Discussion

A. Anti-inflammatory activity

In the present investigation, the seed and root extracts of *Clitoria ternatea* (CT) were studied to evaluate their effects against carrageenan-induced paw edema, carrageenan-induced pleurisy in rats, and cotton pellet granuloma model. To investigate possible mechanism of action, the effects of seed and root extracts were also studied on RBC membrane stabilization, *in vitro* protein denaturation, and *in vivo* anti-histaminic activity.

The inflammatory response is usually quantified by increase in paw size (edema) which is maximal around 3-4 h post carrageenan injection. Varieties of cellular components have been reported to be involved in the inflammatory cascade. Studies have shown that the first phase is associated with elevated PGE$_2$ and thromboxan (TX) B$_2$ levels, and increased expression of COX-2 (Guay *et al.*, 2004). This indirectly support the generation of PGs in the second phase of carrageenan-induced paw edema. Adenosine monophosphate activated protein kinase is a component of a kinase cascade, playing pivotal role in energy homeostasis, and have been involved in carrageenan-induced paw edema (Cheng *et al.*, 2007). The carrageenan-induced edema can also be modulated by the involvement of spinal nitric oxide/cyclic GMP pathway (Brock and Tonussi, 2008).

In the present study, CT seed and root extracts significantly reduced paw edema within 3 h, suggesting their anti-inflammatory effect on the early phase of the acute inflammatory response, which is characterized by release of inflammatory mediators. The anti-inflammatory effect of plant in carrageenan-induced paw edema model could be attributed to inhibition of release of inflammatory mediators like histamine, bradykinin, and tachykinin. The inhibition of leukocyte migration, reactive oxygen and nitrogen species, and PGs synthesis could also be possible mechanism of anti-inflammatory effect of CT in rats.

Carrageenan-induced pleurisy model exhibits a biphasic response (4 and 48 h), and permits the quantification of exudates, cell migration, and certain
enzymes such as MYP and adenosine-deaminase, NO synthase, etc. which act as markers of activated leukocytes. Thus, it is the most suitable model to study the transudative phase of the acute inflammation. The complement activation is believed to be involved in the leukocytes migration (Capasso et al., 1975). The pleural exudate examination has revealed the presence of higher levels of arachidonate metabolites like PGI₂, TXA₂ and PGE₂ (Ghiara et al., 1986). The MYP and adenine-deaminase activities are decreased, and inducible NO synthase activity has been found increased in the carrageenan-induced pleurisy (Dalmarco et al., 2002). Studies have demonstrated the role of IL-6 (Cuzzocrea et al., 1999a), IL-8, TNF-α, and PGE₂ in carrageenan-induced pleurisy (Jia et al., 2009). The 5-lipoxygenase has been found to be actively involved in inflammatory changes observed in the carrageenan-induced pleurisy (Cuzzocrea et al., 2003). The PPAR-α pathway is known to modulate the degree of acute inflammation in carrageenan-induced pleurisy in mice (Cuzzocrea et al., 2006).

Free radicals have been found to be involved in the development of inflammatory process. Diverse group of compounds have been reported to modulate the carrageenan-induced pleurisy like, fructose -1,6-bisphosphate (Filho et al., 2004), 17-β-estradiol (Cuzzocrea et al., 2000), gastrin-releasing peptide receptors antagonists (Petronilho et al., 2010), IFN-β (Ghiara et al., 1986), NO synthase inhibitors (Tracey et al., 1995), NF-kB inhibitors (Cuzzocrea et al., 2002), glycogen synthase kinase-3 β inhibitors (Cuzzocrea et al., 2006), etc. This suggested that diversity of biochemical pathways involved in the inflammatory response in the carrageenan-induced pleurisy.

In the present study, CT seed and root extracts decreased the pleural exudates volume, suggesting their effect on the early phase of pleurisy, which is characterized by release of inflammatory mediators like histamine, serotonin, and leukotrienes. Further, the examination of exudates from both the extracts treated groups showed decrease in the total leukocytes and differential leukocytes counts. Since the leukocyte migration is the crucial step in the carrageenan-induced inflammatory response in lung tissues that is mediated through the

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activation of complementary system and release of ROS. The suppressive effect of CT on leukocyte migration could be attributed to its effects on the chemotactic factors. These findings were supported by the anti-oxidant effects of both the extracts in particular seed extract, which is evident from the decreased lipid peroxidation, and SOD, along with increase in catalase activity and GSH levels in lung tissues. Thus the overall protective effects of CT against carrageenan-induced pleurisy could be attributed to anti-oxidant properties, inhibition of pro-inflammatory mediators, and suppression of leukocytes migration.

The cotton pellet granuloma method has been widely employed to assess the transudative, exudative, and proliferative component of the chronic inflammation. This model is an indicative of the proliferative phase of inflammation. Inflammation involves proliferation of macrophages, neutrophils and fibroblasts, which are basic sources of granuloma formation. The fluid absorbed by the cotton pellet greatly influences the wet weight of the granuloma and dry weight correlate well with the granuloma of the granulomatous tissue formed (Olajide et al., 2000). In the cotton pellet granuloma model, the inflammation develops during the period of several days. The proliferative phase is characterized by the synthesis of collagen. The resident fibroblasts in the connective tissue are found responsible for the production of collagen (Hiramastu et al., 1982). Cytokines such as IL-1, TNF-α, and growth factor influences the proliferation of smooth muscle cells and production of granuloma (Mitchell and Cotran, 2003). Hence, the decrease in the weight of the granuloma indicates that the proliferative phase is effectively suppressed.

In the present study, the weight of granuloma was significantly decreased by CT seed and root extracts. The total WBC counts, polymorphonuclear leukocyte counts, and mononuclear leukocyte counts were significantly decreased by the seed extract. The root extract significantly decreased granuloma weight and total leukocyte counts. However, it did not produce any effects on the polymorphonuclear, and mononuclear leukocyte counts. The findings of our study suggested that both the extracts produced effect on the
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proliferative phase of the inflammation, mainly affecting the leukocyte migration/infiltration and leukocyte counts.

The vitality of cells depends on the integrity of their membrane, exposure of RBC's to injurious substances such as hypotonic medium results in lysis of its membrane accompanied by hemolysis, and oxidation of hemoglobin (Ferrali et al., 1992). The hemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through the free radical-induced lipid peroxidation. It is therefore expected that compounds with membrane-stabilizing properties, should offer significant protection of cell membrane against injurious substances (Vadivu and Lakshmi, 2008). Compounds with membrane-stabilizing properties are further well known for their ability to interfere with the release of phospholipase that trigger the formation of inflammatory mediators (Aitadafoun et al., 1996). The studies have shown that various plant drugs like Gongronema latifolium leaves (Olugbenga et al., 2005), Momordica charantia leaf (Umukoro and Ashorobi, 2006), and Cansjera rheedii (Mounnissamy et al., 2007) are capable of stabilizing RBC membrane, and exert anti-inflammatory activity. CT seed and root extracts have shown significant membrane stabilizing property, which suggests that their anti-inflammatory activity observed in this study, may be related to the inhibition of the release of phospholipases that trigger the formation of inflammatory mediators.

Denaturation of proteins is a well documented cause of inflammation and rheumatoid arthritis (Mizushima and Kobayashi, 1968). Several synthetic anti-inflammatory (Grant et al., 1970), and plant drugs like Barleria cristata leaves have shown dose dependent ability to inhibit thermally induced protein denaturation (Gambhire et al., 2009). Inhibition of protein denaturation has been reported as one of possible mechanism of anti-inflammatory activity (Nargund et al., 2006). Ability of CT seed and root extracts to bring down thermal

denaturation of protein is possibly an important contributing factor for its anti-inflammatory activity.

Based on our above observation, it is suggested that CT seed and root possesses significant anti-inflammatory effects against both acute and chronic inflammation models. The anti-inflammatory effects of CT could be attributed to the inhibition of release of inflammatory mediators, inhibition of leukocyte migration, lysosomal membrane stabilization, prevention of protein denaturation, and anti-oxidant properties. The preliminary phytochemical screening showed presence of flavonoids in seed part of our plant. Since plant flavonoids were reported to have anti-inflammatory activity (Rotelli et al., 2003; Clavin et al., 2007; Aquila et al., 2009; Jin et al., 2010), the anti-inflammatory effects of seed extract could also be attributed to the presence of flavonoids.

B. Hepatoprotective activity

In the present study, the effects of CT seed and root extracts were evaluated against paracetamol (PCM) and carbon tetrachloride (CCI₄)-induced liver injuries in rats. The liver functioning was assessed by measuring serum SGOT, SGPT, and ALP levels. Effects on lipid peroxidation, anti-oxidant enzymes, and GSH were also studied. In addition, the mast cell infiltration, hepatic collagen synthesis, and microsomal enzymes inhibition were determined.

PCM at therapeutic doses is primarily metabolized and detoxified by glucuronidation and sulphation, and subsequently followed by renal excretion (Miner and Kissinger, 1979). However, when PCM is taken in toxic doses, the compound is converted to a toxic form N-acetyl-p-benzo-quinone imine (NAPQI). NAPQI is an electrophilic intermediate, oxidized by cytochrome P₄₅₀, and converted to a highly reactive and toxic metabolite as in the case of PCM overdose (Gupta and Misra, 2006). It can rapidly react with the glutathione, and leads to about 90% total hepatic GSH depletion in the cells and mitochondria, which can result in hepatocellular death and mitochondrial dysfunction (Mitchell et al., 1973). In addition, NAPQI can increase the formation of ROS, and reactive
nitrogen species (RNS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide (H$_2$O$_2$), NO, and peroxynitrite, respectively. Excess levels of ROS and RNS can attack biological molecules such as DNA, protein and phospholipids, which lead to lipid peroxidation, nitrination of tyrosine, and depletion of anti oxidant enzymes that further results in oxidative stress (Michael et al., 1999).

$\text{CCl}_4$ is a well known xenobiotic producing hepatotoxicity in various experimental animals (Lee et al., 2007; Rudnick et al., 2007). Biotransformation of $\text{CCl}_4$ by cytochrome P$_{450}$ produces hepatotoxic metabolites – trichloromethyl free radicals (CCl$_3$• and / or CCl$_3$OO•) (Brattin et al., 1994; Rikans et al., 1994). Covalent binding of trichloromethyl free radicals to cell proteins is considered to be the initial step in a chain of events that eventually lead to membrane lipid peroxidation and finally to cell apoptosis and necrosis (Brautbar and Williams 2002; Basu 2003; Weber et al., 2003). It is also reported that immune cells are activated by hepatotoxins including PCM and $\text{CCl}_4$, leading to infiltration in damaged liver tissues where they secrete various cytokines (Ramaiah and Rittling, 2007). These cytokines are known to trigger hepatotoxicity via a Fas/FasL pathway in $\text{CCl}_4$–induced liver toxicity in rats (Wen et al., 2006).

The membrane lipid peroxidation by $\text{CCl}_4$ metabolites leads to impairment of membrane functions and integrity resulting in release of cytosolic contents into the serum. The magnitude of hepatic damage is assessed by measuring the levels of released cytosolic contents viz. transaminases like SGOT and SGPT, in circulation (Agarwal et al., 2006). SGOT is relatively non-specific and elevated during hepatic, cardiac, and muscle injuries. SGPT is more selectively a liver parenchymal enzyme than SGOT, and is a better parameter for detecting liver injury (Arnaiz et al., 1995). The elevated levels of these serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Drotman and Lawhorn, 1978). In the present study, significant increase in the serum levels of SGOT, SGPT, and ALP in PCM and $\text{CCl}_4$ intoxicated rats, could be attributed to cytosolic leakage, and damaged structural integrity of
hepatocytes, because these are cytoplasmic in location and released into circulation after cellular damage (Recknagel et al., 1991). The results of the present study demonstrated that pretreatment of rats with CT seed extract effectively protected against PCM and CCl₄–induced hepatotoxicity. However, root extract showed significant protection against CCl₄–induced liver toxicity only. This fact was further confirmed by histological observations. CT seed and root extracts dramatically prevented the CCl₄–induced morphological changes in the liver sections.

Lipid peroxidation by free radical derivatives of CCl₄ is one of the principle mechanisms of CCl₄–induced liver injury (Castor et al., 1974). Increased lipid peroxidation is generally believed to be an important underlying cause of the initiation of oxidative stress related various tissue injuries, cell death, and progression of many acute and chronic diseases (Helliwell, 1997). MDA is a major reactive aldehyde that appears during the peroxidation of polyunsaturated fatty acids of biological membrane (Vaca et al., 1988). Increase in MDA levels in the liver suggests enhanced peroxidation leading to tissue damage, and failure of the antioxidant defense mechanisms to prevent the formation of excessive free radicals (Naik, 2003). In the present study, PCM and CCl₄–induced liver toxicity caused an increase in the MDA levels in liver tissues as compared to normal control group. Treatment with CT seed and root extracts significantly reversed these changes and decreased MDA levels.

SOD is an exceedingly effective defense enzyme that converted the dismutation of superoxide anions into H₂O₂ (Reiter et al., 2000). Catalase is a haemoprotein in all aerobic cells that metabolize H₂O₂ to oxygen and water. GSH acts as a non-enzymatic anti-oxidant that reduces H₂O₂, hydroperoxides (ROOH), and xenobiotic toxicity (Kadiska et al., 2000). The glutathione is readily oxidized to glutathione disulphide (GSSG) by any of the selenium-containing glutathione peroxidase isozymes, as well as the reaction with ROOH or xenobiotic compounds that may subsequently cause the reduction of GSH levels. The GSSG is either rapidly reduced by glutathione reductase and NADPH or
utilized in the protein folding process in the endoplasmic reticulum, where it is recycled by protein disulfide isomerase to form glutathione. Because of these recycling mechanisms, it is an extremely efficient intracellular buffer for oxidative stress (Cantin et al., 2007).

In the present study, PCM and CCl₄ produced significant increase in SOD, and catalase activity indicating the oxidative stress condition during PCM and CCl₄ intoxication. CT seed and root extracts produced significant decrease in SOD, and catalase activities in CCl₄ intoxicated rats. However, root extract did not affect MDA, and catalase in PCM intoxicated rats. The GSH levels were also significantly increased by both the extracts in PCM and CCl₄ intoxicated rats. A significantly higher content of GSH in liver would afford the tissue a better protection against an oxidative stress, thus prevent hepatotoxicity. There is report that Azadiracta indica leaf extract showed hepatoprotective effect against PCM-induced hepatic damage in rats that is attributed to the presence of quercetin and rutin compounds of plant - showing antioxidant activity (Chattopadhyay and Bandyopadhyay, 2005). Our results are parallel to this report.

Bilirubin is one of the most useful clinical clues to the severity of hepatic necrosis, and its accumulation is a measure of binding, conjugation and excretory capacity of hepatocytes. Hepatotoxins like PCM have been reported to decrease hepatic conjugation of bilirubin (Davis et al., 1976). The present study showed that CT seed and root extracts significantly decreased both direct and indirect bilirubin levels in serum suggesting their effects on the functional integrity of the hepatocytes.

Creatinine levels are indicative of renal functioning, and are increased in severe renal damage. CCl₄ is known to produce renal toxicity (Ogeturk et al., 2005). Hence, in the present study we investigated the effects of CCl₄ on kidney functioning. All the treatments did not alter creatinine levels in both the models of hepatotoxicity. The finding of the study indicated that the selected dose levels of hepatotoxins did not produce renal damage.
Hepatotoxins like PCM and CCl₄ have been reported to activate immune cells especially mast cells, leading to their infiltration into the damaged liver where they secrete various cytokines like TNF-α, leucotrienes, and interleukins (Ramaiah and Rittling, 2007). The number of mast cells is reported to increase in chronic liver diseases associated with fibrosis (Sugihara et al., 1999). In the present study, we investigated the hepatic mast cell infiltration in paracetamol and CCl₄ intoxicated rats. Pretreatment with CT seed and root extracts significantly prevented hepatic mast cell infiltration. A well known hepatoprotective drug silymarin has been reported to inhibit mast cell infiltration (Jeong et al., 2005). Thus, the inhibition of mast cell infiltration or mast cell stabilization could be one of possible mechanisms attributed to protective effects of CT against PCM and CCl₄-intoxicated rats.

Cirrhotic changes are the hallmark of hepatic damage. Fibrosis is found to be associated with many toxin-induced liver injury including PCM and CCl₄. In the present study we estimated hepatic fibrosis in terms of collagen content measured as P-hydroxyproline levels. CT seed and root extracts significantly decreased hepatic hydroxyproline content in CCl₄ intoxicated rats. The plants from the same family Leguminosae like mung bean, adzuki bean, black bean and rice bean have been reported to decrease hepatic collagen content (Wu et al., 2001).

Barbiturates are the class of xenobiotics that are extensively metabolized in the liver and their clearance is delayed in case of damaged liver functioning (Kulkarni, 1999). Hepatic microsomal enzyme inhibitors also delay their clearance and potentiate barbiturate-induced sleeping time. Hence, to investigate hepatic microsomal enzyme inhibition as possible mechanism of hepatoprotective effect, we studied effects of various extracts on phenobarbitone induced sleeping time. CT seed extract significantly decreased phenobarbitone induced sleeping time instead of potentiation. This can be attributed to brain tonic activity of CT seeds (Mukherjee et al., 2008). The findings suggested that
protective effects of CT seed and roots could be because of effects other than hepatic microsomal enzyme inhibition.

It is therefore, suggested that CT seed extract showed significant hepatoprotective activity against both PCM and CCl₄-induced liver toxicities, which could be due to inhibition of pro-inflammatory changes, leukocyte migration, and generation of free radicals. In addition, both the extracts significantly reduced lipid peroxidation, bilirubin levels, hepatic mast cell infiltration, and hepatic collagen content in CCl₄-intoxicated rats. Thus, it is suggestive of their protective effect on functional integrity of the hepatocytes. Failure of CT root extract to protect against PCM-induced liver toxicity could be partly explained by the absence of flavonoid Kaempferol in root, as observed in HPTLC studies.

**C. Antihyperlipidemic activity**

Hyperlipidemia is a major cause of atherosclerosis and atherosclerosis-associated conditions, such as coronary heart disease (CHD), ischemic cerebrovascular disease, and peripheral vascular disease (Mahley and Bersot, 2001). Among these, hypercholesterolemia and hypertriglyceridemia are closely related to ischemic heart disease (Kumar et al., 2008). Medicinal plants play a major role in having anti-hyperlipidemic activity (Kumar et al., 2008). In the present study, we have investigated the effect of CT seed and root extracts against Poloxamer-407 (P-407) and diet-induced hyperlipidemia in rats.

P-407 is a nontoxic, nonionic surfactant that has been utilized to produce hyperlipidemia due to its convenience, reproducibility, and lack of undesirable underlying pathological conditions (Wout et al., 1992). It has been shown to cause significant elevations in serum cholesterol and triglycerides in various animal models, including rats (Wout et al., 1992; Johnston and Palmer, 1997), mice (Palmer et al., 1998; Johnston et al., 2000, 2001), and rabbits (Blonder et al., 1999). In rats and mice, the elevation in plasma triglycerides appears to be more sensitive to the effects of P-407 as compared to serum cholesterol.
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has been demonstrated to cause significant inhibition of plasma lipoprotein lipase (LPL), both in vitro and in vivo accompanied by an increase in tissue LPL activity (Johnston and Palmer, 1993). The accumulation of plasma triglycerides following the administration of P-407 has, therefore, been suggested to be due to inhibition of capillary-bound LPL activity. Other studies in rats have indicated that increase in serum cholesterol following P-407 i.p. administration may be due to inhibition of cholesterol 7a-hydroxylase, but not due to stimulation of HMG CoA reductase (Johnston et al., 2001). It has been demonstrated that plasma Lecithin-cholesterol acyltransferase (LCAT), and cholesterol ester transfer protein (CETP) activities are markedly elevated, and the plasma LPL and hepatic lipase (HL) activities are significantly inhibited in P-407 treated rats (Wasan et al., 2003). In previous studies, a single i.p. injection of P-407 produced marked hyperlipidemia in rodents (Johnston and Palmer, 1997; Johnston et al., 2001; Kim et al., 2008). This was confirmed in the present study, where the serum TC and TG levels were significantly elevated in the P-407-treated hyperlipidemic control group as compared to the normal group. CT seed extract mainly affected serum TG levels and the root extract affected both TC and TG levels in the P-407-induced acute hyperlipidemia model. Since P-407-induced hyperlipidemia is mainly due to inhibition of extractable pool (heparin releasable) of lipoprotein lipase (Johnston and Palmer, 1993), the serum TG lowering effects can be attributed to the prevention of P-407-induced inhibition of lipoprotein lipase or activation of lipoprotein lipase.

Cholesterol feeding has often been used to elevate serum or tissue cholesterol levels to study the etiology of hypercholesterolemia-related metabolic disturbances (Bocan, 1998). High cholesterol diet increases serum lipid levels and leads to oxidative stress (Bobek et al., 1997; Lu and Chiang, 2001; Balkan et al., 2004). In the present study, the high cholesterol diet significantly increased serum TC, TG LDL-C and VLDL-C, and decreased HDL-C in hyperlipidemic control group when compared with the normal group receiving regular chow diet. These findings were in accordance with results of other investigators (Pande and Dubey, 2009; Rachh et al., 2010). Hydroalcoholic extracts of seed and root
significantly decreased serum TC, TG, LDL-C, and VLDL-C, levels. The AI and HDL-C/LDL-C ratio are the relative indicators of atherogenic risks. The atherogenic index was significantly decreased by both seed and root extracts. However, the HDL-C/LDL-C ratio was significantly increased by root extracts only suggesting reduced risk of hyperlipidemia.

It is reported that cholesterol homeostasis is maintained by the control of the two processes, viz. cholesterol biosynthesis in which HMG-Co-A reductase catalyzes the rate-limiting process and cholesterol absorption of both dietary cholesterol and cholesterol cleared from the liver through biliary secretion. Hence, we investigated effects of CT seed and root extracts on HMG CoA reductase activities indirectly by measuring HMG-CoA/mevalonate ratio, and fecal cholesterol excretion. The HMG-CoA / mevalonate ratio has an inverse relationship to the activity of HMG-CoA reductase (Rao and Ramakrishnan, 1975). The results of the study indicated that the activity of the enzyme is significantly depressed by reference HMG CoA reductase inhibitor – atorvastatin only. The findings of the study suggested that lipid lowering effects of CT seed and root were mediated by mechanisms other that HMG CoA reductase inhibition. Further, we observed significant increase in the cholesterol content of the fecal matter indicating that all the extract either promoted the excretion of cholesterol or prevented the absorption of cholesterol. In diet–induced hypercholesterolemia, the cholesterol pool is diverted to bile acid synthesis. Since the fecal bile acid levels were significantly increased in the present study, it could be suggested that the lipid lowering effect is mainly because of increased cholesterol excretion.

A prominent mechanism that may be involved in the deleterious effects of hyperlipidemia or hypercholesterolemia is the augmented formation of ROS, and increased oxidative stress (Nepoli and Lerman, 2001; Chade et al., 2003). The abundance of ROS in hypercholesterolemia also facilitates the oxidation of LDL and upregulation of its receptor LOX-1, thereby increasing both the availability and uptake of oxidized LDL (Stulak et al., 2001; Nagase et al., 2001). If the fatty
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acid is damaged by ROS, it becomes a free radical itself setting up a chain reaction of lipid peroxidation (Gutteridge, 1995; Valko et al., 2007). Thus, lipid peroxidation is the key factor leading to atherosclerotic plaque formation. It is now assumed that a complex endothelial dysfunction induced by elevated and modified LDL, free radicals, infectious microorganisms, shear stress, hypertension, toxins after smoking or combinations of these and other factors lead to a compensatory inflammatory response (Ross, 1999). Endothelial dysfunction is characterized by decreased nitric oxide synthesis, local oxidation of circulating lipoproteins and their entry into the vessel wall (Davignon and Ganz, 2004). Intracellular reactive oxygen species similarly induced by the multiple atherosclerosis risk factors lead to enhanced oxidative stress in vascular cells and further activate intracellular signaling molecules involved in gene expression (Fuster et al., 2005). The biological oxidative effects of ROS on lipids, DNA, and proteins are controlled by a wide spectrum of enzymatic antioxidants such as SOD, catalase, GSH-peroxidase, and non-enzymatic antioxidants such as vitamin-E, vitamin-C, and glutathione (Valko et al., 2007).

Hence, in the present study we investigated the effects of high cholesterol diet on hepatic lipid peroxidation and oxidative stress. The high cholesterol diet produced significant increase in MDA, SOD, and catalase activities, and decreased GSH levels. These findings are in accordance with the previous studies (Mahfouz and Kummerow, 2000; Wang et al., 2010), suggesting that there is oxidative stress (Balkan et al., 2004). The results of the present study indicated that the lipid peroxidation was significantly reduced by pretreatment with both the extracts. Further, the oxidative stress was reduced by both the extracts, as supported by significant decrease in SOD and catalase activity, and increase in the GSH levels in the liver tissues. The L-ascorbic acid is the only biologically active form playing vital role as natural antioxidant against a variety of stress conditions including lipid peroxidation (Frei et al., 1990). It gets easily converted into the dehydroascorbic acid, thereby regenerating vitamin-E. It is also maintaining high intracellular levels of glutathione (Meister, 1994). The findings of this study showed significant higher levels of the serum total and L-
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Ascorbic acid levels in both the extract pretreated groups, suggesting marked reduction in the oxidative stress. This could also be partly supported by increased glutathione levels in the liver tissues.

Thus, it is suggested that CT seeds and roots extracts possess significant lipid lowering activity against experimentally-induced hyperlipidemia. The TG lowering effects might be attributed to increase in the lipoprotein lipase activities. The cholesterol lowering effects of CT seed and root extracts might be because of increased fecal excretion by promoting biliary excretion and preventing absorption of dietary cholesterol. Further, they also prevented lipid peroxidation and improved natural antioxidants status in the liver tissues. Besides, phytochemical analysis of CT seed and root extracts showed presence of polyphenolic compounds and flavonoids. Because of their anti-oxidative properties, plant flavonoids are able to inhibit the oxidative modification of LDL cholesterol (Yokozawa et al., 2002). The lipid lowering effect of flavonoids has also been demonstrated in many animal (Choi et al., 2001; Kim et al., 2001; Bok et al., 2002; Raedersdorf et al., 2003) and human (Arai et al., 2000) studies. Therefore, the anti-hyperlipidemic effects of CT seed part can also partly be attributed to their polyphenolic and flavonoid compounds. Plant sterols have been found to reduce serum cholesterol levels (Normén et al., 2004). CT seeds have been reported to contain plant sterols like, β-sitosterol and γ-sitosterol (Sinha, 1960; Gupta and Lal, 1968). Therefore, plant sterols could also be responsible for lipid lowering effect of CT seeds.

D. Immunomodulatory activity

In the present study, the immunomodulatory activity of CT seed and root were investigated using experimental models. The humoral response was measured as primary and secondary antibody titers in sheep red blood cells (SRBC) sensitized rats, and the cell- mediated immune response was measured as delayed type of hypersensitivity (DTH) response in SRBC sensitized rats. The neutrophil recruiting and phagocytic activity of reticuloendothelial system were measured as neutrophil adhesion and carbon clearance method. Further, the

effects of CT seed and root extracts on hematological parameters were also investigated.

When animal hosts are non-intravenously sensitized with sheep red blood cells (SRBC), this 'antigen' initially becomes diffused within the extra vascular space and ultimately, via the lymphatic system, enters regional lymph nodes. Macrophages in the lymphoid tissues or lining the sinuses are then able to phagocytosize the antigen, process it for presentation, and become antigen-presenting cells (APC) to many cells, including lymphocytes. Another APC is the B-lymphocyte; like macro-phages, B-lymphocytes are not very effective at presenting this or other antigen to naïve T-lymphocytes. They are, however, effective in presenting antigen to memory lymphocytes, especially when antigen level is low. Once the antigen has been fragmented and processed, helper T_{H2} T-lymphocytes can then interact to assist/stimulate the B-lymphocytes to produce antibody against the SRBC. In general, during a primary response to exposure to the SRBC/antigen, IgM is secreted initially, followed by a switch to IgG (Goldsby et al., 2003). On re-exposure to the antigen, a secondary response is elicited that is characterized by a rapid onset and highly amplified level of antibody production. Thus, antibody molecules, a product of B-lymphocytes and plasma cells, are central to humoral immune responses. IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins, etc. (Miller et al., 1991).

In the present study, anti-SRBC antibody titers - during both primary and secondary responses - were found significantly decreased in the hydroalcoholic extracts-treated rats. The inhibition of the humoral response by CT seed and root extracts indicate that there was decreased responsiveness of macrophages/B-lymphocytes subsets in these hosts.

Phagocytosis represents an important innate defense mechanism against ingested particulates including whole pathogenic microorganisms. The specialized cells that are capable of phagocytosis include blood monocytes, neutrophils and tissue macrophages. Once particulate material is ingested into
phagosomes, the phagosomes fuse with lysosomes and the ingested material is then digested. Thus, it is not only ingesting and removing microorganisms but also malignant cells, inorganic particles and tissue debries (Miller et al., 1991). In general, the rate of in situ carbon particle clearance is frequently used as a measure of reticuloendothelial system (RES) competency. Faster removal of particles is correlated with an enhanced phagocytic activity of RES cellular components (Abbas and Litchman, 2001). In the study here, prophylactic treatment with CT seed and root extracts inhibited the rate of carbon clearance seen in the control group of rats.

Neutrophil, is capable of showing a wide range of responses, in particular chemotaxis, phagocytosis, exocytosis, and both intracellular and extracellular killing (Dale and Foreman, 1989). Normally, a more rapid clearance of exogenous particulates from the blood by macrophages would arise from opsonization of the material with antibodies/complement C3b. The decrease in neutrophil adhesion strongly suggests that the function in the treated rats' phagocytes was inhibited (i.e., immunoinhibited).

Cell-mediated immunity (CMI) involves effectors mechanisms carried out by T lymphocytes and their products (lymphokines). CMI responses are critical to defense against infectious organisms, infection of foreign grafts, tumor immunity and delayed-type hypersensitivity reactions (Miller et al., 1991). Delayed type hypersensitivity reaction is characterized by large influxes of non-specific inflammatory cells, in which the macrophage is a major participant. It is a type IV hypersensitivity reaction that develops when antigen activates sensitized T-cell derived helper T-cells (TDTH). Activation of TDTH cells by antigen presented through appropriate antigen presenting cells results in the secretion of various cytokines including IL-2, IFN-γ, macrophage migration inhibition factor, and TNF-α (Askenase and Van Loveren, 1983).

In addition to the above-noted outcomes, the DTH response - the magnitude of which can be directly correlated with the competence of a host's cell-mediated immune function that was decreased in rats that received CT seed
and root extracts. Apart from the key role of sensitized memory T-lymphocytes in this reaction, the role of local macrophages (initially) and then recruited monocytes/other phagocytes are critical as well. From the data here, no specific conclusions about the functionality of memory T-lymphocytes can be predicted. However, decreases in anti-SRBC titers in CT seed and root extracts treated rats were suggestive of decreased activation of T-lymphocytes. The decreased phagocytic activities of local/recruited phagocytes would also be a major factor for the substantive decrease observed in DTH response among extracts-treated rats.

The majority of the cells involved in the immune system are produced from common hematopoietic stem cells found in the bone marrow. This site also provides a microenvironment for antigen-dependent differentiation of B-lymphocytes (Raphael and Kuttan, 2003). Since CT seed and root extract treatments were seen here to give rise to decreased circulating antibody titers (specifically against the SRBC), it would be expected then that there should have also been decrease in levels of one or more of the cell types involved in the humoral response to this antigen. In the present study, the evaluations of peripheral blood of extracts-treated rats confirmed the suppression of total WBC counts. These outcomes suggested strongly that the potential effect of CT seed and root extracts was an impact on hematopoietic processes and on the bone marrow in particular.

Intensity of inflammatory immune responses is controlled by recruitment of inflammatory cells into the inflammatory lesions. This process is tightly governed by expression of certain inflammatory chemokines, such as monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1a (MIP-1a), macrophage inflammatory protein 1h (MIP-1h), etc. (Kallinich, et al., 2005), and adhesion molecules, such as lymphocyte function-associated antigen 1 (LFA-1), cluster of differentiation 44 (CD44) by the inflammatory cells, and inter-cellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) by the endothelial cells (Cartier et al., 2005). Given the central role of
chemokines and adhesion molecules in orchestrating the immune response, interference with the expression of these mediators substantially alter the quality of the immune response, leading to either enhancement or inhibition of the ongoing immune response. Thus, one potential mechanism that might mediate the inhibitory effect of CT on inflammatory immune responses is an alteration of trafficking of the inflammatory cells via modulating expression of chemokines and/or adhesion molecules.

Thus overall, the immunoinhibitory effect of CT can be explained partly by its inhibitory effects on humoral antibody formation, phagocytosis, delayed type hypersensitivity response, and immune cell activities. The anti-inflammatory activity of CT seed and root extracts observed in our study is also partly supporting inhibition of inflammatory components of immune response by CT.

**E. Wound healing activity**

Wound healing is a natural process of regenerating dermal and epidermal tissue. Whenever there is a wound, a set of overlapping events take place in a predictable fashion to repair the damage (Iba et al., 2004). The process has been conveniently categorized into phases such as the inflammatory, proliferative, and remodeling phases (Stadelmann et al., 1998). In the inflammatory phase, bacteria and debris are phagocytized and removed and factors are released that cause the migration and division of cells involved in the proliferative phase. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction (Midwood et al., 2004) In epithelialization, epithelial cells crawl across the wound bed to cover it (Chin et al., 2000). The wound is closed by a combination of all these and by the process of wound contraction. During wound contraction, the wound is made smaller by the action of myofibroblasts, which establish a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cells. In the maturation and remodeling phase, collagen is remodeled and realigned along tension lines and cells that are no longer needed are removed by apoptosis. In the present study, CT seed and root extracts and
its ointment in simple ointment base significantly decreased the wound area when compared with the control group. Increased wound contraction in CT seed and root extracts treated rats might be due to an enhanced activity of fibroblasts in regenerated wound tissues.

Myofibroblasts are believed to play a key role in wound contraction by exerting tension on surrounding extracellular matrix (ECM) and secreting ECM protein such as collagen to stabilize the contraction. Collagen is a major protein of extracellular matrix and component that ultimately contributes to wound strength (Singer and Clark, 1999). Hexosamine and uric acid are matrix molecules which act as ground substance for the synthesis of new extracellular matrix. There is a report of increase in the levels of these components during the early stage of wound healing followed by restoration of normal levels (Suguna et al., 2002). Therefore, the wound contraction and healing effects of CT could be attributed to increased synthesis of ECM proteins and ground substances. This is also supported by the increase in the tensile strength.

In incision wound model, increase in tensile strength of treated wounds may be due to an increase in collagen formation per unit area and stabilization of the fibers (Mukherjee et al., 2002). The tensile strength depends upon the Van der Waals force interaction among the hydrogen bonds of the triple helix collagen, leading to twisting of the collagen fibers. Twisting of collagen fibers results in greater tensile strength, and hence the better the healing of wounds (Mian et al., 1992). Deposition of newly synthesized collagen at the wound site increases the collagen concentration per unit area and hence, the tissue tensile strength (Suguna et al., 2002). In the present study, CT significantly increased the tensile strength of the wound. This could therefore, be attributed to increased synthesis and deposition of collagen.

In dead space wound model, granulation tissue formation is indicative of proliferative and remodeling phase of wound healing process. The granulation tissue of the wound is primarily composed of edema, fibroblasts, collagen, and new blood vessels. The mesenchymal cells of the wound area adjust themselves
Discussion

into fibroblasts then begin migrating into the wound gap together with the fibrin strands (Pesin et al., 2009). In the present study, CT seed and root significantly increased the granulation weight, indicating that there might be increased protein synthesis and improvement of both proliferative and remodeling phases of the wound healing.

Thus, in the present study, CT plant showed profound wound healing activity against various experimental wound models, affecting all the phases – wound contraction, proliferative and remodeling – of wound healing. The free radicals and oxidative reaction products produce tissue damage and play a major role in the aggravation of tissue damage during wound healing (Sonel et al., 1997). Several antioxidants like curcumin and vitamin-E have been reported to quench oxidative damage to the tissue (Sieradzki et al., 1998). Since, CT extracts showed significant anti-oxidant and anti-inflammatory activities in our previous studies, the wound healing activity could also be attributed to these activities. Further, the flavonoids are reported to have therapeutic uses due to their anti-inflammatory, anti-fungal, anti-oxidant and wound healing properties (Okuda, 2005; Nayak et al., 2009). Flavonoids are also known to endorse the wound healing process primarily due to their anti-microbial and astringent properties, which appears to be responsible for wound contraction and elevated rate of epithelialization (Tsuchiya et al., 1996). CT plant is also reported to contain flavonoids (Kulshrestha and Khare, 1968), and have antimicrobial activity (Kelemu et al., 2004). We also found presence of flavonoids in seed part of this plant. Hence, the wound healing activity of CT plant could be attributed to the presence of flavonoids and phenolic compounds.

In summary, our observations suggest mainly anti-inflammatory action of CT plant against various experimental conditions. Anti-inflammatory activity of our plant can be attributed to inhibition of inflammatory mediators and migration of leukocytes at the site of inflammation. This activity of CT plant could be playing major role for protection or prevention of hepatotoxicity, hyperlipidemia, immunomodulatory, and wound healing activity. In addition to inhibition of
inflammation, anti-oxidant properties observed in different models under the present study could also be important mechanism for the diverse pharmacological activities of CT plant. Based on the phytochemical findings of the present study, the therapeutic potential of CT plant observed against different experimental models could be attributed to the presence of flavonoids in the hydroalcoholic extract of seed part of CT plant. Flavonoids are already reported to possess anti-inflammatory and anti-oxidant potential (Aquila et al., 2009; Jin et al., 2010). Thus, anti-oxidant potential of our plant could be correlated with the presence of flavonoids namely kaempferol. Besides anti-inflammatory and anti-oxidant mechanisms, modulation of immune response by CT plant could not be ruled out. Therefore in conclusion, the present investigation demonstrated the therapeutic potential of CT against inflammation, hepatic dysfunction, and related conditions, and provided evidence for the therapeutic uses of CT in traditional Indian system of medicine.