CHAPTER II

MATERIALS AND METHODS

CHEMICALS

Diethyl phthalate was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India and was of analytical grade. All the other chemicals used in the present study were of analytical grade and purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India, Sisco Research Laboratories Pvt. Ltd., Mumbai, India and Sigma Aldrich, St. Louis, MO, USA. Olive oil was obtained from Figaro, Madrid, Spain.

HOUSING AND CARE OF ANIMALS

All animal studies were sanctioned by Institutional Animal Ethics Committee of Gujarat University, Ahmedabad and approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India. Healthy young female albino mice of Swiss strain weighing 30-35 gm were obtained from Zydus Research Centre, Ahmedabad, India. The animals were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India under controlled conditions (Temperature 25±2°C, relative humidity 50-55% and 12h light/dark cycle). They were fed with certified pelleted rodent feed supplied by Amruti Feeds, Pranav Agro Industries Ltd., Pune, India and potable water ad libitum. Animals were handled according to the guidelines published by the Indian National Science Academy, New Delhi, India (1991).
PLAN OF STUDY

The present study was carried out in three parts:

PART-I: To study the toxic effects of diethyl phthalate both in vitro and in vivo conditions

PART- II: Phytochemical analysis of the Nigella sativa seed extract

PART-III: To study the ameliorative effect of Nigella sativa seed extract on toxicity induced by diethyl phthalate
PART-I

TO STUDY THE TOXIC EFFECTS OF DIETHYL PHTHALATE

BOTH IN VITRO AND IN VIVO CONDITIONS

A. In vitro study

*To evaluate the toxicity of diethyl phthalate in vitro conditions lipid peroxidation and protein content was studied.*

Healthy adult Swiss strain female albino mice were humanly sacrificed by cervical dislocation. Liver and kidney were isolated, blotted free of blood and used in the study.

*Lipid peroxidation assay:*

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 0.2 mL of tissue homogenates and phosphate buffered saline.
2. DMSO control tubes: These tubes contained 0.2 mL of tissue homogenates and DMSO.
3. Antidote control tubes: These tubes contained 0.2 mL of tissue homogenates and *Nigella sativa* seed extract (200 µg/mL).
4. DEP - treated tubes: These tubes contained 0.2 mL of tissue homogenates with different concentrations of DEP (10-40 µg/mL) in DMSO.
5. DEP and *Nigella sativa* seed extract tubes: These tubes contained 0.2 mL of 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$_2$O$_2$ 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 tubes were measured by quantification of thiobarbituric acid reactive substance (TBARS) by the method of Ohkawa *et al.* with slight modifications. This method is based on the formation of a red chromophore that absorbs light at 532 nm following the reaction of thiobarbituric acid (TBA) with products of lipid peroxidation like malondialdehyde (MDA) and others collectively called as thiobarbituric acid reactive substances (TBARS). The results were expressed as n moles MDA formed/mg protein/60 min.

**Protein assay:**

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

Following sets of tubes were prepared.

1. Control tubes: These tubes contained 0.2 mL of tissue homogenates and distilled water.
2. DMSO control tubes: These tubes contained 0.2 mL of tissue homogenates and DMSO.
3. Antidote control tubes: These tubes contained 0.2 mL of tissue homogenates and *Nigella sativa* seed extract (200 µg/mL).

4. DEP - treated tubes: These tubes contained 0.2 mL of tissue homogenates with different concentrations of DEP (10-40 µg/mL).

5. DEP plus *Nigella sativa* seed extract – treated tubes: These tubes contained 0.2 mL of 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 tubes were estimated by the method of Lowry *et al* with slight modification. Bovine serum albumin was used as a standard. Reaction of protein with Folin Ciocalteau reagent results in blue colour which is due to two reactions occurring simultaneously i.e., the reaction of alkaline copper sulphate solution with peptide bonds and reduction of phosphomolybdic and phosphotungstic acids by aromatic amino acids present in the protein. Resulting blue colour was measured at 540 nm. The protein contents were expressed as mg/100 mg tissue weight.

B. *In vivo study*

To evaluate the toxicity of diethyl phthalate (DEP) in vivo conditions following parameters were studied.

**STUDY DESIGN**

Fifty animals were randomly divided into five different groups and caged separately. Animals of *Group 1* (untreated control) were maintained without any
treatment. Animals of Group 2 (vehicle control) received olive oil (0.2 mL/animal/day) for 30 days as olive oil was used as vehicle to dissolve DEP. Animals of Group 3, 4 and 5 were treated with low (310 mg/kg body weight/day), mid (620 mg/kg body weight/day) and high (1240 mg/kg body weight/day) dose of DEP respectively. All treatments were given using a feeding tube attached to a hypodermal syringe for 30 days. Doses of DEP were based on LD_{50} value i.e. 6200 mg/kg body weight (NTP, 2006) (Table 2.1).

Behavioral and clinical changes throughout the experiments were recorded. The body weight of control and all treated groups of mice were recorded to the nearest gm on an animals weighing balance. At the end of treatment, the animals were weighed and humanely sacrificed by cervical dislocation. 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. Obtained non – haemolysed serum samples were stored at -4˚C and used for biochemical analysis. The liver and kidneys were dissected out, blotted free of blood and weighed to the nearest mg on a balance and used for histopathological and biochemical analysis.

BIOCHEMICAL ANALYSIS

Protein, lipid, carbohydrate and nucleic acid contents

Protein content:

Methodology used for protein estimation was as described in part I in vitro study section. The protein content was expressed as mg/100 mg tissue weight.

Glycogen content:

The glycogen content in the liver was estimated by the method of Seifter et al. (1950). The glycogen present in tissue is converted to glucose, which reacts with
anthrone reagent to give a green coloured product which was read at 620 nm. The glycogen content was expressed as mg/100 mg tissue weight.

Total lipid content:

Total lipid content in the liver and kidney was estimated according to the method of Fringes et al. (1972) using olive oil as a standard. Lipid on being heated with sulphuric acid followed by addition of vanillin and phosphoric acid produces a pink colour whose optical density is measured at 530 nm. The total lipid content was expressed as mg/100 mg of tissue weight.

Total cholesterol content:

The concentration of cholesterol was estimated in the liver and kidney by the method of Zlatki et al. (1953). Cholesterol forms a coloured complex with FeCl₃ in the presence of concentrated sulphuric acid and glacial acetic acid which can be measured at 540 nm. The cholesterol content was expressed as mg/100 mg tissue weight in liver and mg/dL.

Estimation of nucleic acid:

Extraction:

A known weight of fresh tissue was homogenized in 5 mL of cold 5% TCA and the homogenate was kept at 0-4°C for 30 min. The precipitate obtained after centrifugation (10 min at 1000 x g) was dissolved in 5 mL of cold 5% TCA and left for 30 min at 0-4°C. Thereafter, centrifugation (10 min at 1000 x g) was carried out and the precipitate obtained was dissolved in alcohol: ether (1:3, v/v) mixture and left for 30 min at 50°C. This process was repeated once again. The tubes were centrifuged
at 1000 x g for 10 min and the supernatant was discarded. The lipid free pellet obtained was dissolved in 5 mL of 0.1 N KOH and incubated at 37°C for 16-18 h. Then 0.17 mL of 6 N HCl and 5 mL of 10% TCA were added to the incubated suspension and precipitate was allowed to be formed at 4°C for 30 min. After centrifugation at 1000 x g for 10 min the supernatant was separated and used for estimation of RNA. The pellet containing DNA and protein was heated at 90°C for 15 min after adding 5 mL of 5% TCA. The supernatant was then separated by centrifugation (10 min at 1000 x g) after cooling at 4 °C for 30 min and used for estimation of DNA.

(i) Deoxyribonucleic acid (DNA):

The estimation of DNA in the liver and kidney was carried out by the method of Giles and Meyer (1965). The DNA in the supernatant reacts with diphenylamine to give a blue coloured complex whose optical density was read at 620 nm. The DNA content was expressed as µ moles/100 mg tissue weight.

(ii) Ribonucleic acid (RNA):

The estimation of RNA in liver and kidney was carried out by the method of Schneider (1939). The RNA in the supernatant reacts with the orcinol reagent to give a greenish colour, whose absorbance was read at 670 nm. The concentration of RNA was expressed as µ moles/100 mg tissue weight.

Enzymatic assays

Alanine transaminase (EC 2.6.1.2) activity:
The alanine transaminase (ALT) activity in liver and serum was assayed by the method of Reitman and Frankel (1957). A buffered solution of α-ketoglutarate and L-alanine were made to react with the liver homogenate for 30 min. The pyruvate formed from L-alanine by the enzymatic reaction reacts with 2, 4-dinitrophenyl hydrazine (DNPH) in alkaline medium and formazon formed was measured at 540 nm. The enzyme activity was expressed as mU/mg protein/30 min in case of liver and mU/mL in case of serum.

**Aspartate transaminase (EC 2.6.1.1) activity:**

The aspartate transaminase (AST) activity was assayed by the method of Reitman and Frankel (1957). Assay method is similar as described in ALT activity assay, except buffered solution which contained L-aspartate instead of L-alanine and allowed to react for 1 hr. The enzyme activity was expressed as mU/mg protein/60 min in case of liver and mU/mL in case of serum.

**Alkaline phosphatase (E.C.3.1.3.1) activity:**

The alkaline phosphatase (ALP) activity in liver and serum was determined by the method of Bessey *et al.* (1946). Alkaline phosphatase at optimum pH 10.5 catalyzes the hydrolysis of p-nitrophenyl phosphate (disodium salt) to p-nitrophenol and inorganic phosphate. The liberated p-nitrophenol reacts with sodium hydroxide to form yellow coloured complex which was measured at 410 nm. The ALP activities in liver were expressed as μmoles p-nitrophenol released/mg protein/30 min and IU/mL in serum.

**Acid phosphatase (E.C.3.1.3.2) activity:**
The acid phosphatase (ACP) activity was assayed in the liver, kidney and serum by the method as described in Sigma Technical Bulletin (Sigma Technical Bulletin, MO, USA). Acid phosphatase at optimum pH 4.8 catalyzes the hydrolysis of p-nitrophenyl phosphate (disodium salt) to p-nitrophenol and inorganic phosphate. The liberated p-nitrophenol reacts with sodium hydroxide to form a yellow coloured complex which was measured at 420 nm. The enzyme activity was expressed as µmoles p-nitrophenol released/mg protein/30 min in liver and kidney and IU/mL in serum.

**Adenosine triphosphatase (EC.3.6.1.3) activity:**

The adenosine triphosphatase (ATPase) activity in the liver and kidney was assayed by the method of Quinn and White (1968). ATPase causes hydrolysis of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and inorganic phosphate (i.p.). The liberated inorganic phosphate was estimated by the method of Fiske and Subbarow (1925). The optical density was read at 660 nm. The enzyme activity was expressed as µmoles inorganic phosphate released/mg protein/30 min.

**Succinic dehydrogenase (EC.1.3.99.1) activity:**

The succinic dehydrogenase (SDH) activity in the liver and kidney was assayed by the method of Beatty et al. (1966) using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl- 2H – tetrazolium chloride (INT) as an electron acceptor. The electrons released by the enzyme SDH from the substrate are taken up by INT, which was reduced to a red coloured formazan. This was extracted in ethyl acetate and the absorbance was read at 420 nm. The enzyme activity was expressed as µg formazan formed/mg protein/15 min.
**Phosphorylase (EC 2.4.1.1) activity:**

The liver phosphorylase activity was assayed by the method of Cori *et al.* (1943). The inorganic phosphate (i.p.) formed at the end of the reactions was estimated by the method of Fiske and Subbarow (1925). The enzyme phosphorylase hydrolyses the substrate glucose -1-phosphate. The inorganic phosphate formed at the end was treated with an acidic molybdate solution; it forms phosphomolybdic acid which on addition of 1-amino-2-naphthol-4-sulphonic acid (ANSA) is quantitatively reduced to a blue coloured complex which is measured spectrophotometrically at 660 nm. The enzyme activity is expressed as µg phosphorus released/100 mg fresh tissue/15 min.

**LIPID PEROXIDATION AND ANTIOXIDATIVE DEFENSE MECHANISM**

**Lipid peroxidation:**

The level of lipid peroxidation in the liver and kidney was measured by the method as described by Ohkawa *et al.* (1979). This method is based on the formation of a red chromophore that absorbs light at 532 nm following the reaction of thiobarbituric acid (TBA) with products of lipid peroxidation like malondialdehyde (MDA) and others collectively called as thiobarbituric acid reactive substances (TBARS). The results were expressed as n moles MDA formed/mg protein/60 min.

**Non-enzymatic antioxidants:**

**Glutathione content:**

The glutathione (GSH) content in the liver and kidney was measured by the method of Grunert and Philips (1951). In saturated alkaline medium, the GSH present in the tissues reactes with sodium nitroprusside to give a red coloured complex which
was measured at 520 nm. The glutathione content was expressed as µg/100 mg tissue weight.

**Total ascorbic acid content:**

Total ascorbic acid (TAA) content was estimated in the liver and kidney by the method of Roe and Kuether (1943). TAA is oxidized to dehydroascorbic acid (DHA) by Norit reagent in the presence of TCA. This couples with 2, 4-dinitrophenyl hydrazine in the presence of thiourea and sulphuric acid to yield a red coloured complex which was read at 540 nm. TAA content was expressed as mg/gm tissue weight.

**Enzymatic antioxidants:**

**Catalase (EC.1.11.1.6) activity:**

The catalase (CAT) activity was assayed in the liver and kidney by the method of Luck (1939). The assay mixture consisted of 50 mM phosphate buffer (pH 7.0), aliquot and 10 mM H₂O₂ which was added to initiate the reaction. The decrease in absorbance was noted every 5 seconds at 240 nm. The enzyme activity was expressed as µ moles H₂O₂ consumed/mg protein/min.

**Superoxide dismutase (EC.1.15.1.1) activity:**

The superoxide dismutase (SOD) activity in the liver and kidney was assayed by the method of Kakkar *et al.* (1984) with slight modification. This method is based on the NADH-phenazine methosulfate-nitroblue tetrazolium formazon inhibition. The formazon formed at the end of the reaction was extracted into butanol layer, upon inactivation of the reaction with acetic acid. The enzyme activity was expressed as
units/mg protein. One unit of enzyme activity is defined as the enzyme concentration required to inhibit the optical density of chromogen production at 560 nm by 50% in 1 min under the assay condition. The enzyme activity was expressed as units/mg protein.

**Glutathione reductase (E.C.1.6.4.2) activity:**

The glutathione reductase (GR) activity in liver and kidney was assayed by the method of Mavis and Stellwagen (1968). 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.

**Glutathione-S-transferase (EC 2.5.1.18) activity:**

The glutathione-S-transferase (GST) activity was assayed by the method of Habig et al. (1974). 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.

**Glutathione peroxidase (EC.1.11.1.9) activity:**

The glutathione peroxidase (GSH-Px) activity in the liver and kidney was assayed by the modified method of Pagila and Valentine (1967). The enzyme activity was expressed as units/mg protein/min, where 1 unit of GSH-Px equals to nmoles of NADPH consumed/mg protein/min.

**Serum parameters**
Methods for measurement of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and acid phosphatase (ACP), as well as protein and cholesterol contents were as described earlier in part I section.

**Serum creatinine content:**

Creatinine present in the serum reacts with picric acid in alkaline medium to form an orange red colour which can be measured colourimetrically (Jaffe reaction). Estimation of creatinine was performed by the alkaline picrate method of Bonsnes and Taussky (1945) as described by Varley (1988). The optical density of the orange colour developed was read at 520 nm. The creatinine content was expressed as mg/dL serum.

**HISTOPATHOLOGICAL STUDIES:**

Histopathological studies were carried out using the standard technique of hematoxylin and eosin (H & E) staining. Liver and kidney tissues of all control and treated animals were preserved in 10% neutral buffered formalin immediately after the autopsy. The tissues were dehydrated by passing through ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (58 to 60°C mp). 5 µm thick sections were cut on a rotary microtome and stained with H & E, dehydrated in alcohol, cleared in xylene, mounted in DPX and examined under a light microscope.
PART II

PHYTOCHEMICAL ANALYSIS OF THE NIGELLA SATIVA
SEED EXTRACT

Extract preparation

Seeds of *Nigella sativa* was purchased from LVG, Ahmedabad and extract was prepared according to the method of Bhargava and Singh (1981) with slight modification. The finely ground seed powder was mixed with 50% methanol in water 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 residue which was stored under refrigerated conditions. Percent yield of the extract was calculated. Polyphenolic contents of the extract was qualitatively and quantitatively analyzed using standardized methods. The antioxidative potency of both the extracts was estimated using various chemical assay systems as described below:

**Qualitative and quantitative analysis of polyphenolic content:**

**Qualitative analysis**

Qualitative analysis for determining the presence of tannins, saponins, flavonoids and alkaloids in the plant extracts were carried out using standard methods as described by Harborne (1973), Trease and Evans (1989) and Sofowara (1993).

**Test for tannins:**
0.5 gm of extract was dissolved in 20 mL distilled water in a test tube and then filtered. A few drops of 0.1% FeCl₃ was added and observed for brownish green or blue black colour.

**Test for saponins:**

2 gm of the extract was boiled in 20 mL of distilled water in a water bath and filtered. 10 mL of filtrate was mixed with 5 mL of distilled water and shaken vigorously. The forth was mixed with 3 drops of olive oil and shaken vigorously and then observed for formation of emulsion.

**Test for flavonoids:**

5 mL of dilute ammonia solution was added to plant extract, followed by addition of concentrated H₂SO₄. A yellow colouration indicated presence of flavonoids.

**Test for alkaloids:**

The test was performed with Mayer’s, Wagner’s and Dragendorff’s reagents. Observation of white, brown, orange colouration indicated the presence of alkaloids.

**Quantitative analysis:**

The quantitative analysis of the plant extract was performed using standard protocols as mentioned below:

**Total phenolic content (TPC):**

Total phenolic content of hydro-alcoholic extract was estimated by the method as described by Singleton *et al.* (1999). Briefly extract react with Folin-Ciocalteu
reagent in the presence of sodium carbonate to form a blue coloured complex which was read at 760 nm. Total phenolic content of the extract was expressed as mg gallic acid equivalents/gm dry weight of extract.

**Flavonoid content:**

The flavonoid content in the extract was estimated by the method of Lamaison and Carnat (1990). Briefly 1 ml of plant extract was mixed with 1 ml of aluminium chloride reagent and resultant colour was read at 430 nm. The flavonoid content of the extract was expressed as mg quercetin equivalents/gm dry weight of extract.

**Tannin content:**

The tannin content of the extract was estimated by the method as described by Price and Butler (1977). Plant extract was allowed to react with K$_3$Fe(CN)$_6$-FeCl$_3$ reagent for five min and the intensity of colour developed was measured spectrophotometrically at 720 nm. The tannin content of the extract was expressed as mg rutin equivalents/gm dry wt. of extract.

**Ascorbic acid content:**

Ascorbic acid, also known as vitamin C, is one of the most abundant antioxidant present in plant, was quantified by the method of Jagota and Dani (1982). The ascorbic content of plant extract was expressed as µg/gm dry weight of extract.

**Antioxidative potency:**

**Superoxide radical scavenging assay:**

Superoxide radical scavenging activity was assessed by the method of Liu *et al.* (1997). In the PMS/NADH-NBT system, superoxide anion derived from dissolved
O₂ by PMS/NADH coupling reaction reduces NBT. The addition of various concentrations of hydro-alcoholic extract resulted in decreased colour intensity which was read at 560 nm against blank to determine the quantity of the formazan generated. IC₅₀ value of the extract (concentration required to scavenge 50% of the radicals) was calculated.

**Hydroxyl radical scavenging assay:**

The hydroxyl radical scavenging activity of the extract was estimated by the method of Halliwell et al. (1987), where radicals were generated from Fe³⁺/ascorbate/EDTA/H₂O₂ from Fenton’s reaction. Briefly different concentrations of plant extract was made to react with 2-deoxy-2-ribose, H₂O₂, FeCl₃ and EDTA. The reaction was initiated by the addition of ascorbic acid. After incubation of 90 min the reaction was estimated by addition of thiobarbituric acid (TBA) and resulting colour was red at 590 nm. Percent inhibition by various concentrations of plant extract and IC₅₀ was calculated.

**Nitrous oxide radical scavenging assay:**

Nitrous oxide radical scavenging activity was measured using method of Sreejayan and Rao (1997). Various concentrations of plant extract was incubated with 10 mM sodium nitroprusside and incubated for 150 min. After incubation, Griess reagent was added to the tubes and absorbance of chromophore formed was read at 590 nm. IC₅₀ value and percent inhibition by various concentrations of plant extract was calculated comparing the absorbance of control and test compounds against blank.
**DPPH radical scavenging assays:**

Ability of plant extract to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured by the method of Gymfi et al. (1999). DPPH is a purple colour radical compound which changes to stable compounds having yellow colour by reacting with antioxidant compounds. Addition of 0.1 mM DPPH solution in various concentrations of extract in presence of tris-HCl buffer (50 mM, pH-7.4) resulted in decreased absorbance which was measured at 517 nm. Percent inhibition was calculated by measuring the absorbance of plant extract treated samples against blank. IC_{50} value for the extract was calculated.
PART III

TO STUDY THE AMELIORATIVE EFFECT OF NIGELLA SATIVA SEED EXTRACT ON TOXICITY INDUCED BY DIETHYL PHTHALATE

STUDY DESIGN

Based on the results of part – I, the high dose (HD) of DEP was chosen further to evaluate the hepatoprotective effect of Nigella sativa seed extract. Fifty animals were divided into five groups. Animals of Group 1 received 0.2 mL olive oil/animal/day for 30 days and marked as vehicle control. Antidote control group (Group 2) animals were orally administered with Nigella sativa seed extract (300 mg/kg body weight/day) for 30 days. Animals of Group 3 received HD (1240 mg/kg body weight/day) of DEP for 30 days. Animals of Group 4 and 5 were treated with HD of DEP along with 150 and 300 mg/kg body weight/day of Nigella sativa seed extract for 30 days.

Behavioural and clinical changes throughout the treatment were observed in the animals of all groups. On the completion of the treatment (30 days), animals were humanely sacrificed on 31st day by cervical dislocation. The liver and kidney were dissected out carefully, blotted free of blood, weighed to the nearest mg and utilized for the study. For studying serum parameters, the blood collected by cardiac puncture was allowed to clot and centrifuged at 1000 x g for 10 min to obtain serum. The obtained serum samples were stored under refrigerated conditions and used within 24 h.
BIOCHEMICAL ANALYSIS

*Protein, lipid, carbohydrate and nucleic acid contents*

The protein, glycogen, total protein, cholesterol, DNA and RNA contents were estimated using standard protocols as described in part I materials and methods section.

*Enzymatic assays*

The activities of ALT, AST, ACP and ALP in liver and kidney were done using standard methods as described in part I materials and methods section.

*Lipid peroxidation and antioxidative defense mechanism:*

*Lipid peroxidation:*

The lipid peroxidation was estimated using standard methods as described in part I materials and method section.

*Non-enzymatic antioxidants:*

Methods used for measurement of glutathione and total ascorbic acid contents were described in part I material method section.

*Enzymatic antioxidants:*

Methods used for assessment of superoxide dismutase, catalase, glutathione reductase, glutathione transferase and glutathione peroxidase were described in part I materials and method section.

*Histopathological studies:*

Tissues of all control and treated animals were preserved in 10% neutral buffered formalin for histopathological examination after autopsy. The standard technique of H & E was followed as described in part I material methods section.
**Hepato/reno-protective index:**

The liver/kidney protecting activity of the *Nigella sativa* seed extract was expressed as hepatoprotective/renoprotective percentage (H/R) (Prakash *et al.* 2008) which was calculated using the formula:

\[
H/R = \left(1 - \frac{T-V}{C-V}\right) \times 100
\]

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, linear regression analysis and Pearson correlation analysis of relationship between experimental parameters 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. The IC₅₀ values were calculated by probit analysis.