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Protective Effect of Cool Extraction of Black Seed (*Nigella Sativa)* Oil Against CCl_4-Induced Oxidative Damages in Wistar Rats Testis.

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ABSTRACT: *Over many centuries humans have been mining the bounties of nature for discovering substances that have been used for the treatment of all human diseases. Traditionally various human diseases of kidneys, hormonal imbalance and sexual diseases are treated with Nigella sativa oil. In the present study, protective effects of cool extraction of Nigella sativa oil was evaluated against CCl4-induced oxidative damages in rat testis. To examine the protective effects of cool extraction of Nigella sativa oil on testis against oxidative stress of carbon tetrachloride in male rats, 35 male albino rats were equally divided into 7 groups (5 rats). Group 1 rats were administered normal Saline (volume per body weight) orally. Group 2 rats were administered olive oil 4ml/kg body weight orally. Group 3 rats were administered 2ml/kg body weight of N. sativa oil orally. Group 4 rats were administered 4ml/kg body weight of N. sativa oil orally for 2 weeks. Group 5 rats were administered 4ml/kg body weight CCl4 (30% CCl4 in 70% olive oil) orally. Group 6 rats were administered 2ml/kg body weight of N. sativa oil plus 4ml/kg body weight CCl4 (30% CCl4 in 70% olive oil) orally. Group 7 rats were administered 4ml/kg body weight of N. sativa oil plus 4ml/kg body weight CCl4 (30% CCl4 in 70% olive oil) orally. Protective effects of Nigella sativa oil were observed on the biochmical (SOD, CAT, GSH and LPO) and histological paramters. Results of the present study revealed that treatment of CCl4 caused elevated level of TBARS and significantly reduced sperm concentration comparatively to control. N. sativa treatment positively protects the alterations in biochemical variables SOD, CAT, GSH and LPO in the CCl4 + N. sativa-treated rats. N. sativa markedly reduced elevated TBARS. Histopathological changes were observed. Testis tissues in group 1, 2, 3 and 4 showed normal testicular architecture. There was a widespread degeneration in the spermatogenic cell lines of the seminiferous epithelium of group 5 rats. N .sativa oil showed protective effect on the histological section of the testis in groups 6 and 7. From the results it is suggested that cool extraction of Nigella sativa oil extract has the ability to protect testis against oxidative and histological damages, possibly through antioxidant effects of its bioactive compounds.*
KEYWORD: *CCl4, N. sativa, Biochemical Assay, Testis, Wistar Rats*

I. INTRODUCTION

Carbon tetrachloride (CCl_4) is one of the oldest and most widely used toxins for experimental induction of liver fibrosis in laboratory animals (Tsukamoto *et al.,* 1990). This model has been used in various studies on examined the deposition of extracellular matrix in the fibrotic and cirrhotic liver (Hernandez-Munoz *et al.,* 1994; Muriel *et al.,* 1998). CCl_4 is a selective hepatotoxic chemical agent. CCl_4- induced reactive free radicals initiate cell damage through two different mechanisms of covalent binding to the membrane proteins and cause lipid peroxidation. A number of investigators have utilized this chemical to produce liver cirrhosis in experimental animals (Parola *et al.,* 1992). Production of reactive oxygen species and lipid peroxidation induced by iron overload (Bacon *et al.,* 1990), cholestatic injury (Parola *et al.,* 1996) and intoxication by ethanol (Kamimura *et al.,* 1992) and CCl4 (Parola *et al.,* 1992) is associated with liver fibrosis and cirrhosis. These effects are partially prevented by antioxidant compounds including α-tocopherol (Parola *et al.,* 1992; Halim *et al.,* 1997), silymarin (Mourelle *et al.,* 1989) and salvianolic acid (Hu *et al.,* 1997). 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. NS contains more bthan 30 of a fixed oil and 0.40-0.45 w/w of a volatile oil. The volatile oil has been shown to contain 18.4-24% thymoquinone and 46% many monoterpenes such as p-cymene, and α-pinene (El Tahir *et al.,* 1993). Recently conducted clinical and experimental researches have shown many therapeutic effects of NS extracts such as immunomodulator, antiinflammatory and anti-tumour, antibacteria agents (Rogozhin *et al.,* 2011; Alam *et al.,* 2010; Houghton *et al.,* 1995; El Daly *et al.,* 1998; El-Kadi et al., 1987).

II. MATERIALS AND METHODS

Collection of Plant Nmaterial

The plant was obtained at Sabon Gari market, Zaria in November, 2012. The plant was identified and authenticated in the herbarium section in the Department of Biological Science, Ahmadu Bello University, Zaria and then processed and extracted at National Reseach Institute of Technology (NARIT).

Preparation of extract

Cool Water extraction of N. sativa seed was prepared. N. sativa seeds were collected, and pulverized with pestle and mortar. About 100 g of the powder was mixed with 50mls of water. The mixture was made in to several mold and dried in a dessicator for 10 minutes. Each mold was rap and tied in white pieces of cloth and then subjected to pressure using hydrolic machine. Oil was collected in air-tight container and stored in the refrigerator prior to the commencement of the experiment.

Experimental Animals

A total number of 35 young adult Wistar rats were purchased from the Department of Pharmacology, Ahmadu Bello University Zaria. The animals were housed in the animal house of the Department of Human Anatomy, Ahmadu Bello University Zaria. The animals were between the ages of six and seven weeks and weighed between 130-180g. The animals were kept and maintained on standard laboratory condition of room temperature, humidity and under twelve hours dark – light cycle. The animals were fed with standard pellet diet and water. The animals were allowed to acclimatize to their new condition for two weeks before the commencement of the experiment.

Experimental Protocol

The 35 animals were randomly divided in to seven groups. Each group comprised of 5 rats and each rat in every group was marked for identification. The experimental animals weighed between 130-180 g and were randomly divided in to seven groups. Each group comprised of 5 rats. Group 1 rats were administered normal Saline (volume per body weight) orally for 2 weeks. Group 2 rats were administered olive oil 4m1/kg body weight orally for 2 weeks. Group 3 rats were administered 2ml/kg body weight of *N. sativa* oil orally for 2 weeks. Group 4 rats were administered 4ml/kg body weight of *N. sativa* oil orally for 2 weeks. Group 5 rats were administered 4ml/kg body weight CCl4 (30% CCl4 in 70% olive oil) orally for 2 weeks. Group 6 rats were administered 2ml/kg body weight of *N. sativa* oil plus4ml/kg body weight CCl4 (30% CCl4 in 70% olive oil) orally for 2 weeks. Group 7 rats were administered4ml/kg body weight of *N. sativa* oil plus 4ml/kg body weight CCl4 (30% CCl4 in 70% olive oil) orally for 2 weeks.

SACRIFICE OF ANIMALS

On day 15, all animals were humanely sacrificed. Blood was collected from the jugular vein of the animals under deep anaesthesia with chloroform for biochemical analysis. The testis tissues were harvested for histological examination.

III. ESTIMATEMATION OF OXIDATIVE PARAMETERS

Determination of Catalase Activity

Catalase activity was determined spectrophotometer at 570 nm using the method described by Sinha (1972). 5 % Potassium heptaoxochromate (VI), $K_2Cr_2O_7$: 5 g of $K_2Cr_2O_7$ was dissolves in little quantity of distilled water and made up to 100 ml. 0.2 M H_20_2: 0.6 ml of H_20_2 will be in dissolved in little quantity of water distilled and made up to 100 ml. it is stores at 4°C. 0.0lM phosphate buffer: 1.2g of NaH_2P0_4 and 1.4lg of Na_2HPO_4 was dissolved in distilled water and made up to 1000 ml mark in a volumetric flask. The buffer was adjusted to pH 10.2. Diebroinate/Acetic acid solution 5 % Potassium heptaoxochromate (VI) $K_2Cr_2O_7$ was mixed with gacial acetic acid in the ratio 1:3, and was stored in brown bottle at room temperature.0.9 ml or distilled water was added to 0.1 ml of microsome and mixed thoroughly. 2.5 ml of phosphate buffer was put in a small conical flask; 0.5 ml of microsome was added; and 2.0 ml of H_20_2 added, starting the stop watch. The reaction mixture will be thoroughly mixed and the reaction will be stopped after every 60 seconds for 3 minutes with Dichroniate/Acetic acid solution. It was heated in water bath for 10 minutes at 80°C. Absorbance was read at 570 nm.

Determination of superoxide dismutase activity

Superoxide Dismutase (SOD) activity was determined spectrophotometer by a method described by Fridovich (1989). The ability of superoxide dismutase (SOD) to inhibit auto oxidation of adrenaline at pH 10.2, form the basses of this assay. 0.05 M phosphate buffer: 6.97 g of diphosphate K_2HPO_4 and 1.36 g of KH_2PO_4 was dissolved in distilled water and made up to 1000 ml mark in a volumetric flask. The buffer was adjusted to

pH 7.8. 0.05 Carbonate buffer: 14.3g of Na_2CO_3) and 4.2g Of $NaHC0_3$) was dissolved in distilled water and made up to 1000 ml mark in a volumetric flask. The buffer was adjusted to pH 10.2. 0.3 mM Adrenaline: 0.01 g of adrenaline was dissolved in 17 ml of distilled water. The solution was prepared fresh. 0.1 ml of microsome will be diluted in 0.9 ml of distilled water to make 1:10 dilution of microsome. An aliquant mixture of 0.20 ml of the diluted mierosome was added to 2.5 ml of 0.05 M Carbonate buffer. The reaction started with the addition of 0.3m1 of 0.3 mM adrenaline. The reference mixture contains 2.5 ml of 0.05 M Carbonate buffer. 0.3rn1of 0.3mM adrenaline and 0.20 ml of distilled water. Absorbance was measured ever 30 up to 150 s at 48Onm. 1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adenochrome in 1 minute.

Assessment of Lipid Peroxidation

Lipid peroxidation as evidenced by the formation of TBARS was measured by the modified method of Niehaus and Samuelson (1968) and described by Akartji et al. (2009). To 150µl of serum, (O.25M sucrose solution) were treated with 2m1 of (1:1:1 ratio) TBA-TCA-HCL reagent (thiobarbituric acid 0.37%, 0.25N HCL and 15% TBA) and place in water bath for l hour at 90°C. The mixture was cooled and centrifuged at 3000rpm for 5mm at 4°C. The absorbance of the pink supernatant 2.0ml was measured against a reference blank using spectrophotometer at 535nm.

Assay of Reduced Glutathione Concentration

Reduced glutathione (GSH) concentration measurements was done according to ElIman (1959) as described by Rajagopalan et al. (2004). 0.2M phosphate buffer: 8.40g of NaH_2PO_4 and 9.94 of Na_2HPO_4 was dissolved in distilled water and made up to 1000 ml mark in a volumetric flask. The buffer was adjusted to pH 8. To 150µ1 of serum or tissue homogenate (in phosphate -saline PH 7.4), 1.5m1 of 10% TCA was added and centrifuged at 1 500g for 5mm. 1 ml of supernatant was treated with 0.5 ml of ElIman's reagent (19.8 mg of *5*, 5'-clithiobis (nitro benzoic acid) (DNTB) in 100 ml of 0.1% sodium nitrate) and 3 ml of phosphate buffer (0.2 M, p1-I 8). The absorbance was read at 412 nm.

IV. HISTOLOGICAL ANALYSIS

Testis specimens were extracted, placed in 10% formalin solution, and processed routinely by embedding in paraffin. Tissue sections (4-5 µm) were stained with haematoxylin-eosin and examined under light microscope (Celestron).

V. STATISTICAL ANALYSES

Data are presented as mean ± standard deviation. For establishing significant differences between groups, data were analyzed by the One-way ANOVA of variance followed by the Tukey *post hoc* test. Values were considered statistically significant if P value is less than 0.05 ($p < 0.05$), using sigmastat 2.0 for Window (soft stat, jan Raff, CA) and Microsoft Excel 2007.

VI. RESULT

Antioxidant status in the blood serum was estimated by determining the activities of superoxide dismustase (SOD), catalase (CAT), thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) levels. Oxidative stress was characterized by increased lipid peroxidation and/or altered non-enzymatic and enzymatic antioxidant systems. The liver antioxidant activity of SOD, CAT and GSH significantly decreased, while testis TBARS significantly increased in the CCl4- treated, group of rats. The control group of rats maintained optimal value activity of the antioxidants studied. Administration of *Nigella sativa* significantly decreased the elevated TBARS, and also significantly increased the reduced antioxidant enzyme activities. Furthermore, *Nigella sativa* proved significantly better in restoring the altered activity of antioxidant enzymes like SOD, CAT, GSH and TBARS towards their normal values in the kidney homogenates. The animals treated with *Nigella sativa* alone showed no significant change in the levels of SOD, CAT, TBARS and GSH (Fig. 1).

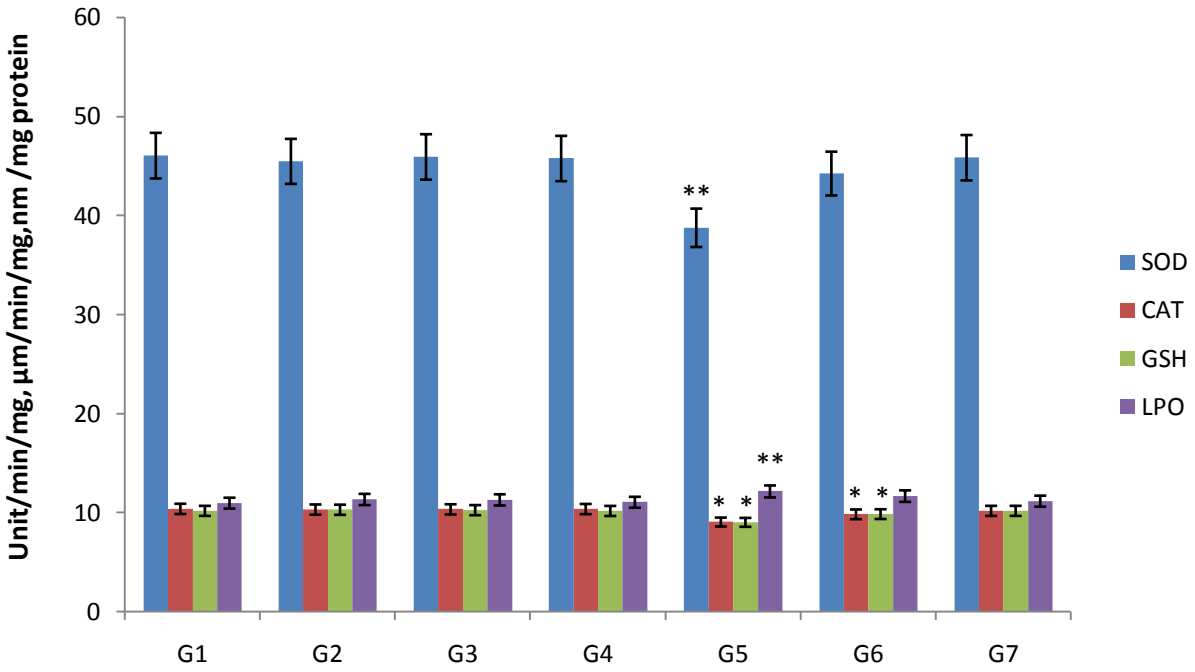

Fig 1 Effect of *N.sativa* and CCl_4 on the Testis Biochemical Parameters
**= p<0.01
*=p<0.05

Epididymis histopathology
Epididymis showed normal histology in control, olive oil as well as N. sativa treated groups (plate 1, 2, 3 and 4). CCl_4 treatment (plate 5) caused degeneration of the membranes and lumens of epididymis was devoid of sperms. However, treatment of rats with N. sativa CCl_4 (plate 6 and 7) retard the toxicity of CCl_4 and the testis histopathology was protected towards the control architecture in a concentration dependent manner. These effects were more pronounced especially at the high dose of N. sativa (4 ml/kg body weight).

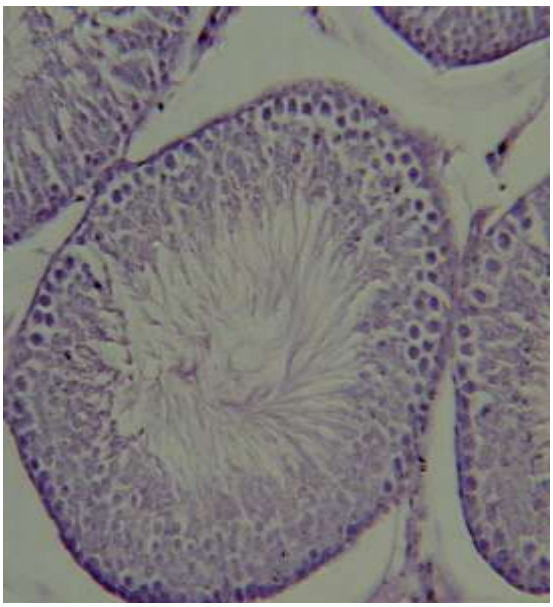

Plate 1: Microscopic section of testis of group 1 (control) rats; showing normal architecture of the seminiferous tubuls with defined basement membrane and germinal layers. H & E X100.

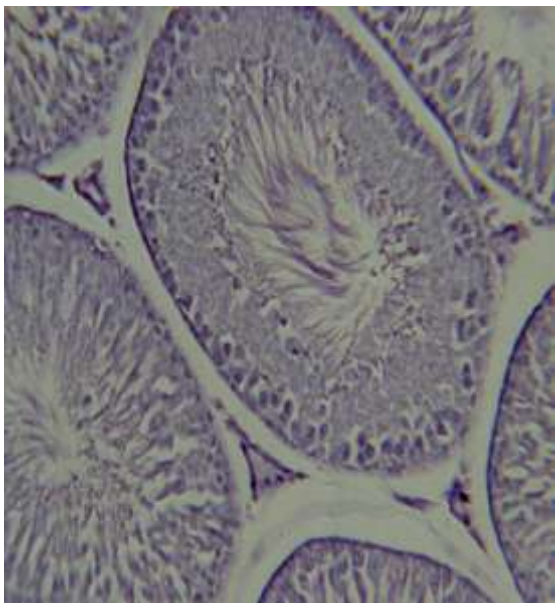

Plate 2: Microscopic section of the testis of group 2 (2ml/kg olive oil) rats; showing normal architecture of the seminiferous tubuls. H & E X100.

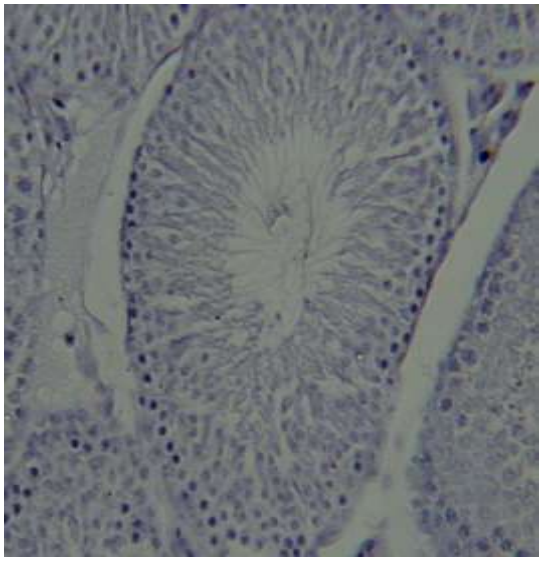

Plate 3: Microscopic section of testis of group 3 (2ml/kg *N. sativa*) rats; showing normal architecture of the seminiferous tubuls cells; basement membrane and germinal layers are well developed. H & E X100.

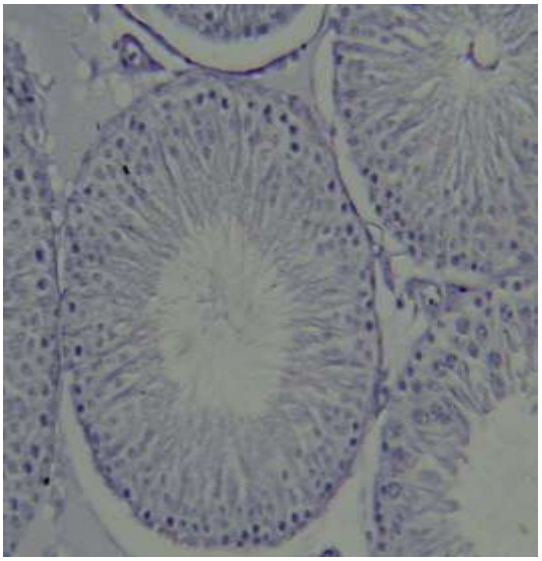

Plate 4: Microscopic section of testis of group 4 (4ml/kg *N. sativa*) rats; showing normal architecture of the seminiferous tubuls. H & E X100.

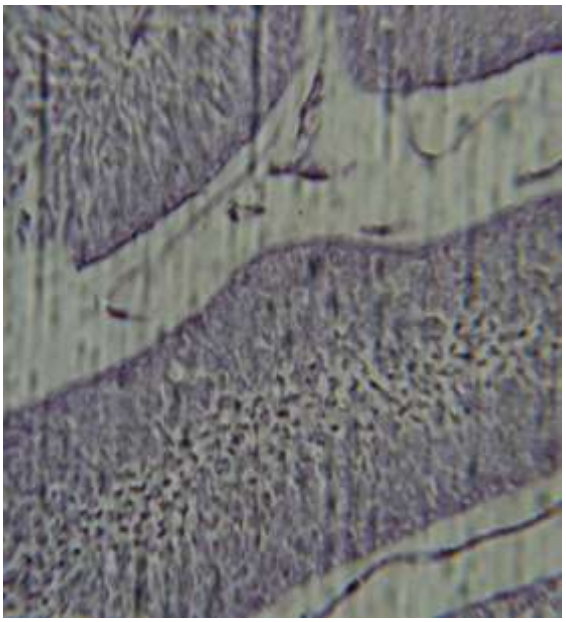

Plate 5: MicroscopicSection from the CCl_4 group **5 (4ml/kg CCl_4) rats;** showing deterioration of the seminiferous tubuls; germinal layers, basement membrane is absent and seminiferous tubule is infiltrated with the inflammatory cells. H & E X100.

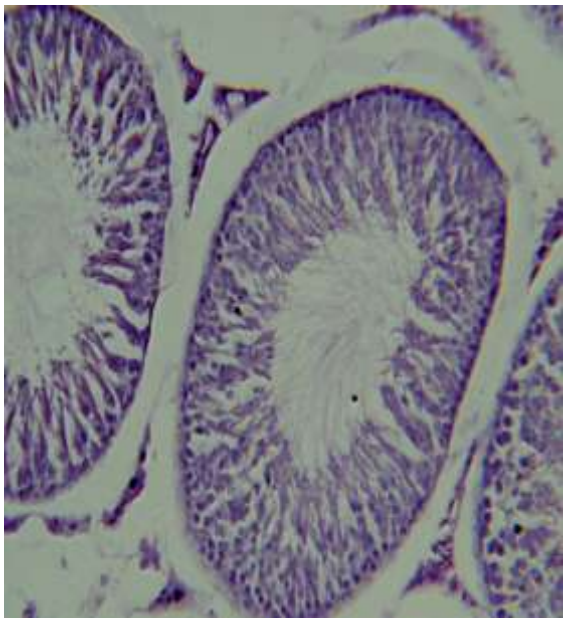

Plate 5: Microscopic Section from group 6 **(2ml/kg *N. sativa* + 4ml/kg CCl_4) rats;** showing mild deterioration the seminiferous tubuls. H & E X100.

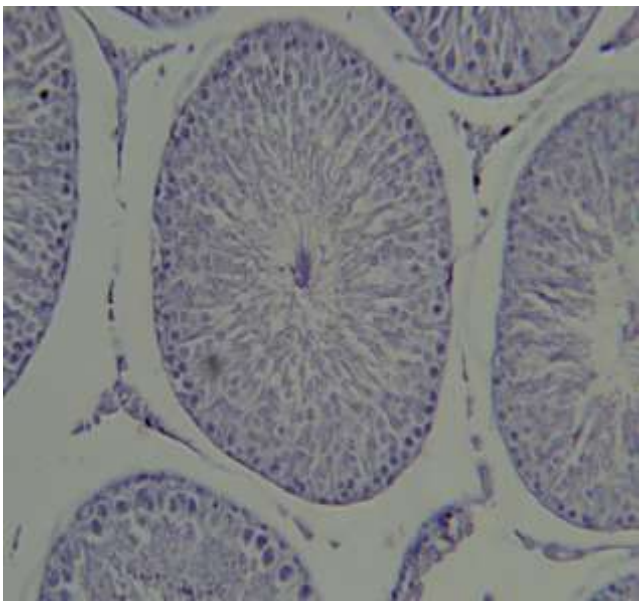

Plate 7: Microscopic section of testis of group 7 (4ml/kg *N. sativa* + 4ml/kg CCl_4) rats; showing normal structure of seminiferous cells; basement membrane and germinal layers are well developed. H & E X100.

VII. DISCUSSION

In this study, we demonstrated testicularprotective effects of N. sativa against oxidative stress induced by CCl_4 exposure in rats. CCl4 treatment enhanced lipid peroxidation through trichloromethyl radical (CCl_3) formation from the metabolic conversion of CCl4 by cytochrome P450. As O_2 tension rises, a greater fraction of CCl_3 present in the system reacts very rapidly with O_2 and many orders of magnitude more reactive free radical, peroxy trichloromethyl (CCl3OO) has been generated from CCl_3. This radical is more reactive and is capable of abstracting hydrogen from polyunsaturated fatty acids (PUFA) to ignite the process of lipid peroxidation (Yuan et al., 2008). Reactive oxygen species (ROS) play a major role in the development of a wide variety of oxidative diseases (Halliwell and Whiteman, 2004). Any interference in these enzymes leads to biochemical alteration and lesions of the tissue and cellular function (Khan et al., 2001). The reduction in the levels of these parameters toward the respective normal values by N. sativa at two doses (2ml and 4ml /kg body weight) is an indication of the stabilization of plasma membranes as well as pretecting of testicular tissue damage caused by CCl_4. This indicates that the anti-lipid peroxidation and/or adaptive nature of the systems brought about by N. sativa acted against the damaging effects of free radicals produced by CCl_4. In this study CCl_4 treatment inhibits the activities of antioxidant enzymes; CAT, SOD, and GSH contents (figure 1). Szymonik-Lesiuk et al. (2003) have shown that CCl_4 intoxication can lead to alteration in gene expression and depletion of CAT and SOD levels. The CAT, SOD and GSH activity was brought to increase by the treatment of N. sativa to CCl4-treated rats (figure 1). Treatment of various doses of N. sativa (2ml and 4ml / kg body weight) possibly inhibited the conversion of CCl4 into its reactive metabolites, decreased the oxidative stress and protected the antioxidant enzymes of testis as revealed by the enhanced level of CAT, SOD and GSH in this experiment (figure 1). GSH is an important protein thiol which coordinates body defense system against oxidative stress (peroxide scavenger), could eliminate superoxide anion and hydrogen peroxide. The maintenance of sufficient glutathione levels is important for the prevention of CCl4-induced damages. Reduced glutathione (GSH) effectively scavenge free radicals and other reactive free oxygen species. Nitrites can maintain tissue protective reactions as well as prooxidant effects. Nitrite reacts with superoxide radical (O2) result in peroxynitrite which is a strong oxidant that reacts with thiols and initiates lipid peroxidation (Rubbo et al., 1996; Khan et al., 2009; Khan et al., 2010). In the present study, CCl4-induced testicular txicity was identical to that previously reported in rat (Khan and Ahmed, 2009). This action of CCl4 on the testis may be a direct toxic action of CCl4 on the tissues and is likely to affect gonadal response to FSH and LH; diminished production of testosterone. The histopathological findings of testes in the CCl4-treated group showed a decrease in size and germinative cell layer, thickness of seminiferous tubules suggesting spermiotoxicity through oxidative damage to biomolecules (plate 5). Interestingly, N. sativa protects the histological alterations induced by CCl4 treatment (plate 6 and 7). However it was dose dependent.

VIII. ACKNOWLEDGMENT

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