOWING EFFECT OF *NIGELLA SATIVA* SEED EXTRACT ON DEP- INDUCED ALTERATIONS IN LIPID PEROXIDATION AND PROTEIN CONTENT IN KIDNEY HOMOGENATES OF MICE

Treatment	Kidney homogenate	
	Lipid peroxidation (n moles MDA formed/mg protein/60 min)	Protein content (mg/100 mg tissue weight)
(I) Control		
1.Untreated control	13.31 ± 0.57	9.14 ± 0.38
2. DMSO control	13.78 ± 0.41	9.34 ± 0.35
3. Antidote (200 µg/ml)	13.80 ± 0.38	9.90 ± 0.19
(II) Diethyl phthalate - Treated		
4. DEP (10 µg/ml)	19.04 ± 1.63[a]	7.38 ± 0.20 [a]
5. DEP (20 µg/ml)	26.63 ± 0.90 [a]	6.97 ± 0.38 [a]
6. DEP (30 µg/ml)	36.73 ± 1.48 [a]	6.02 ± 0.29 [a]
7. DEP (40 µg/ml)	51.75 ± 1.18[a]	5.16 ± 0.19 [a]
(III) DEP1240(HD)+ Nigella sativa extract - Treated		
8. DEP (40 µg/ml) + NS (25 µg/ml)	46.77 ± 1.04 [ab]	6.70 ± 0.42 [ab]
9. DEP (40 µg/ml) + NS (50 µg/ml)	40.94 ± 0.40 [ab]	11.44 ± 0.19[ab]
10. DEP (40 µg/ml) + NS (75 µg/ml)	36.09 ± 0.77 [ab]	12.40 ± 0.48 [ab]
11.DEP (40 µg/ml) + NS (100µg/ml)	33.77 ± 0.70 [ab]	13.28 ± 0.44 [ab]
12. DEP (40 µg/ml) + NS (150 µg/ml)	28.42 ± 0.48 [ab]	14.87 ± 0.65 [ab]
13. DEP (40 µg/ml) + NS (200 µg/ml)	21.63 ± 0.47 [ab]	16.34 ± 0.90a [b]

Results are expressed as Mean ± S.E.M., n = 10. No significant difference was noted between control tubes. a $p<0.05$ as compared to Control. [b] $p<0.05$ as compared to Toxin treated. Level of significance $p < 0.05$

CONCLUSION: From this study, it can be concluded that different concentrations of DEP leads to concentration- dependent significant increase in lipid peroxidation and decrease protein content and *Nigella sativa* effectively mitigates the toxic effect of DEP- induced lipid peroxidation and protein content in liver tissue.

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