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# Evaluation of hepatoprotective potential of functional food formulations using in vitro and in vivo models of $CCl_4$ radical induced toxicity

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## Abstract

Carbon tetra chloride ($CCl_4$) induced acute hepatotoxicity causes severe damage to hepatocytes and affects the liver functions which resemble various liver ailments like hepatitis, jaundice, cancer etc. Using 12-different fruits, formulation F1 and F2 were prepared. Hepatoprotective potential of the formulations was assessed using HepG2 cell (in vitro) and rat model (in vivo). Biochemical parameters like alkaline phosphatase, lactate dehydrogenase, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, bilirubin, blood urea nitrogen, plasma TBARS, trolox equivalent antioxidant capacity, total cholesterol, triglycerides were studied. Various markers of liver functions viz., super oxide dismutase, catalase, glutathione peroxidase, reduced glutathione, tissue Thio barbituric acid reactive substances (TBARS) were assessed. Significant decrease in the activity of aminotransferases, alkaline phosphatase and bilirubin was observed in formulation F1 followed by F2 groups as compared with $CCl_4$ treated group. The biochemical and histopathological observations supported hepatoprotective effect. Antioxidant enriched polyherbal formulations F1 and F2 effectively ameliorated $CCl_4$ induced acute hepatotoxicity by improving antioxidant status in rats.

**Keywords:** Functional food formulations F1 & F2, in vitro model, in vivo model, HepG2.

## Introduction

'Functional food' can be defined as a food that delivers a health benefit beyond basic nutrition and makes a claim about this benefit. Functional foods can be whole foods or foods that naturally contain or have been fortified with nutrients and/or bioactive substances that provide a specific benefit to health. Development of plant based hepatoprotective drugs has received significant attention in the global market. Herbal drugs are traditionally used in the treatment of liver diseases, especially in India. Researchers have examined the effect of plants used traditionally by indigenous healers and herbalists to support liver function and treat diseases of the liver [1]. A large number of plants and formulations have been claimed to have hepatoprotective activity. However, only few plant materials have received systematic investigations. Further, hepatoprotective activity in food materials has been studied less extensively.

$CCl_4$ is the most extensively used model to study effect of experimental materials against the oxidative stress and liver toxicity developed in rats [2]. Use of this model, as a preventive measure against radical toxicity to the liver, is needed to develop functional food supplements having hepatoprotective activity. Therefore, it was thought worthwhile to confirm the hepatoprotective efficacy of both the polyherbal formulations F1 and F2 against $CCl_4$ induced toxicity in vivo using rat model.

The objectives of this study was to evaluate hepatoprotective effect as a consequence of consumption of antioxidants rich polyherbal formulations against $CCl_4$ induced acute liver damage in rats and to observe the changes in the renal function, antioxidative defense enzymes and their possible prophylactic action.

## Materials and methods

Chemicals and reagents:

Kits for the enzymatic assays like alkaline phosphatase (ALP), lactate dehydrogenase (LDH), serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), and non enzymatic assays viz., bilirubin, blood urea nitrogen (BUN), urea, total cholesterol, triglycerides, HDL, were procured from Accurex Biomedical Pvt. Ltd. (India). Fetal bovine serum (FBS) was purchased from Hi media Ltd. and Dulbeco's Minimum Essential Medium (DMEM) from Sigma Ltd. All the chemicals used were of highest purity grade available.

In vitro Hepatoprotective activity using HepG2 Cells (Human liver hepatocellular carcinoma cell line) :

**Preparation of extracts from the formulations F1 and F2:**

To mimic the physiological conditions, overnight extractions of both the polyhebral formulations F1 and F2 were done in (PBS) phosphate buffer saline (0.1 g in 10 ml PBS pH 7.4). The extracts were filtered through 0.2µ membrane filters under sterile conditions and used for their hepatoprotective activity at flask level.

**Study design for in vitro hepatoprotective activity of formulations:**

HepG2 cells (passage No: 40) were trypsinized and uniform single cell suspension having approximately (4-5 x $10^5$ cells/ml) in DMEM (with 10 % FBS) were seeded in 12 new (25 $cm^2$) flasks. HepG2 cells from all 12 flasks were allowed grow confluent up to 80-90 % and were grouped into 4 (3 flasks in each). To each flask 4 ml DMEM with 10% FBS was added. After that to each flask from control group, 1 ml of (1%) DMSO solution was added. To the flasks from $CCl_4$ control group, $CCl_4$ solution (20 mM $CCl_4$ in 1% DMSO) was added so that the effective concentration of $CCl_4$ in the medium would be 4 mM. Similarly to the flasks

from F1 and F2 group, $CCl_4$ solution (effective concentration 4 mM) was added along with 1 ml of buffer extract of respective formulations F1 and F2.

After 14 h treatment of $CCl_4$, medium was decanted and monolayer of cells from respective flask was washed with HBSS (Hank's Balanced Salt Solution) twice. Cells were scrapped, dissolved in 1 ml Tris HCl buffer, lysed completely using homogenizer. The homogenates were centrifuged at 3000X g for 30 min at 4°C and the supernatant was used for the estimation of total soluble protein, different enzymes viz. catalase, GPx, SOD, LDH and ALP.

**Protein content:**

The protein concentration of liver tissue homogenates was determined by the method of Lowry et al (1951) [4] using bovine serum albumin (BSA) as standard protein.

**Catalase assay:**

Catalase activity was determined by a method of Claiborne and Fridovich (1979) [5] and was expressed as nM of $H_2O_2$ decomposed $min^{-1}$ $mg^{-1}$ protein. The specific activity was calculated using the molar extinction coefficient for $H_2O_2$ as 43.6 $M^{-1}cm^{-1}$ at 240 nm in the following equation: Specific activity (Units/min/mg protein) = $\Delta A_{240nm}$ (1 min) X 1000/43.6 X mg protein.

**Superoxide dismutase (SOD) assay:**

The activity of SOD of tissue homogenate was estimated by using the method of Sun et al 1988 [6] and Kono 1978 [7]. The method is based on the principle of the inhibitory effect of SOD on reduction of nitro blue tetrazolium (NBT) dye by superoxide anions, which were generated by the photo-oxidation of hydroxylamine hydrochloride. The rate of NBT reduction was calculated and one unit of enzyme was expressed as inverse of the amount of the protein required to inhibit the reduction rate of NBT by 50% (U/mg protein).

**Glutathione peroxidase (GPx) assay:**

GPx estimation was done by using the method of Mohandas et al (1984) [8]. The non-enzymatic reaction rate was correspondingly assayed by replacing the tissue homogenate with phosphate buffer. The enzyme activity was expressed as nM NADPH oxidation $min^{-1}$ $mg^{-1}$ protein and was calculated using an extinction coefficient of (6.22 $mM^{-1}$ $cm^{-1}$).

**Alkaline phosphatase (ALP):**

Levels of ALP are reported to be elevated during the oxidative stress or cellular damage to the HepG2 cells. ALP activity of respective cell lysates were measured using assay kit in accordance with manufacturer's instructions using the formula: (absorbance/min.) x 2720 and expressed in terms of IU/l. For this experiment, ALP activity of HepG2 cell homogenate as IU/mg protein.

**Lactate dehydrogenase (LDH):**

The LDH assay is based on the reduction of NAD by the action of LDH and the activity was measured using assay kit in accordance with manufacturer's instructions, using the formula: (absorbance/min.) x 8109 and expressed in terms of (IU/l) and was expressed as IU/mg protein.

Experimental design for in vivo rat model:

Modified AIN-1993G diet was prepared as per American institute of Nutrition (AIN) guidelines [3], containing casein as the source of protein, wheat bran as a source of fiber, whereas corn starch and sucrose as the sources of carbohydrates. Calorific value of formulations was similar to that of corn starch, hence, 33.3 % of the corn starch of AIN -93 diets was replaced with the respective formulation F1 and F2. Thus pellets of isocaloric AIN-93 diets viz., Control, F1 and F2 were prepared for respective groups just one week before use.

Three month old adult male albino Wistar rats (326.9 ± 10.5 g) were issued from the animal house facility of Agharkar Research Institute, Pune and were divided into four groups of six each. They were individually housed in well ventilated cages and maintained under standardized environmental conditions (22–28°C, 60–70% relative humidity, 12h dark/light cycle). Appropriate guidelines of the local animal ethics committee were followed for the animal experiments. To get acclimatize to new diet; rats were fed on AIN-93 diet (control diet) and water ad-libitum for one week. Based on the preliminary experiments on HepG2 cell line, the hepatoprotective dose of both formulations F1 and F2 were decided. 15 g of feed pellets per day were administered to each animal. Food treatment was continued for 30 days. On 31$^{th}$ day, $CCl_4$ 1:1 diluted in olive oil (2.0 ml/kg body weight) was administered intra peritoneal (I.P.) to the rats from $CCl_4$ group, F1 and F2 groups. To the control group, olive oil 1:1 diluted with saline was administered as a vehicle. Permission from Institute ethical committee was taken prior to this experiment on animals.

**Tissue Sampling:**

After 24 h of $CCl_4$ administration, animals were anaesthetized by anesthetic ether, blood was withdrawn by cardiac puncture and immediately animals were dissected, livers excised and rinsed in PBS, total weight of liver tissue was measured. A Small section of each liver (same lobe for each animal) was placed in 10% phosphate-buffered formalin for histopathological analysis and the remaining tissue was kept in ice cold tris-buffer (50 mM, pH 7.4) and processed for various biochemical estimations. Plasma and serum were separated from the blood samples and processed for biochemical estimations.

**Estimation of markers of liver function in plasma:**

The enzyme activities of ALP, LDH, GOT, GPT and non-enzymatic markers like bilirubin (total and direct), urea, BUN were estimated using commercial kits. Plasma lipid profile was also done using commercial kits. Plasma TBARS was done by using the method of Placer et al (1966) [9].

**Estimation of hepatic antioxidant enzymes:**

1 g of liver tissue samples was homogenized in 10 ml of 50 mM Tris–HCl buffer pH 7.4 to obtain 10% liver homogenate. The homogenates were centrifuged at 3000 X g for 30 min at 4°C and the supernatants were used for the estimation of protein content, GSH, TBARS and different enzymes viz. catalase, GPx and SOD. Antioxidant assays viz., catalase, SOD, GPx and protein content of liver tissue homogenate were done as described above.

**Estimation of GSH:**

The assay was performed within 1 h after sacrificing the animals to avoid errors due to oxidation of GSH from the liver tissue homogenate[10], [11]. Assay of known amounts of GSH in the presence of 0.1–0.2 ml of 5% TCA instead of the sample were also done which demonstrated that at these concentrations, TCA did not interfere with the GSH-DTNB complex formation. For each set of assays, a standard curve for GSH was prepared. The GSH contents were expressed in terms of nM of GSH/ mg protein (mmol $g^{-1}$ tissue).

Statistical analysis:

The statistical significance of the difference was analyzed through one way analysis of variance (ANOVA). If ANOVA showed F-value greater than F-critical value, critical differences (CD) at different levels of significance was computed and then compared with differences within the mean values different groups. Results are summarized as graphs and tables. Levels of significance are indicated as ($p< 0.05$, *), ($p<0.02$, **), ($p<0.01$, ***), ($P<0.001$, ****).

## Results and Discussion

Exposure to $CCl_4$ results in hepatic steatosis, centrilobular necrosis and ultimately, cirrhosis in the liver and acute tubular necrosis in the kidney[12]. $CCl_4$ induces oxidative stress in many settings and it also inhibits the activity of antioxidant enzymes in renal tissue [13]. Lipids peroxidation is a major mechanism by which free radicals can induce tissue injury [14]. Against such oxidative injuries, tissues have a variety of defense mechanisms including the non-enzymatic glutathione (GSH) and the enzymatic superoxide dismutase scavenger systems [15].

(In vitro) Hepatoprotective activity of formulations F1 and F2 on HepG2 cells:

A) Catalase Assay**:** Formulation F1 ($p<0.01$) significantly lowered the catalase activity followed by F2 ($p<0.02$) than that of $CCl_4$ control group but also showed the tendency to normalized the levels of catalase as that of normal control group ($p>0.1$) (Figure 1-A). As catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules, it can be inferred that both the formulations are efficient enough to minimize the hydroxyl radical toxicity generated during radical induced hepatic injury.

B) SOD Assay**:** SOD catalyzes the breakdown of the superoxide anion into oxygen and hydrogen peroxide. It was observed that both the formulation F1 ($p< 0.02$) and F1 ($p<0.05$) significantly reduced the SOD levels in HepG2 cells than that of $CCl_4$ control group (Figure 1-B).

C) GPx Assay**:** Both the formulation F1 and F2 significantly ($p<0.01$) lowered the glutathione peroxidase levels than that of $CCl_4$ control group (Figure-1-C).

D) LDH Assay: It was observed that the levels of LDH were significantly increased in the $CCl_4$ control group than that of normal control group whereas significantly lowered by formulation F1 ($p<0.001$) and F2 ($p<0.001$) as shown in Figure 1- D. As the LDH activity is used as an indicator of relative cell viability as a function of membrane integrity, it can be said that both the formulations F1 and F2 showed significant hepatoprotective activity by lowering the $Cl^-$ radical toxicity to HepG2 cells.

E) ALP Assay**:** ALP levels were also found to be lowered significantly by both the formulation F1 and F2 ($p<0.001$) as shown in (Figure 1- E). Results are in agreement with the Shah et al (2011) [16] and Brindha et al (2010) [17], proves the hepatoprotective potential of both the formulations F1 and F2.

Morphological changes in HepG2 cells (Figure 2) also indicated that the cell architecture was found to be comparatively less disturbed than that of $CCl_4$ control in presence of buffer extracts of both the formulations F1 and F2.

(In vivo) Hepatoprotective activity of formulations F1 and F2 in rats:

**Changes in Blood parameters:**

Administration of a single dose of $CCl_4$ to a rat produces centrilobular necrosis and fatty changes within 24 h. $CCl_4$ is metabolized by cytochrome P-450 in endoplasmic reticulum and mitochondria with the formation of $CCl_3O^-$, a reactive oxidative free radical, which initiates lipid peroxidation. The poison reaches its maximum concentration in the liver within 3 h of administration. Thereafter, the level falls and by 24 h there is no $CCl_4$ left in the liver. The development of necrosis is associated with leakage of hepatic enzymes in plasma viz., ALP, LDH, SGPT, SGOT. Similarly, levels of total bilirubin and direct bilirubin were also found to be elevated during hepatic injury. Levels of these enzymes reflect the severity of liver injury [18]. In agreement to this study, level of all these markers were found to be significantly elevated in $CCl_4$ control group than that of normal control group and formulations (F1 and F2) groups (Table 1).

Formulation F1 followed by F2 significantly ($p<0.001$) lowered the levels of ALP than that of $CCl_4$ control group. Similarly levels of LDH, SGOT were observed to be significantly decrease by formulations F1 ($p<0.01$) and F2 ($p<0.02$) as that of $CCl_4$ control group. Levels of SGPT were also observed to be significantly decreased by formulations F1 ($p<0.001$) and F2 ($p<0.01$) than $CCl_4$ control group. Hence it can be inferred that the hepatic injury due to $CCl_4$ might me less in formulation groups than $CCl_4$ control group. For all these enzymatic parameters of liver function, there was no significant difference ($p>0.1$) observed in respective levels of normal control group and F1, F2 groups; indicating preventive role of both formulations against chloride radical toxicity and hence comparatively less damage than $CCl_4$ control group.

Similarly significant reduction ($p<0.01$) in the levels of total bilirubin and direct bilirubin from the F1 and F2 groups as compared to $CCl_4$ group supported the hepatoprotective role of both the formulations and less hepatic damage.

Levels of blood urea and blood urea nitrogen were found to be significantly ($p<0.01$) higher in $CCl_4$ group than control, F1 and F2 groups indicating the renal dysfunctioning and disturbed protein metabolism in $CCl_4$ intoxicated groups. But both formulations significantly prevented the damage by lowering the blood urea and BUN levels as that of $CCl_4$ control group.

There was no significant difference observed in levels of total cholesterol, triglycerides among all experimental groups. However levels of HDL were found to be significantly ($p<0.001$) lowered in $CCl_4$ control group, F1 and F2 than that of normal control group.

Due to severe radical toxicity to the liver, fatty acid metabolism of liver might be lowered down. This might be the reason for no significant change total cholesterol and triglycerides levels among all $CCl_4$ treated animal. Cholesterol level of normal control group was found to be significantly higher than that of $CCl_4$ intoxicated groups.

Levels of hemoglobin in all $CCl_4$ treated groups were found to be significantly elevated than normal control group. This observation might be due to the impairment hemoglobin metabolism in liver. But in both experimental groups, hemoglobin content was found to be significantly decreased F1 ($p<0.05$) and F2 ($p<0.05$) than $CCl_4$ control group indicating better hemoglobin metabolism and hence less damage to liver.

Random glucose levels of control, F1 and F2 groups were found to be significantly increased ($p<0.001$) than the $CCl_4$ control group. Due to severe liver damage to $CCl_4$ control group rats, they didn't consume food after $CCl_4$ intoxication where as other groups did. This might be the reason for higher glucose levels in formulations and control groups.

Trolox equivalent antioxidant capacity (TEAC) of plasma samples were found significantly higher in formulation groups F1 ($p<0.01$) and F2 ($P<0.02$) than the control and $CCl_4$ groups. This might be due to improved antioxidant status of animals consuming polyphenols enriched (F1 and F2) diet.

**Changes in liver tissue parameters:**

Lipid peroxidation has been implicated in the pathogenesis of hepatic injury by the chloride ($Cl^-$) radical derivatives of $CCl_4$ and is responsible for cell membrane damage and consequent release of marker enzymes of heptotoxicity[19]. In the present study, significantly elevated levels of TBARS, products of membrane lipid peroxidation, observed in $CCl_4$ administered rats indicated hepatic damage. Pretreatment of formulations F1 ($p<0.01$) and F2 ($p<0.02$) significantly lowered the levels of liver TBARS than that of $CCl_4$ control group indicating the prevention of lipid peroxidation which could be attributed to the radical scavenging antioxidant constituents of formulations. GSH is the major non-enzymatic antioxidant and regulator of intracellular redox homeostasis, ubiquitously present in all cell types[20]. Mechanistic studies on $CCl_4$ induced studies revealed that GSH conjugation plays a critical role in eliminating the toxic metabolites[21]. $CCl_4$ administration leads to a significant decrease in the glutathione level. The mechanism of hepatoprotection and significant improvement in GSH levels by formulations F1 ($p<0.01$) and F2 ($p<0.05$) against $CCl_4$ induced toxicity might be due to restoration of the GSH level or may possibly be due to enhancement of GSH synthesizing enzyme activities such as c-glutamyl cysteine synthetase (c-GCS) and GSH synthetase but this needs further studies.

SOD catalyses the dismutation of superoxide anion to $H_2O_2$ and $O_2$. Because $H_2O_2$ is still harmful to cells, catalase and GPx further catalyzes the decomposition of $H_2O_2$ to water ($H_2O$). In the reaction catalyzed by GPx, GSH is oxidized to GSSG, which can then be reduced back to GSH by glutathione reductase [22]. Thus, the coordinate actions of various cellular antioxidants in mammalian cells are critical for effectively detoxifying free radicals. $CCl_4$ administration to rats declined antioxidant capacity of the rat liver as evinced in decreased activity of the antioxidant enzymes viz. catalase, GPx, SOD, which is in agreement with earlier reports [23]. Simeonova et al (2013)[24] found that ROS, produced by $CCl_4$, decrease the activities antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST).

Pretreatment of formulations F1 and F2 prevented the reduction in the levels of antioxidant enzyme (SOD, Catalase & GPx) against oxidative damage to the liver as shown in Table 2. Levels of SOD were found to be significantly improved by formulation F1 ($p<0.01$) and formulation F2 ($p<0.05$); levels of catalase were found to be improved significantly by F1 ($p<0.01$) and F2 ($p<0.2$); levels of GPx were found to be improved significantly by F1 ($P<0.001$) and F2 ($p<0.02$) than that of $CCl_4$ control. No significant difference ($P>0.1$) was observed between the formulations groups (F1 and F2) and normal control group as far as levels of SOD, catalase and GPx are concerned. This again confirmed the comparatively less damage to liver in formulation groups than $CCl_4$ control group. Similar studies have shown a positive effect of different classes of polyphenols on antioxidant enzyme activities in vivo [25, 26].

Histology:

The leakage of large quantities of enzymes into the blood stream was associated with centrilobular necrosis and ballooning degeneration of the liver which was further confirmed by comparing the cell architecture of four groups by histological observations (Figure 1). Images showing congestion (red arrow), fatty changes (yellow arrow), MNC infiltration (blue arrow) and necrosis of hepatocytes (black arrow). The reduced amount of histological injuries were observed in group F1 (grade-II i.e. up to or less than 50% damage) followed by group F2 (grade-III i.e. up to or less than 75% damage) compared with $CCl_4$ group (grade-IV i.e. more than 75% damage) Table 3). Severe centrilobular necrosis was observed in $CCl_4$ group whereas extent of centrilobular necrosis was significantly decreased in both the formulation groups. Significant protective effect of formulation F1 followed by F2 was observed because of the comparatively less fatty changes around the central vein, less derangement of hepatic cords, less infiltration of inflammatory cells (MNCs) than $CCl_4$ group. The histological changes induced by $CCl_4$ were markedly ameliorated by treatment with the formulation F1 followed by F2 as shown in Figure 1. Present results are in agreement with the previous studies [27].

## Conclusions

In conclusion, antioxidants containing polyherbal formulations F1 and F2 effectively prevented $CCl_4$-induced acute hepatotoxicity in rats which proves their potential to boost antioxidant status of rats and amelioration of $CCl_4$ (radical) induced

liver damage. Polyphenols and the bioactive antioxidants of the respective plant materials present in the formulations F1 and F2 might be synergistically responsible for the observed hepatoprotective effect.

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**References**

1. Luper S. A review of plants used in the treatment of liver disease, Part 2. Alt. Med. Rev., 1999, 4(3): 178-89.
2. Simeonova R, Kondeva-Burdin M, and Mitcheva M. Some in vitro/in vivo Chemically-Induced Experimental Models of Liver Oxidative Stress in Rats. BioMed Res. Int., 2014, Article ID 706302, 6 pages.
3. Reeve, PG, Nielsen FH, Fahey GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A diet. J. Nut., 1993, 123: 1939–51.
4. Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 1951, 193: 265–75.
5. Claiborne A, Fridovich I. Purification of the o-dianisidine peroxidase from Escherichia coli B. Physicochemical characterization and analysis of its dual catalatic and peroxidatic activities. J. Biol. Chem., 1979; 254: 4245-52.
6. Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clinic. Chem., 1988, 34: 497-500.
7. Kono Y. Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. Arch. Biochem. and Biophy. 1978, 186 (1): 189–95.
8. Mohandas M, Marshall JJ, Duggin GG, et al., Differential distribution of glutathione and glutathione related enzymes in rabbit kidney. Cancer Res., 1984, 44: 5086-91.
9. Placer ZA, Cushman LL, Johnson BC. Estimation of product of lipid peroxidation (malondialdehyde) in biochemical systems. Analyt. Biochem., 1966, 16: 359-364.
10. Moron MJ, Dipierre JW, Mannerv KB. (1979). Levels of glutathione, glutathione reductase and glutathione-s-transferase activities in rat lung and liver. Biochimica et Biophysica Acta, 1979, 582: 67–71.
11. Sidhu P, Garg ML, Dhawan DK. Protective effects of zinc on oxidative stress enzymes in liver of protein-deficient rats. Drug and Chem. Toxicol., 2005, 28: 211-30.
12. Handa SS, Sharma A. Hepatoprotective activity of andrographolide from andrographis paniculata against carbon tetrachloride. Indian J. Med. Res., 1990; 92: 276-92.
13. Shirwaiker A, Sreenivasan KK, Krishnanand BR., et al. Chemical investigation and anti hepatotoxic activity of the root bark of Caparis spinosa. Fitoterapia, 1996, 67(3): 200-204.
14. Zimmerman MD, Hayman J. Function and integrity of the liver. Clin. Diag. And Management By Lab. Meth., 1976, 17:217-50.
15. Agarwal AK, Mehendale JK. Potentiation of carbon tetrachloride hepatotoxicity and lethality by chlordecone in female rats. Toxicology, 1983; 26: 231-42.
16. Shah VN, Shah MB, Bhatt PA. Hepatoprotective activity of punarnavashtak kwath, an Ayurvedic formulation, against CCl4-induced hepatotoxicity in rats and on the HepG2 cell line. Pharma. Biol., 2011, 49(4): 408-15.
17. Brindha D, Saroja S, Jeyanthi GP. Protective potential [correction of potencial] of Euphorbia hirta against cytotoxicity induced in hepatocytes and a HepG2 cell line. The J. Basic and Clinic. Phys. and Pharmac., 2010: 21(4): 401-13.
18. Lin SC, Yao CJ, Lin CC, et al. Hepatoprotective activity of Taiwan folk medicine: Eclipta prostrate Linn. against various hapatotoxins induced acute hapatotoxicity. Phototherapy Res., 1996, 10: 483–90.
19. Danni O, Chiarpotto E, Aragno M, et al., Lipid peroxidation and irreversible cell damage: Synergism between carbon tetrachloride and 1,2-dibromoethane in isolated rat hepatocytes. Toxic. and Appl. Pharmac., 1991; 110: 216–22.
20. Meister A, Anderson ME. Glutathione. Annual Rev. Biochem., 1983, 52: 711-60.
21. Lee KK, Shimoji M, Hossain QS, et al. Novel function of glutathione transferase in rat liver mitochondrial membrane: role for cytochrome-C release from mitochondria. Toxic. and Appl. Pharmac., 2008, 232 (1):109-18.
22. Hayes JD, Flanagan JU, Jowsey IR. Glutathione Transferases. Annual Review of Pharmac. and Toxic., 2005, 45: 51-88.
23. Shahjahan M, Sabitha KE, Jainu M. et al. Effect of Solanum trilobatum against carbon tetrachloride induced hepatic damage in albino rats. Indian J. Med. Res., 2004, 120:194-98.
24. Simeonova R., Krasteva I, Kondeva-Burdina M, et al., Effects of extract from Astragalus Glycyphylloides on Carbon tetrachloride-induced hepatotoxicity in Wistar rats. Int. J. Pharma. and Bio. Sci., 2013, 4(3): 179–186.
25. Molina MF, Sanchez-Reus I, Iglesias I. Quercetin, a flavonoid antioxidant, prevents and protects against ethanol-induced oxidative stress in mouse liver. Biological and Pharmaceutical Bulletin, 2003, 26: 1398-1402.
26. Hubber WW, Scharf, G., Nagel G, et al. Coffee and it's chemo preventive components Kahweol and Cafestol increase the activity of O-6 methylguanine DNA methyltransferase in rat liver-comparision with phase-II xenobiotic metabolism. Mutation Res., 2003, 522: 57-68.
27. Orhan DD, Orhan N, Ergun E. et al. Hepatoprotective effect of Vitis vinifera L. leaves on carbon tetrachloride-induced acute liver damage in rats. J. Ethnopharmac., 2007, 112: 145-51.

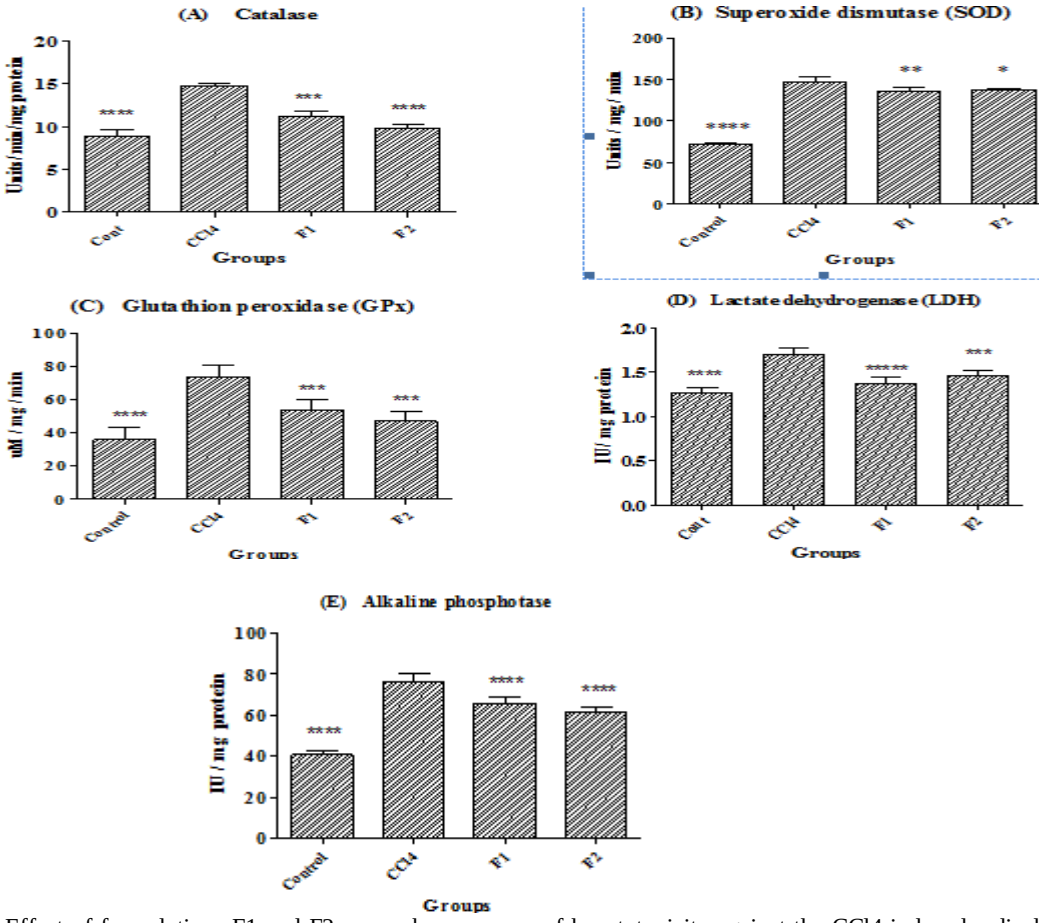



**Figure 1**: Effect of formulations F1 and F2 on marker enzymes of hepatotoxicity against the CCl4 induced radical toxicity to HepG2 cells. (Note:*, **, *** and **** represent the p values less than 0.05, 0.02, 0.01 and 0.001 respectively).


**Figure 2**: Morphological changes in HepG2 cells after intoxication with $CCl_4$ and protective effect of formulations F1 and F2.

**Table 1**: Effect of formulation F1 and F2 on biochemical parameters of blood in $CCl_4$ induced acute hepatic injury to Wistar rats.

| Blood parameters | | | | |
|---|---|---|---|---|
| | **Control** | **$CCl_4$** | **Formulation-1 + $CCl_4$** | **Formulation-2 + $CCl_4$** |
| Alkaline phosphatase (IU/l) | 129.7±13.7 *** | 249.3±17.5 | 155.4±11.6 *** | 172.9±12.4 *** |
| LDH (U/l) | 612.2±27.9 *** | 1587.9 ± 62.8 | 1204.2±113.1 *** | 1274.9±101.6 ** |
| SGOT(U/l) | 73.6±6.7 *** | 241.1±15.3 | 173.2±11.1 *** | 192.6±12.7 ** |
| SGPT(U/l) | 36.3±1.7 **** | 158.4±10.3 | 78.7±3.5 **** | 132.4±4.6 *** |
| Total Bilirubin (mg/dl) | 0.21+0.05 *** | 1.14+0.1 | 0.37+0.03 *** | 0.45+0.02 *** |
| Direct Bilirubin (mg/dl) | 0.101+0.031 *** | 0.367+0.08 | 0.11+0.03 *** | 0.101+0.06 *** |
| Urea (mg/dl) | 50.2±3.8 *** | 90.6±7.4 | 57±2.6 *** | 65.8±3.8 *** |
| BUN (mg/dl) | 24.4±1.7 *** | 44.2±3.7 | 22.9±1.4 *** | 28.1±1.2 *** |
| Triglycerides (mg/dl) | 87.7±7.0 NS | 59.2±3.7 | 66.4±4.1 NS | 65.5±4.3 NS |
| Total cholesterol (mg/dl) | 58.7±4.4 *** | 43.7±3.5 | 41.8±4.0 NS | 46.7±3.9 NS |
| HDL (mg/dl) | 45.2±3.1 *** | 24.7±1.3 | 30.8±2.2 NS | 27.1±1.9 NS |
| Hb (mg/dl) | 13.6±0.5 *** | 15±0.4 | 14.3±1.0 * | 14±0.9 * |
| Glucose (mg/dl) | 141.5±9.3 **** | 71.8±3.7 | 163.2±8.7 **** | 127.7±11.9 **** |
| Plasma TEAC (mg/dl) | 9.9±0.7 | 9.8±0.6 | 13.7±1.1 *** | 11±0.8 ** |

Note: The levels of significance were indicated as ($p< 0.05$, *), ($p<0.02$, **), ($p<0.01$, ***), ($p<0.001$, ****) and (non significant as NS).

**Table 2**: Effect of polyphenols enriched formulations F1 and F2 on enzymatic and non enzymatic marker of liver tissue against $CCl_4$ induced hepatotoxicity to rats.

| Liver tissue parameters | | | | |
|---|---|---|---|---|
| | **Control** | **$CCl_4$** | **F1 + $CCl_4$** | **F2 + $CCl_4$** |
| SOD (U/mg protein) | 71.3±5.6 **** | 46.2±3.4 | 67 ±2.3 *** | 54±2.6 * |
| Catalase U/min/mg protein) | 41.7±3.2 **** | 34.7±2.4 | 36.9±3.2 *** | 34.9±2.4 ** |
| GPx (U/min/mg protein) | 123±8.9 **** | 78±7.2 | 103 ±8.3 **** | 88.6±8.3 ** |
| GSH (µM/mg protein) | 1.1±0.09 **** | 0.6±0.04 | 0.72±0.04 *** | 0.66±0.03 * |
| Tissue TBARS (nM/mg protein) | 6.6±0.5 **** | 10.8±0.7 | 7.7±0.6 *** | 7.8±0.2 ** |

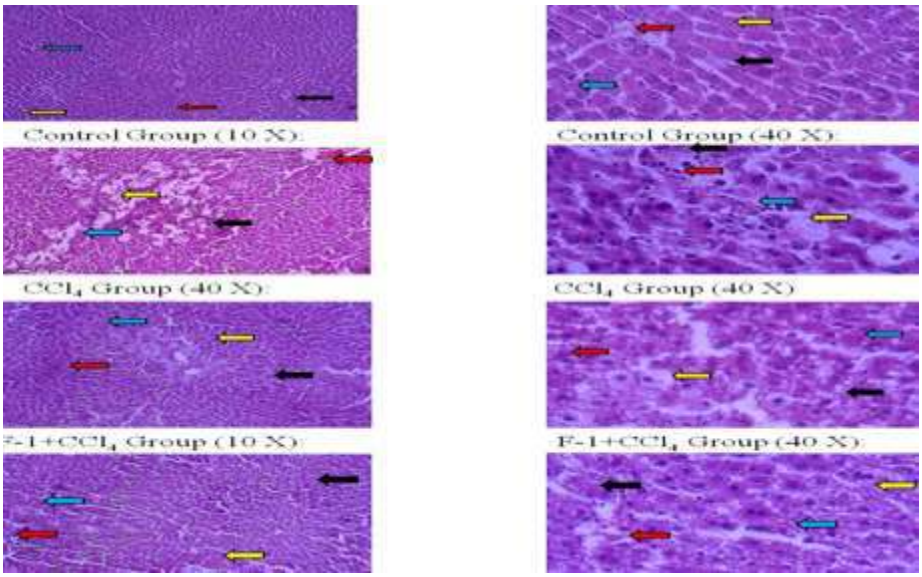
The levels of significance were indicated as ($p< 0.05$, *), ($p<0.02$, **), ($p<0.01$, ***), ($p<0.001$, ****) and (non significant as NS).

**Table 3**: Comparison of histopathological changes indicating extent of damage in liver tissues from different experimental groups.

| Groups | Derangement of hepatocytes and hepatic cord | Degeneration of hepatocytes | Congestion of central vein and simusoids | Focal necrosis | Inflitration of lymphocytes (MNC) |
|---|---|---|---|---|---|
| Control | + | + | + | 0 | + |
| $CCl_4$ | +++ | ++++ | ++++ | +++ | +++ |
| $CCl_4$ + F1 | ++ | +++ | ++ | ++ | ++ |
| $CCl_4$ + F2 | +++ | +++ | +++ | ++ | +++ |

Histological damages or active changes were scored as follows: 0: absent; +: mild (i.e. up to less than 25%); ++: moderate (i.e. up to less than 50%); and +++: severe (i.e. up to less than 75%) and ++++: (i.e. up to more than 75%).

**Figure 3**: Histological changes in liver tissues due to $CCl_4$ intoxication and amelioration of liver damage due to hepatoprotective formulations F1 and F2.




**Highlights**:

1. Functional food formulations F1 and F2 are potential enough to increase the antioxidant status in rat.
2. Both the formulations showed significant prevention of radical toxicity
3. Pre clinical trial on rat model confirmed the potential of formulations F1 and F2 as functional food supplements with antioxidant and hepatoprotective activity.