Research article

Protective effect of *Helianthus annuus* seeds extract against CCl_4-induced hepatocellular damage.

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Abstract: Carbon tetrachloride (CCl_4) is a hepatotoxin that causes toxicity in animals on its exposure. Hepatotoxins lead to hepatic demage that is treated by generating antioxidant effect. Synthetic and as well as natural drugs are available for this purpose but phyto-based herbal medicines got paramount importance against drug induced hepatotoxicity Current research was conducted to inspect the protective effect of aqueous extract of *Helianthus annuus*seeds pre-treatment on the carbon tetrachloride-induced hepatotoxicity in Balb C mice. Study covered the valuation of the enzymatic activity such as alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (ALP) and Lactate dehydrogenase (LDH) and other biochemical components like bilirubin, total protein, catalase, glutathione (GSH), malondialdehyde bis-(dimethyl acetal) tetra ammonium (MDA). The induction of carbon tetrachloride (CCl_4)caused rise in plasma ALAT, ASAT, ALP and LDH. *Helianthus annuus*seeds extract (HA extract) pre-treatment obliterated CCl_4-induced deviations in the activities of these enzymes significantly. Bilirubinlevel increased whereas total protein contents decreased after the induction of CCl_4 this effect was reversed by HA extract. Induction of CCl_4 caused increased in MDA level while decrease in GSH and catalase. *Helianthus annuus* seeds extract also abolished these changes.*Helianthus annuus* seeds extract pre-treatment also prevented CCl_4-induced changes in bilirubin and total protein contents. Carbon tetrachloride treatment resulted in huge hepatic damage. This was prevented by *Helianthus annuus* seeds extract. These results show that *Helianthus annuus* seeds extract pre-treatment prevented the mice from CCl_4-induced hepatic damage, which visibly shows its defensive effects against hepatic damage.

Keywords: Carbon tetrachloride, Hepatotoxicity, *Helianthus annuus* seeds extract, Balb C mice, Pre-treatment

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1. INTRODUCTION

Many toxic compounds enter the body which are detoxified and metabolized by the liver. Hepatic injury may occur in this process leading to fatal diseases [1]. Most of the toxicological problems are linked to the liver [2]. Hepatotoxicants affect the hepatocytes by oxidative damage [3].

Carbon tetrachloride (CCl_4) is released into water from many industries and animals get toxicity on its exposure [4]. Normal physiological progressionscan also produce free radicals [5] however cell and tissue damage is caused when there is an imbalance between ROS and free radical scavenging [6].

Hepatic damage in animal model is usually tested by CCl_4, a hepatotoxin.Free radical generation, decreased antioxidant enzymes activity and lipid peroxidation are the basic reasons behind CCl_4-induced hepatopathies[7-9]. Hepatotoxins attack on the fatty acids of cell membranes which lead to rise of lipid peroxides which loose functional integrity of hepatic mitochondria leading to hepatic demage [10]. Hepatopathies are treated by producing antioxidant effect or by the reduction of free radicals generation [11]. Synthetic and as well as natural drugs are available for the treatment of liver diseases [12]but phyto-based herbal medicines got paramount importance against drug induced hepatotoxicity[13]. Phyto-based medicines enriched with antioxidants are the compulsion of the hour due to toxicity mattersallied with certain drugs[14]. In this regard many plants are used to treat liver diseases.

The *Helianthus annuus* belongs to the Asteraceae family. Its seeds are the rich sources of folate, copper, selenium, zinc, iron, and vitamins especially vitamin E.It is a potential proteinsupplement for human diet due to good nutritional contents of seeds. These sulfur-rich seeds of *Helianthus annuus* are used for various metabiological needs of human, including insulin production, and development of skeletal and muscular cell [15].

It has various chemical components especially phenolic component with anti-oxidant activity. *Helianthus annuus* seeds might be considered in the prevention of degenerative diseases associated with free radical damage [16].Sproutand seeds of sunflower have beneficial effects such as wound-healing, antioxidant, anti-inflammatory, antimicrobial, antihypertensive .anticancer as well as cardiovascular benefits due to the presence of its flavonoids, phenolic compounds, vitamins,and polyunsaturated fatty acids[17]. Phytochemicals (polyphenols and flavonoids) of *Helianthus annuus* play an important role in prohibit the development of cancer cells bu scavenging the free radicals [18].Seeds of *Helianthus annuus* have been widely usedfor treatmentof various disorders includingwhooping cough,colds, pulmonary infections, and cardiovascular disorders [19].

The major objective of this study was to assess the pharmacological intervention of *Helianthus annuus* seed extracts against CCl_4-induced hepatotoxicity.Hepatotoxicity was determined by calculating the biochemical parameters blood serum of mice.

2. MATERIALS AND METHODS

Ethical statement

Animal trials were directed in accord with indigenous (law of Government College University, Lahore, Pakistan) and international law (Wet op de dierproeven, Wod, Article 9 of Dutch Law as mentioned in our previous studies **[20-24]**.

Animal Management

Mice of different groups were housed in different polypropylene cages. Standard laboratory pellet feed and purified drinking water was provided *ad libitum*. The temperature of the experimental room was maintained at 22 ± 3 °C. Relative humidity was controlled to be within 70% and a 12 h light/12 h dark cycle was maintained.

Preparations of the aqueous extract of *Helianthus annuus seeds*

The *Helianthus annuus*seedswere collected from Muzaffarabad city, Azad Kashmir, Pakistan.Shade dried seeds were grinded into a uniform powder. The powder (10 g) was added to 100 ml distilled water. The solution was agitated and permitted to soak for 24 hrs. This solution was filtered through Whatsman filter paper No. 1. Filtrate was stored for further activities.

Chemicals

Carbon tetrachloride was acquired from Sigma, Aldrich (USA).

Experimental animals grouping and dose preparation

Twenty Balb C mice were obtained with average weight of 50 g from Foot and Mouth Disease Research Center (FMDRC), Veterinary Research Institute (VRI), Lahore, Pakistan. The mice were treated gently, placed in cages in a well-ventilated and hygienic animal house under appropriate conditions. They were provided with a standard mouse pellets and drinking water ad libitum. Lethal dose (LD50) of CCl_4 was taken as 1 ml/kg b. w. These animals were allocated into four groups of five mice each namely I, II, III and IV. Group I served as control group. Group II was administered with CCl_4 (0.4 ml/kg b. w). Group III was given *Helianthus annuus* seeds extract and group IV was given *Helianthus annuus* seeds extract and CCl_4 (0.4 ml/kg b. w). All the injections were intra-peritoneal. Animals were anesthetized after 24 hour of last dosing, sacrificed and blood was collected. Blood was kept in heparinised (20 µl heparin/1 ml of blood) tubes and was centrifuged at 3000 rpm for 20 mins for the isolation of plasma to estimate biochemical components and enzymatic activities.

Estimation of ALP:

Determination of Alkaline Phosphatase (ALP) was done by Bowers method (1966) [26].

Estimation of LDH:

Activity of lactate dehydrogenase (LDH) was assessed by the method described by (King, 1965) [27].

Estimation of bilirubin:

Total bilirubin level (TBL) was determined by modified dimethyl sulfoxide (DMSO) method (Dangerfield and Finlayson, 1953) [28] on the basis of sulfanilic acid reaction with sodium nitrite to produce deoxidized sulfanilic acid.

Estimation of total protein:

Level of total protein was estimated by method of Lowry *et al*., (1951) [29].

Estimation of catalase activity.

Catalase activity was assessed by the standard protocol of Luck [30]with some modifications, wherein degradation of substrate H_2O_2 by catalase in the liver tissue samples was measured. 50 mg of tissue samples were homogenized in 0.05Mof 1 ml Tris-HCl buffer (pH 7.0) and centrifuged at 10,000 rpm for 10 min at 4^0C for the study of catalase activity. The supernatant was collected. In a spectrophotometric cuvette, 500 µl of 0.34 mM H_2O_2, 2.5 ml H_2O and 40 µl supernatant were added and change in absorbance was noted six times at 30 sec intervals at 240 nm.

Estimation of reduced glutathione (GSH).

Reduced glutathione activity was measured according to the standard protocol [31]. An aliquot of 1 ml liver tissue supernatant was treated with 0.5 of Elman reagent (19.8 mg DTNB dissolved in 100 ml of 0.1% sodium nitrate). After the treatment with Elman reagent, 3 ml of phosphate buffer was added and the absorbance was measured at 412 nm.

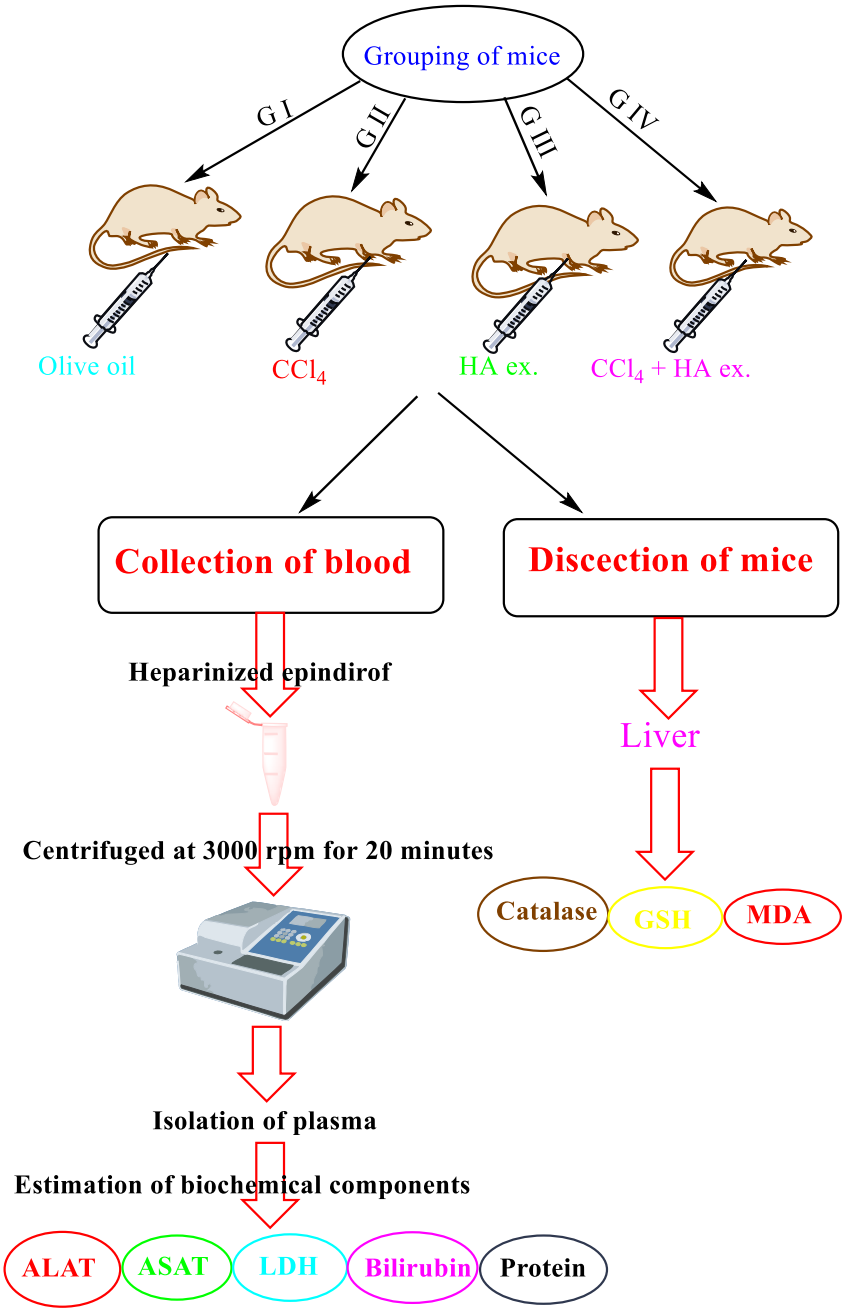

Figure1: Schematic overview of work done.

Estimation of liver markers:

Estimation of ALAT/SGPT:

The ALAT/SGPT level was estimated as per Reitman and Frankel (1957) method using SGPT [25].

Estimation of ASAT/SGOT:

The ASAT/SGOT activity was determined according to the method of Reitman and Frankel (1957) [25].

Estimation of MDA:

The method described by Okkawa ***et al. 1979*** [32]was followed. The liver tissues were homogenized in aqueous KC1 solution and incubated with thiobarbituric acid reagent at *90°C* for **1** hour. Mixture was allowed to cool and after centrifugation, the optical density of the clear pink supernatant was read at 532nm. Malondialdehyde bis-(dimethyl acetal) tetra ammonium was used as an external standard.

Statistical analysis

All statistical analyses were performed by GraphPad prism. All values were expressed as mean±SEM. Statistical difference among different groups was assessed by one way ANOVA with bonferroni test. Values were considered statistically significant at $p \leq 0.05$.

3. RESULTS

Effect on ALAT/SGPT

Intraperitoneal administration of CCl_4 (0.4 ml/kg body weight) caused highly significant increase in levels of ALAT /SGPT (control: 42.4±1.9 IU/L; CCl_4: 153.2±5.1IU/L). Administration of HA extract alone caused no significant change. But when HA extract was injected intraperitoneally (150 mg/kg body weight) in CCl_4 treated mice, highly significant decrease in level of ALAT/SGPT (CCl_4+ HA extract: 83.2±4.9 IU/L) was observed. Figure. 2

Effect on ASAT/SGOT:

Intraperitoneal administration of CCl_4 (0.4 ml/kg body weight) caused highly significant increase in levels of ASAT /SGOT (control: 84.2±1.5 IU/L; CCl_4: 462.2±4.2 IU/L). Administration of HA extract alone caused no significant change. But when HA extract was injected intraperitoneally (150 mg/kg body weight) in CCl_4 treated mice, highly significant decrease in level of ASAT/SGOT (CCl_4 + HA extract:166.4±7.9 IU/L) was observed. Figure. 2

Effect on ALP:

Intraperitoneal administration of CCl_4 (0.4 ml/kg body weight) caused highly significant increase in levels of ALP (control: 111.4±3.6 IU/L; CCl_4: 303±15.4 IU/L). Administration of HA extract alone caused no significant change. But when HA extract was injected intraperitoneally (150 mg/kg body weight) in CCl_4 treated mice, highly significant decrease in level of ALP (CCl_4+ HA extract:220.8±7.2 IU/L) was observed. Figure. 2

Effect on LDH:

Intraperitoneal administration of CCl_4 (0.4 ml/kg body weight) caused highly significant increase in levels of LDH (control: 329.9±19.33 IU/L; CCl_4: 995±64.9 IU/L). Administration of HA extract alone caused no significant change. But when HA extract was injected intraperitoneally (150 mg/kg body weight) in CCl_4 treated mice, highly significant decrease in level of LDH (CCl_4 + HA extract:643.6±16 IU/L) was observed. Figure. 2

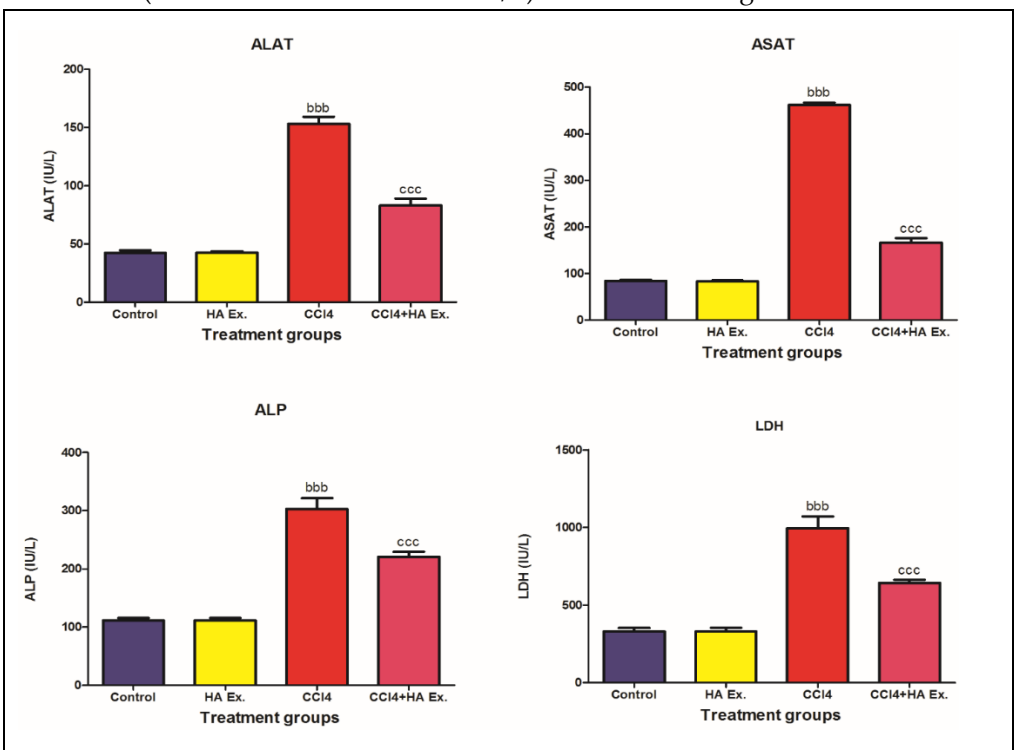

Figure 2: Analysis of enzymes (ALAT, ASAT, ALP and LDH) in liver of Balb C mice. Abbreviations: "HA ex." stands for*Helianthus annuus* extract and CCl_4 stands for carbon tetrachloride. **Keys:** (b) indicates the significance difference between control and CCl_4. (c) indicates significance difference between CCl_4 and CCl_4 + HA extract. Each bar signifies the mean value of five replicates and SEM. Statistical icons: bbb, ccc= $p \leq 0.001$.

Effect on MDA

Intraperitoneal administration of CCl_4 (0.4 ml/kg body weight) caused highly significant increase in levels of MDA (control: 46.2±4.45 mmol/g liver; CCl_4: 652.2±20.19 mmol/g liver). Administration of HA extract alone caused no significant change. But when HA extract was injected intraperitoneally (150 mg/kg body weight) in CCl_4 treated mice, highly significant decrease in level of MDA (CCl_4 + HA extract: 349.6±31.75 mmol/g liver was observed. Figure. 3

Effect on GSH:

Intraperitoneal administration of CCl_4 (0.4 ml/kg body weight) caused highly significant increase in levels of GSH (control: 3.89±0.19 μ mol/g liver; CCl_4: 2.04±0.12 μ mol/g liver). Administration of HA extract alone caused no significant change. But when HA extract was injected intraperitoneally (150 mg/kg body weight) in CCl_4 treated mice, highly significant decrease in level of GSH (CCl_4 + HA extract: 3.08±0.44 μ mol/g liver) was observed. Figure. 3

Effect on CATALASE

Intraperitoneal administration of CCl_4 (0.4 ml/kg body weight) caused highly significant decrease in levels of CATALASE (CCl_4: 112.8±3.44 mmol/min/g liver) as compared to control (182±7.76 mmol/min/g liver). Administration of HA extract alone caused no significant change. But when HA extract was injected intraperitoneally (150 mg/kg body weight) in CCl_4 treated mice, no significant change in level of CATALASE (CCl_4 + HA extract: 143.2±10.59 mmol/min/g liver was observed. Figure. 3 Effect on total bilirubin:
Intraperitoneal administration of CCl_4 (0.4 ml/kg body weight) caused highly significant increase in levels of bilirubin (control: 2.6±0.19 mg/dl; CCl_4: 4.28±0.16 mg/dl). Administration of HA extract alone caused no significant change. But when HA extract was given no significant change was observed (CCl_4 + HA extract: 3.48±0.2 mg/dl). Figure. 3Effect on total protein:

Intraperitoneal administration of CCl_4 (0.4 ml/kg body weight) caused highly significant increase in levels of total protein (control: 8.42±0.5 g/dl; CCl_4: 4.18±0.15 g/dl). Administration of HA extract alone caused no significant change. But when HA extract was given to CCL_4 treated group no significant change was observed (CCl_4 + HA extract: 5.28±0.42 g/dl). Figure. 3

DISCUSSION

Saini et al.(2011) defined the traditional uses of *Helianthus annuus* like source of treatment for different diseases and food [33]. It is used for the treatment of kidney diseases, chest pain, pulmonary troubles, asthma, malaria, lung ailments, diabetes, high fever, swellings, snakebites, spider bites, and wound healing. It is also used all over the world as a lubricant, stimulant, anti-diarrheal, dermatological aid and as a disinfectant.

Carbon Tetrachloride is used as dry cleaning agent, refrigerant and as a solvent for oils and fats. Its inhalation causes damage of the liver and kidneys. Carbon Tetrachloride is also a human carcinogen [34]. Major detoxifying organ present in our body is liver [1] that's why most of the toxicological problems are associated to it [2]. Liver cells are harmed by oxidative damage through hepatotoxicants [3]. This indicates the necessity of studying the hepatotoxicity induced by CCl_4 and possible preventive opportunities.

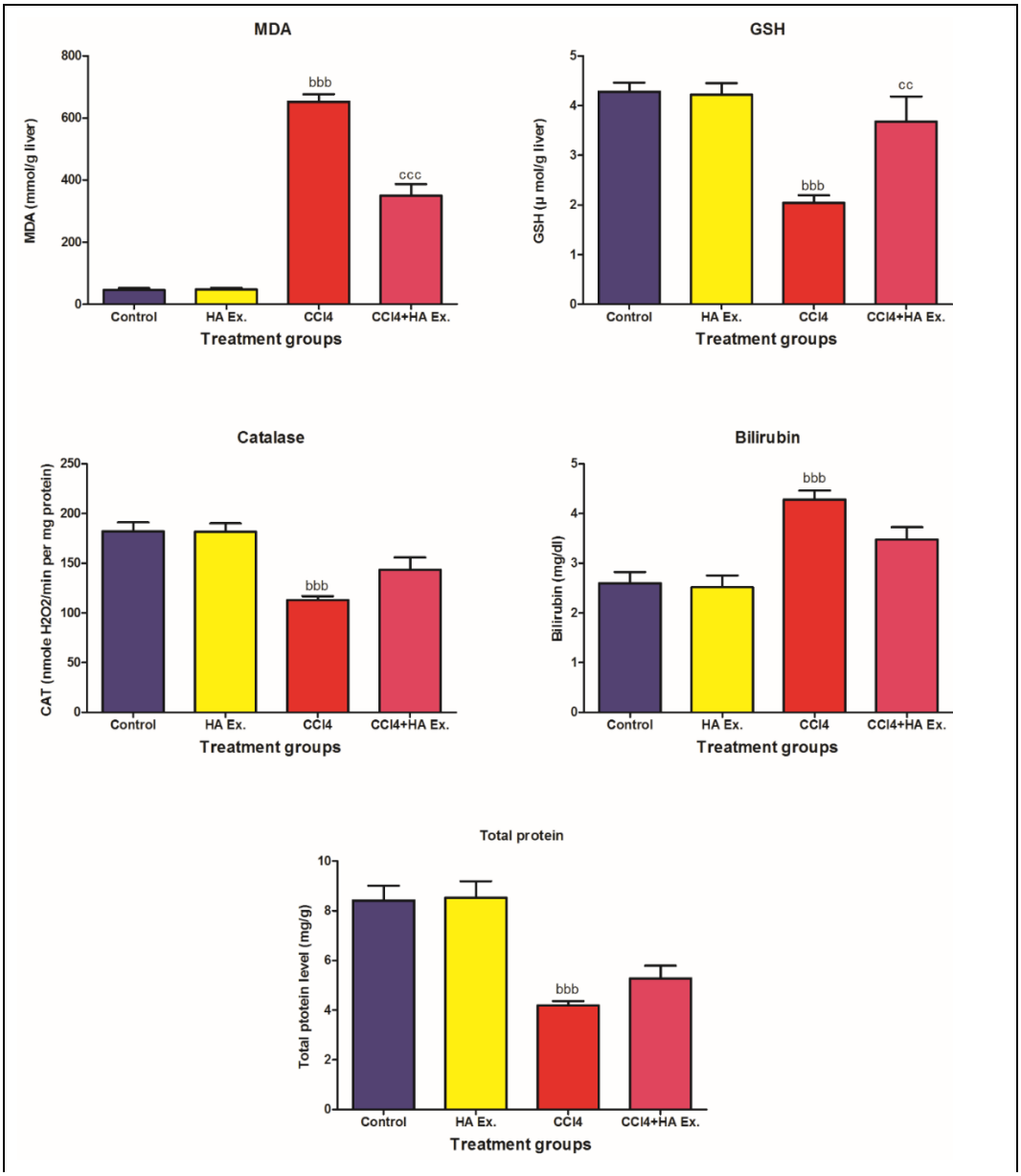

Figure 3: Analysis of enzymes (MDA, GSH, CATALASE, total bilirubin and total protein) in liver of Balb C mice. Abbreviations: "HA ex." stands for*Helianthus annuus* extract and CCl_4 stands for carbon tetrachloride. **Keys:** (b) indicates the significance difference between control and CCl_4. (c) indicates significance difference between CCl_4 and CCl_4 + HA extract. Each bar signifies the mean value of five replicates and SEM. Statistical icons: c = $p \leq 0.05$, bb, = $p \leq 0.01$, bbb, ccc= $p \leq 0.001$.

Administration of a single dose of CCl_4 to Balb C mice resulted in extremely significant rise in plasma ALAT, ASAT, ALP and LDH level as compared to control. When HA extract was given in combination with CCl_4 it caused highly significant fall in plasma ALAT, ASAT, ALP and LDH as compared to CCl_4 (Figure 3). Increase in plasma ALAT, ASAT, LDH were also monitored in rats and Balb C mice [20] after CCl_4 administration. Carbon tetrachloride administration in rats for 24 h induced surge in plasma ASAT and ALAT activities, prime liver lipid peroxidation, depleted sulfhydryl contents, impaired total antioxidant

capabilities and induced genotoxicity. Carbon tetrachloride increases ALAT and ASAT level along with lipid peroxidative enzymes, for example superoxide dismutase and catalase in liver.These may reflect damage to the liver tissues resulted in leakage of these enzymes into the blood. When hepatotoxicity in mice were induced by CCl_4 significant elevation in the level of LDH and liver trasaminases was observed with respect to control group while pretreatment with carrot extract caused a significant decline in the level of LDH as compared with CCl_4 treated group [35]. According to Al-Snafi et al. (2019), when carbon tetrachloride induced hepatotoxicity mice were treated significant ($p<o.oo1$) reduction in the level of ALP, AST, ALT was found with respect to CCl_4 treated group [36]. This reduction might be occur due to decrease the oxidative stress enhanced by carbon tetra chloride. These results supported our conducted research. Similar results were found when carbon tetrachloride induced hepatotoxicity rabbits were treated with alcoholic roots extracts of *Cynodon dactylon* (100mg/kg bw.) significant decline in the level of bilirubin and liver transaminases enzymes was observed that was increased in CCl_4 treated rabbits. In present research in CCl_4 intoxicated mice level of MDA and bilirubin was increased while that of total protein, catalase and GSH decreased. These conditions were reversed on treatment with BLR-extract (Figure 3). These results are in line with Al-Snafi (2017) who stated that when rats were treated with thioacetamide to induce the oxidative stress significant decline in the level of CAT, GSH and SOD was found while, when these mice were treated with seed extract of *Dacus carota*, significant elevation in the level of CAT, GSH and SOD was observed with respect to that thioacetamide group [37]. Rahate and Rajasekaran (2015) had found that when female rats intoxicated with tamoxifen to induce hepatotoxicity significant elevation in the level of MDA and reduction in the level of total protein was found with respect to control group. whereas, polyphenolic fraction of *Desmostachia bipinnata* root bring down the level of MDA and elevated the total protein, SOD and GSH level in serum of female rats [34].

CONCLUSION

Results revealed that hepatic damage was caused by the administration of carbon tetrachloride in Balb C mice. Many biochemical parameters (ALAT, ASAT, ALP, LDH, GSH, MDA, catalase, bilirubin and protein etc) were disturbed that were normalized by *Helianthus annuus* seed extract.*Helianthus annuus* seeds extract has the ability to prevent the damage caused by carbon tetrachloride.

Authors' contributions

Tafail Akbar Mughal andShaukat Ali designed the study; Tafail Akbar Mughalconducted the experimentations; Tafail Akbar Mughal and Shaukat Ali analysed the data. All authors wrote and approved the manuscript.

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This research received no funding.

Conflict of interest

All authors declare there is no conflict of interest.

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