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Reduction of Carbon Tetrachloride-Induced Rat Liver Injury by Coffee and Green Tea

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Abstract: Cirrhosis is one of the most degenerative, world wide diseases and can be lead to an inability of liver functions. Green Tea (GT) and Coffee are natural products and considered as powerful antioxidant, chemoprotective, antiinflammatory and antitumorigenic agent. The present study was designed to investigate the protective effect of green tea and coffee against Carbon tetrachloride (CCl_4)-induced liver cirrhosis by using biochemical and histopathological parameters. 24 Male Albino Wistar rats were randomly divided into 6 groups; each group consists of 4 rats. Group I comprises normal healthy rats remains untreated; Group II comprises of CCl_4 (0.8 mg/kg) induced Cirrhotic rats; Group III was administered coffee orally (1 gm/100 mL) daily; Group IV administered CCl_4 (0.8 mg/kg) intraperitoneally once a week for 8 weeks+ 1% oral administration of coffee; Group V was administered 5% Green tea orally; Group VI comprises of CCl_4 (0.8 mg/kg) intraperitoneally once a week for 8 weeks+ 5% oral administration of Green Tea. The volume of green tea and coffee ingested by rats of group III and V was measured daily. The effect of antioxidants on CCl_4-induced liver cirrhosis were estimated by plasma ALT, ALP, total and direct bilirubin, tissue MDA, tissue SOD, tissue catalase. CCl_4-induced cirrhosis is indicated by increased level of plasma ALT, direct bilirubin, tissue MDA and decreased level of tissue SOD. Pathological changes induced by CCl_4 were characterized by fibrotic scar tissue as well as regenerative nodules, the parenchyma deteriorates; the lobules are infiltrated with fat and structurally altered; dense perilobular connective tissue. Coffee and green tea reduced these changes and also restored antioxidant and liver enzymes. Our results showed the possible protective effect of coffee and green tea in association with liver and antioxidant enzymes, indicated that administration of coffee and green tea not only reversed the pathological effects of CCl_4 but also counteracted on deleterious effects of CCl_4- induced liver injury.

Key words: Carbon tetrachloride liver injury, green tea, coffee, antioxidant enzymes, liver enzymes

INTRODUCTION

The liver disorders are a world problem. Despite its frequent occurrence, high morbidity and high mortality, its medical management is currently in adequate, no therapy has successfully prevented the progression of hepatic diseases, even though newly developed drugs have been used to treat chronic liver disorders these drugs have often side effects. Therefore, that is an essential research about suitable herbal drugs, that could replace the chemical ones (Bruck *et al*., 1996). Plant extracts have been used by traditional medical practioners for the treatment of liver disorders for centuries (Schuppan *et al*., 1999). It is being acknowledged that plants contain non-nutritional constituents with beneficial health effects, such as anti-inflammatory and anti-carcinogenic properties (Bissell *et al*., 1998). Cirrhosis is a consequence of chronic liver disease (Wolf *et al*., 2008). Previous studies showed that oxidative stress and DNA damage can initiate the tumor formation and the normal process of oxidation produces highly reactive free radicals (Kyung *et al*., 2007). An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. These free radicals readily react and damage biomolecules and DNA (Knight, 1998). Antioxidants "mop up" these free radicals and eliminate them before they can damage healthy tissue and lead to tumor formation. As antioxidants inhibit free radical damage, they also may block tumor formation (Suganuma *et al*., 1999). Beneficial effects can be achieved by increased antioxidant capacity in the body may be the reduction of oxidative damage to important molecules (Hara, 1994). Antioxidants are present in variety of foods (fruits, vegetables, green tea, coffee, chocolate and soya) and also present in body (glutathione, melatonin, SOD, catalase enzyme). There are two richest antioxidant regimes Green tea and Coffee. Components of green tea include Epigallocatechingallate (EGCG) Polyphenols, Catechin and Caffeine. Mechanisms of action of green tea may be inhibition of cancer cell proliferation and induction of apoptosis (Fujiki *et al*., 1999). Green tea polyphenols are also antimutagenic and is effective in reducing the formation of carcinogens in the body and reducing chromosomal damage during mutagen exposure (Bushman, 1998). Green tea catechins, act as an

antioxidant scavenger of reactive oxygen species as superoxide, hydroxyl and peroxyl radicals, inhibition of lipid peroxidation and inhibition of 2'-deoxyguanosine oxidation in DNA to 8-hydroxy-2'-deoxyguanosine (Esterbauer and Cheeseman, 1990). The scientist suggested that green tea and coffee help the liver health in 2 ways. By protecting liver cells and triggering immune system (Yang *et al*., 1999). By considering the multifunctions of caffeine and green tea the present study is conducted to evaluate the hepatoprotective effects of caffeine and green tea against CCl_4-induced cirrhosis.

MATERIALS AND METHODS

Animals and diet: Wistar albino rats of male sex (200-250 g b.w.), purchased from the animal house of ICCBS, (Karachi, Pakistan), were taken for the study. Animals were acclimatized to the laboratory conditions 1 week before the start of experiment and caged in a quite temperature controlled room (23±4°C). Rats had free access to water and standard rat diet. The experiments were conducted in accordance with ethical guidelines for investigations in laboratory animals.

Experimental design: Animals were allocated in to six experimental groups (n=4):

Group I: These animals were designed as controls and given standard diet and water.

Group II: Received Ccl_4 at a dose of 40% CCl_4 (0.8 mg/kg b.w,i.p) at 11:45 am, once a week for 8 weeks.

Group III: Received 5% green tea extracts (Tapal Gulbahar green tea) prepared in distilled water, orally on daily basis and the volume of green tea consumed by each rat was measured on 11:30 am every morning. Mean intake of green tea extract in these rats was 48.7±10.58 mL on the first day which was increased to 120.3±8.86 mL on 45th day of treatment.

Group IV: Green Tea+ CCl_4 treated group: received 40% CCl_4 (0.8 mg/kg b.w., i.p) weekly for 8 weeks + 5% green tea extract (5%) orally on daily basis and the volume of green tea consumed by each rat was measured on 11:30 am every morning. Mean intake of green tea extract in these rats was 40.5±12.56 mL on the first day which was increased to 110.5±15.45 mL on 45th day of treatment.

Group V: Received 1% coffee (Nestle, Nescafe) prepared by taken 1gm of coffee dissolved in 100 mL of distilled water and the volume of coffee consumed by each rat was measured on 11:30 am every morning. Mean intake of coffee in these rats was 30.40±8.43 mL on the first day which was increased to 98.9±13.55 mL on 45th day of treatment.

Group VI: Coffee + CCl_4 treated group; received 40% CCl_4 (0.8 mg/kg b.w, i.p) weekly for 8 weeks + 1% coffee orally on daily basis. The volume of coffee consumed by each rat was measured on 11:30 am every morning. Mean intake of coffee in these rats was 50.8±9.98 mL on the first day which was increased to 108.11±14.64 mL on 45th day of treatment.

Sample collection: After 6 weeks treatment, animals were decapitated and blood was sample collected from head wound in the lithium heparin coated tubes. A portion of blood was taken in the separate tube to collect the plasma. Liver were excised, trimmed of connective tissues, rinsed with saline to eliminate blood contamination, dried by blotting with filter paper and weighed. The tissues then kept in freezer at -70°C until analysis. A portion of liver fixed in 10% formalin for histological examination. Blood samples were collected for determination of biochemical analysis of plasma ALT, ALP total and direct bilirubin.

Preparation of liver homogenate: A portion of liver was weighed, perfused with saline and homogenized in chilled potassium chloride (1.17%) using a homogenizer. The homogenates were centrifuged at 800 g for 5 min at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 g for 20 min at 4°C to get the post mitochondrial supernatant which was used to assay SOD (super oxide dismutase), CAT (catalase) and MDA (malonyldialdehyde) activities.

Histological examination: A portion of liver were quickly removed, immerse in 10% formalin, dehydrated and embedded in paraffin, sectioned at 3 µm, stained with hematoxylin and eosin (H&E) and evaluated by light microscopy.

Analytical methods

Assessment of antioxidant enzymes

Estimation of Catalase activity (Sinha, 1972): Catalase activity was assayed by the method of Sinha *et al.* (1972). Briefly, the assay mixture consisted of 1.96 mL phosphate buffer (0.01 M, pH 7.0), 1.0 mL hydrogen peroxide (0.2 M) and 0.04 mL PMS (10%) in a final volume of 3.0 mL. About 2 mL dichromate acetic acid reagent was added in 1 mL of reaction mixture, boiled for 10 min, cooled. Changes in absorbance were recorded at 570 nm.

Estimation of Super Oxide Dismutase (Kono, 1978) Levels of SOD in the cell free supernatant were measured by the method of Kono (1978). Briefly, Solution A: 1.3 mL of solution A (0.1 mM EDTA containing 50 mM Na_2CO_3, pH 10.5). Solution B: 0.5 mL of solution B (90 mm NBT-nitro blue tetrazolium dye). Solution C: 0.1 mL of solution C (0.6% TritonX-100 in solution A). Solution D: 0.1 mL of solution D (20 mM

Hydroxylamine hydrochloride, pH 6.0) was mixed and the rate of NBT reduction was recorded for one minute at 560 nm. 0.1 mL of the supernatant was added to the test cuvette as well as reference cuvette, which do not contain solution D. Finally, the percentage inhibition in the rate of reduction of NBT was recorded as described above. One enzyme unit was expressed as inverse of the amount of protein (mg) required inhibiting the reduction rate by 50% in one minute.

Assessment of oxidative status

Assessment of tissue Lipid peroxide: 10 µL of BHT (0.5 M in acetonitrile) was added to prevent homogenate from oxidation and the homogenate was stored at -70°C until analysis for MDA.

Estimation of malonyldialdehyde (MDA): The Malonyldialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of Thiobarbituric Acid Reacting Substances (TBARS) Ohkawa *et al.* (1979). Briefly, the reaction mixture consisted of 0.2 mL of 8.1% sodium dodecyle sulphate, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 mL of 10% (w/v) of PMS. The mixture was brought up to 4.0 mL with distilled water and heated at 95°C for 60 min. After cooling with tap water, 1.0 mL distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1 v/v) was added and centrifuged. The organic layer was taken out and its absorbance was measured at 532 nm and compared with those obtained from MDA standards. The concentration values were calculated from absorption measurements as standard absorption.

Assessment of liver enzymes: Plasma ALT (alanine aminotransferase) (Reitman and Frankel, 1957), ALP (alkaline phosphatase) (Englehardt, 1970) total and direct bilirubin (Sherlock, 1951) were analyzed using commercially prepared reagent kits from Randox.

Statistical analysis: Results are presented as mean± SE. Statistical significance and differences from control and test values were evaluated by Student's t-test. Statistical probability of $p<0.01$, <0.05 were considered to be significant.

RESULTS

Histology of liver tissue in control, CCl_4-treated, coffee, green tea, CCl_4+coffee and CCl_4+green tea treated rats: The liver of CCl_4-treated rats showed fibrotic scar tissue as well as regenerative nodules as compared to control (Fig. 1a). The most characteristic features were parenchyma deteriorates; the lobules are infiltrated with fat and structurally altered; dense perilobular connective tissue forms; and often areas of regeneration develop leading to progressive loss of liver function (Fig. 1b). Treatment with coffee and G.T. markedly prevented alterations in damaged tissues (Fig. 1c and d).

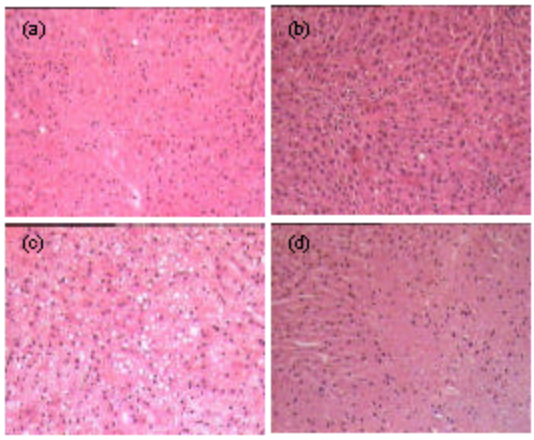

Fig. 1: Histology of liver tissue in control, CCl_4-treated, coffee, green tea, CCl_4+coffee and CCl_4+green tea treated rats. a) Control, b) CCl_4 treated rats, c) CCl_4+Coffee, d) CCl_4+ G.T

Effect of Green tea extract and Coffee on Catalase activity in control, CCl_4 -treated, coffee, green tea, CCl_4+coffee and CCl_4+green tea treated rats: Catalase activity in CCl_4- treated rats was significantly decreased as compared to control ($p<0.05$) (Fig. 2). Coffee treated rats showed significant decreased catalase activity as compared to control ($p<0.01$) while slightly increased in green tea treated rats but results were not significant. Coffee + CCl_4-treated rats showed significant slightly increased catalase activity ($p<0.01$), while the increased activity of catalase was observed in CCl_4+Green tea treated rats but results were not significant.

Effect of Green tea extract and Coffee on SOD activity in control, CCl_4-treated, coffee, green tea, CCl_4+coffee and CCl_4+green tea treated rats: Fig 3 showed a significant decreased activity of SOD in CCl_4-treated rats as compared to control ($p<0.01$). SOD was also decreased significantly in alone coffee and green tea treated rats as compared to control ($p<0.01$). Activity of SOD was found to be decreased significantly in CCl_4+Coffee treated rats as compared to control ($p<0.01$), while marked increased SOD activity was observed in CCl_4+Green tea treated rats but results were not significant.

Effect of Green tea extract and Coffee on tissue MDA level in control, CCl_4-treated, coffee, green tea, CCl_4 + coffee and CCl_4+green tea treated rats: Level of MDA was found to be increased in CCl_4-treated rats as compared to control but results were not significant (Fig. 4). Decreased MDA level was observed in coffee

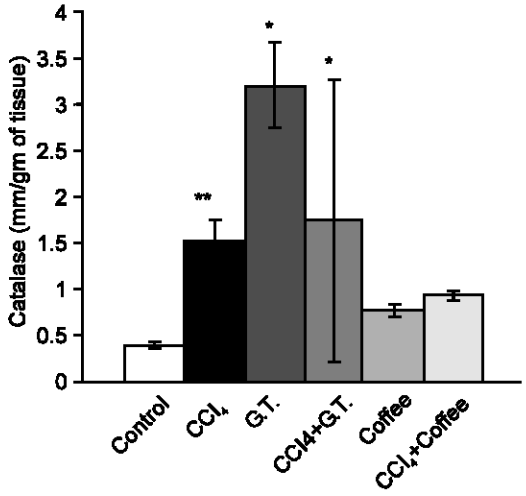

Fig. 2: Effect of Green tea extract and Coffee on Catalase activity in control, CCl_4 treated, coffee alone, green tea alone, CCl_4+coffee and CCl_4+green tea treated rats (*p<0.01, **p<0.05)

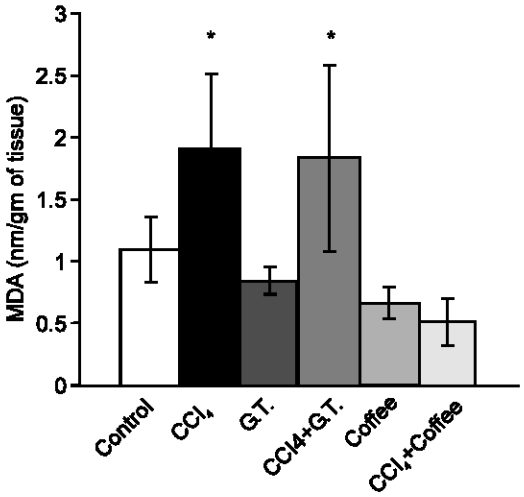

Fig. 4: Effect of Green tea extract and Coffee on tissue MDA level in control, CCl_4 treated, coffee alone, green tea alone, CCl_4+coffee and CCl_4+green tea treated rats (*p<0.01)

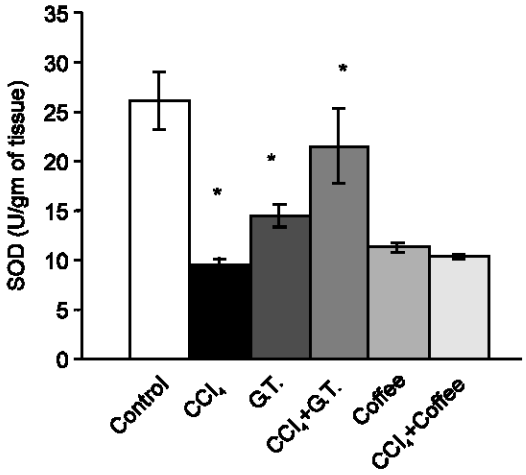

Fig 3: Effect of Green tea extract and Coffee on SOD activity in control, CCl_4 treated, coffee alone, green tea alone, CCl_4+coffee and CCl_4+green tea treated rats (*p<0.01)

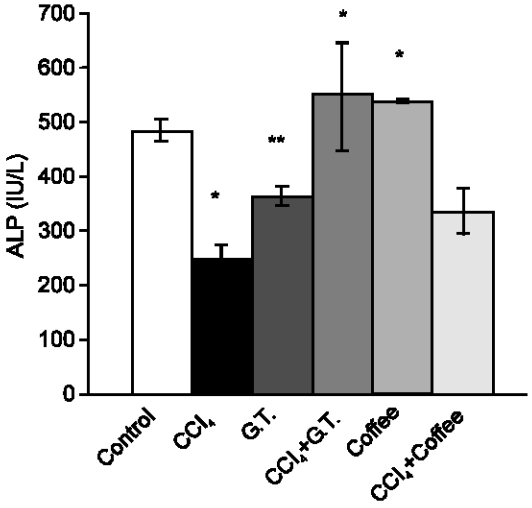

Fig. 5: Effect of Green tea extract and Coffee on plasma ALP activity in control, CCl_4 treated, coffee alone, green tea alone, CCl_4+coffee and CCl_4+green tea treated rats (*p<0.01, **p<0.05)

and green tea treated rats but results were not significant. Level of MDA was decreased significantly in CCl_4 + Coffee treated rats as compared to control (p<0.01) while insignificantly increased in CCl_4+Green tea treated rats.

Effect of Green tea extract and Coffee on plasma ALP activity in control, CCl_4-treated, coffee, green tea, CCl_4 + coffee and CCl_4+green tea treated rats: Fig. 5 showed a significant decreased ALP level in CCl_4 -treated rats as compared to control (p<0.01). Significant increased activity of ALP was observed in coffee and green tea a treated rats as compared to control (p<0.05), (p<0.01) respectively. Significant partially decreased activity of ALP was observed in CCl_4+Coffee treated rats as compared to control (p<0.01), while in CCl_4 + Green tea treated rats showed increased activity of ALP but results were not significant.

Effect of Green tea extract and Coffee on plasma ALT activity in control, CCl_4-treated, coffee, green tea, CCl_4+coffee and CCl_4 + green tea treated rats: A significant increased of plasma ALT level was observed in CCl_4-treated rats as compared to control (p<0.01)

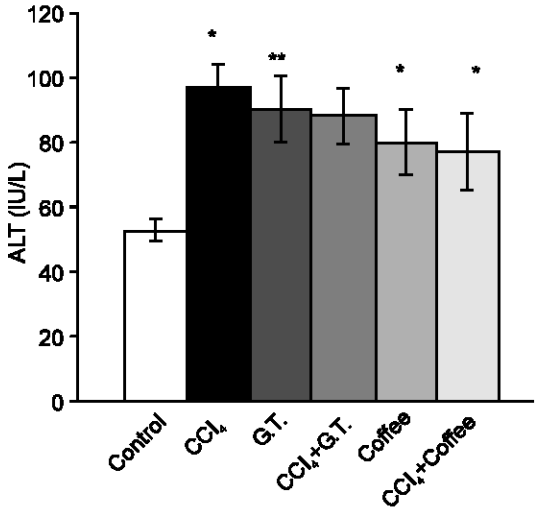

Fig. 6: Effect of Green tea extract and Coffee on plasma ALT activity in control, CCl_4 treated, coffee alone, green tea alone, CCl_4+coffee and CCl_4+green tea treated rats (*p<0.01, **p<0.05)

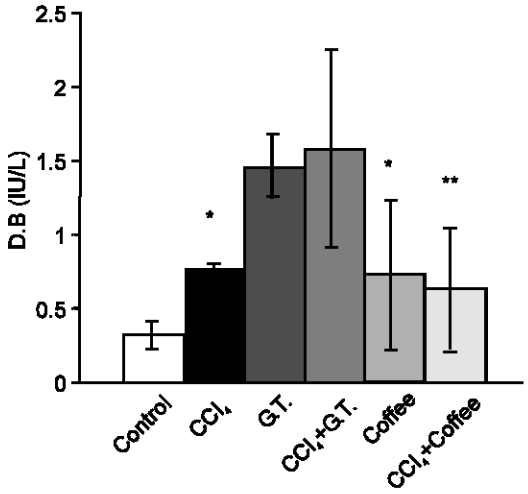

Fig. 7: Effect of Green tea extract and Coffee on plasma direct bilirubin level in control, Ccl_4- treated, coffee alone, green tea alone, CCl_4+coffee and CCl_4+green tea treated rats (*p<0.01, **p<0.05)

(Fig. 5). Coffee and green tea treated rats showed significant increased level of ALT as compared to control (p<0.05), (p<0.01) respectively. Partially increased activity of ALT was observed in CCl_4 + Coffee treated rats while a partial increased activity of ALT was observed in CCl_4 + Green tea treated rats as compared to control (p<0.01).

Effect of Green tea extract and Coffee on plasma direct bilirubin level in control, CCl_4-treated, coffee, green tea, CCl_4 + coffee and CCl_4 + green tea treated rats: CCl_4-treated rats showed significant increased in plasma Direct bilirubin level as compared to control

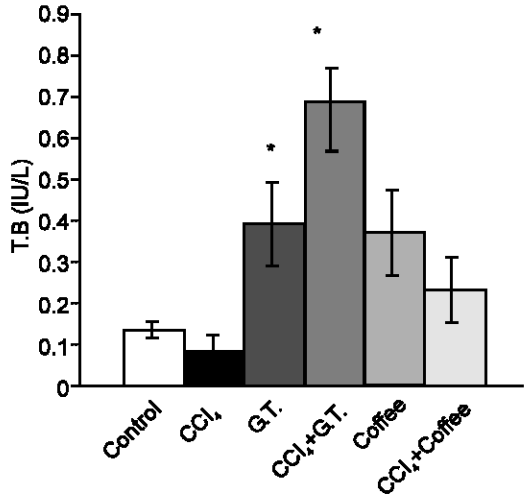

Fig. 8: Effect of Green tea extract and Coffee on plasma total bilirubin level in control, CCl_4 treated, coffee alone, green tea alone, CCl_4+coffee and CCl_4+green tea treated rats (*p<0.01, **p<0.05)

(p<0.01). An increased level of plasma direct bilirubin was observed in coffee treated rats and a significant increased level of direct bilirubin was observed in green tea treated rats as compared to control (p<0.01). An Increased level of plasma direct bilirubin was observed in CCl_4 + Coffee treated rats while significant increased in CCl_4 + Green tea treated rats as compared to control (p<0.05) was observed (Fig. 7).

Effect of Green tea extract and Coffee on plasma total bilirubin level in control, CCl_4 -treated, coffee, green tea, CCl_4 + coffee and CCl_4 + green tea treated rats: Fig. 8 showed decreased plasma total bilirubin level in CCl_4-treated rats but results were insignificant. A significant increased level of plasma total bilirubin was observed in coffee treated rats as compared to control (p<0.01) whereas, an increased level was observed in green tea and result was insignificant. A marked increased level was observed in CCl_4 + Coffee treated rats whereas a marked significant increased was observed in CCl_4+Green treated rats as compared to control (p<0.01).

DISCUSSION

Liver injuries induced by CCl_4 are the best-characterized system of the xenobiotic-induced hepatotoxicity and is a commonly used model for the screening the anti-hepatotoxic/hepatoprotective activity of drugs (Brautbar and Williams, 2002; Brent and Rumack, 1993).

In this study our results demonstrates that at a dose of 0.8 mg/kg b.w. of CCl_4, antioxidant enzymes; SOD and catalase activity (Fig. 2 and 3) was lower and MDA level (Fig. 4) was elevated in liver tissue, altered liver enzymes (Fig. 5-8) and induced liver cirrhosis.

The mechanism of CCl_4-induced liver damage is considered to be due to the enzymatic activation

(cytochrome P_{450}) by CCl_4 into the trichloromethyl free radical (CCl_3) within the membrane of the endoplasmic reticulum. This highly reactive compound reacted with sulfhydryl group, protein-thiol and reduced Glutathione (GSH), thus covalently bind with the cell membrane and leads to membrane lipid peroxidation and this end at necrosis of the cell (Kyung *et al*., 2007). This is followed by chloromethylation, saturation, peroxidation and progressive destruction of the unsaturated fatty acid of the endoplasmic reticulum membrane phospholipids (Nalan *et al*., 2007).

Gallus *et al*. (2007) reported that the oxidative tissue damage in cirrhosis causes a significant lowering in the level of catalase. Alia *et al*. (2006) reported that during the process of inflammation ROS increases and produces the oxidative stress due to this, a significant decrease in antioxidant enzyme system. The main target of ROS are the poly unsaturated fatty acids in cell membranes causing lipid peroxidation and formation of MDA which may lead to damage the cell structure and function (Kuper *et al*., 2000).The tissue SOD and catalase enzymes were significantly lower in CCl_4 treated rats and these activities were significantly restored by G.T and coffee treated rats.

Figure 4-7 indicates an altered ALT, ALP, total and direct bilirubin level which strongly claimed liver tissue injury. The increased serum levels of AST and ALT have been attributed to the damaged structural integrity of the liver, because these are cytoplasmic in location and are released into circulation after cellular damage (Recknagel *et al*., 1989).

In present study, ALT level was significantly increased in CCl_4 treated rats where the cells of liver have been inflamed and ALT leaked into blood stream (Giboney 2005), while ALP is significantly decreased in CCl_4 treated rats. According to the Zaidi *et al*. (2005), ALP synthesized in the bile canalicular cells and appeared into blood stream only whenever biliary duct inflamed or blocked. It might be possible that CCl_4 produced only hepatic damage not biliary. Low ALP level is associated with magnesium deficiency as ALP activity is almost inhibited due to chelation of zinc and magnesium, an enzyme cofactors (Baldi *et al*., 1993). Direct bilirubin concentrations was elevated in CCl_4 treated rats, indicated that either due to an increased production, decreased uptake by the liver, decreased conjugation, decreased secretion from the liver or blockage of bile ducts (Bun *et al*., 2006), decreasing amount of reducing equivalents i.e., NADPH reductase, reduced glutathione (GSH). GSH maintain the integrity of RBCs membrane, its reduced level increases the hemolysis and increases the bilirubin level (Oberti *et al*., 1997). Both coffee and G.T. increases the biliary flow and bile helps to eliminate the bile salts, fats, toxins from the body.

Herbal polyphenolic compounds in the cell can function as an antioxidant and prooxidant by scavenging reactive oxygen species via enzymatic and non-enzymatic reactions (Pyo *et al*., 2004).

Coffee components cafestol, kehweol, cholinergic acid and caffeine have an antioxidant (Son and Lewis, 2002), chemoprotective and anitinflammatory property (Sudina *et al*., 1993) reverse lipid peroxidation, enzymatic leakage and enhance cellular antioxidant defense mechanism, reported by Dreosti *et al*. (1997). Similarly, Green tea polyphenol EGCG (epigallocatechingallate) and catechin are promising anticancer potential (Gaetani *et al*., 1996). However, prolonged administration of green tea and coffee may cause liver enzymes alterations as showed in our results.

Treatment with coffee and GreenTea leads to CCl_4, decreases MDA, ALT, bilirubin level and increases the antioxidant enzymes. Our results preclude the protective effects of coffee and green tea against CCl_4-induced liver injury. Green tea and coffee has a potent potential to reduce the severity of cirrhosis in association with decreased lipidperoxidation, restored antioxidant and liver enzymes. Both are useful supplements in the treatment of liver cirrhosis.

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