CHAPTER 5

In Vivo Hepatoprotective Activity of Nigella sativa L. Seed in Various Germination Phases

“One thing I have learned in a long life: that all our science, measured against reality, is primitive and childlike – and yet it is the most precious thing we have.”

~Albert Einstein
5.1 Rationale

Largest and complex organ in the body is liver which deals multiple and diverse functions to safeguard internal environment of the body. Liver is involved in synthesizing number of various plasma proteins such as albumin, fibrinogen and the intermediate metabolism of protein, fat and carbohydrates. It is also involved in synthesis of clotting factors and also produces a number of enzyme, formation and excretion of bile. It acts as a storage depot for proteins, glycogen, various vitamins and metals. It also has a role in the regulation of blood volume by transferring the blood from portal to systemic circulation and its reticule endothelial system and participates in immune mechanism. It plays a central role in detoxification and excretion of many endogenous and exogenous compounds. Hence, any injury to it or impairment of its function has grave implication for the health of the affected person.

Liver diseases are affecting humans of all ages and one of the major causes of morbidity and mortality in public. About 20,000 deaths occur every year due to liver disorder. Hepatocellular carcinoma is one of the ten most common tumors in the world with over 2,50,000 new cases each year (Gupta and Misra, 2006). According to WHO estimates, globally 170 million people are chronically infected with hepatitis C alone and every year 3–4 millions are newly added into the list. Also, there are more than 2 billion people infected by hepatitis B virus (Negi et al., 2008). Every year about 18,000 people are reported to die due to liver cirrhosis caused by hepatitis (Handa, 1991).

Usually liver fibrosis is initiated by hepatocyte damage and various biologic factors such as hepatitis virus, bile duct obstruction, overload of cholesterol, schistosomiasis or chemical factors such as CCl₄ administration, alcohol intake etc. were known to contribute to liver fibrosis. Hepatic fibrosis is a major feature of wide range of chronic liver injuries including metabolic, viral, cholestatic and genetic diseases. The failure of bile salt excretion in cholestatic leads to retention of hydrophobic bile salts within the hepatocytes and causes apoptosis and/or necrosis.
Oxidative stress has been implicated in the pathogenesis of various liver diseases including alcoholic liver disease, nonalcoholic fatty liver disease, and chronic hepatitis C (Seki et al., 2005; Kitase et al., 2005). The liver diseases are classified as acute or chronic depending on the duration of the disease. Acute viral hepatitis and drug reactions account for the majority of cases of acute liver diseases. Chronic liver damage is a worldwide common pathology characterized by inflammation and fibrosis that can lead to chronic hepatitis, cirrhosis and cancer (Tessitore and Bollito, 2006; Kohle et al., 2008). Chronic hepatitis or long term intoxicification can severely injure hepatic cells.

Drug/chemical-mediated hepatic injury is the common sign of drug toxicity (Lee, 2003) and accounts for greater than 50% of acute liver failure cases. Hepatic damage is the largest obstacle to the development of drugs and is the major reason for withdrawal of drugs from the market (Cullen and Miller, 2006). Drug-induced liver disease can be predictable (high incidence and dose-related) or unpredictable (low incidence and may or may not be dose-related). Conventional drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects. Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological and medicinal activities, higher safety margins and lesser costs (Chattopadhyay et al., 2007). Presently a few hepatoprotective drugs and that too from natural sources are available for the treatment of liver disorders. Hence, people are looking at the traditional systems of medicine for remedies to hepatic disorders.

The present study is aimed to evaluate the hepatoprotective and antioxidant activity of methanolic extract of the Nigella sativa L. from different germination stages of seed against CCl₄-induced hepatotoxicity in rats as hepatotoxin to prove its claim in folklore practice against liver disorders with no side effects.
5.2 REVIEW OF LITERATURE

5.2.1 Hepatotoxicity (causes and pattern of liver damage)

As the major drug metabolizing and detoxifying organ in the body, the liver is subjected to potential damage from an enormous array of pharmaceutical and environmental chemicals. More than 900 drugs have been implicated in causing liver injury (Keeffe et al., 2004) and it is the most common reason for a drug to be withdrawn from the market. Chemicals often cause subclinical injury to liver which manifests only as abnormal liver enzyme tests. Drug-induced liver injury is responsible for 5% of all hospital admissions and 50% of all acute liver failures (Ostapowicz et al., 2002). Liver is susceptible to injury from drugs and other substances. 75% of blood coming to the liver arrives directly from gastrointestinal organs and then spleen via portal veins which bring drugs and xenobiotics in near-undiluted form. There are many mechanisms behind liver damage, many chemicals damage mitochondria and its dysfunction releases excessive amount of oxidants which in turn injured hepatic cells. Activation of some enzymes in the cytochrome P-450 system such as CYP2E1 also leads to oxidative stress (Jaeschke H et al., 2002). Injury to hepatocyte and bile duct cells lead to accumulation of bile acid inside liver that promotes further liver damage (Patel T et al., 1998).

Drugs/chemicals produce a wide variety of clinical and pathological hepatic injury. Biochemical markers (e.g. alanine transferase, alkaline phosphatase and bilirubin) are often used to indicate liver damage. Liver injury is defined as a rise in either ALT level more than three times of upper limit of normal, ALP level more than twice upper limit of normal or total bilirubin level more than twice upper limit of normal when associated with increased ALT or ALP (Mumoli N et al., 2006). Three important characteristics of the P450 system have role in drug induced toxicity (Figure 5.1).
a. Direct toxicity

b. Hepatic conversion of xenobiotics to an active toxin
c. The immune mechanism usually by a drug or a metabolite acting as hepatic to convert cellular protein into an immunogen.

**Figure 5.1**: Drug and toxin induced hepatic injury.

Exposure to various organic compounds including a number of environmental pollutants and drugs can cause cellular damages through metabolic activation of those compounds to highly reactive substances such as reactive oxygen species (ROS). Drugs or toxins that have a pharmacological hepatotoxicity are those that have predictable dose-response curves (higher concentrations cause more liver damage) and well characterized mechanisms of toxicity such as directly damaging liver tissue or blocking a metabolic process. Idiosyncratic injury occurs without warning when agents cause non-predictable hepatotoxicity in susceptible individuals which is not related to dose and has a variable latency period (Zimmerman, 1978).
Idiosyncratic hepatotoxicity has led to the withdrawal of several drugs from market even after rigorous clinical testing as part of the FDA approval process.

5.2.2 Carbon tetrachloride: A most commonly used hepatotoxin

Chemical toxicity comprises an important source of reactive oxygen species (ROS), which may occur through processes such as inhibition of mitochondrial electron transport chain and subsequent accumulation of intermediates inactivation of antioxidant enzymes and deletion of radical scavengers (Mates, 2000). Carbon tetrachloride (CCl₄) is a well known hepatotoxin and exposure to this chemical is known to induce oxidative stress and causes liver injury by the formation of free radicals (Sil et al., 2007). Carbon tetrachloride is one of the most commonly used hepatotoxin in the experimental study of liver disease (Johnston et al., 1998).

Carbon tetrachloride is used in the synthesis of chlorinated organic compounds, including chlorofluorocarbon refrigerants. It is also used as an agricultural fumigant and as a solvent in the production of semiconductors, in the processing of fats, oils and rubber and in laboratory applications (Lewis, 1993; Kauppinen et al., 1998). CCl₄ continues to provide an important service today as a model substance to elucidate the mechanisms of action of hepatotoxic effects such as fatty degeneration, fibrosis, hepatocellular death, and carcinogenicity. CCl₄ has been extensively used to study liver injury induced by ROS in the mouse model, which is closely analogous to hepatotoxicity in humans.
Mechanism of CCl₄ Toxicity:

\[ \text{CCl}_4 \]

\[ \text{sER} \]

\[ \text{CCl}_3^* \]

\[ \text{Lipid radicals} \]

\[ \text{Lipid peroxidation} \]

(Autocatalytic spread along microsomal membrane)

Membrane damage to rER

Polysome Detachment

\[ \text{H}_2\text{O, Ca}^{2+} \]

Apo protein synthesis

Fatty liver

Release of products of lipid peroxidation

Damage to plasma membrane

Permeability to Na⁺,

Cell swelling

Massive influx of Ca^{2+}

Inactivation of Mitochondria, cell enzymes, Denaturation of proteins
The hepatotoxic effect of CCl₄ are largely due to its active metabolites, CCl₃ (trichloro methyl radicals) CCl₃⁻ (tri chloro methyl per oxy radicals) is most reactive species and causes damage to biological macromolecules by combining with them there by causing covalent modification and setting the chain reactions of lipid peroxidation and ultimately cell death (Benedetti et al., 1987). Among various mechanism involved in hepatotoxic effect of carbon tetrachloride, one is oxidative damage through free radicals generation (Deleve et al., 1995). The hepatotoxicity of CCl₄ is attributed to the formation of trichloromethyl and trichloromethyl peroxyl radicals, initiating lipid peroxidation and resulting in fibrosis and cell necrosis (Kadiiska et al., 2000).

At the molecular level CCl₄ activates tumor necrosis factor TNF-alpha, nitric oxide (NO), transforming growth factors, TGF-alpha and TGF-beta in the cell processes that appear to direct the cell primarily toward self destruction or fibrosis. TNF-alpha pushes toward apoptosis whereas the TGFs appear to direct toward fibrosis. CCl₄ intoxication also leads to hypomethylation of cellular components; in the case of RNA the outcome is thought to be inhibition of protein synthesis, in the case of phospholipids it plays a role in the inhibition of lipoprotein secretion (Weber et al., 2003). The over production of ROS and therefore oxidative stress can be initiated by a variety of factors including exposure to xenobiotics, such as acetaminophen and carbon tetrachloride (Ronsein et al., 2005). Of concern here is the adverse effect of carbon tetrachloride to the liver, having visualized the prominent functions of the liver for survival. According to Liu et al., (1995), ingestion of carbon tetrachloride can lead to marked hepatotoxicity.

5.2.3 Free radicals and oxidative damage in liver

A free radical can be defined as any species which is capable of independent existence and contains one or more unpaired electrons (Halliwell and Gutteridge, 1989). In excess, free radicals can damage cell membranes and cause cell necrosis; they can cause damage to all macromolecules, lipids, proteins
mitochondrial and nuclear DNA molecules of the cells causing inflammation or lesion on various organs (Beckman et al., 1990; Halliwell and Gutteridge, 1984). It is increasingly being realized that majority of disorders/diseases is mainly due to the imbalance between pro-oxidant and antioxidant homeostatic phenomenon in the body. Pro-oxidants conditions either dominate due to increase in the generation of free radicals and/or their inadequate quenching or scavenging in the body (Tiwari, 2001). The reactive oxygen species play an important role related to the degenerative or pathological processes of various serious diseases such as aging (Burns et al., 2001), cancer, coronary heart disease, Alzheimer’s disease (Smith et al., 1996; Diaz et al., 1997), neurodegenerative disorders, atherosclerosis, cataracts and inflammation (Aruoma, 1998).

ROS such as superoxide radical, hydroxyl radical, peroxyl radical and nitric oxide radical attack biological molecules such as lipids, proteins, enzymes, DNA and RNA. This may be lead to cell or tissue injury associated with aging, atherosclerosis, carcinogenesis (Keli Chen et al., 2005) and development of chronic diseases related to the cardio and cerebrovascular systems. More than one hundred disorders in humans including atherosclerosis, arthritis, ischemia, reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS associate with reactive oxygen species (Cook and Samman, 1996).

ROS play an important role in fibrogenesis throughout increasing platelet-derived growth factor. Most hepatocellular carcinomas occur in cirrhotic livers and the common mechanism for hepatocarcinogenesis is chronic inflammation associated with severe oxidative stress; other risk factors are dietary aflatoxin B1 consumption, cigarette smoking and heavy drinking. Ischemia–reperfusion injury affects directly on hepatocyte viability particularly during transplantation and hepatic surgery. Ischemia activates Kupffer cells which are the main source of ROS during the reperfusion period (Pablo Muriel, 2009). Hepatocellular carcinoma is one of the most malignant and frequent worldwide spreading diseases. It is the third most common cause of cancer deaths (Pisani et al., 1999; Parkin et al., 2005). Most Hepatocellular
carcinoma occurs in cirrhotic livers and the common mechanism for hepatocarcinogenesis is chronic inflammation associated with severe oxidative stress (Seitz et al., 2006). There is a large body of evidence indicating that ROS play a pathogenesis role in carcinogenesis in liver (Marx et al., 2004).

5.2.4 Antioxidant

Almost all organisms are well protected against free radical damage by antioxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT) or chemical compounds such as α-tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione. When the mechanism of antioxidant protection becomes unbalanced by factors such as ageing, deterioration of physiological functions may occur resulting in diseases and accelerated ageing. However, antioxidant supplements or antioxidant containing foods may be used to help the human body to reduce oxidative damage (Mau et al., 2001; Gulcin et al., 2002).

All living organisms have endogenous defense systems against oxidative damage such as lipid peroxidation DNA damage (Lee et al., 2005) and inhibition of cell communication due to reactive oxygen species (ROS). Antioxidants protect against chemotherapy toxicity and local toxic effects of tumors or surrounding tissues. The protection of cells against damage from oxygen and its metabolites can be accomplished through enzymatic and non-enzymatic means. Superoxide dismutase, catalase and glutathione peroxides (GPx) are considered to be the primary antioxidant enzymes, since they are involved in the direct elimination of reactive oxygen species. Glutathione-S-transferase (GST), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) are secondary antioxidant enzymes which help in the detoxification of reactive oxygen species by decreasing peroxide levels by GST or by maintaining a steady supply of metabolic intermediates like glutathione as by GR and NADPH by, G6PD for the primary antioxidant enzymes. The non-enzymatic small molecular antioxidants include
sulfhydryl compounds such as glutathione (GSH) and thiols, NADPH, ascorbate, α-tocopherol etc., (Halliwell and Gutteridge 1984). Phytochemical components such as polyphenols, ascorbic acid, and carotenoids also serve as antioxidants (Rice-Evans et al., 1997). Oxygen is essential for aerobic life process. However, cells under aerobic condition are threatened with the insult of reactive oxygen molecules that are efficiently taken care of by the powerful antioxidant system in human body.

5.2.5 Hepatoprotective activities of *Nigella sativa* L.

A large number of medicinal plants have been tested and found to contain active principles with curative properties against a variety of diseases (Lewis, 1977). Liver protective plants contain a variety of chemical constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotinoids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthenes. Recent experience has shown that plant drugs are relatively non-toxic, safe and even free from serious side effects (Momin, 1987).

*Nigella sativa* L. is one of the most revered medicinal seed in history. A large number of *in vitro* and *in vivo* studies have been conducted on laboratory animals and humans in order to investigate its pharmacological properties like immunostimulation, anti-inflammatory, hypoglycemic, antihypertensive, antiasthmatic, antimicrobial, antiparasitic, antioxidant as well as anticancer properties (Randhawa and Alghamdi, 2002). Acute and chronic toxicity studies on laboratory animals have reported that *N. sativa*, its oil and thymoquinone the most widely studied active principle are quite safe particularly when given orally (Badary et al., 1997; Mansour et al., 2001; Ali et al., 2003).

It has been reported that *N. sativa* oil possesses hepatoprotective effects in some models of liver toxicity. However, it is *N. sativa* seed that is used in the treatment of liver ailments in folk medicine rather than its oil. Clinical and experimental investigations have shown that *N. sativa* has a protective effect against
oxidative damage in isolated rat hepatocytes (Daba et al., 1998). It was found that the fixed oil of *N. sativa* has both antioxidant and anti-eicosanoid effects greater than thymoquinone which is its active constituent (Houghton et al., 1995). Furthermore, *N. sativa* has antioxidant activity by suppressing the chemiluminescence in phagocytes (Haq et al., 1995).

Administration of oil extract of *N. sativa* to CCl₄ intoxicated animals showed significant hepatoprotective activity by restoring the hepatocellular activity. It has been reported that thymoquinone (the active component of black cumin) presents in high level in *N. sativa* possesses antioxidant properties (Hesham et al., 2002). Thymoquinone prevents the formation of toxin stable complex by the combination of CCl₃O₂ free radical and the glycolipid component of cell membrane and therefore restores cellular architecture and prevent the leakage of its enzymes (Ali BH et al., 2003). Significant hepatoprotective effects of *N. sativa* in carbon tetrachloride (Mastour and Al-Ghamdi et al., 2003), D-galactosamine and turpentine oil- induced liver damage (Subodh et al., 2011) were noted.

This was also reported that *N. sativa* has a significant hepatoprotective effect in CCl₄ intoxicated rabbits. *N. sativa* protects liver against fibrosis possibly through immunomodulator and antioxidant activities. *N. sativa* seed extract reduced total bilirubin, serum enzymes level in CCl₄ treated animals and also prevent liver fibrosis and cirrhosis, suggested that *N. sativa* protects liver against fibrosis possibly through immunomodulator and antioxidant activities. (Turkdogan et al., 2003). *N. sativa* seeds appeared to be safe and possibly protective against CCL₄-induced hepatotoxicity (Mastour and Al-Ghamdi, 2003).
5.3 MATERIALS AND METHODS

5.3.1 Collection of *N. sativa* seeds

Seeds of *N. sativa* were procured in September, 2010 from a herbal shop in Lucknow, India and authenticated by a botanist at National Botanical Research Institute, Lucknow. A voucher specimen of the seeds was kept in the museum of the Department for future reference.

5.3.2 Germination of *N. sativa* seeds

Germination was done according to the method of Ahmad *et al.*, (2010). Seed lots used for different experiments showed germination capacities ranging from 80 to 98%. The seeds were surface sterilized with 0.1% HgCl$_2$ for 3 min. They were rinsed thoroughly with double distilled water and soaked in de-ionized water for 30 min. For germination of seeds, they were placed on four folds of damp filter paper at 25°C and incubated in dark till the initiation of sprouting after which they were placed at a light intensity of 100 µmol m$^{-2}$ s$^{-1}$ (that was measured by LI-190SA quantum Sensor, Li-COR Co., USA) and a 14/10 h (day/night) photoperiod till the complete plantlet with two leaves were obtained. The complete germination took eleven days with emergence of epicotyl, hypocotyl, roots and green leaves. Germination, defined as 1 mm radicle emergence, was followed for 11 days. No contamination by microorganisms was observed during this time period.

5.3.3 Harvest of germinated seeds

The germinated seeds of different days were harvested with sterilized forceps and were kept on blotting sheet to remove excess water. The germinated seeds collected for different experiments were used immediately for preparing extracts.
Seeds were considered to be germinated after the radicle emerged from the testa. All the samples were stored at -80°C in a deep freezer until used further.

5.3.4 Preparation of distilled extracts

The samples of seed and germinated phases 5th, 7th and 11th day were shade-dried and ground to a fine powder. The powder (20gm) was extracted with 200 ml methanol solvent for 48 h in order to extract bioactive compounds using soxhlet apparatus (AOAC method 1980). The extracts were filtered using Whatman filter paper (No.1) and methanol was evaporated using rotary distillation apparatus to obtained pure extract. Oily fraction of extracts was stored at 4°C until use.

5.3.5 Animals

Male Wistar rats, weighing 150 - 200 g, were purchased from Central Drug And Research Institute (CDRI), Lucknow, India and housed in a temperature controlled room (22±2°C) with a 12 hour light-12 hour dark cycle and allowed free access to a standard rat chow and filtered tap water for 7 days for acclimatization. The study received the approval of the Institutional Animal Ethics Committee (IAEC) of Era’s Lucknow Medical College & Hospital. Animals were cared for in accordance with the internationally accepted principles for laboratory animal use and care and the procedures followed were in accordance with the standards set forth in the Guide for the Care and Use of Laboratory Animals (published by the National Academy of Science, National Academy Press, Washington, D.C.). They were housed under controlled conditions of temperature of 23±20C, relative humidity of 30–70% and 12 h light–12 h dark cycle. The animals were housed individually in polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the experiment. All animals were fed with sterile commercial pelleted rat chow supplied by Hindustan Lever Ltd. (Mumbai, India) and had free access to water. Animals were kept under fasting for overnight and weighed before the experiment.
5.3.6 Study design

Hepatoprotective effects of *N. sativa* methanolic extracts of different germination stages were studied against carbon tetrachloride (CCl₄) induced hepatotoxicity in Wistar rats. Dose of *N. sativa* extracts was selected as per study of Mehta *et al.*, (2009). Silymarin (50 mg/kg b.w.) was used as standard hepatoprotective drug (Nayak *et al.*, 2012, Palanivel *et al.*, 2008).

5.3.6.1 Carbon tetrachloride induced hepatotoxicity

Carbon tetrachloride (CCl₄) toxicity was induced according to the method of Asif Mir *et al.*, 2010. Acute CCl₄ toxicity was induced by treating rats for 24 hrs by oral administration of CCl₄ mixed with olive oil as vehicle in 1:1 ratio (3 ml/kg b.w.).

5.3.6.2 Hepatoprotective effects of *N. sativa*

To study hepatoprotective effects of *N. sativa* during different phases of its germination in carbon tetrachloride induced (CCl₄) induced hepatotoxicity, animals were divided in following groups containing 6 rats each.

**Group-I (control)** Received single dose of distilled water orally from day 1 to day 7 followed by olive oil (1.5ml/kg) on day 7.

**Group-II (CCl₄)** Received distilled water for six days followed by CCl₄ with olive oil (3 ml/kg) orally on day 7 (Oral administration of CCl₄ mixed with olive oil as vehicle in 1:1 ratio (3 ml/kg b.w.)

**Group-III** Received Silymarin (50 mg/kg) orally from day 1 to day 7 followed by CCl₄ with olive oil (3ml/kg) on day 7 after 2 hrs.

**Group- IV (Seed extract)** Received *N. sativa* seed extract 1 g/kg orally from day 1 to day 7 followed by CCl₄ with olive oil (3ml/kg) on day 7 after 2 hrs.
**Group-V (5th day extract)** Received *N. sativa* extract of 5th day of germination phase (1 g/kg) orally from day 1 to day 7 followed by CCl₄ with olive oil (3ml/kg) on day 7 after 2 hrs.

**Group-VI (7th day extract)** Received *N. sativa* extract of 7th day of germination phase (1 g/kg) orally from day 1 to day 7 followed by CCl₄ with olive oil (3ml/kg) on day 7 after 2 hrs.

**Group-VII (11th day extract)** Received *N. sativa* extract of 11th day of germination phase (1 g/kg) orally from day 1 to day 7 followed by CCl₄ with olive oil (3ml/kg) on day 7 after 2 hrs.

**Figure 5.2:** Study design to investigate hepatoprotective effect of *N. sativa* extracts of different germination phases in CCl₄-induced hepatotoxicity.
5.3.7 Blood sampling

**Cardiac puncture (Diaphragmatic approach):** General anesthesia was administered and the animal placed on solid surface with its ventrum exposed. The xyphoid process was palpated at the caudal aspects of the animal sternum. A notch was present on both sides of this process. A 1.5O, 22 gauge needle attached to a 5 ml syringe was inserted into either notch and directed towards the heart as determined by palpating the apex beat. Negative pressure was applied by placing slight backward pull of the plunger, once it has been inserted beneath the skin. Reflux of blood was apparent once the needle penetrated the heart.

**Plate 5.1:** Blood sampling by cardiac puncture and collection of liver by dissection.
5.3.8 Biochemical estimation

5.3.8.1 Liver enzymes

Estimation of serum levels of glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP). Total bilirubin was measured by standard method using semi-auto analyzer.

5.3.8.2 Tissue Homogenate Preparation

10% (w/v) homogenate of rat liver was prepared with the aid of York’s homogenizer fitted with Teflon plunger in 0.1 M phosphate buffer (pH 7.5). The whole homogenate was first centrifuged at 2500xg for 10 minutes in a refrigerated centrifuge. The pellet consisting of nuclear fraction and cell debris was discarded. The supernatant was further centrifuged at 11,000xg for 15 min and mitochondrial fraction was separated. The clear supernatant was further centrifuged at 100,000xg for 90 min and the resulted supernatant was used for enzyme activities.

5.3.8.3 Lipid peroxide level (Ohkawa et al., 1979)

Procedure: 0.2 ml of each sub-cellular fraction having 3 to 10 mg protein, was mixed with 1.0 ml of 20% acetic acid followed by the addition of 0.2 ml of 8% aqueous SDS. The mixture was adjusted to pH 4 by addition of concentrated NaOH solution if needed. After adjusting the pH of reaction mixture, 1.5 ml of 0.8% TBA solution and sufficient amount of distilled water was also added to achieve the final volume of 4 ml. The reaction mixture was incubated in a boiling water bath for 1 hour. After cooling to room temperature, 3 ml of n-butanol was mixed. The reaction mixture was centrifuged at 10,000 x g for 15 min. A clear butanol fraction obtained from centrifugation was used for measuring the absorbance at 532 nm.

Calculation: An appropriate standard made up of malondialdehyde (MDA) 2.5 nmol was run simultaneously. Result was expressed in nmol MDA/gm.
5.3.8.4 Superoxide dismutase (McCord Fridovich, 1969)

**Procedure:** 2 ml of homogenate was dispensed in centrifuge tube. The tube was placed in a refrigerated centrifuge and spin at 10,000x g for 15 min. To the supernatant for each sample, 313mg/ml ammonium sulphate was added to the final concentration of 50%. The tube was shaken thoroughly and kept for 4 hours in cold (4°C). Thereafter, the tube was centrifuged at 14,000x g for 30 min at 4°C. The supernatant sample was dialyzed against cold tripled distilled water with 3 changes, each change after 3 hrs interval. The content of dialysis bags were subsequently used as enzyme source. Experimental tubes was contain 0.3 ml of 1.5mM nitrobluetetrazolium (NBT), 0.2 ml of 0.93 mM phenazinemethosulphate, 1 ml of 20.4mM pyrophosphate buffer (pH 9.2), 1 ml triple distilled water and 0.2ml enzyme source. The second setup reference tubes received the above reagents except the enzyme source. The reaction was started simultaneously in two sets by the addition of 0.1 ml 2.34mM NADH. After an interval of 90 sec, 1 ml glacial acetic acid was added to each tube for checking the reaction then 0.2 ml enzyme source was added for checking the reaction followed by addition of 0.2 ml enzyme source in the reference tubes. The absorbance of these tubes was read at 560 nm in a spectrophotometer against blank (NBT+PMS+Buffer+TDW).

**Calculation:** The unit of enzyme activity was defined as the amount of enzyme required to inhibit the reduction in optical density of nitro blue tetrazolium up to 50%, in 1 min at 560 nm under the assay condition. Results were expressed as unit/mg protein

5.3.8.5 Catalase (Aebi, 1974)

**Procedure:** 3.0 ml of H₂O₂ phosphate buffer was pipette into the cuvette followed by addition of required amount of tissue supernatant (cytosolic fraction) as enzyme source and the content was mixed thoroughly. The decrease in absorbance at
240 nm recorded after every 30 sec for 3 min using UV spectrophotometer. Result was expressed as unit/mg protein.

5.3.8.6 Reduced glutathione (Eliman et al., 1959)

**Procedure:** 1 ml liver (10%) homogenate was deprotenized by adding 1 ml of 10% TCA and centrifuged at 6000x g for 5 min. 0.5 ml aliquots for supernatant was mixed with 0.5 ml of double distilled water thereafter 2 ml of 0.4 M Tris buffer and 0.1 ml DTNB were added to it with proper stirring. The absorbance was read at 412 nm within 5 min of the addition of DNTB.

**Calculation:** GSH in the sample was calculated using the standard curve and results were expressed as µmol/g tissue.

5.3.9 Histopathological study

For histopathology half of liver tissue of each rat was stored in 10% formalin solution for 48 hours or until processing. For block preparation, liver were processed using a graded ethanol series and embedded in paraffin. 4 µm paraffin sections were cut and stained with Haematoxylin and eosin. The study was conducted in consultation with the trained pathologist under light microscope with 10x, 40x, and 100x magnifications.

**Tissue fixation, embedding & sectioning**

**Tissue Fixation**

The rats were sacrificed after the completion of the period of the dose and the organs were collected in normal saline. The tissue was cut in small pieces (L.Section/T.Section, approx 2.3 mm in size). The tissue was fixed in 10% formalin for 24 hours and washed in running tap-water after that tissue was kept in distilled
water for 10-15 minutes. Tissue was dehydrated in following graded series of alcohol.

- 30% alcohol for 45 minutes.
- 50% alcohol for 45 minutes.
- 70% alcohol for 45 minutes.
- 90% alcohol for 45 minutes.
- 100% alcohol for 45 minutes.

Then, this tissue was transferred in absolute alcohol + xylene (1:1) for 35 minutes and then after in pure xylene for 30 minutes.

Following chemicals are transferred in incubator at 60°C
Xylene + wax in 1:1 ratio for 45 minutes and pure wax for 45 minutes.

**Tissue Embedding**

The processed tissues were then transfer to the molten wax poured into the L-block at 60°C, allowed to solidify for 10-15 minutes and removed from the L-block. The casted wax blocks were left overnight at room-temperature to uniform solidification. (Precautions were taken to minimize the appearance of air bubbles in the blocks and to maintain the orientation of the tissue for transverse section (TS) or longitudinal section (LS) while placing it into the molten wax in L-molds).

**Section Cutting**

With the help of microtome the section of the tissue has been cut (2-5 µm in size). The thin section has been taken in clean slide coated with a mixture of egg albumin + glycerol (1:1). Then it was unfolded with slight warm distilled water, then heat fixed and the slides were kept for 12 hours.
Slide Staining

The tissue was immersed in xylene for 10 min.

\[ \text{Immersed in 100\% alcohol for 10 min.} \]

\[ \text{Immersed in 90\% alcohol for 10 min.} \]

\[ \text{Immersed in 70\% alcohol for 10 min.} \]

\[ \text{Immersed in 50\% alcohol for 5-8 min.} \]

\[ \text{Immersed in 30\% alcohol for 5-8 min.} \]

\[ \text{Immersed in distilled water for 5 min.} \]

\[ \text{Immersed in Haematoxylin for 2-3 min.} \]

\[ \text{Immersed in tap water for 10-15 min.} \]

\[ \text{Immersed in acid water 3-5 min.} \]

\[ \text{Immersed in distilled water for 5 min.} \]

\[ \text{Immersed in 30\% alcohol for 5-8 min.} \]

\[ \text{Immersed in 50\% alcohol for 5-8 min.} \]

\[ \text{Immersed in 70\% alcohol for 10 min.} \]

\[ \text{Immersed in acid for 3-5 min.} \]

\[ \text{Immersed in 90\% alcohol for 15 min.} \]

\[ \text{Immersed in 100\% alcohol for 15 min.} \]

\[ \text{Immersed in xylene for 5-10 min.} \]

Then mounted on the slide with DPX.

Then left for 12 hours & seen under microscope.
5.3.10 Statistical analysis

Statistical significance was determined by One Way Analysis of Variance (ANOVA) followed by Dunnet’s t-test to compare group means. The level of significance was $P < 0.001$.

5.4 RESULTS AND DISCUSSION

5.4.1 Hepatoprotective effect of *N. sativa* extracts of different germination phases against CCl$_4$ induced hepatotoxicity.

Administration of carbon tetrachloride CCl$_4$ caused a significant increase in liver enzymes and total bilirubin level which was attenuated by oral administration of *N. sativa* extracts from different phases of seed germination.

**Table 5.1:** Effect of *N. sativa* extracts of different germination phases on liver enzymes in CCl$_4$ induced hepatotoxicity.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALP (U/L)</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>Total bilirubin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>74.87±5.09</td>
<td>44.77±3.37</td>
<td>39.57±5.04</td>
<td>0.19±0.01</td>
</tr>
<tr>
<td>Group-II</td>
<td>267.22±18.4$^a$</td>
<td>1217.98±156.38$^a$</td>
<td>434.89±71.37$^a$</td>
<td>0.668±0.08$^a$</td>
</tr>
<tr>
<td>Group-III</td>
<td>75.2±5.9$^b$</td>
<td>45.1±6.12$^b$</td>
<td>49.14±4.32$^b$</td>
<td>0.2±0.04$^b$</td>
</tr>
<tr>
<td>Group- IV</td>
<td>110.5±6.95$^b$</td>
<td>63.96±7.67$^b$</td>
<td>100.22±7.1$^b$</td>
<td>0.285±0.01$^b$</td>
</tr>
<tr>
<td>Group-V</td>
<td>74.74±5.7$^b$</td>
<td>45.42±3.33$^b$</td>
<td>49.33±3.54$^b$</td>
<td>0.206±0.02$^b$</td>
</tr>
<tr>
<td>Group-VI</td>
<td>86.77±3.44$^b$</td>
<td>45.88±4.47$^b$</td>
<td>59.1±5.9$^b$</td>
<td>0.208±0.02$^b$</td>
</tr>
<tr>
<td>Group-VII</td>
<td>100.94±3.4$^b$</td>
<td>64.44±7.8$^b$</td>
<td>81.33±4.2$^b$</td>
<td>0.2±0.022$^b$</td>
</tr>
</tbody>
</table>

$^*$Group-I (control); Group-II (CCl$_4$); Group-III (silymarin); Group- IV (Seed extract); Group-V (5$^{th}$ day extract); Group-VI (7$^{th}$ day extract); Group-VII (11$^{th}$ day extract). *Values in Mean ±SEM, N=6. $^{a}$p < 0.001 compared with Group I (control) and $^{b}$p<0.001 compared with Group II (CCl$_4$ treated).
5.4.1.1 ALP

Results of present study showed that level of ALP in control group was 74.87±5.09 U/L serum. There was a significant (P<0.001) increase in mean ALP level in rats administered with CCl₄ (3ml/kg) for 24 hrs (267.22±18.4 U/L serum). Oral administration of N. sativa seed extract (non-germinated) and extracts of different germination phases (5th day, 7th day and 11th day) ameliorated this increase in ALP level. In all the four groups receiving N. sativa extracts rise in ALP level was prevented (Table 5.1 and Figure 5.3). Among all extracts 5th day germination extract showed significant protective effect and reduced serum ALP level (74.74±5.7 U/L serum) which was equal to control group (74.87±5.09 U/L serum) and the standard drug silymarin (75.2±5.9 U/L serum). Extract of 7th day germination was on second position (86.77±3.44 U/L serum) followed by 11th day extract (100.94±3.4 U/L serum) and seed extract (110.5±6.95 U/L serum).

**Figure 5.3:** Effect of N. sativa extracts of different germination phases on ALP level against CCL₄-induced toxicity.

*Group-I (control); Group-II (CCL₄); Group-III (silymarin); Group- IV (Seed extract); Group-V (5th day extract); Group-VI (7th day extract); Group-VII (11th day extract).*
5.4.1.2 SGOT

Table 5.1 and Figure 5.4 showed that administration of CCl₄ (3ml/kg) for 24 hrs significantly (P<0.001) increase SGOT level as compared to control. The SGOT value of CCl₄ group (1217.98±156.38 U/L serum) was very high than control group (44.77±3.37 U/L serum). Administration of N. sativa seed extract (non-germinated) and extracts from different germination phases of seed (5th day, 7th day and 11th day) ameliorated the increase in SGOT level due to CCl₄ toxicity. Among all extracts 5th day and 7th day germination extract showed significant protective effect against CCl₄-induced toxicity and reduced serum SGOT level (45.42±3.33 U/L serum and 45.88±4.47 U/L serum respectively) which was significantly (P<0.001) equal to control group (44.77±3.37 U/L serum) as well as standard group (45.1±6.12 U/L serum) followed by 11th day extract (64.44±7.8 U/L serum) and seed extract (63.96±7.67 U/L serum).

Figure 5.4: Effect of N. sativa extracts of different germination phases on SGOT level against CCl₄-induced toxicity.

*Group-I (control); Group-II (CCl₄); Group-III (silymarin); Group- IV (Seed extract); Group-V (5th day extract); Group-VI (7th day extract); Group-VII (11th day extract).
5.4.1.3 SGPT

Results showed SGPT level in control group was 39.57±5.04 U/L serum. There was a significant (P<0.001) increase in mean SGPT level in rats administered with CCl₄ (3ml/kg) for 24 hrs (434.89±71.37U/L serum). Oral administration of *N. sativa* non-germinated seed extract and extracts from different germination phases of seed (5th day, 7th day and 11th day) ameliorated the increase in SGPT level due to CCl₄ toxicity. In all the four groups, receiving *N. sativa* extracts rise in SGPT level was prevented (Table 5.1 and Figure 5.5). Among all extracts 5th day germination extract showed significantly protective effect against CCl₄-induced toxicity and reduced serum SGPT level (49.33±3.54 U/L serum) which was approximately equal to control group (39.57±5.04 U/L serum) in comparison to the toxic group. Level of SGPT in group which received silymarin was (49.14±4.32 U/L serum) which was equal to 5th day germination extract. 7th day extract was on second position (59.1±5.9 U/L serum) followed by 11th day extract (81.33±4.2 U/L serum) and seed extract (100.22±7.1 U/L serum).

**Figure 5.5**: Effect of *N. sativa* extracts of different germination phases on SGPT level against CCL₄-induced toxicity.

*Group-I (control); Group-II (CCL₄); Group-III (silymarin); Group-IV (Seed extract); Group-V (5th day extract); Group-VI (7th day extract); Group-VII (11th day extract).
5.4.1.4 Total bilirubin

Mean value of total bilirubin in control group was 0.19±0.01 (mg/dl serum). There was a significant (P<0.001) increase in value of total bilirubin level in rats administered with CCl₄ (3ml/kg) for 24 hrs (0.668±0.08 mg/dl serum). Administration of N. sativa seed extract (non-germinated) and extracts from different germination phases (5th day, 7th day and 11th day) ameliorated the increase in total bilirubin level due to CCl₄ toxicity. In all the four groups receiving N. sativa extracts rise in total bilirubin level was prevented (Table 5.1 and Figure 5.6). 7th day extract was on second position followed by 11th day extract and seed extract to ameliorate the level of bilirubin.

**Figure 5.6:** Effect of N. sativa extracts of different germination phases on bilirubin level against CCL₄-induced toxicity.

*Group-I (control); Group-II (CCL₄); Group-III (silymarin); Group- IV (Seed extract); Group-V (5th day extract); Group-VI (7th day extract); Group-VII (11th day extract).*
5.4.2 Effect of *N. sativa* extracts of different germination phases on antioxidant enzymes level against CCL₄-induced hepatotoxicity.

Carbon tetrachloride (CCL₄) administration caused a significant oxidative stress as evident by increase in MDA level and decrease in SOD, CAT and GSH levels. This altered redox status was attenuated by administration of *N. sativa* extracts of different phases of seed germination.

**Table 5.2:** Effect of *N. sativa* extracts of different germination phases on antioxidant level against CCL₄-induced hepatotoxicity.

<table>
<thead>
<tr>
<th>Groups</th>
<th>nmol MDA/gm tissue</th>
<th>SOD U/mg protein</th>
<th>CAT U/mg protein</th>
<th>GSH µmol/g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>289.4±19.23</td>
<td>12.5±0.56</td>
<td>9.5±0.23</td>
<td>38.12±0.52</td>
</tr>
<tr>
<td>Group-II</td>
<td>363.22±56.12</td>
<td>4.56±0.48</td>
<td>3.46±0.25</td>
<td>25.39±0.52</td>
</tr>
<tr>
<td>Group-III</td>
<td>295.2±7.42</td>
<td>11.02±0.61</td>
<td>8.91±0.45</td>
<td>37.1±0.21</td>
</tr>
<tr>
<td>Group-IV</td>
<td>321.01±6.67</td>
<td>8.24±0.99</td>
<td>6.24±0.25</td>
<td>35.1±0.31</td>
</tr>
<tr>
<td>Group-V</td>
<td>299.32±10.33</td>
<td>11±0.56</td>
<td>8.9±0.56</td>
<td>36.99±0.4</td>
</tr>
<tr>
<td>Group-VI</td>
<td>303.12±6.47</td>
<td>10.19±0.88</td>
<td>8.1±0.61</td>
<td>36±0.12</td>
</tr>
<tr>
<td>Group-VII</td>
<td>316.1±8.8</td>
<td>9.97±0.88</td>
<td>6.97±0.64</td>
<td>35.89±0.52</td>
</tr>
</tbody>
</table>

*Group-I (control); Group-II (CCL₄); Group-III (silymarin); Group-IV (Seed extract); Group-V (5th day extract); Group-VI (7th day extract); Group-VII (11th day extract).*

*Values in Mean ±SEM, N=6.*

5.4.2.1 MDA LEVEL

Level of MDA in control group was 289.4±19.23 nmol MDA/gm tissue. There was a significant (P<0.001) increase in mean MDA level in rats administered with CCL₄ (3ml/kg) for 24 hrs (363.22±56 nmol MDA/gm tissue). This effect of CCL₄
toxicity was ameliorated in rats which received *N. sativa* extracts from different germination phases of seed. In all the four groups receiving *N. sativa* extracts rise in MDA level was prevented (Table 5.2 and Figure 5.7). Among all extracts 5th day germination extract showed significant protective effect against CCl₄-induced toxicity and reduced MDA level as control (299.32±0.33 nmol MDA/gm tissue). Silymarin also reduced MDA level (295.2±0.42 nmol MDA/gm tissue) significantly. 7th day, 11th day and seed extract (non-germinated) also reduced the MDA level.

**Figure 5.7:** Effect of *N. sativa* extracts of different germination phases on MDA level against CCl₄-induced toxicity.

![Graph showing MDA level against CCl₄-induced toxicity]

*Group-I (control); Group-II (CCl₄); Group-III (silymarin); Group-IV (Seed extract); Group-V (5th day extract); Group-VI (7th day extract); Group-VII (11th day extract).

### 5.4.2.2 Superoxide dismutase

There was a significant (P<0.001) decrease in SOD level observed in rats administered with CCl₄ (4.56±0.48 U/mg) in comparison to control group (12.5±0.56 U/mg). Extracts of *N. sativa* raise the level of superoxide dismutase enzyme and maintain the level of catalase as in control group. The mean value of 5th day, 7th day, 11th day and seed extract was 11±0.568 U/mg, 10.19±0.88 U/mg, 9.97±0.88 U/mg
and 8.24±0.99 U/mg respectively (Table 5.2 and Figure 5.8). Results showed that 5th day germinated extract significantly increased the level of SOD. Extract of 7th day germination phase was on second position followed by 11th and seed extract (non-germinated).

**Figure 5.8:** Effect of *N. sativa* extracts of different germination phases on SOD level against CCL₄-induced toxicity.

![SOD Level Graph](image)

*Group-I (control); Group-II (CCL₄); Group-III (silymarin); Group- IV (Seed extract); Group-V (5th day extract); Group-VI (7th day extract); Group-VII (11th day extract).*

### 5.4.2.3 Catalase

There was a significant (P<0.001) decrease in CAT enzyme level in rats administered with CCl₄ (3ml/kg) for 24 hrs (3.46±0.25 U/mg) in comparison to control group (9.5±0.23 U/mg). Extracts of *N. sativa* from germination phases of seed raise the level of catalase and maintain the level of catalase as was in control group. The mean value of silymarin, 5th day, 7th day, 11th day and seed extract (non-germinated) was 8.91±0.45 U/mg, 8.9±0.56 U/mg, 8.1±0.61 U/mg, 6.97±0.64 U/mg.
and 6.24±0.25 U/mg respectively (Table 5.2 and Figure 5.9). Extract of 5th day germination phase increased catalase activity as in control group.

**Figure 5.9:** Effect of *N. sativa* extracts of different germination phases on CAT level against CCL4-induced toxicity.

*Group-I (control); Group-II (CCL4); Group-III (silymarin); Group- IV (Seed extract); Group-V (5th day extract); Group-VI (7th day extract); Group-VII (11th day extract).*

**5.4.2.4 Reduced glutathione GSH**

There was a significant (P<0.001) decrease in GSH level in rats administered with CCl4 (3ml/kg) (25.39±0.52 µmol/gm protein) in comparison to control group (38.12±0.52 µmol/g protein). Extracts of *N. sativa* raise the level of GSH. All the extract showed protective effect in CCL4-induced toxicity and increased the level of GSH significantly. Extract of 5th day germination phase increased the level of GSH significantly (36.99±0.4 µmol/gm protein), this was higher than non-germinated seed extract (35.1±0.31 µmol/gm protein).
**Figure 5.10:** Effect of *N. sativa* extracts of different germination phases on GSH level against CCL$_4$-induced toxicity.

![Graph showing GSH (µmol/g protein) levels across different groups.]

*Group-I (control); Group-II (CCL$_4$); Group-III (silymarin); Group- IV (Seed extract); Group-V (5$^{th}$ day extract); Group-VI (7$^{th}$ day extract); Group-VII (11$^{th}$ day extract).*

### 5.4.3 Histopathology studies

In control group animals showed normal liver parenchyma and normal architecture of hepatocytes, vasculature, distinct hepatic cords and central vein (Plate 5.2). However, animals treated with carbon tetrachloride showed extensive necrosis, fraying of cell margins, and portal triditis with mononuclear lymphoplasmocytic inflammatory infiltration (Plate 5.3). This effect was significantly decreased in animals pretreated with *N. sativa* extracts from different germination phases of seed.

**Plate 5.2:** Photomicrograph from control (Group-I) rat showing normal liver parenchyma and normal architecture of hepatocytes and vasculature, distinct hepatic cords and central vein. Haematoxylin and eosin stained (H and E) 5 micron thick section (H&E 40X).
Plate 5.3: Photomicrograph of CCl₄ treated rats (Group-II) showing extensive necrosis, fraying of cell margins, portal triditis with mononuclear lymphoplasmocytic inflammatory infiltration, strip focal necrosis, feathery degeneration (in the mid zone) involving several hepatocytes, necrosis of several hepatocyte extended in band like fashion in the mid zonal area and occasional apoptotic bodies.
Plate 5.4: Liver section from rat orally treated with seed extract (non-germinated 1g/kg) showing a slight protective effect but shows some lymphoplasmocytic inflammatory infiltration.

Plate 5.5: Liver section from rat orally treated with 5th day germination extract (1 g/kg), showing highly protective effect without lymphoplasmocytic inflammatory infiltration.
Plate 5.6: Liver section from rat orally treated with 7\textsuperscript{th} day germination extract (1 g/kg) showing slight protective effect with some inflammatory cells.

Plate 5.7: Liver section from rat orally treated with 11\textsuperscript{th} day germination extract (1 g/kg) showing protective effect with reduced necrosis.
A number of chemicals including various environmental toxicants and clinically useful drugs can cause severe cellular damages in different organs of our body through the metabolic activation to highly reactive substances such as free radicals. CCl$_4$ is one of such widely studied environmental toxicant (Sil et al., 2006). Up to the present time the etiology and treatment of most liver diseases are not known. The liver is the commonest site affected during the toxic manifestation of many drugs. Toxicity in liver due to CCl$_4$ and other chemicals is attributed to the toxic metabolites formed which are responsible for the initiation of CCl$_4$ dependent lipid peroxidation (Arulkumaran et al., 2007). In the liver CCl$_4$ is metabolized by the cytochrome P450-dependent monooxygenase systems followed by its conversion to more chemically active form namely trichloromethyl radical (CCl$_3$•) that bind covalently to the macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids which leads to the formation of lipid peroxides. This lipid peroxidative degradation of biomembranes is one of the principle causes of hepatotoxicity of CCl$_4$ (Kaplowitz et al., 1986). CCL$_4$ also causes depletion of glutathione content and reduced the level of SOD and CAT enzymes (Kamiyama et al., 1993).

In the assessment of liver damage by CCL$_4$ the determination of enzyme levels such as SGOT (AST), SGPT (ALT) and bilirubin is largely used. Necrosis or membrane smash up releases the enzyme into circulation and hence they can be measured in the serum. Increase levels of SGOT indicate liver damage in the same manner caused by viral hepatitis as well as cardiac infarction and muscle injury. This enzyme catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore SGPT is more specific to the liver and is a better parameter for detecting liver injury. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Drotman et al., 1978). On the other hand serum ALP, bilirubin and total protein levels are related to the function of hepatic cell. Increase level of ALP in serum is due to increased synthesis in presence of increasing biliary pressure (Muriel et al., 1992).
As the results showed that oral administration of CCl4 caused a significant (P<0.001) elevation of enzyme levels such as ALP, SGPT, SGOT, total bilirubin and decrease in antioxidant system increased in MDA level and depletion of glutathione content, SOD and CAT activity when compared to control group. There was a significant (P<0.001) restoration of these enzyme levels on oral administration of the N. sativa extracts from different germination phases in day-dependant manner and also by silymarin at a dose of 50 mg/kg. Among all tested extracts 5th day germination extract showed significant protective effect like standard drug silymarin. Extract of 7th day germination was on second position followed by 11th day and seed extract. The reversal of increased serum enzymes in CCl4-induced liver damage by the extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases returns to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew et al., 1987). Effective control of ALP and bilirubin levels point towards an early improvement in the secretory mechanism of the hepatic cells.

The worth of any hepatoprotective drug is reliant on its capability of either reducing the harmful effect or restoring the normal hepatic physiology that has been disturbed by a hepatotoxin. Silymarin, the standard hepatoprotective drug used in the present study exerted marked protective effects against CCl4-induced liver injury. It has been reported in several in vitro and in vivo studies (Letteron et al., 1990). The crucial protective mechanism of silymarin is an inhibition of lipid peroxidation by its free radical scavenging properties (Farghali et al., 2000). Silymarin prevented CCl4 induced lipid peroxidation and hepatotoxicity in mice by decreasing the metabolic activation of CCl4 and by acting as a chain-breaking antioxidant (Letteron et al., 1990). Both silymarin and the N. sativa extracts of germination phases (specially of 5th day germination extract) decreased CCl4 induced elevated enzyme levels in tested groups indicating the protection of structural integrity of hepatocyte cell membrane or regeneration of damaged liver cells as they showed in histopathology results. The increase in MDA (LPO) level in liver induced by CCl4 suggests enhanced lipid
peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. Treatment with *N. sativa* significantly reverses these changes. Hence it is likely that the mechanism of hepatoprotection of *N. sativa* is due to its antioxidant effect. *N. sativa* reduced lipid peroxidation significantly during germination phases by scavenging free hydroxyl radical was reported in our previous research (Kamal *et al.*, 2010).

Superoxide dismutase (SOD) has been reported as one of the most important enzymes in the enzymatic antioxidant defense system which scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical. Decrease in enzyme activity of SOD is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in live injury (Curtis *et al.*, 1972). Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity is found in the red blood cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Chance *et al.*, 1992). *N. sativa* extracts from germination phases cause a significant (P<0.001) increase in hepatic SOD and CAT activity and thus reduces reactive free radical induced oxidative damage to liver. Previous research has been proved that *N. sativa* shows significant high antioxidant activity during germination phases as compared non-germinated seed (Kamal *et al.*, 2010). Glutathione is one of the most abundant tripeptide, non-enzymatic biological antioxidant present in the liver. It removes free radical species such as hydrogen peroxide, superoxide radicals and maintains membrane protein thiols. Also it is substrate for glutathione peroxidase (Prakash *et al.*, 2001). Decreased level of GSH is associated with an enhanced lipid peroxidation in CCl₄ treated rats. Administration of *N. sativa* significantly (P<0.001) increased the level of GSH especially in 5th day germination extract treated group.

Many studies have demonstrated hepatoprotective effect of *N. sativa* seeds and reported significant improvement in liver functional test. After giving *N. sativa* oil significant improvement of deranged liver functional test parameters against
various hepatotoxic compounds (Mohideen et al., 2003; Al-Ghamdi et al., 2003; Kanter et al., 2005; El-Gharieb et al., 2010; Janakat et al., 2010). *N. sativa* administration in several model of induced hepatotoxicity has been shown to exert hepatoprotective effect along with restoration of reduced glutathione, superoxide dismutase, lactate dehydrogenase, catalase and decrease in lipid per oxidation reaction (Al-Ghamdi et al., 2003; Mahmoud et al., 2002; Kanter et al., 2005; El-Gharieb et al., 2010). Present study is the first study to investigate hepatoprotective effect of *N. sativa* during germination phases of seed. As results showed that *N. sativa* possesses better hepatoprotective effect during germination phases (especially during 5th day) than non-germinated seed.

Protective effect of *N. sativa* may be due to the presence of high amount of bioactive compounds such as thymoquinone (TQ), thymol, carvacrol and other phytochemicals like flavonoids, phenols and tannins. Both biochemical and histopathological data showed that there was no significant difference in extract treatment when compared to control group. Extract of 5th day germination phase was highly protective among all the extracts due to presence of higher quantity of secondary metabolites, TQ, thymol and other active constituents were higher in 5th day germination extract (chapter 2).

TQ has been reported to have potent superoxide anion (O$_2^-$) scavenging abilities and to inhibit iron-dependent microsomal lipid peroxidation (Badary et al., 2003). TQ may protect against CCl$_4$-induced hepatotoxicity by a combination of two mechanisms, i.e. TQ antioxidant potential (Houghton et al., 1995) and a previous report of the quinone to have in vitro and in vivo superoxide anion radical scavenging ability (Nagi and Mansour, 2000). TQ provided good protection against lipid peroxidation and the oxidative damage caused by several toxic agents, as in cisplatin nephrotoxicity (Badary et al., 1997), doxorubicin cardiotoxicity (Al-Shabanah et al., 1998) and benzo(a)pyrene-induced forestomach carcinogenesis (Badary et al., 1999). Burits and Bucar (2000) found that TQ had a scavenger effect against the OH’ radical in vitro. Moreover, Houghton et al. (1995) reported that TQ
had potent anti-inflammatory and inhibitory effects on non-enzymatic peroxidation of brain phospholipid liposomes. This was also reported by Abdulrahman L. Al-Malki (2010) that pretreatment with thymol ameliorates the deleterious effect of CCl₄. It was speculated that thymol exert their effects by decreasing lipid peroxidation and enhancing the activities of antioxidant enzymes. For this reason, thymol could be used as hepatoprotective agent with free medication side effects (Abdulrahman L. Al-Malki, 2010). Carvacrol, another constituent of *N. sativa* affords significant hepatoprotective and antioxidant effect against D-GalN-induced hepatotoxicity rats (Baser et al., 2008; Aristatile et al., 2009). The protective effects of alpha-Hederin (constituent of *N. sativa*) on carbon tetrachloride-induced hepatotoxicity were also observed by Jeong et al., (1998). *N. sativa* seed have been shown to significantly decrease TNF-α (tumor necrosis factor α), IFN-γ (interferon γ), IL-β (interleukin β) in CCl₄-induced hepatotoxicity (Al-Ghamdi, 2003). These hepatoprotective effects were probably due to antioxidant activities of *N. sativa* and their active constituent TQ.

The above observation shows that *N. sativa* produces hepatoprotective effect by restoration of anti oxidation enzyme system of the liver, reduction of lipid peroxidation and inhibition of neutrophil infiltration and release of cytokines from the inflammatory cells. Our study is also in conformity with Mahmoud et al., (2002) who reported that active components of *N. sativa* increased hepatic antioxidant enzymes activities (superoxide dismutase, catalase, glutathione) and reduces the hepatic lipid peroxidation.
5.5 Conclusion

It may be concluded the *N. sativa* methanolic extracts from different germination phases showed high protective activity in CCl₄-induced hepatotoxicity. The extracts of germination stages were more protective than non-germinated seed extract. This may be due to the fact that during germination many phytoconstituents are produced in high quantities like flavonoids, phenols, tannins, which increase the level of antioxidant enzymes and reduce the toxicity of CCl₄. In the present study 5ᵗʰ day germination phase followed by 7ᵗʰ day extracts possesses significant hepatoprotective activity and reduces the CCl₄-induced hepatotoxicity compared than non-germinated seed extract. The activity seems to be due to active component thymoquinone which increase the antioxidant enzyme level. Further study is required in this area as preliminary results are very encouraging to investigate the mechanism of action of the compound to minimize toxicity.
5.6 REFERENCES


• Liu, S.L., Degli Esposti, S., Yao, T., Diehl, A.M., Zern, M.A. (1995). Vitamin E therapy of acute CCl4-induced hepatic injury in mice is associated


