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MITIGATION OF DIETHYL PHTHALATE - INDUCED HEPATOTOXICITY BY *NIGELLA SATIVA* SEED EXTRACT IN MICE

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ABSTRACT

The aim of the present investigation was to evaluate hepatoprotective activity of *Nigella sativa* seed extract against diethyl phthalate (DEP) – induced toxicity in mice. Colony inbred Swiss strain adult female albino mice were orally administered with 310, 620 and 1240 mg/ kg body weight /day (low, mid, high dose respectively) for 30 days. Treatment caused, as compared with the control, significant ($p < 0.05$) decrease in body weight and significant ($p < 0.05$) increase in absolute and relative weights of liver. Also, DEP treatment caused significant ($p < 0.05$), dose – dependent increase in lipid peroxidation in liver and liver marker enzyme activities measured in serum such as ACP, ALP, AST and ALT than that of vehicle control. Histopathological studies revealed, intracellular vacuolation, necrosis and loss of hepatic architecture in DEP - treated animals. *Nigella sativa* seed extract (150 and 300 mg/kg body weight/day) treatment along with HD of DEP , caused significant ($p<0.05$) restoration in body weight, organ weight, lipid peroxidation in liver as well as liver marker enzymes in serum as compared to DEP alone treated mice. In addition histopathological changes were completely ameliorated in high dose extract treated mice. It is concluded from the present study that supplementation of *Nigella sativa* extract can be beneficial in positively modulating DEP – induced alterations in liver.

KEY WORDS: **Diethyl phthalate, enzymes, hepatotoxicity, histopathology, liver, *Nigella sativa***

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INTRODUCTION

Diethyl phthalate (DEP), a plastic additive, has been used as a plasticizer as well as solvent and fixative for cosmetic products[1]. In Indian subcontinent, DEP is also extensively used in the manufacture of incense sticks and as a perfume binder[2]. DEP exposure to human beings is likely to occur through oral, dermal as well as respiratory routes. Treatment with higher concentrations of DEP results in mitochondrial proliferation as well as accumulation of glycogen, cholesterol and triglycerides within the liver, but exposure to lower concentrations for a longer period result in an increase in the number of peroxisomes leading to severe hepatocellular changes[3]. DEP enhance oxidative stress such as that induced by reactive oxygen species[4]. Dietary antioxidant intake may be an important strategy for inhibiting or delaying the oxidation of susceptible cellular substrates, and is thus relevant to disease prevention in many paradigms. Phenolic compounds such as flavonoids, phenolic acids and tannins have received attention for their high antioxidative activity[5]. Various synthetic antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) have been approved and routinely used as food preservatives[6]. However, these food additives have been reported to posses possible toxic and carcinogenic properties[7]. Hence the recent growing public demand for replacement of synthetic with natural antioxidants has initiated intense research activity[8]. Plants, including herbs and spices, have many phytochemicals which are potential sources of natural antioxidants. Among various medicinal plants, one of them *Nigella sativa* is an anuual *Ranunculaceae* herbaceous plant, has been used traditionally for centuries in the Middle East, Northern Africa, Far East and Asia for the treatment of asthma.It is very popular in various traditional systems of medicine like Unani and Tibb, Ayurveda and Siddha. Seeds and oil have a long history of folklore usage in various systems of medicines and food[9].Recently concluded clinical and experimental researches have shown many therapeutic effects of *Nigella sativa* extracts such as neuroprotective effect[10], anti-oxidant effect[11], anti-inflammatory[12] and anti-tumour agents[13]. With these promising results of numerous research studies on *Nigella sativa* seed extracts, it was selected to evaluate its mitigatory effect under DEP toxaemia. Present study deals with the evaluation of protective effect of *Nigella sativa* seed extract on DEP-induced hepatotoxicity under *in vivo* conditions.

MATERIALS AND METHODS

Chemicals

Analytical grade diethyl phthalate (CAS No. 84-66-2) was procured from Sisco Research Laboratories, Mumbai, India. Olive oil was obtained from Figaro, Madrid, Spain. All other chemicals used in the present study were of analytical grade.

Nigella sativa seed extracts preparation

Seed of *Nigella sativa* was purchased from local markets and extract was prepared according to Bhargava and Singh with slight modification [14]. Finely ground seed 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. Pooled filtrate was evaporated to dryness (below 50˚ C) and stored under refrigerated condition.

Experimental animals

Colony Inbred adult healthy Swiss strain female albino mice (Mus musculus) 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 ± 2˚C, 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 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 the Indian National Science Academy, New Delhi, India (1991).

Treatment

Eighty mice weighing 30 – 35 gm were divided into eight groups and treated with DEP alone and DEP along with *Nigela sativa* as per the given experimental plan (Table 1). Dosages of DEP treatment were based on the LD_{50} value i.e., 8600mg/kg[15]. DEP was dissolved in olive oil and administered by gavaging to mice for 30 days. Behavioural and clinical changes were observed throughout the experimental period. The body weight of each group of mice was recorded individually and mean weights were calculated. After completion of the treatment animals were sacrificed using over dose of diethyl ether.Blood samples collected by cardiac puncture in non - anticoagulant added tubes were allowed to clot and centrifuged at 1000 x g for 10 min at 4˚C. Non – haemolysed serum samples were stored at -4˚C and used for biochemical analysis. The liver was dissected out, blotted free of blood and weighed to the nearest mg on a balance and used for determination of histopathology and biochemical parameters. Relative organ weights were calculated.

$$\text{Relative organ weight} = \frac{\text{Absolute weight}}{\text{Body weight}} \times 100$$

Lipid peroxidation

Lipid peroxidation (LPO) in liver homogenate was measured by estimating malondialdehyde (MDA) - an intermediary product of lipid peroxidation by thiobarbituric acid reactive substances (TBARS) by the method as described by Ohkawa *et al*[16]. 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. Protein content was measured in the liver by the method of Lowry *et al*.[17] using bovine serum albumin as a standard. Resulting blue colour was measured at 540 nm.

Measurement of serum enzyme activity

Serum sample was used for measuring liver marker enzymes. The activities of serum enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed according to a method of Reitman and Frankel [18]. Alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were assayed by the method as described by Bessey *et al* [19]. According to International Union of Biochemistry the serum ALT and AST were expressed in mU/mL and ALP, ACP was expressed in IU/mL.

Histopathology

Histopathological studies were carried out using the standard technique of hematoxylin and eosin staining. The fresh pieces of liver were fixed for 18 h in alcoholic Bouin's fixative. The tissue was dehydrated by passing through ascending grades of alcohol, cleared in xylene and embedded in paraffin wax. 5 µm thick sections were cut on a rotary microtome and stained with Ehrlich's hematoxylin - eosin (alcohol soluble), dehydrated in alcohol, cleared in xylene, mounted in DPX and examined microscopically.

DPPH radical Scavenging activity

Scavenging ability of plant extract for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured by the method of Gyamfi*et al*[20].

Hepatoprotective index

The liver protecting activity of the *Nigella sativa* seed extract was expressed as hepatoprotective percentage (H)[21] which was calculated using the formula:

$$H = \left(1 - \frac{T - V}{C - V}\right) \times 100$$

Where T is the mean value of plant extracts along with the DEP, C is the mean value of DEP alone, and V is the mean value of vehicle control animals.

Statistical analysis

All the data are expressed as the means ± standard error mean (SEM). Statistical analysis and was 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. Linear regression was also calculated. The level of significance was accepted with $p<0.05$.

RESULTS

Body weight

Oral administration of DEP caused significant ($p< 0.05$), dose – dependent reduction in body weight (Groups 4, 5 and 6) as compared with vehicle control. However, administration of DEP along with an extract of *Nigella sativa* seeds significantly ameliorated reduction in DEP induced body weight (Table 2).

Organ weight

DEP treatment in animals of groups 4, 5 and 6 caused significant ($p<0.05$), dose - dependent increase in absolute and relative liver weights (Table 3).Cotreatment of *Nigella sativa* extract along with HD of DEP (Groups 7 and 8). significantly mitigated the DEP – induced change in liver weights. Amelioration was more in high dose of *Nigella sativa* extract than that of low dose (Table 3).

Lipid peroxidation

The results shown in Table 4 revealed that DEP treatment for 30 days caused significant ($p< 0.05$) and dose - dependent elevation in lipid peroxidation in liver of mice. However, cotreatment of DEP (HD) along with *Nigella sativa* seed extract significantly reduced lipid peroxidation in liver as compared with DEP (HD) alone treated mice. 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.

Serum parameters

Oral administration of DEP caused significant ($p < 0.05$) increase in activities of ACP, ALP, ALT and AST in serum (Groups 4, 5 and 6) as compared to that the vehicle control (Group 2). The effect was dose – dependent ($r^2 \leq 0.835$). DEP induced effect were reversed on oral administration of *Nigella sativa* extract along with HD of DEP (Group 7 and 8). All parameters remained within normal levels as that of the control groups (Group 3) (Table 5). The coefficient of regression r^2 was obtained by linear regression. All results exhibited coefficient of regression $r^2>9$ ($p< 0.05$). Linear regression shows that lipid peroxidation in liver strongly correlates with ACP, ALP, ALT and AST in serum respectively ($r^2 \leq 0.991$).

Histopathological study

Light microscopic study of liver of untreated, vehicle control (Plate1) and antidote (Plate2) control mice showed normal histo- architecture. However, treatment with low dose (310 mg/ kg body weight/day) and mid dose (620 mg/ kg body weight/ day) of DEP caused mild to moderate damage in hepatocytes and slightly showed intracellular vacuolation in mid dose. On the other hand, oral administration of high dose (1240 mg/kg body weight/day) of DEP for 30 days caused severe intracellular vacuolation and loss of hepatic architecture as compared to control (Plate 3). Cotreatment of *Nigella sativa* seed extract (150 and 300 mg/kg body weight/day) along with HD of DEP caused almost complete amelioration in mice as hepatocellular necrosis, intracellular vacuolation were almost absent (Plates4and5).

DPPH activity

Figure 8 shows dose - dependent curve of DPPH radical scavenging activity of extract of *Nigella sativa* seeds. It was observed that extract of *Nigella sativa* seeds and BHT had highest activity 62.22% and 97.10% respectively at 250 µg/ml. IC50 value of extract of *Nigella sativa* seeds and BHT was found to be 2.3 µg/ml and 1.7 µg/ml respectively.

Table 1
Experimental protocol.

Experimental Groups	No. of animals	Time of treatment (days)	Day of autopsy
(I)Control			
1. Untreated	10	30	31st
2. Vehicle	10	30	31st
3. Antidote (NS300)	10	30	31st
(II) Diethyl phthalate - Treated			
4. DEP310 ; LD	10	30	31st
5. DEP620 ; MD	10	30	31st
6. DEP1240 ; HD	10	30	31st
(III) DEP1240 (HD)+ Nigella sativa extract - Treated			
7. HD + NS150	10	30	31st
8. HD + NS300	10	30	31st

Table 2
Effect of Nigella sativa seed extract on diethyl phthalate-induced changes in the body weight (gm) of mice.

Experimental Groups	Day of autopsy	
	0th	31st
(I)Control		
1. Untreated	33.90 ± 0.34	33.56 ± 0.62
2. Vehicle	33.69 ± 0.37	33.06 ± 0.37
3. Antidote (NS300)	33.80 ± 0.24	33.57 ± 0.49
(II) Diethyl phthalate - Treated		
4. DEP310 ; LD	33.86 ± 0.33	30.15 ± 0.57[a]
5. DEP620 ; MD	34.33 ± 0.30	29.11 ± 0.98 [a]
6. DEP1240 ; HD	35.58 ± 0.38	27.84 ± 0.81 [a]
(III) DEP1240 (HD)+ Nigella sativa extract - Treated		
7. HD DEP + NS150	33.83 ± 0.24	30.71 ± 042[ab]
8. HD DEP + NS300	33.08 ± 0.26	32.05 ± 0.42[ab]

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)

Table 3
Effect of Nigella sativa seed extract on diethyl phthalate-induced changes in the liver weight.

Experimental Groups	Liver weight	
	Absolute weight (gm)	Relative weight (gm/100gmb.wt.)
(I)Control		
1. Untreated	1.37 ± 0.02	4.33 ± 0.11
2. Vehicle	1.38 ± 0.02	4.34 ± 0.19
3. Antidote (NS300)	1.37 ± 0.03	5.47 ± 0.27
(II) Diethyl phthalate - Treated		
4. DEP310 ; LD	1.64 ± 0.02 [a]	5.68 ± 0.25 [a]
5. DEP620 ; MD	1.79 ± 0.04 [a]	6.02 ± 0.48 [a]
6. DEP1240 ; HD	1.95 ± 0.11 [a]	6.92 ± 0.23 [a]
(III) DEP1240(HD)+ Nigella sativa extract - Treated		
7. HD DEP + NS150	1.71 ± 0.03[ab] (42.10%)	5.08 ± 0.20[ab] (71.31%)
8. HD DEP + NS300	1.55 ± 0.05[ab] (71.92%)	4.84 ± 0.18[ab] (80.62%)

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 4

Effect of Nigella sativa seed extract on diethyl phthalate-induced changes in lipid peroxidation in mice liver.

Experimental Groups	Lipid peroxidation (n moles MDA formation/mg protein/60 min)
(I)Control	
1. Untreated	2.12 ± 0.17
2. Vehicle	2.09 ± 0.09
3. Antidote (NS300)	2.27 ± 0.11
(II) Diethyl phthalate - Treated	
4. DEP310 ; LD	2.67 ± 0.14 [a]
5. DEP620 ; MD	3.24 ± 0.09 [a]
6. DEP1240 ; HD	4.57 ± 0.13 [a]
(III) DEP1240(HD)+ Nigella sativa extract - Treated	
7. HD DEP + NS150	3.28 ± 0.19[ab] (52.01%)
8. HD DEP+ NS300	2.66 ± 0.15[ab] (77.01%)

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 5

Effect of Nigella sativa seed extract on diethyl phthalate-induced changes in serum parameters

Experimental Groups	Acid phosphatase (IU/mL	Alkaline phosphatase (IU/mL)	Alanine aminotrasferase (mU/mL)	Aspartate aminotrasferase (mU/mL)
(I)Control				
1. Untreated	0.30 ± 0.02	0.12 ± 0.01	8.60 ± 0.30	25.00 ± 0.29
2. Vehicle	0.30 ± 0.03	0.12 ± 0.01	8.50 ± 0.37	26.00 ± 0.44
3. Antidote(NS300)	0.34 ± 0.03	0.13 ± 0.01	8.20 ± 0.24	25.60 ± 0.42
(II) Diethyl phthalate - Treated				
4. DEP310 ; LD	0.49 ± 0.08 [a]	0.19 ± 0.01 [a]	11.20 ± 0.44 [a]	29.80 ± 0.32 [a]
5. DEP620 ; MD	0.63 ± 0.08 [a]	0.25 ± 0.01 [a]	21.00 ± 0.78 [a]	38.80 ± 1.46 [a]
6. DEP1240 ; HD	0.95 ± 0.09 [a]	0.31 ± 0.01 [a]	36.00 ± 0.95 [a]	51.90 ± 1.07 [a]
(III) DEP1240(HD)+ Nigella sativa extract - Treated				
7. HD DEP + NS150	0.72 ± 0.03[ab] (35.80%)	0.24 ± 0.01[ab] (36.84%)	22.70 ± 0.88[ab] (37.83%)	41.90 ± 0.76[ab] (38.61%)
8. HD DEP + NS300	0.50 ± 0.02[ab] (69.23%)	0.18 ± 0.01[ab] (68.42%)	14.70 ± 0.81[ab] (60.59%)	30.40 ± 0.76[ab] (83.01%)

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).

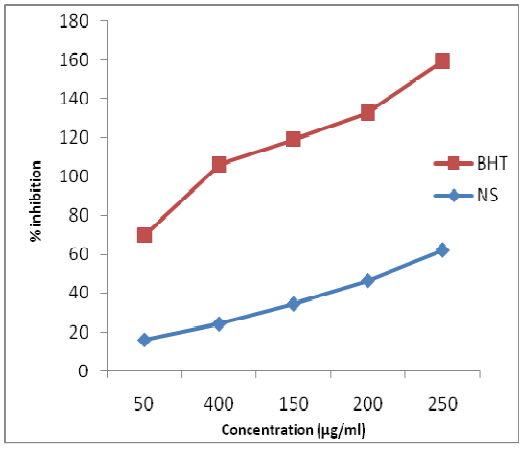

Figure 1

DPPH radical scavenging activity of hydro – alcoholic extract of Nigella sativa seeds. light micrograph (haematoxylin and Eosin staining) of liver.

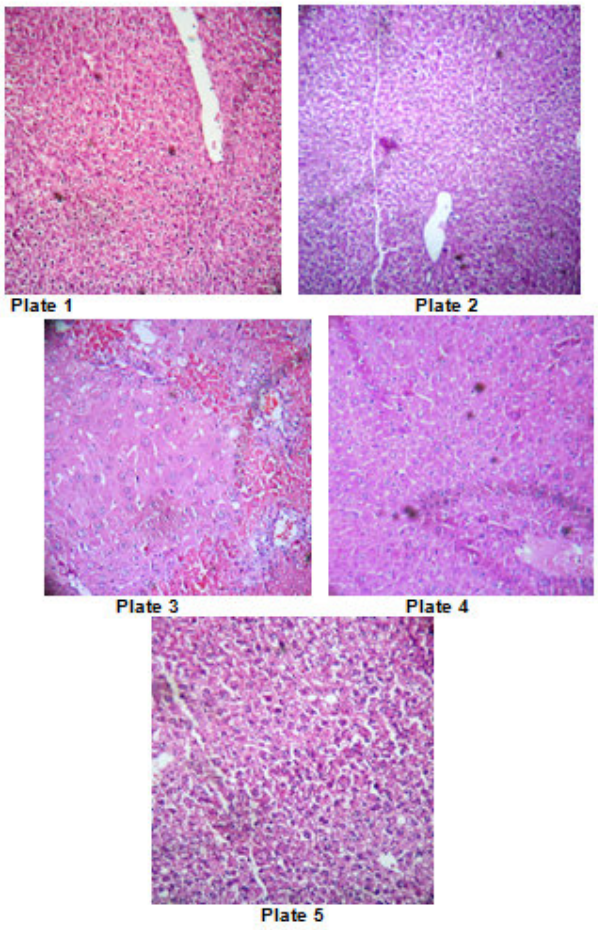

Plate 1:Histology of vehicle control mice, normal hepatic architecture (100x).
Plate 2:Histology of Antidote control mice, normal hepatic architecture (100x).
Plate 3:Histology of HD – diethyl phthalate – treated mice, severe intracellular vacuolation and loss of hepatic architecture and necrosis (100x).
Plate 4:Histology of HD – diethyl phthalate along with 150 mg Nigella sativa seeds extract – treated mice, intracellular vacuolations, and necrosis absent (100 x).
Plate 5:Histology of HD – diethyl phthalate along with300 mg Nigella sativa seeds extract – treated mice, normal hepatic architecture and almost complete recovery (100x).

DISCUSSION

Result of the present study revealed that DEP treatment for 30 days caused significant reduction in body weight of mice (Table 2). The decrease in body weight is because of the reduced feed intake and anorexia which was seen throughout the treatment period. Reduction in body weight was also reported by Brown *et al*[22]. Oral administration of DEP for 30 days in the present study caused an increase in absolute and relative weights of liver of mice (Table 3) because of accumulation of lipid. These data agreed with the reports of many other investigators. According to Pereira *et al*[3], liver weight to body weight ratio showed significant increase in DEP treated rats as compared to control rats. Barse *et al*[23] also reported increased liver size with DEP treatment in *Cyprinus carpio* fish. Also reported by Brown *et al*[22], liver increased in rat with DEP treatment. Lipid peroxidation is a major harmful consequence of reactive oxygen species (ROS) formation[24]. Results shown in Table 4 revealed, significant dose - dependent increase in lipid peroxidation in liver of treated mice. Increased level of lipid peroxidation was also reported by Pereira *et al* [3] in male Wistar rats and Kang *et al*[25] *in* olive flounder *paralichthys olivaceus*, a marine culture fish. Increased lipid peroxidation could also lead to severe cell organelle damage leading to impairment in the various metabolic functions of the cell[26]. Elevation of oxidative stress in liver indicates

high level of ROS production due to liver damage by varying exposure of DEP. Acid phosphatase (ACP) activity in its own case is a marker enzyme for the lysosomal integrity[27]. The present study revealed significant, dose – dependent increase in ACP activity in serum of DEP - treated mice (Table 5). It might be due to the consequence of damage to lysosomal integrity in the liver. The significant rise seen in the ACP activity after toxin administration may be attributed to an increase in cellular degeneration and other pathological liver injury[28]. Many investigators[3,29,30] have reported increase in serum acid phosphatase activity.

Alkaline phosphatase (ALP) activity significantly and dose - dependently increased in serum of DEP - treated animals (Table 5). Alkaline phosphatase is a marker enzyme for plasma and endoplasmic reticulum. It is often employed to assess the integrity of plasma membrane[31.] Lipophilic DEP interact with plasma membrane and thereby could have led to increase in ALP activity[23].Pereria*et al*[3], Ghorpade *et al*[29] and Barse *et al*[23] also reported increase in ALP activity. The present study revealed increase in ALT and AST activities in DEP - treated animals (Table 5). These two enzymes are localized normally within the cells of the liver, heart, kidney, gill, muscle and other organs[32]. The enzymes are important markers in assessing and monitoring liver damage[33]. According to Gao *et al*[34] ALT activity is an important index to measure the degree of cell membrane damage, while AST is an indicator of mitochondrial damage since it contains 80% of this enzyme. Ghorpade *et al*[29]reported increased ALT and AST activities in liver and muscle of freshwater fish *Cirrhina mrigala*, Mapuskar *et al*[30] and Barse *et al*[23]also reported level of ALT and AST increased in mice, *Cyprinus carpio* respectively. Elevated levels of serum enzymes are indicators of cellular leakage and loss of functional integrity of the cell membrane in liver [35]. These are major importance in assessing and monitoring functional status of liver. Thus, their increased presence in serum may give information on organ dysfunction[36]. Histopathological studies also support the biochemical analysis.Examination of liver sections of mice received DEP revealed disruption of the normal histo architecture of normal liver cell. Many hepatic cells were damaged and lost their characteristic appearance while others showed marked cytoplasmic vacuolization and necrosis (Plate 3). Previous studies revealed that, DEP – treated mice showed severe intracellular vacuolation and mild loss of hepatic architecture in the hepatocytes in centriobular and periportal area of liver[23,30]. DEP exposure in liver brings hepatocelluler damage due to oxidative stress[4]. It revealed by significantly dose – dependent increase in lipid peroxidation in liver (Table 4). Damage to the liver cell membrane might have resulted in increased serum enzyme activities such as AST, ALT, ACP and ALP.

Treatment with *Nigella sativa* significantly declined the effects of DEP induced damage and it was evidenced by the decreased level of liver enzymes measured in serum and lipid peroxidation as well as restoration of normal histo architecture of liver. All these changes could be due to higher antioxidative potential of *Nigella sativa* seed extract as evidenced by DPPH radical scavenging activity (Figure8) Histoprotective effect of *Nigella sativa* against CCl4 induced hepatotoxicity[37], liver fibrosis and cirrhosisas well as hepatic damage induced by *Schistosoma mansoni* infection[38] have been reported. Thymoquinone is the active compound of the *Nigella sativa*. The hepatoprotective activities of this was evidenced by: (1) a significant reduction in elevated levels of serum enzymes, (2) a significant decrease in the hepatic malondialdehyde content (represent hepatic lipid peroxidation), and (3) a significant decrease in the total sulfhydryl content (decrease in sulfhydryl group signifies hepatotoxicity) 24 h after CCl4 administration [39].More recently, it has been shown that thymol, one of the constituents of *Nigella sativa* seeds also exhibit hepatoprotective effects in rodents[40].

CONCLUSION

In conclusion, DEP oral administration caused alteration in oxidative stress marker lipid peroxidation as well as serum enzymatic activities and histopathology in liver, which could be a principal mechanism responsible for its hepatotoxicity. *Nigella sativa* seed extract reduced DEP induced hepatic changes mainly due to its phytochemicals having antioxidative properties.

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